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Mechanisms of Cytotoxicity of the AIDS Virus

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13. ABSTRACT (Maximum 200 words)

Regulatory genes of HIV-1, HIV-2, and SIV are important in modulating virus infection and infection and transmission in vivo. Viral proteins R (VPR) and X (VPX) and the negative factor (NEF) are three of the least well characterized regulatory proteins. Identification of their functions, their mechanisms of actions, and structure-function relationships of each protein, in vitro and in vivo will assist in our understanding of the pathogenesis of HIV induced disease. This information will be critical in defining therapeutic approaches to suppression HIV-1 infection, replication, and transmission.

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The current study examined the functions of the Vpr, Vpx, and Nef regulatory proteins of human immunodeficiency viruses. Vpr was found to be important for productive infection of monocytes by HIV-1 but could be replaced by Vpu. Several Vpr mutants have been constructed in viral clones and expression vectors and a Vpr antibody raised for further structure-function studies of this protein. Cellular localization studies for Vpr suggest that a significant proportion of the protein accumulates in the nucleus. Studies of Vpx have been initiated focusing on requirements for its packaging, possible RNA binding, and role in infectivity. Vpx was found to be associated with gag p24 in the virus particle. Furthermore, Vpx played a role in virus infection of primary lymphocytes at low multiplicities of infection. Studies of Nef demonstrated an inhibition of transcription of the HIV-1 LTR and IL2 promoter mediated by inhibition of NF-kB and AP-1 binding. Studies of Nef mutants demonstrated a role for myristoylation in anchoring the protein to the cytoskeleton.
FOREWORD

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For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45 CFR 46.

In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

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2. HIV-1 Nef Protein Inhibits NF-kB Induction in Human T Cells.

3. HIV-1 Nef Protein Inhibits the Induction of AP-1 DNA Binding Activity in Human T Cells.

4. Functional Analysis of HIV-1 and SIV Nef Proteins

5. The Biology and Molecular Biology of HIV-1
Mechanisms of Cytotoxicity of the AIDS Virus

P.I.: Lee Ratner, M.D., Ph.D.

a) Technical Objectives

1. To define the function of viral protein R (VPR)
   a. Express HIV-1, HIV-2, and SIV vpr genes in E. coli
   b. Develop antibodies to recombinant VPR products
   c. Determine size(s) of VPR products in acute and chronically infected lymphoid and monocytoid cells infected with HIV-1, HIV-2, and SIV
   d. Determine cellular localization of VPR
   e. Assess co- and post-translational modifications of the VPR proteins
   f. Isolate cDNAs encoding VPR
   g. Determine role of VPR in HIV-1, HIV-2, and SIV replication in a variety of lymphoid and monocytoid cells
   h. Determine mechanism of action of VPR in enhancing HIV-1 infectivity and/or replication in MT4 lymphoid cells
   i. Determine role of VPR in vivo with appropriate model systems
   j. Determine role of VPR in modulating disease in HIV-1 infected humans

2. To determine the function of viral protein X (VFX)
   a. To assess effects of VFX on replication and cytopathicity of HIV-2 in T lymphoid and monocytoid cells
   b. To determine cellular localization of VFX in HIV-2 infected cells
   c. To determine if there are co- or post-translational modifications of VFX
   d. To assess whether VFX proviral mutants can be complemented in trans by a VFX expression clone
   e. To determine structure-function-relationships of VFX
   f. To assess effects of VFX on replication and cytopathicity of SIV in T lymphoid and monocytoid cells
   g. To assess role of VFX in vivo with animal model systems

3. To determine function and mechanism of action of NEF
   a. To determine relative effects of HIV-1 NEF on viral RNA transcription, degradation, and nuclear-cytoplasmic transport
   b. To characterize NEF responsive sequences
   c. To characterize mechanism of transcriptional suppression by NEF
   d. To determine role of phosphorylation, GTP binding, GTPase activity, and myristoylation acceptor activity in NEF activity
   e. To determine effects of NEF on cellular proteins including those which may modulate HIV-1 infectivity or replication
   f. To determine role of NEF in HIV-2 and SIV replication
   g. To determine role of NEF in vivo with animal model systems
   h. To determine role of NEF in modulating manifestations of HIV-1 infection in humans
   i. To determine therapeutic role of a retrovirus expressing NEF
b) Hypotheses

Regulatory genes of HIV-1, HIV-2, and SIV are important in modulating virus infection and transmission in vivo. Viral proteins R (VTR) and X (VPX) and the negative factor (NEF) are three of the least well characterized regulatory proteins. Identification of their functions, their mechanisms of actions, and structure-function relationships of each protein, in vitro and in vivo will assist in our understanding of the pathogenesis of HIV induced disease. This information will be critical in defining therapeutic approaches to suppressing HIV-1 infection, replication, and transmission.

c) Background

i) Basis

HIV-1 and HIV-2 cause a slowly progressive immunosuppressive disorder in humans. One species of simian immunodeficiency virus (SIV) derived from rhesus macaques, SIV-MAC, can cause a similar disorder in this species of monkeys (Chakrabarti et al., 1987). Related lentiviruses are found in other species of monkeys including mandrills (Tsujimoto et al., 1989), sooty mangabeys (Hirsch et al., 1989), and African green monkeys (Fukasawa et al., 1988). More distantly related lentiviruses causes immunosuppression in cats (feline immunodeficiency virus) (Pederson et al., 1987; Luciv et al., 1989), sheep (visna virus) (Haas et al., 1985), goats (caprine-arthritis encephalitis virus) (Narayan & Cork, 1985), and horses (equine infectious anemia virus (Issel et al., 1986).

These viruses are biologically and structurally related. They differ from avian and murine retroviruses in the complex nature of their genomes. In addition to genes encoding structural and enzymatic virion proteins, GAG, POL, and ENV, these viruses all encode a number of regulatory proteins (Haseltine et al., 1988). Seven regulatory proteins have been identified thus far.

TAT is a positive feedback regulator of expression of virion and regulatory proteins, working primarily at the level of transcriptional initiation or elongation, and to a lesser degree at a post-transcriptional level.

REV is a differential regulator that increases expression of virion proteins at the expense of regulatory proteins by increasing the transport of unspliced and singly spliced mRNAs from the nucleus to the cytoplasm and by increasing their stability. In addition, a fusion protein between TAT and REV has recently been described (Felber et al., 1989b); its function is unknown.

VIF is important for the infectivity of the virus particle by a post-translational mechanism that remains to be defined. VPU is important for mature virus assembly at the cell surface. VPR, VPX, and NEF are additional regulatory proteins whose functions will be the focus of this study, and will be discussed below.

Regulatory proteins are likely to be important in determining the level of virus replication at different stages of disease, in determining the types of interaction with the immune system, and in modulating virus infectivity and transmission. A better understanding of their structure, expression, and mechanism of action will undoubtedly improve our understanding of pathogenesis, lead to the development of new diagnostic assays, provide new insights into therapeutic maneuvers which may suppress virus replication and/or cytopathicity, and assist in the development of a vaccine for HIV prevention.
Viral Protein R (VPR)

The vpr gene is found in the genomes of HIV-1, HIV-2, simian immunodeficiency virus (SIV) of rhesus macaques (SIV-MAC), SIV of sooty mangabeys (SIV-SM), but not SIV of African green monkeys (SIV-AGM), or SIV of mandrills (SIV-MN) (Wong-Staal et al., 1987; Guyader et al., 1987; Chakrabarti et al., 1987; Fukasawa et al., 1987; Tsujimoto et al., 1989; Hirsch et al., 1989). An open reading frame is also found in a similar position in the visna virus genome (Sonigo et al., 1985). The conservation of the predicted VPR proteins among these different lentiviruses is almost as great as that of GAG and POL proteins.

The HIV-1 VPR protein is 78 amino acids long in several strains, and 96 amino acids long in the remaining strains (Meyers et al., 1989). Functional proviral clones of HIV-1 have been identified with either form of the vpr gene (Dedera et al., 1989, Adachi et al., 1989). Among the first 70 amino acids, 50% conservation of amino acid sequences are noted (Meyers et al, 1989). The HIV-2 VPR protein is 105 amino acids in length, whereas that of SIV-MAC is 97 amino acids long (Guyader et al., 1987; Chakrabarti et al., 1987).

The VPR proteins of HIV-1, HIV-2, and SIV-MAC are expressed in vivo as evidenced by the presence of antibodies reactive with recombinant VPR products in 33-67% of infected humans or rhesus macaques (Wong-Staal et al., 1987; Lange et al., 1989; Yu et al., 1989). A single antibody to the 96 amino acid form of the HIV-1 VPR product has been developed and claimed to detect a 13 kD VPR protein in cells acutely infected with HIV-1 (Lange et al., 1989). The poor quality of the radioimmunoprecipitation analyses using this antibody suggest that the specificity and avidity of this antibody are poor. An antibody to the SIV-MAC VPR product has also recently been developed (Yu et al., 1989), but results with this antiserum have not yet been reported. No antibody to the HIV-2 VPR product has yet been developed.

Work from our laboratory has demonstrated that the HIV-1 and HIV-2 VPR products are dispensable for virus infectivity, replication, and cytopathicity (Dedera et al., 1989). Proviral clones have been constructed expressing a 2 (R2), 22 (R22), 31 (R31), 40 (R40), 78 (R78 or X), or 96 (R96) amino acid form of the VPR product. No differences in the above noted parameters were detected in H9, MOLT 3, CEM, U937, or SUP T1 cell lines, or peripheral blood lymphocytes. HIV-2 proviral clones have been constructed which express either a 105 (MR105 or SE) or 6 amino acid (MR7) form of VPR. No alterations in infectivity, replication, or cytopathicity were noted with viruses derived from these clones in H9, MOLT 3, CEM, SUP T1, Jurkat, or U937 cell lines, or primary human lymphocytes or monocytes.

However, recent data suggests a cell-type dependent effect of vpr expression or action. In MT4 cells, the kinetics of virus replication of virus derived from R2 were significantly different from that of R78, with retarded and diminished virus yield from the vpr mutant. This finding has now been obtained in 6 replicate experiments with 3 different preparations of R2 and R78. Cytopathicity was comparably depressed. The novel feature of this cell line which may account for this effect is unknown; there may be a relationship to human T-lymphotropic virus type 1 (HTLV-I) expression in MT4 cells.

In addition, we have noted subtle morphological differences of virus derived from vpr mutant infected cell lines compared to those infected with the parental virus. R2 virus particles appeared to be less homogenous and more immature than those derived from R78. This may suggest an effect of VPR in virus assembly or maturation. Possible alterations in the structure of the virus particle could account for possible changes in infectivity of the virus.
Viral Protein X (VPX)

The vpx gene is found in HIV-2, SIV-MAC, SIV-AGM, SIV-SM, but not HIV-1 or SIV-MN (Guyader et al., 1987; Chakrabarti et al., 1987; Fukasawa et al., 1988; Hirsch et al., 1989; Ratner et al., 1985a; Tsujimoto et al., 1989). It is a immunogenic protein expressed in vivo, to which 85% of HIV-2 infected humans and 20% of SIV-MAC infected rhesus macaques generate antibodies (Kappes et al., 1988; Yu et al., 1988).

The VPX product is a 112 amino acid proline-rich protein which is found in the virion in equimolar ratio to the GAG capsid (CA; p24) antigen (Henderson et al., 1988). It has also been found to be a nucleic acid binding protein, though specificity for this property remains to be investigated.

Four groups of investigators, including our own group, have now reported on findings of SIV or HIV-2 viruses with alterations in vpx (Yu et al., 1988; Guyader et al., 1989; Hu et al., 1989; Kappes et al., 1989). All groups agree that VPX is dispensable for virus infectivity, replication, and cytopathicity. For example, we have found no effect of VPX on replication of HIV-2 in CEM, H9, U937, SUP T1, and Jurkat cell lines. These studies were carried out with HIV-1 proviral clones capable of coding for the full-length VPX protein, or a clone with a serine substitution for the initiator methionine (MX1), a clone with a termination codon at position 22 (MX22), and a clone with the same mutation present in MX1 as well as a frameshift mutation at position 62 and another termination codon at position 70 (MX1+62).

However, divergent results were obtained in studies of vpx mutant replication on primary T lymphocytes. Whereas Guyader reported a 10-fold decrease in HIV-2 replication in the absence of VPX on T lymphocytes, our own studies have repeatedly failed to detect an alteration in infectivity, replication, or cytopathicity of HIV-2 viruses in primary human lymphocytes with or without vpx (Guyader et al., 1989; Hu et al., 1989). A more comprehensive examination of kinetics of HIV-2 and SIV replication in primary human and macaque lymphocytes and in primary monocytes is clearly indicated to resolve these potentially important discrepancies.

Our group has recently detected a particularly intriguing property of VPX to direct HIV-2 budding to particular sites in the cell. In the presence of VPX, HIV-2 was found in H9 cells to bud intracellularly and at the plasma membrane. The morphology of the virus particles was generally mature and rather homogeneous. In the absence of VPX, HIV-2 buds exclusively at the plasma membrane. The virus particles were generally less mature and less homogeneous.

Negative factor (NEF)

The nef gene, unlike the vpr and vpx genes, is poorly conserved between different strains of HIV-1 and other lentiviruses (Ratner et al., 1985; Meyers et al., 1989). A similar open reading frame, however, has been described also in HIV-2, SIV-MAC, SIV-AGM, SIV-MN, and SIV-SM (Guyader et al., 1987; Chakrabarti et al., 1987; Fukasawa et al., 1987; Tsujimoto et al., 1989; Hirsch et al., 1989).

The NEF protein is immunogenic in vivo in infected humans. Both humoral and cell-mediated immune responses have been detected to this protein (Allan et al., 1985; Arya et al., 1986; Franchini et al., 1986 and 1987). Perhaps the most intriguing finding is the identification by several different investigators of antibodies reactive with NEF early after infection and frequently prior to the detection of other anti-HIV-1 antibodies (Ameisen et al., 1989a and b; Sabatier, et al., 1989; Reiss, et al., 1989; Ronde et al., 1989; Chengsong-Popov et al., 1989; Laure et al., 1989). This
suggests that NEF may be the first viral protein to be expressed in vivo. Recent data from tissue culture experiments confirm that NEF mRNA is expressed after infection earlier than mRNAs for other regulatory and structural proteins (Klotman et al., 1989).

Five laboratories, including our own, have now reported that NEF is a negative regulator of virus replication (Luciw et al., 1986; Terwilliger et al., 1986; Ahmad & Venketassen et al., 1988; Niederman et al., 1989 & Levy et al., 1989). However, one laboratory has failed to detect an effect of NEF (Kim & Baltimore, 1989). Differences in sequence of the NEF product expressed or other technical difficulties may explain the discrepancy. Differences in NEF expression, NEF action, or NEF responsiveness have been demonstrated for different HIV-1 clones (Levy et al., 1989). Kim & Baltimore have not yet carried out similar experiments with proviral clones obtained from any of the five laboratories which have described down-regulatory effects of NEF.

In studies of SIV-MAC clones, all of those which are capable of giving rise to virus in macaque lymphocytes have a defect in nef, whereas the single clone which is not functional has an intact nef gene (Desrosiers, personal communication).

Our own laboratory has recently localized the effect of NEF to an effect on viral RNA levels (Niederman et al., 1989). This effect is at least partially due to an effect on viral transcription as determined by nuclear run-off experiments. Effects on RNA transport or degradation have not been excluded. Confirmation of these results was reported by Ahmad & Venketessan (1988).

The NEF protein has also been reported to down-regulate human CD4 expression (Guy et al., 1987). However, this study was carried out with vaccinia expressed NEF, and only a single control experiment was done examining another lymphocyte surface antigen, 4B4, which was only minimally down-regulated. However, differences in stability of different antigens on the surface of lymphocytes could be reflected in vaccinia infected cells, and the effect may not be specific to NEF. Further studies of the effects of NEF on cellular protein expression and growth are warranted.

Nef is expressed as two proteins from the same mRNA due to utilization of different AUG codons (Ahmad & Venketessan, 1988). When the first AUG codon is recognized a 206 amino acid, N-myristoylated 27 kd protein is expressed. This protein may be phosphorylated at the threonine at position 15 by protein kinase C (Guy et al., 1988). A second NEF product is expressed from utilization of the second AUG codon to produce a 187 amino acid, 25 kd protein (Ahmad & Venketessan, 1988). Both proteins can bind and cleave GTP, and can autophosphorylate at a carboxyl terminal serine residue in the presence of GTP (Guy et al., 1988). Sequence similarities between amino acids 95 and 111 of NEF and other nucleotide binding proteins are readily apparent (Samuel et al., 1987; Guy et al., 1988). The possible relationships of these interesting biochemical activities with NEF activity remain to be investigated.
1) To define the function of viral protein R (VPR)

a) Express HIV-1, HIV-2, and SIV vpr genes in E. coli

In the original application, we had proposed to express each form of vpr in E. coli, in order to obtain proteins that could be used for immunization of rabbits for antibody development. We have essentially bypassed this undertaking, by obtaining a purified synthetic HIV-1 Vpr protein from Dr. Gras-Maase. Furthermore, the current availability of antisera to the HIV-2 and SIV mac Vpr proteins (kindly provided by Dr. T.H. Lee, Harvard) also precludes the requirement for the expression of these proteins in E. coli for this purpose.

b) Develop antibodies to recombinant Vpr products

We have inoculated a single rabbit with the synthetic HIV-1 Vpr protein have now obtained over 100 ml of antiserum over the last six months. This antiserum shows better reactivity with HIV-1 vpr than those provided by Dr. T.H. Lee, AIDS Repository, or Dr. George Shaw, and was utilized in all studies described below.

We have infected CEM cells with HIV-2 strains with or without mutations in vpr, and we will use lysates from these cells to test the reactivity of the anti-HIV-2 Vpr antiserum. We will also examine the reactivity of this antiserum with HIV-2 Vpr expressed in reticulocyte lysates and in transfected BSC40 cells.

c) Determine the size(s) of Vpr products in acute and chronically infected lymphoid and monocytoid cells infected with HIV-1, HIV-2, and SIV

We have used the anti-vpr antibody to analyze 3H-leucine labeled proteins from reticulocyte lysates, HIV-1 infected primary lymphocytes, and vaccinia virus expressed vpr and have found no significant differences in sizes of the single 16 kDa product (Fig. 1). No evidence for a precursor of a different size has been detected or for coimmunoprecipitation of other labeled proteins. We will use the anti-HIV-2 vpr and anti-SIV vpr antisera to analyze proteins from these viruses as well in the next year.
d) Determine the cellular localization of Vpr

In BSC40 cells infected with vTF7-3 (vaccinia virus expressing T7 RNA polymerase) and transfected with pTM3(vpr), we have found that the majority of vpr was localized with the nuclear fraction (Fig. 1a, left). Co-expression of gag did not significantly effect the localization of vpr (Fig. 1a, middle). In contrast, gag p24 was found primarily in the cytosol, and to a lower extent in the membrane and cell-free supernatant under these labeling conditions (Fig. 1b).

**Fig. 1. Cellular localization of vpr.** BSC40 cells were infected with vTF7-3, followed by transfection of pTM3(vpr) alone or with pTM3(gag). pTM3 vector was used as a control. Cells were labeled with 3H-leucine overnight. Cells were fractionated into total cell lysates (L), nuclei (N), cytosol (C), membrane (M), and cell-free supernatant (S). Equal amounts of each fraction were used for immunoprecipitation with antiserum against a) vpr or b) p24 gag.
We have also analyzed the cellular localization of Vpr expressed in HIV-1 infected primary lymphocytes and from proviral constructs in transfected BSC40 cells. In each case, predominant nuclear localization was seen in the biochemical fractionation experiments.

We have also analyzed the cellular localization of mutant Vpr proteins (Fig. 2) using this vaccinia virus expression system. The distribution of the SRIG mutant, with a deletion of amino acid residues 79-82, closely mirrors that seen with the parental form of Vpr, with primarily nuclear localization (Fig. 3, left). In contrast, the CRST mutant, truncated after amino acid residue 77, is approximately equally distributed between nuclear, cytosol, and membrane fractions. This suggests that sequences between amino acids residues 78 and 96 may contribute to the localization with the nuclear fraction.

To assess the purity of each of these fractions, similar experiments will be performed with marker proteins, for nuclear, membrane, Golgi, endoplasmic reticulum, and cytosolic proteins.

Immunofluorescence experiments were also performed with VTF7-3 infected BSC40 cells transfected with pD3(vpr), fixed in ethanol-acetone, and stained with the anti-vpr antiserum and counter-stained with an anti-rabbit antibody conjugated with fluorescein. These experiments could not clearly determine whether vpr was localized within the nucleus or in a perinuclear localization, consistent with endoplasmic reticulum or Golgi localization. Disruption of the cell morphology in the above experiments was a significant problem in data interpretation.

To more clearly define the cellular localization of vpr by immunofluorescence, we plan to perform similar experiments with paraformaldehyde fixed cells. In addition, we have cloned the vpr gene into the SRalpha expression vector which utilizes an HTLV-I promoter and SV40 enhancer. Immunoblot assays have demonstrated Vpr expression from this vector after transfection of BSC40 cells. This expression system will be utilized to ask whether vaccinia virus alters the localization of Vpr. This expression system will be used in both fractionation experiments and immunofluorescence studies. Antibodies to marker proteins will also be used in the immunofluorescence experiments.

We have also performed immunogold localization studies with Vpr expressed in primary lymphocytes. First, we examined several vpr+ HIV-1 strains, and found in each case both intracellular (in vacuoles that resembled Golgi remnants) and extracellular virus particles (Fig. 4, top). The average number of gold particles per virion was determined to be 1.15-1.79. For extracellular virions, the average number of gold particles per virion was 1.34-1.77, whereas for intracellular virions, the average number of gold particles per virion was 0.75-0.99. Insufficient number of virions were counted for virus 127 infected lymphocytes for an accurate determination. No significant effects on vpr packaging in the virus particle were found in the presence compared to the absence of co-expression of Vpu. The finding of decreased levels of Vpr per virion for intracellular versus extracellular virions was a consistent and interesting finding.
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Carboxyl terminal sequence of vpr

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(underline = conserved in HIV-1/HIV-2/SIVmac)

**Fig. 2. Vpr Mutants.**

In many cases the localization of Vpr in the virus particle appeared in a circular array, that resembled envelope localization (Fig. 5). No such localization was seen with anti-vpx or anti-gag antisera. Experiments are underway to perform similar labeling experiments with anti-gp120 antisera to determine whether a similar array of gold particles is demonstrated with anti-gp120 antisera as anti-vpr antisera. In addition, we have constructed mutant proviruses with frameshift deletions in envelope to examine whether vpr can still be packaged into the virions in cells transfected by this clone. BSC40 cells transfected with such proviruses are currently being examined by immunogold electron microscopy.

The Vpr mutants (Fig. 2 and Fig. 6) were also examined by immunogold electron microscopy. In the case of SRIG, with a deletion of amino acid residues 79-82, no significant effect was seen on Vpr localization in intracellular or extracellular virions (Fig. 4, bottom). However, in the case of CRST, truncation after residue 77; 62, truncation after residue 62; and LQ, deletion of residues 64-65, Vpr could not be detected in intracellular or extracellular virus particles (Fig. 4, bottom). To confirm these findings, we are examining the amount of 3H-leucine labeled vpr in
Fig. 3. Localization of vpr mutants. BSC40 cells were infected with vTF7-3, followed by transfection with pTM3(SRIG) or pTM3(CRST). pTM3 vector was used as a control. Cells were labeled with 3H-leucine overnight. Cells were fractionated into nuclei (N), cytosol (C), membrane (M), and cell-free supernatant (S). Equal amounts of each fraction were used for virus particles released from each of these mutants. These results are not likely due to alterations in recognition by the antibody, since each of the mutant forms of Vpr has been expressed in reticulocyte lysates and is detectable with the antiserum.

e) Assess co- and post-translational modifications of the Vpr protein

Experiments to examine whether HIV-1 or HIV-2 Vpr proteins are phosphorylated, O-glycosylated, sulfated, or palmitoylated will be initiated during the next year of study.
**Mechanisms of Cytotoxicity of the AIDS Virus**  
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Basic

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**Figure 4. Distribution of Vpr on HIV-1 particles in primary lymphocytes after 7 days of infection.**

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<th>Samples (N)</th>
<th># of Vpr-G/Virion</th>
<th>Average # of Vpr-G/Virion</th>
<th>% of Wild Type (83)</th>
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<td>127 r+/+ (12)</td>
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<td>125 r+/+ (39)</td>
<td>3922/2195</td>
<td>1.79</td>
<td>119.33</td>
<td>1.77</td>
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<tr>
<td>159 r+/+ (36)</td>
<td>1524/1326</td>
<td>1.15</td>
<td>76.67</td>
<td>1.36</td>
<td>0.75 (55.21)</td>
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<td>Mutants (77)</td>
<td></td>
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<tr>
<td>SLig (33)</td>
<td>1428/1292</td>
<td>1.15</td>
<td>77.24</td>
<td>0.39</td>
<td>0.85 (220.98)</td>
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<td>62 (11)</td>
<td>23/372</td>
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<td>4.18</td>
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<tr>
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<td>3.64</td>
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<td>Control (5)</td>
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Vpr-G: Vpr-gold complex; EC: Extracellular virus; IC: Intracellular virus.  
Most IC data were obtained from SR group.  
*Low in sample number (1)

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f) Isolate cDNAs encoding Vpr

The experiments on isolation of Vpr cDNAs are being carried out jointly with investigators on contract DAMD17-90C-0057. For this purpose, primers have already been synthesized, and are currently being tested. Such cDNAs will be useful for two purposes. First, we will determine if there are mRNAs which may encode fused proteins with the Vpr open reading frame and some other HIV-1 open reading frame. Second, we will examine sequence heterogeneity and correlate it with functional differences in Vpr sequences obtained from patients at different stages of disease.

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g) Determine role of Vpr in HIV-1, HIV-2, and SIV replication in a variety of lymphoid and monocytoid cells

Previously, we and other others have demonstrated that a discrete env determinant, including the V3 loop, but not the CD4-binding domain, is necessary and sufficient for HIV-1 infection of monocytes. Additionally, we have identified three virus replication phenotypes in monocytes in vitro using molecular defined proviral clones. These include productive infection, with the generation of high virus replication levels; silent infection, with low to undetectable virus replication in monocytes, despite ultimate virus recovery from infected monocytes following cocultivation with uninfected, phytohemagglutinin stimulated peripheral blood mononuclears (PBMCs [lymphoblasts]); and no infection, with neither virus replication in nor virus recovery from monocytes observed. In the present study,
we investigated the role the HIV-1 "accessory" genes vpr and vpu, which are dispensable for virus replication in primary and immortalized CD4+ T-lymphocytes. We demonstrated that vpr and vpu are central to the regulation of virus replication in primary monocytes and together mediate the expression of silent versus productive infection.

To study viral regulation of monocyte infection, we utilized a panel of chimeric HIV-1 clones, constructed from the nonmonocytotropic clone HXB2, and the monocytotropic clone ADA, as previously described. To correct a vpr defect in each of these clones, the result of a single base insertion in HXB2, 2.7 kb Sal I-Bam HI HYADA DNA fragments (nucleotides 5785-8474) were subcloned into the full-length
**Fig. 6. Additional vpr mutants.**

Provirial clone NL4-3, in which the vpr open reading frame is intact. The resultant NLXADA clones contained the ADA-derived env determinant previously localized to nucleotides 7040–7323, flanked by other portions of env and vpu and small portions of tat and rev. A clone in which the entire 5785–8474 sequence was HXB2 derived (thus lacking a monocytoprotic env determinant) was used as a negative control for these experiments. Because HXB2 lacks a vpu initiator methionine codon, clones in which vpu was HXB2 derived were defective for that product, in contrast to clones with an ADA-encoded vpu. Finally, a vpr mutant corresponding to each NLXADA clone was generated by introducing a frameshift mutation at codon 63.

Virions from the recombinant clones, generated by transfection, were assayed for their ability to infect and replicate in primary monocytes by the presence of reverse transcriptase (RT) activity in culture supernatants and by the ultimate recovery of virus following cocultivation of monocytes with uninfected PBMCs. The results are summarized in Fig. 7. All clones containing the ADA-derived env determinants with an intact vpr gene generated high virus replication levels in monocytes. Inactivation of vpr in these clones, however, generated divergent results, depending upon the derivation of nucleotide sequences 5999–6345 (SK fragment). Clones in which this portion of the genome was ADA derived generated lower (but readily detectable) virus replication levels than did their wild-type vpr counterparts. However, vpr mutants in which the SK fragment was HXB2 derived typically failed to generate virus replication levels detectable above background in monocytes, despite subsequent virus recovery from these cultures onto uninfected PBMCs. The negative control clone, which carried a wild-type vpr but lacked the monocytoprotic env determinant generated virions which neither replicated in nor were recovered from monocytes, as previously demonstrated. No significant differences were seen in the replication of each virus strain on PBMCs obtained from several different donors.
Mechanisms of Cytotoxicity of the AIDS Virus

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Basic

Fig. 7

(A) Replication of recombinant HIV-1 clones with both wild-type and mutant vpr genes. (A) The panel of recombinant NL4-3 clones is represented diagrammatically. The region of the genome corresponding to the NL4-3 fragment (nucleotides 5761 to 8474) is expanded to highlight the relative positions of NL4-3 and ADA-derived sequences. The open reading frames in this portion of the genome are represented above. Recombinant clones were generated by reciprocal DNA fragment exchanges of ADA- and HXB2-derived sequences into a StuI-donut fragment (5761 to 8474) from HXB2 subcloned into an intermediate shuttle vector, utilizing the restriction enzyme sites indicated on top. The resultant chimeric StuI-donut fragments were then subcloned into the clone NL4-3 to generate
Monocytes were infected with recombinant HIV-1 clones containing a functional vpr gene, stained with toluidine blue, and examined by light microscopy (1 μm thick plastic sections). Cultured infected with a noncytolytic virus, NLXADA-SK which contains a functional vpu gene, were indistinguishable from uninfected cells, with rare, small multinucleated cells (Fig. 8a). Cultured productively infected with virus containing the noncytolytic env determinant and a functional (NLXADA-SM, Fig. 2b) or nonfunctional (NLXADA-GG, Fig. 8c) vpu gene showed characteristic cytopathic effects. These consisted of the formation of multinucleated giant cells, often containing 10 or more nuclei per cell, and cell lysis. The frequencies and sizes of these cells were comparable in the NLXADA-SM and NLXADA-GG infected monocyte cultures. Virus production and cellular degeneration and necrosis were primarily confined to multinucleated cells. Transmission electron microscopy examination demonstrated typical budding and mature virions in intracellular vacuoles that were associated with the plasma membrane, in both the presence and absence of vpu, but not in the NLXADA-SK infected cells (Fig. 8d). Freeze fracture scanning electron microscopy demonstrated budding of virion particles from the plasma membrane of monocytes infected with virus which lacked a functional vpu (Fig. 8e). No virus could be detected in monocytes infected with recombinant clones lacking both vpr and vpu (data not shown).

The SK fragment encoded the entire vpu gene product, 14 amino acids at the C termini of both the tat and the rev first exons, and the N-terminal 41 amino acids of env (Fig. 9). Although the absence of a vpu initiator methionine codon in HX2 is the most obvious difference between the SK portions of HX2 and ADA, a role for tat, rev, or env could not be ruled out. The env sequences differ at 7 of 41 predicted amino acid positions, not including the nonaligned insertion of 3 residues and deletions of 4 residues in ADA. All but three of these differences are confined to the signal peptide, which varies by up to 30% between different HIV-1 clones. Furthermore, tat and rev both differ at 3 of 14 amino acid positions between the ADA and HX2 SK fragments with four of these six changes being conservative in nature. Therefore, it is unlikely that these alterations in env, tat, or rev alter their function. However, to formally determine the specific requirement for vpu during HIV-1 infection of monocytes, the vpu initiator methionine codon of the silent infection clone NLXADA-GG (vpr mutant) was restored by site-directed mutagenesis. The resultant clone was found to generate virus capable of productive infection of monocytes (data not shown).

HIV-1 and related lentiviruses are distinct from most other retroviruses in that besides the structural gag, pol, and env genes common to all retroviruses, they also encode a number of genes whose functions have been shown or are speculated to be regulatory in nature. In HIV-1, these genes include tat, rev, vif, nef, vpu, and vpr. While tat, rev, and vif are essential for viral gene expression or virion infectivity, the precise role and overall importance of vpr, vpu, and nef are unclear, since these genes are dispensable for virus infection and replication in CD4+ lymphocytes in vitro. The availability of molecular HIV-1 clones which infect and replicate in monocytes at levels comparable to those observed with many monocytotropic virus isolates has facilitated investigation of the role that these viral genes may play in regulating the virus life cycle in monocytes. In the
Mechanisms of Cytotoxicity of the AIDS Virus
P.I.: Lee Ratner, M.D., Ph.D.

Contract No. DAMD17-90-C-0125

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FIG. 9. Light, transmission, and freeze fracture scanning electron microscopy of infected monocytes. Light micrographs of toluidine blue-stained, sectioned plastic sections showing typical fields of primary monocytes infected by xenomycoid retrovirus clone MLVXAda-SK (A) and monocyte/macrophage clone MLVXAda-G (B) and MLVXAda-GG (C) are shown (15). The small, elongated giant cells were fewer and smaller in panel A than in panels B and C. Magnification, x480. Infected adherent cultured cells were carefully washed twice with phosphate-buffered saline (PBS), fixed in situ with 2% glutaraldehyde (pH 7.4) in PBS, scraped free with a rubber policeman, transferred to a 15-ml plastic conical tube, and pelleted for 10 min at 600 x g centrifugation. The cells were mixed with warm agar, repositioned in the microscope for 1 min, and refrigerated overnight to form a firm agar block. The cell block was divided into small pieces and processed into Spurr’s plastic, after osmium tetroxide and bleached section mounting (15). Sections (1-μm thick) were stained with toluidine blue for light microscopy, while thin sections (0.05-0.06 μm) were stained with uranyl acetate and lead citrate for transmission electron microscopy. (D) Transmission electron micrograph of a small portion of a monolayered cell from MLVXAda-GG-infected monocytes showing a cytoplasmic vacuole (lower left) containing intact and mature virions and numerous typical r- and v-particles associated with a stretch of plasma membrane. Magnification, x44,000. (E) Transmission electron microscopy view of MLVXAda-GG-infected monocytes, stabilized by barium hyaluronic acid before quick-freezing, freeze-drying, and platinum replication (18). Sectioning from the cryostat surface are several 50-nm-diameter brightly outlined spherical virus particles. At higher magnification (not shown), these display characteristic surface coats of 22-nm “wings.”

In the present study, we observed moderately decreased levels of virus replication in the absence of either vpr or vpu, whereas in the absence of both genes, virus replication in monocytes dropped to levels barely at or below the level of detection by the RT assay, such that infection of these cells usually could be detected only by virus rescue into PBMCs.

The vpr open reading frame encodes a protein of 96 amino acids in most HIV-1 clones and is conserved in other lentiviruses, including visna-maedi virus. Previous studies have shown that vpr is not required for HIV-1 infection or replication in CD4+ lymphocytic cell lines in vitro, although its inactivation led to slower replication kinetics and delayed cytopathogenicity in these cells. A recent study involving HIV-2 has shown that vpr is likewise dispensable during infection of PBMCs and T-cell lines but essential for productive infection of monocytes. The vpr protein has been demonstrated by radioimmunoprecipitation to be
virus-associate, and thus it is speculated to function either late in the virus life cycle, during particle assembly or maturation, or early, during the initial stages of infection. The vpu gene encodes an 80-82 amino acid protein. It has not been reported whether the vpu protein is found in virion particles. vpu has been shown to augment virion particle release from infected cells, without affecting levels of viral RNA or protein synthesis. In the absence of vpu, a higher ratio of immature to mature particles has been seen, with a shift in capsid formation from the plasma membrane to intracellular membranes. In monocytes, however, particle assembly and release occur both at the plasma membrane and in intracellular vacuoles in the presence or absence of vpu as shown in Fig. 8d.

It is intriguing that HIV-2 and SIV lack a vpu open reading frame but instead carry a gene designated vpx, which encodes a protein of 114-118 amino acids in these viruses. vpu and vpx occupy similar positions in their respective viral genomes, between pol and env, but have only distant amino acid homology. Recently, it has been suggested that vpx and vpr arose by duplication from a common progenitor in HIV-2 and SIV, and on the basis of predicted amino acid sequence homology between the genes. To investigate the possibility of a similar link between vpr and vpu in HIV-1, the predicted amino acid sequences of both vpu and vpx were aligned with that of vpr. Although less compelling than the homology between vpr and vpx, a 38% identity was observed between vpr and vpu over a 24 residue overlap at the C-terminus of vpu and the N terminus of vpr (Fig. 10, top). These sequences were particular rich in acidic residues. Similarity in the hydrophilicity profiles of these portions of the vpu, vpr, and vpx products was also noted (Fig. 10, bottom). The striking effect on virus replication levels in monocytes observed only when both genes were defective suggests that their gene products may perform similar roles and thus provide partial functional complementation. Alternatively, since lower replication levels were observed in the absence of either gene, the nearly complete attenuation observed in the absence of both may result from a compound effect of the
loss of two relatively important but functionally unrelated genes. More detailed studies to determine the precise mechanisms of action of the vpr and vpu gene products will be required to address these alternatives. In either case, our data indicate that together, vpr and a second determinant, vpu, are more important for efficient HIV-1 infection and replication in primary monocytes than was observed previously in lymphocytes. These observations provide a rationale for designing potential antiviral therapies to block the action of these gene products during HIV-1 infection of monocytes.

Persistent infection of tissue macrophages plays an important role in the pathogenic effects of other lentiviruses, including equine infectious anemia virus, visna-maedi virus, and caprine arthritis-encephalitis virus, providing a sanctuary for continuous virus replication in the face of a vigorous host immune response. The onset of increased virus replication has been correlated with the onset of clinical disease manifestations, such as encephalitis, pneumonitis, arthritis, and hemolytic anemia. The existence of poorly replicative HIV-1 variants may be essential for establishment of persistent macrophage infection during the early, asymptomatic stage of disease. Several studies have suggested a relationship between the in vitro replicative properties of HIV-1 isolates in T lymphocytes and clinical disease stage, with earlier isolates tending to replicate more slowly and to lower levels ("slow, low") than isolates from later stages of disease ("rapid, high"). Nonessential regulatory genes are ideally suited to act as "molecular switches" for control of replication phenotypes by their activation or inactivation, particularly in viruses such as HIV-1, which characteristically generate high levels of sequence heterogeneity. We demonstrate here that discrete genetic alterations in such accessory genes result in profoundly different replication rates in monocytes in vitro, which suggests a mechanism for transition from subclinical to clinical disease in vivo. These findings thus provide a rationale for addressing on a wider scale whether functional status of vpr and/or vpu correlates with disease stage or serves as a potential prognostic indicator of disease progression and outcome.

To further test the role of vpu and vpr in monocyte infection, several additional vpu mutant proviruses have been constructed. These include NLUXADA-SM and NLUXADA-SK/GG plasmids with a site-directed mutation at the initiator methionine codon of vpu. In addition, we have constructed NLUXADA-GG, NLUXADA-GP, NLUXADA-12-GP, and NLUXADA-GP plasmids in which we have inserted the vpu initiator methionine codon. Each of these clones were constructed with or without a frameshift mutation at the Eco RI site of vpr. Mutations in these plasmids will be confirmed by nucleotide sequencing and then assayed for virus replication kinetics on monocytes. In addition, we will use primary lymphocytes infected with these viruses to label with 3H-leucine and immunoprecipitate with anti-vpu or anti-vpr antiserum.

Additional vpr mutants have also been constructed to further assess the role of vpr in HIV-1 replication in monocytes (Fig. 2 and 6). Preliminary results with some of these mutations are listed in Fig. 11. These data suggest that the C-terminus of vpr is not essential for productive infection in macrophages. Replication studies with the remaining clones will be performed over the next several months.
To further assess the nature of the vpr requirement in macrophage infection, trans-complementation studies will be performed. In this case, we will ask whether virions generated from COS cells co-transfected with SRAphα(vpr) + fs62 can produce virions with vpr protein, and whether these virions are now capable of infection of macrophages. In a second approach, we will transfet macrophages which are already silently infected with fs62 with SRAphα(vpr) to ask whether complementation with vpr can convert silent infection to productive infection.

Electron microscopy studies will also be performed with macrophages and lymphocytes infected with NLH or NLHADA-GP viruses with or without vpu and vpr determinants. In addition, we will perform, electron microscopy studies of macrophages at different time points after activation with primary lymphocytes.

To identify the function of vpr and vpu in macrophages, we have constructed a recombinant vaccinia virus that expresses vpr, and are in the process of constructing a recombinant vaccinia virus that expresses the vpu protein together with either the ADA or HXB2 envelope. The latter strategy was taken in light of the fact that vpu and envelope are expressed from a single bicistronic mRNA. We will examine the effect of vpu or vpr on env synthesis, processing, stability, cell surface transport, CD4 binding, release, oligomerization, and gp120 cleavage.
h) Determine mechanism of action of Vpr in enhancing HIV-1 infectivity and/or replication in MT4 lymphoid cells

The contract application describes 3-10-fold differences in the ability of HIV-1 clones with or without vpr to replicate in HIV-1 infected cell lines, MT4 and MT2. However, the much more dramatic differences in the presence of vpr in monocytes, and the particular relevance of monocytes to disease pathogenesis, suggested to us that much greater emphasis should be placed on examining the mechanism of action of Vpr in monocytes than in MT4 cells. Thus, the same analyses described in the original contract application for MT4 cell studies will still be performed, but these studies will take a lower priority to pursuing the much more exciting monocyte infection studies.

i) Determine effects of Vpr on HIV-1 infectivity

Initial experiments have been performed to determine if Vpr affects the level of HIV-1 infection in monocytes. For this purpose, HIV-1 strains with or without the monocyte-tropic envelope determinant, vpr, and/or vpu were expressed from COS-7 cells and used to infect monocytes or lymphocytes. One day after infection, cellular RNA was harvested and quantitative PCR was performed with primers that were designed to detect the earliest reverse transcriptase products, i.e. R and U5 sequences. Controls were performed with heat-treated virus in order to insure that signals were not due to contaminating RNA in the virus preparations. These data demonstrated that the monocyte-tropic envelope determinant was essential for infection. Clone NL43 gave rise to no detectable viral RNA synthesis in monocytes, whereas viral RNA could be detected in primary lymphocytes. Similar levels of viral RNA were seen in the presence of the monocyte-tropic envelope determinant in the presence or absence of vpr or vpu determinants. This experiment must be confirmed with other virus preparations and isolates. However, it suggests that vpr and vpu function at a step distinct from that of the envelope determinant after the initiation of reverse transcription. If similar findings are obtained in additional experiments of this type, other steps in virus replication will be examined in the presence or absence of the vpr and vpu determinants.

ii) Determine effects of Vpr on HIV-1 production

Studies of the effects of vpr on HIV-1 production from monocytes have not been initiated, and will await the results of I.h.i. If the results described above for HIV-1 RNA levels in monocytes are confirmed, we will repeat the assays examining HIV-1 RNA levels to assess whether vpu and/or vpr affect steps in the replication cycle between these two points (i.e. completion of reverse transcription, integration, transcription, RNA transport, RNA stability). Other studies will include those examining HIV-1 protein expression in infected monocytes by immunoblot and immunofluorescence.
We have examined by transmission electron microscopy, monocytes infected productively or silently by a number of the virus strains described above. No virus particles were detected in silently infected monocytes, but this is being re-examined as described above. Thus, either the replication cycle is aborted prior to the step of virus assembly, or the level of expression was too low to be detected by electron microscopy.

iii) Determine if Vpr effects can be complemented in trans

These experiments are underway as described in 1.g.

iv) Determine if Vpr effects are independent of other regulatory proteins

We have already demonstrated a potential interaction between vpr, vpu, and vpx. Additional experiments will be initiated to examine the nature of this interaction.

v) Determine the structure-function relationships of Vpr

Several additional vpr mutants have been constructed and are described in Fig. 2 and 6. Each of these mutants will be examined after synthesis in a cell-free reaction in reticulocyte lysates, in vaccinia virus expression systems, and in HIV-1 proviral clones in infected lymphocytes as described in 1.d. and 1.g.

i) Determine the role of Vpr in vivo with appropriate model systems

We have initiated studies in scid/hu mice with constructs NLHX, NLHXADA-GG, and NLHXADA-GG vpr mutant in collaboration with R. Markham (Johns Hopkins). The first experiment showed successful replication and recovery from inoculated mice with each virus. Quantitative assays are now developed using in situ hybridization and PCR and will be applied in additional in vivo experiments of this type.

We have deferred work in the rabbit model system as advised by our collaborators, Drs. Tom Folks and Michael Laimmore (CDC and Ohio State University), since consistent infection results could not be reproduced with this model system. Studies of vpr mutants in SIVmac239 are already underway in Dr. Desrosiers group at the New England Primate Research Center.

j) Determine the role of Vpr in modulating disease in HIV-1 infected humans

In collaboration with Dr. Richard Markham (Johns Hopkins), we have obtained samples from seven patients followed over the course of several years with three PEMC samples from each patient at times when the CD4>1000, CD4=250-750, and CD4<250. PCR primers for amplifying RNA or DNA sequences from these samples are currently being evaluated.
2. To determine the function of viral protein X (Vpx)

a) To assess the effects of Vpx on replication cytopathicity of HIV-2 in T lymphoid and monocyteid cells

i) To determine effects of Vpx on site of virus assembly in the cell

We have utilized immunogold electron microscopy to examine Vpx incorporation into virus particles. No detectable gold particles were found on virions generated from vpx mutant HIV-2 virus, MX, and vpx/vpr double mutant, MR/MX (Fig. 12). As with Vpr expression, intracellular particles were relatively depleted of Vpx compared to extracellular particles. Localization of Vpx in viruses released into the medium is currently being examined, using concentrated preparations of cell-free virus. Background gold labeling in cellular sites was too high to determine the localization of free vpx in the cell. These experiments will be repeated under more stringent labeling conditions. Vpx had no effect on the site of virus assembly in PBLS or CEM cells.

| Table 1. Expression of Vpx on HIV-2 in CEM after 14 day-infection |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Samples (N) | Vpx-G/Vision | Average # of Vpx-G/Vision | % of ES | Vpx-G/Vision | IC Average # of Vpx-G/Vision (% of IC) |
| ES (16) | 2244/1209 | 1.86 | 100 | 1.92 | 1.42 (74.20) |
| MR7 (6) | 535/343 | 1.56 | 83.99 | 1.62 | 1.30 (83.18) |
| MX (10) | 9/527 | 0.03 | 1.51 | / | / |
| MR/MX (7) | / | / | / | / | / |
| Control (3) | -0 | -0 | -0 | -0 | -0 |

Vpx-G: Vpx-gold complex; IC was obtained mostly from ES/PBL.
/ not available

| Table 2. Expression of Vpx on HIV-2 in PBL after 14 day-infection |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Samples (N) | Vpx-G/Vision | Average # of Vpx-G/Vision | % of ES | Vpx-G/Vision | IC Average # of Vpx-G/Vision (% of IC) |
| ES (37) | 5831/3495 | 1.66 | 100 | 1.44 | 1.70 (118.31) |
| MR7 (8) | 485/437 | 1.11 | 66.69 | 1.29 | 0.87 (67.65) |
| MX (12) | 14/504 | 0.02 | 1.38 | 0.021 | 0.029 (I) |
| MR/MX (7) | / | / | / | / | / |
| Control (9) | -0 | -0 | -0 | -0 | -0 |

Vpx-G: Vpx-gold complex; IC was obtained mostly from ES/PBL.
/ not available
ii) To determine the cellular localization of Vpx in HIV-2 infected cells

Immunogold electron microscopy experiments are underway to identify the cellular localization of Vpx, as described above. In addition, we will clone vpx into the SRalpha vector for expression in the absence of other viral proteins to determine cellular localization in fractionation experiments and immunofluorescence experiments as described above for vpr. In addition, we will examine Vpx localization using the vaccinia virus expression system.

iii) To determine if there are co- or post-translational modifications of Vpx

The clones described in the previous section will be used for these studies. In addition, studies will be performed in HIV-2 infected cells and cells transfected with Vpx from the SRalpha vector.

d) To assess whether Vpx proviral mutants can be complemented in trans by a Vpx expression clone

These experiments are underway and are described in 1.h.iii.

e) To determine structure-function relationships of Vpx

In addition to the studies of Vpx outlined above, a number of other clones are being constructed at this time to answer the following questions:

i) Does vpx bind HIV-2 RNA with specificity?

ii) What HIV-2 viral components are required for packaging Vpx?

To examine Vpx binding to HIV-2 RNA, as noted previously, we have cloned HIV-2 vpx and vpr genes into pIM3 expression vectors to allow expression in reticulocyte lysates and in VTF7-3 infected BSC40 cells, and into pMD vectors for E. coli expression. In addition, we are cloning the HIV-2 genome in both orientations into pGBM1ZP+. We have already cloned a portion of the HIV-2 genome into pGEM in both orientation including the packaging signal. Northwestern blots with the reticulocyte lysate expressed and vaccinia virus expressed Vpx proteins failed to demonstrate specific binding to viral RNA, but the amounts of protein expressed in these systems was quite low. Northwestern blot conditions have been established with the E. coli expressed proteins. As a control, we have shown that pMDN expressed HIV-1 gag binds specifically HIV-1 RNA. We have therefore prepared blots with HIV-2 gag p55 and vpx proteins to examine binding to the HIV-2 RNAs that have been prepared. Additional experiments will include filter binding assays and sucrose gradient sedimentation studies. If specific RNA binding can be detected, we will first localize the sequences required for binding in the HIV-2 RNA. Second, we will construct mutations in Vpx to determine which sequences are required for binding.
To investigate issues related to Vpx packaging, two approaches are currently underway. First, we are constructing clones of HIV-2 which are incapable of synthesizing envelope, by introducing of a frameshift mutation at the Nsi I site in the env gene. This will be useful in asking whether the envelope protein is required for Vpx packaging into virus particles. We are also constructing a packaging mutant of HIV-2. For this purpose, we have cloned the 5' Nsi I fragment of HIV-2 into a subclone. A deletion in the leader sequences expected to contain the packaging sequence has been constructed through the use of PCR. We will then clone the segment with the deletion back into the full clone to determine if the RNA packaging signal is required for Vpx packaging.

An additional method to examine the requirement for Vpx packaging is to determine if virus particles with only Gag and Pol proteins can package Vpx. For this second approach, the gag and pol genes were cloned in pIM3. This clone was then transfected into BSC40 cells infected with VTF7-3. Co-transfection with pIM3(Vpx) led to packaging of Vpx in virus particles as determined by three parameters. First, Vpx was released into the cell-free supernatant only if co-expressed with Gag. Second, Vpx released into the cell-free supernatant was found in particles as determined by its ability to sediment through a 20% sucrose cushion and to band on sucrose gradients at a density of 1.14-1.16 together with gag p24 protein. Third, we have succeeded in co-immunoprecipitating vpx with gag p24 with a monoclonal antibody to HIV-2 p24.

Additional work will examine whether the vpx and gag containing particles include RNA. This will be approached in two ways. First, we will perform dot blot hybridization with an HIV-2 genomic DNA probe with the fractions from the sucrose gradient to determine if any HIV-2 RNA is present in the particles. Second, we will label VTF7-3 infected BSC40 cells cotransfected with pIM3(gag-pol) and pIM3(Vpx) with 3H-uridine and determine if any 3H incorporation is found in the viral particles. This experiment will ask whether either cellular or viral RNA is present in these particles.

We will also examine the gag-pol requirements for vpx packaging. Will the HIV-2 gag gene alone suffice to form particles and package vpx? Can the HIV-2 gag gene be truncated at the N-terminus (without affecting the myristoylation acceptor signal) or the C-terminus without affecting particle formation or vpx packaging? In addition, we will ask whether the HIV-1 gag gene can replace the HIV-2 gag gene in packaging vpx. The advantage of the latter system is two-fold. First, we have already constructed many mutations in the HIV-1 gag gene that can be analyzed for vpx packaging if the parental construct leads to vpx packaging. Second, we can construct chimeric HIV-1/HIV-2 gag proteins to identify critical determinants required for packaging if only HIV-2 gag proteins allow vpx packaging.

We have also begun construction of a series of vpx mutations to ask what portions of vpx are essential for packaging. In one clone, we have deleted amino acids 102-112, including the proline-rich tail. In the second clone, we have deleted amino acids 73-112, including the proline-rich tail, and a cysteine-histidine-rich region of the protein. In the third clone, we have deleted amino acids...
acids 73-102, including the cysteine-histidine-rich region. In the fourth clone, we have deleted amino acids 40-102. In the fifth clone, we have deleted amino acids 20-102 including the above domains plus the amphipathic helix. In the sixth clone, we have deleted the amphipathic helix alone, between residues 20-40.

f) To assess effects of Vpx on replication and cytopathicity of SIV in T lymphoid and monocytoid cells

We have focused our initial work on the HIV-2 Vpx protein. Studies of the SIVmac Vpx protein will be done subsequently. We have found that mutation of vpx in HIV-2 leads to a decrease in HIV-2 replication in PBUs and HeLa/T4 cells, particularly at low multiplicities of infection. Minimal or no effects were found in CEM cells.

Fig. 13. Replication of HIV-2 clones with vpr (MR), vpx (MX), or double mutations (MR/MX) in HeLa/T4 cells.
To further define the nature of the vpx defect in PB~s versus CEM cells, several experiments are underway. We will determine if there is a difference in HIV-2 infectivity in these cells with or without vpx, by serial dilutions of equivalent numbers of virus particles. Infectivity experiments will also be performed in HeLa/T4/beta-galactosidase indicator cells.

We are examining if there is a difference in envelope-CD4 interactions in the presence compared to the absence of vpx co-expression. This will be performed initially with the vaccinia virus expression system.

We are examining if there is a difference in fusogenicity of the HIV-2 envelope in the presence compared to the absence of vpx co-expression. This will be performed with both the vaccinia virus expression system, and chronically infected CEM cell lines.

We are examining if there is a difference in viral DNA synthesis in the presence compared to the absence of vpx, using a quantitative DNA PCR assay.

g) To assess the role of Vpx in vivo with animal model systems

Animal model studies will await initial data with the HIV-1 viruses in scid/hu mice (see 1.i.) and the initial data with the HIV-2 virus studies outlined in this section.
3. To determine the function and mechanism of action of negative factor (NEF)

a) To determine relative effects of HIV-1 Nef on viral transcription, degradation, and nuclear-cytoplasmic transport

We have demonstrated that HIV-1 and SIVmac Nef affect viral transcription but not degradation or processing of viral transcripts. This work was reported in last year's annual report and in our manuscript (Niederman, Hu, Ratner, Simian immunodeficiency virus negative factor (NEF) suppresses viral mRNA accumulation in COS cells. J. Virol. 65:3538-3546, 1991).

We have discussed the controversy concerning these results in a recent review by Niederman and Ratner, Functional analysis of HIV-1 and SIV nef proteins. Research in Virology 143:43-46, 1992. We found that the multiplicity of infection was critically important in assessing the effects of Nef in virus replication studies. It appeared reasonable to assess the relevance of the moi considering the relatively moderate effects of Nef compared to the effects of Tat or Rev. The hypothesis is that at relatively high moi, subtle effects of Nef may be masked due to an overload of viral DNA templates. That is, Nef may require a limited pool of cellular factors in order to maintain transcriptional suppression. Once a cascade of virus replication has occurred, and high-titer virus results, it may be impossible for Nef to reverse or even halt the cascade.

To assess the effect of the moi on Nef's suppressive capacity we compared the replication of Nef+ and Nef- viruses in lymphoid cells under conditions of varied moi. We found that at relatively high moi, replication of the Nef+ and Nef- viruses was indistinguishable as measured by reverse transcriptase activity. However, upon serial 10-fold dilutions of the initial virus inoculum, the replication of the Nef+ virus was significantly inhibited (up to 25-fold) in several T-cell lines compared to its Nef- counterpart. In addition to generating lower levels of reverse transcriptase activity, the Nef+ viruses lagged by 4-8 days in comparison to the Nef- virus with regards to detection of reverse transcriptase activity. This lag period may represent an in vitro form of latency. Similar experiments will be carried out in macrophages. Recently other investigators compared the replicative capacities of SIV Nef+ versus Nef- viruses. They also found Nef-mediated suppression to be dependent upon low moi conditions and also observed a lag period for viral growth in the presence of Nef. Finally, there are other examples demonstrating the relevance of the moi when determining the function of other HIV-gene products, including Vpr and Vpx.

Other factors contributing to the differences observed between our laboratory and that of Kim and coworkers may involve different modes of virus transmission. In our system, Jurkat cells were cocultivated with COS cells which were constantly shedding extremely low concentrations of virus, too low to be detected by reverse transcriptase activity. Viral transmission may be either through cell-to-cell contact or via cell-free virus. In contrast, Kim and colleagues used virus derived from lymphocytes and adjusted the level of the virus inocula based on measurable reverse transcriptase activity; additionally, there are several amino acid
differences between our nef allele and that of Kim et al, and it is interesting to note that the Nef proteins differed with respect to the mobilities on SDS PAGE.

b) To characterize Nef responsive sequences

Stimulation of T cells by T-cell-specific stimuli (e.g. antigen and antibody to CD2 or CD3) or nonspecific mitogens (e.g. phytohemagglutinin [PHA] and phorbol 12-myristate 13-acetate [PMA]) results in the induction of the DNA binding activity of the host transcription factor, NF-kB. The NF-kB family of proteins normally regulates the expression of genes involved in T-cell activation and proliferation, such as interleukin 2 (IL-2) and the alpha subunit of the IL-2 receptor. The human immunodeficiency virus type 1 (HIV-1) promoter possesses two adjacent NF-kB-binding sites, which allows the virus to subvert the normal activity of NF-kB to enhance its own replication.

Previous work suggests that the HIV-1-encoded Nef protein serves as a negative regulator of HIV-1 replication. Furthermore, we and others have found that Nef may suppress both HIV-1 and IL-2 transcription. To investigate whether Nef affects the DNA binding activity of NF-kB or other transcription factors implicated in HIV-1 regulation, we used the human T-cell lines stably transfected with the nef gene. Jurkat (J25) human T-cell clone 133 constitutively expresses the NL4-3 nef gene. 22F6 cells represent another antibiotic-resistant clone of J24 cells; however, these cells do not contain nef sequences and do not express Nef. Additionally, we used oligoclonal Jurkat E6-1 and HPB-ALL cells expressing the SF2 nef gene either in the correct orientation (Jurkat/LenfSN and HPB-ALL/LenfSN1 cells) or in the reverse orientation (Jurkat/LenfSN and HPB-ALL/LenfSN cells) with respect to the Moloney murine leukemia virus promoter. These cells represent a mixed population of cells expressing Nef to various degrees and were used to exclude the possibility that clonal selection accounts for Nef effects observed in the J25 clones.

To determine the impact of T-cell activation on the expression of Nef, the human T-cell lines were stimulated with PMA and PMA. Cells were maintained in logarithmic growth in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM glutamine. J25 and Jurkat E6-1 cells (5 x 10⁶) and HPB-ALL cells (1.5 x 10⁶) were either not stimulated or stimulated with 13 μg of PHA-P (Sigma) and 75 ng of PMA (Sigma) per ml for 4 h. The cells were lysed in RIPA buffer, and lysates were immunoprecipitated with rabbit polyclonal anti-Nef sera. The immunoprecipitates were subjected to SDS-PAGE (12% polyacrylamide), and the proteins were transferred to nitrocellulose for Western blot analysis. The primary antibody was the rabbit anti-Nef serum, and the secondary antibody was alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin, specific for the heavy chain (Promega). The proteins were visualized by color development with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate toluidinium (Promega). Band intensity was determined by laser densitometry scanning of the Western blot and was in the linear range of analysis as established by a standard curve. Jurkat E6-1 cells were obtained from the AIDS Repository, American Type Culture Collection (Arthur Weiss), and were stably transduced with the SF2 nef gene as previously described.
Immunoblot analysis with anti-Nef antibodies showed that stimulation caused a two- to threefold increase in Nef expression in clone 133 cells (Fig. 14). This increase was probably due to the inducibility of the chimeric simian virus 40 (SV40)-human T-cell leukemia virus type I promoter used to direct Nef expression. However, Nef expression was not induced in the Jurkat E6-1 or HPB-ALL cells (Fig. 14). The level of Nef expressed in these cells is comparable to the amount of Nef generated by HIV-1 in productively infected CEM human T cells (data not shown). The difference in the apparent molecular weight of the Nef produced in clone 133 cells and those produced in the Jurkat/INefSN and HPB-ALL/INefSN1 cells is due to the presence of an alanine at amino acid position 54 in the NLA-3 nef gene compared with the presence of an aspartic acid at that position in the SF2 nef gene. The amount of lysate equivalents loaded in the HPB-ALL/INefSN1 lanes was threefold higher than that of the Jurkat/INefSN lanes. Nevertheless, the amount of Nef expressed in the HPB-ALL/INefSN1 cells was approximately fourfold higher than the amount produced in the Jurkat/INefSN cells (Fig. 14). Nef did not appear to be toxic in that Nef-producing cells exhibited the same doubling time and morphology as the control cells.

Gel shift assays were performed with nuclear extracts prepared from stimulated and unstimulated cells. Nuclear extracts were prepared from 5 x 10⁷ cells with a modified version of the method of Dignam and colleagues as adapted by Montminy and Bilezikjian. Following ammonium sulfate precipitation, nuclear proteins were resuspended in a 100 ul solution containing 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.9), 20 mM KCl, 1 mM MgCl₂, 2 mM DTT, and 17% glycerol with the addition of 10 mM NaF, 0.1 mM sodium vanadate, and 50 mM beta-glycerophosphate. Cytoplasmic extracts consisted of the supernatant resulting from the lysis of cells in hypotonic lysis solution, douncing, and low-speed centrifugation to pellet nuclei. Binding reaction mixtures contained 2 ul (2 ug) of nuclear protein.
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Fig. 15

FIG. – Gel shift analysis of NF-κB activity in nuclear extracts prepared from IZ5 (a and b), Jurkat E6-1 (c), or HPB-ALL (d) cells. (a) IZ5 and Jurkat E6-1 cells were stimulated with PMA (13 ng/ml) and PHA (75 ng/ml) for 0, 40, 80, 120, or 240 min. 217S and 230S, Jurkat E6-1 (c), or HPB-ALL (d) cells were not stimulated (0) or were stimulated with PHA and PMA as described above for 4 h (b). DNA probes used for binding are specified on the top of each panel. (c) Cytoplasmic protein extract (17 μg each) from the indicated cells were incubated with the NF-κB DNA probe as described in the text, in the presence of 0.6% denaturing acid (Sigma). N, S, and P, NF-κB-specific binding. S′, Y-specific binding, and free probe, respectively. SP-1 binding served as a control for extract quality and specificity of NF effects. C indicates that 100-fold molar excess of unlabeled DNA was added for competition. ns, unspecific binding. Data represent at least three independent experiments.
extract (Fig. 15a through d) or 6 ul (7 ug) of cytoplasmic extract (Fig. 15e), 2 ug of poly(dI-dC) (Pharmacia), 100-fold molar excess of unlabeled NF-κB mutant oligonucleotide (ACACTCTTTCCCTGCTTCCTTGGGAGGAGAG), and 20,000 cpm of end-labeled oligonucleotide probe in DNA binding buffer in a final volume of 22 ul. Reactions were performed at 30°C for 25 min, immediately loaded on a 4.5% polyacrylamide gel with 0.5 X Tris-borate-EDTA, and run at 200 V. Oligonucleotides used were as follows: NF-κB, ACAAGGCAGCCGCGCTGGGCTTGGCAG; SP-1, CAGGAGCGGAGCCCTGGGGGGGGCTTGGGCTTGGCAG. All DNA probes were gel purified and end labeled with gamma-32P-ATP. The intensity of the indicated bands was determined by laser densitometry and by measuring the radioactivity of excised bands in a liquid scintillation counter. There was a linear relationship between the amount of extract used and DNA-binding activity (data not shown). There was no NF-κB DNA binding activity with the cytoplasmic extracts in the absence of deoxycholic acid (data not shown). Protein concentration was determined with the Bradford reagent (Biorad) with bovine serum albumin as a standard. Nuclear extract preparations and binding reactions were repeated on three separate occasions with similar results.

The induction of NF-κB activity in stimulated 133 cells was suppressed 5-7-fold compared with that of the 22F6 cells. This inhibition was evident 40 min poststimulation and was sustained throughout the 4 h stimulation period (Fig. 15a). J25 clone 22D8 cells represent a distinct clonal cell line, which, like the 133 cells, also stably express Nef. NF-κB induction was suppressed 4-5-fold in the 22D8 cells compared with that of the 22F6 cells (Fig. 15b). NF-κB suppression was more profound in the 133 cells than in the 22D8 cells, which correlates with the observation that Nef expression was higher in the 133 cells. Similar to the Nef-expressing J25 clones, Nef inhibited NF-κB induction 3-4-fold in the Jurkat/NefSN and HPB-ALL/NefSN1 cells compared with their non-Nef-expressing counterparts (Fig. 15c and d). Nef-mediated NF-κB suppression was more profound in the Jurkat/NefSN cells than in the HPB-ALL/NefSN1 cells, even though the HPB-ALL/NefSN1 cells expressed severalfold higher levels of Nef. This result is likely due to the biological differences that exist between the two cell lines. That is, Jurkat cells may be more sensitive to the effects of Nef than HPB-ALL cells because of differential expression of proteins involved in signal transduction. That Nef-mediated NF-κB suppression in the 133 and 22D8 cells was greater than in the Jurkat/NefSN and HPB-ALL/NefSN1 cells may be due to the expression of a different nef allele in the 133 and 22D8 cells. Alternatively, this result could be due to the fact that every cell in the culture of 133 and 22D8 cells produced a relatively high level of Nef, whereas the Jurkat/NefSN and HPB-ALL/NefSN1 cells represent a mixed population of cells expressing low and high levels of Nef or no Nef at all.

NF-κB activity in nuclei from unstimulated cells was extremely low but detectable, and no differences between the Nef-expressing and control cells were observed (data not shown). Additionally, when cytoplasmic extracts from unstimulated cells were treated with deoxycholic acid (which releases NF-κB from its cytoplasmic inhibitor IκB), they exhibited NF-κB activity independent of Nef expression (Fig. 15e). Finally, that Nef suppressed the level of NF-κB induction after only 40 min of stimulation suggests that Nef does not suppress p10 or p65 NF--
kb mRNA expression. These observations indicate that Nef affects the recruitment and not the cytoplasmic concentration of NF-κB. The binding of SP-1 was independent of Nef expression ad stimulation, and the amount of SP-1 probe used in these gel shift assays was not limited (Fig. 15a through e). In addition, no differences in binding to NF-AT-, USF-, and URS-specific probes between 22F6 and 133 cells were observed (data not shown). These data suggest that Nef specifically inhibited the induction of NF-κB activity.
To further demonstrate Nef's suppressive effect on NF-κB recruitment, 22F6 cells were transiently transfected with DNA plasmids expressing Nef from the SV40 early promoter, pSVF/N, or the cytomegalovirus immediate-early promoter, pCMVF/N, or with plasmids containing frameshift mutations in the nef gene (pSVF/N and pCMVF/Nfs, respectively). Nuclear extract preparation and DNA binding reactions were as described above. 22F6 cells (2 x 10⁶) (Fig. 16a) were transfected with 30 μg of the indicated plasmid DNA by using DEAE-dextran. Briefly, cells (10⁶) were incubated with plasmid DNA suspended in a solution containing 10 ml of serum-free RPMI 1640, 0.25 M Tris, pH 7.3, and 125 μg of DEAE-dextran (Sigma) per ml at 37°C for 40 min. Following centrifugation at 2,000 x g for 7 min., the cells were maintained in growth medium for 60 h prior to stimulation and cell harvesting.

Plasmid pSVF/N is similar to plasmid pSVF, except that HIV-1 nucleotides 8994–9213 (indicating the NF-κB recognition sites) and 3' flanking cellular sequences were deleted. Plasmid pSVF/N was digested at the unique Bgl II site at codon 88 of the nef gene, the sticky ends were filled in with the Klenow fragment of DNA polymerase I, and the plasmid was religated with T4 DNA ligase. This plasmid was called pSVF/Ns to indicate the introduction of a frameshift in the nef gene. The Bam HI fragment from pSVF/N and pSVF/Nfs, which includes the entire length of the HIV-1 sequences present in these clones was inserted into the vector pCB6 in the correct and incorrect orientation with respect to the cytomegalovirus immediate-early promoter to generate pCMVF/N and pCMVF/Nfs, respectively. Cells transfected with plasmids pSVF/N and pCMVF/N express Nef protein, but cells transfected with pSVF/Nfs and pCMVF/Nfs do not, as determined by Western blot and immunoprecipitation analysis (data not shown). Transfection efficiency was determined by cotransfection with 2 μg of pSV2-CAT. Chlamydomoncas acetyltransferase (CAT) activity (reported as the percent conversion to acetylated products) was determined as described below, and the values for the pSVF/N fs-0-, pSVF/N fs-4-, pSVF/N-4-, pCMVF/N fs-4-, and pCMVF/N-4-transfected cells were 51, 60, 61, 58, and 61%, respectively. A USF-specific DNA probe (corresponding to nucleotides -159 to -173 of the HIV-1 long terminal repeat, CGCGCTGACATTGTCAGGAGGACGC) was used as a control for the specificity of Nef effects and extract integrity. L-α-lysine (Sigma) (Fig. 16b) was used at a concentration of 2 μM. Anti-p50, anti-p65, anti-v-rel and prebleed sera (Fig. 16c) were kindly provided by Mark Hannik (University of Missouri, Columbia, Mo). Because the gels in Fig. 16a and b and Fig. 15 were run for a shorter length of time, the two bands indicated in Fig. 16c appear as one band in Fig. 16a and b, and Fig. 15.

NF-κB induction was consistently inhibited at least two-fold in cells transfected with either pSVF/N or pCMVF/N compared with cells transfected with their nef mutant counterparts (Fig. 16a). Transfection efficiencies in these experiments were determined by cotransfecting cells with the pSV2-CAT plasmid and measuring CAT activity. No significant differences in transfection efficiency between the nef-expressing and the nef mutant plasmids were observed (Fig. 16a). The suppressive effect of Nef in these transiently transfected cells was not as dramatic as the effects observed in the stably transfected and transduced cells. The more subtle effect of Nef in this experiment may be due to the expression of a nef allele which was derived from an HIV-1 isolate distinct from either the NL43 or the SF2 isolates.
In addition, cells which did not receive the nef expression plasmid during the transient-transfection process were not eliminated (by antibiotic selection) from the total cell population.

To explore the relative contributions of individual mitogens to the recruitment of Nef-inhibitable complexes, cells were stimulated with either PHA, PMA, or ionomycin alone, or in combination. The maximal induction of NF-κB activity occurred when PHA was combined with PMA (Fig. 16b). This result coupled with the observation that PHA mimics the effects of the natural ligand for the T-cell receptor (TCR) complex suggests that Nef may inhibit signal transduction emanating from the TCR complex. The addition of the Ca²⁺ ionophore, ionomycin, when coupled with PMA treatment partially substituted for the absence of PHA with respect to NF-κB induction (Fig. 15b). However, ionomycin treatment did not significantly reduce Nef's inhibitory effects, suggesting that events other than Ca²⁺ mobilization may be disrupted by Nef. Using antibodies against the p50 and p65 NF-κB subunits, we found that Nef-inhibitable complexes included both p50-p50 homodimers and p50-p65 heterodimers (Fig. 16c).

To determine whether Nef-mediated inhibition of NF-κB binding activity correlated with a decrease in transcriptional activity, cells were transfected with DNA plasmids by using the HIV-1 long terminal repeat to direct expression of a heterologous gene product, T. Jurkat cells were transfected, and...
described above, with 15 ug of the CAT constructs indicated in Fig. 17. Following tranfection, the cells were maintained in growth medium for 24 h. Cells were or were not treated with PHA-P (13 ug/ml) and PMA (75 ng/ml) and incubated for an additional 18 h. Cell extracts were prepared, and CAT activity was assessed by standard methods. Extract equivalent to 3 x 10^6 cells was used for each 18 h reaction. CAT activity was in the linear range of analysis with respect to extract amount and incubation time (data not shown). CAT assays were normalized to a noninducible control plasmid, RSV-CAT (2 ug), which was transfected in parallel with the HIV-1-CAT plasmids as described above. Assays were also normalized to protein concentrations as determined by Bradford reagent analysis (Bio-Rad). The amount of CAT activity was quantitated by excising the spots corresponding to the unacetylated and acetylated forms of 14C-chloramphenicol and measuring radioactivity in a liquid scintillation counter. CAT activity is expressed as the percent of radioactivity in the acetylated forms compared with the sum of that of the acetylated and unacetylated forms. The wild-type HIV-1-CAT (CD12-CAT) was derived by a small deletion in the nef coding sequence upstream of the long terminal repeat start site of clone C15-CAT), mutant NF-kB HIV-CAT and IL-2-CAT plasmids were generously provided by Steven Joesphs, Gary Nabel, and Gerald Crabtree, respectively. DeltaNRF-HIV-1-CAT was generated by excising the Ava I-Ava I fragment from HIV-1-CAT and therefore lacks the negative regulatory element sequences present in HIV-1-CAT.

CAT activity correlated well with DNA-binding activity in that 133 cells exhibited a capacity to induce CAT activity that was fivefold less than that of 22F6 cells (Fig. 17a). Similarly, CAT activity induction was suppressed twofold in the Jurkat/I nefSN cells compared with that in the Jurkat/I nefSN cells (Fig. 17b). This inhibition was demonstrated with both wild type HIV-1-CAT and the negative regulatory element deletion clone, deltaNRF-HIV-1-CAT, which lacks nucleotides -453 to -156 of the HIV-1 long terminal repeat (Fig. 17a and b). This result suggests that negative regulatory element sequences are not primary targets of Nef regulation in stimulated T cells. An HIV-1-CAT plasmid containing mutated NF-kB sequences was induced, at most, only twofold above basal levels, and induction was independent of cell type and Nef expression (Fig. 17a and b).

The importance of NF-kB with respect to the induction of IL-2 by T-cell mitogens was demonstrated by Hoyos and colleagues. These authors showed that the induction of CAT activity was prevented up to 80% with IL-2-CAT constructs bearing mutations in the NF-kB site compared with that of IL-2-CAT constructs containing wild-type NF-kB recognition sequences. As previously reported, we found that Nef profoundly suppressed the induction of CAT activity directed by the IL-2-CAT plasmid in the 133 cells (Fig. 17a). Where there was a 50- to 60-fold induction of CAT activity in the 22F6 cells, there was only a 2- to 3-fold induction in the 133 cells (Fig. 17a). Although NF-kB appears to play an important role in IL-2 induction, it is possible that Nef blocks other factors in addition to NF-kB which may be required for the efficient induction of IL-2 gene expression. This possibility may explain the dramatic suppressive effect of Nef on IL-2 induction compared with the results of Hoyos and colleagues. CAT activity generated by the IL-2-CAT construct was induced to a much lower extent in the Jurkat E6-1 cells. This result is likely due
to differences that exist between Jurkat B6-1 and J25 cells. Despite the low level of induction of the IL-2 promoter in the Jurkat B6-1 cells, CAT activity was higher in the Jurkat/LfenSN cells than in the Jurkat/LnefSN cells (Fig. 17b). Nef did not affect CAT activity driven by the SV40 early promoter or the promoters from Rous sarcoma virus, the cytomegalovirus, or the Mason-Pfizer monkey virus, indicating that Nef specifically suppressed the HIV-1 and IL-2 promoters (data not shown). The Jurkat B6-1 cells were transfected with equivalent efficiency; however, the Nef-expressing 131 cells were more easily transfected than were the control cells (22F6 cells). Therefore, CAT activity generated by an RSV-CAT plasmid that was transfected in parallel was used to assess the transfection efficiency and to normalize the CAT activity derived from the HIV-1-CAT and IL-2-CAT constructs.

The observation that Nef prevents IL-2 induction (Fig. 16a), coupled with the demonstrations that IL-2 induction requires CD4 and p56Ik and NF-kB recruitment, provides additional evidence to suggest that Nef uncouples signals originating from the TCR. Furthermore, the TCR complex induces NF-kB activity after treatment with antibodies to either CD2 or CD3. Nef inhibits the induction of IL-2 by both of these stimuli.

Interestingly, Nef has been reported to down-modulate the surface expression of CD4. Although Nef did not affect the rate of CD4 transcription or translation, the mechanism by which Nef mediates the down-modulation of CD4 at the cell surface remains unclear. The connection between Nef-mediated negative effects on CD4 cell surface expression and HIV-1 and IL-2 regulation has not yet been established.

Previously, we and others reported that HIV-1 Nef mediated HIV-1 transcriptional suppression. Some investigators were unable to confirm this effect; however, differences in experimental approaches may explain the apparent discrepancy. For the first time, the data presented here suggest that the primary underlying event in Nef-mediated transcriptional repression in activated T cells is the inhibition of induction of NF-kB activity. In vivo, this suppression may limit the production and cell surface expression of viral gene products in infected cells, thereby allowing the cells to evade clearance by the cellular and humoral arms of the immune response. This model for Nef-mediated viral persistence in vivo may be consistent with the results of Kestler and colleagues which demonstrated that the presence of an intact nef gene was required to prolong SIV infection and induce pathogenesis in infected macaques. Furthermore, we and others demonstrated that SIV Nef inhibited SIV replication in vitro in a way that was analogous to the way in which HIV-1 Nef inhibited HIV-1. It is possible that high-level Nef expression early after infection is sufficient to maintain HIV-1 in a relatively latent state, which may be critical for establishing a reservoir of HIV-1 infected cells and the eventual development of AIDS.

We also explored gel retarded complexes using a 32P-oligonucleotide corresponding to the HIV-1 AP-1 DNA recognition site. In the parental 22F6 Jurkat cells, an induced AP-1/DNA complex which was not present in unstimulated cells, was detected between 1 and 2 hr poststimulation and was abundant 4 hrs post-stimulation.
In contrast, the recruitment of the same AP-1/DNA complex was inhibited 5-fold at 2 hr and 9-fold at 4 hr in the Nef expressing 133 cells compared to the 22F6 cells (Fig. 18). Addition of 100-fold molar excess of unlabeled AP-1 specific oligonucleotide inhibited the appearance of the major inducible complex (Fig. 18). However, an oligonucleotide with three nucleotide substitutions in the AP-1 recognition site did not compete away the inducible complex, and we included a 100-fold excess of the unlabeled mutant AP-1 oligonucleotide in all binding reactions as a non-specific inhibitor.

The presence of the constitutive AP-1/DNA complex (the slowest migrating complex in Fig. 18) was minimally if at all, affected by Nef and may be due to the constant presence of serum in the cell growth media. Moreover, this complex was not inducible (Fig. 20b). In addition, the constitutively active transcription factor SP-1 was not affected by the presence or absence of Nef, and was used as a control for extract quality (Fig. 18). Therefore, Nef inhibited the inducible AP-1/DNA complexes, specifically.

Gel shift analysis with extracts prepared from stimulated and unstimulated HPB-ALL cells afforded results similar to those obtained with the Jurkat cells (Fig. 19). However, in contrast to the Jurkat cells, the mitogen-inducible AP-1/DNA complexes were present in unstimulated cells as well as the stimulated cells. Whereas the stimulatable AP-1/DNA complexes in the HPB-ALL/LfensN cells were induced
approximately 5-fold after 4 hrs of PHA and PMA treatment, there was no significant induction of these complexes in the HPB-ALL/LenSNS1 cells (Fig. 19). In this experiment, the amount of AP-1 activity in the unstimulated HPB-ALL/LenSNS1 cells was lower than the HPB-ALL/LenSNS1 cells, however, this was not a consistent finding. In this experiment, we included another Nef non-responsive transcription factor, USF to demonstrate the specificity of Nef action and the integrity of the extract. The apparent difference in migration of the major inducible AP-1/DNA complexes between the HPB-ALL and the Jurkat cells probably reflects differences that exist between the different T-cell lines.

Previous studies indicated that c-fos expression is induced by PHA, the calcium ionophore A23187, and PMA. In order to determine the signalling pathway required to induce AP-1 DNA binding activity, we assessed the role of PHA, PMA, and the calcium ionophore, ionomycin, alone or in combination (Fig. 20a). Interestingly, the induction of AP-1 activity was maximal with PHA treatment alone and addition of PMA did not significantly increase AP-1/DNA complex formation. The level of inducible AP-1 activity was 18-fold higher in the 22F6 cells compared to the Nef expressing 133 cells with PHA alone (Fig. 20a). PMA alone only slightly induced AP-1 activity in the 22F6 cells, however, no detectable AP-1 activity was observed in the 133 cells treated with PMA alone (Fig. 20a). Ionomycin alone was not sufficient
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Basic

Fig. 20.

T-cell activation is mediated by increased Ca²⁺ influx and PKC activation which both occur as a consequence of phospholipase-C activation by the T-cell receptor (TCR) complex. Treatment of the Jurkat cells with a combination of ionomycin and PMA, which both bypass the TCR complex, led to significant induction of AP-1 activity, albeit 2.5-fold less efficiently than PHA alone. Whereas there was an 18-fold higher level of induced AP-1 DNA-binding activity in the 22F6 cells compared to the 133 cells using PHA alone, there was only a 3-fold difference using the combination of ionomycin and PMA. Since PHA mimics the normal activation signal
(i.e. antigen binding to TCR) of T cells, it appeared that Nef exerted its effects primarily (although not exclusively) on TCR initiated signalling, as has been suggested previously.

To determine whether the induction of AP-1 activity required the activation of pre-existing complexes or new protein synthesis, cycloheximide was added 30 min. before mitogen treatment. That cycloheximide treatment inhibited the recruitment of AP-1 activity, suggests that de novo protein synthesis must be involved (Fig. 20b). This result was consistent with the observation that two hours of stimulation were required before significant induction of AP-1 DNA binding activity (Fig. 18).

To identify the polypeptides present in the inducible AP-1 complex, we incubated nuclear extracts derived from the 22F6 cells with anti-c-Fos and anti-c-Jun antibodies, prior to the addition of labeled oligonucleotides (Fig. 20c). Antisera to both c-Fos and c-Jun inhibited complex formation approximately 3-fold, suggesting the presence of c-Fos and c-Jun in the complex. However, these antibodies did not cause a super-shift, presumably because antibody binding to c-Fos and c-Jun caused conformational changes which are not permissive for DNA binding activity. In these experiments, normal rabbit serum and anti-Nef antibodies were used as negative controls.

We will also perform Northern blot assays to confirm that the effects of Nef on AP-1 induction occur at the level of transcription of c-jun and c-fos.

Previous studies indicated that the binding of AP-1 to the HIV-1-LTR AP-1 recognition sites play little, if any, role in affecting transcriptional activity. Zeichner and coworkers generated several HIV-1-LTR-CAT linker-scanning mutants in the region of the AP-1 recognition sites and transfected the mutant plasmids into Jurkat cells. There were no significant differences in CAT activity between the wild-type HIV-1-LTR-CAT plasmid and the AP-1 mutant plasmids in cells that were or were not stimulated with PHA and PMA.

However, the intragenic AP-1 recognition sites were capable of mediating transcriptional activation following phorbol ester treatment. Therefore, we cloned a synthetic oligonucleotide, corresponding to the two adjacent AP-1 sites with the pol gene, or an oligonucleotide which contained three nucleotide substitutions in these AP-1 consensus sites, into the polylinker of the enhancer-less pCAT promoter plasmid (Promega). These plasmids were called pCAT-IG-AP1 and pCAT-MIG-AP1, respectively. The pCAT promoter construct, in the absence of the AP-1 sites, contains the SV40 core promoter, afforded low basal chloramphenicol acetyl transferase (CAT) activity in T-cells, and was not inducible in T-cells following treatment with T-cell mitogens (data not shown).

The pCAT-IG-AP1 and pCAT-MIG-AP1 constructs were transiently transfected into the Jurkat 22F6 and 133 cells, as well as Jurkat 25 clone 22D6 cells. The 22D6 cells represent a distinct clonal cell line which, like the 133 cells, also stably express the nef gene from HIV-1 isolate NL43. Transiently transfected cells were either not stimulated or were stimulated with PHA and PMA for 18 hr and CAT activity
was then measured. CAT activity in transfected cells was relatively low, between 1-3% conversion to acetylated products. However, we found an average fold induction in CAT activity of 3.6 ± 0.4 in the 22F6 cells transfected with the pCAT-IG-AP1 plasmid, compared to an average fold induction of 1.4 ± 0.2 in the 133 cells and no induction in the 22D8 cells (Fig. 21). Transfection efficiencies were higher in the
Nef expressing cells and were determined by parallel transfection with non-Nef responsive promoters including Rous sarcoma virus-CAT, cytomegalovirus-CAT, and simian polyoma virus 40-CAT (data not shown). These determinations were statistically significant, with 95% confidence intervals, with respect to fold-induction, of 2.8-4.4 for the 22F6 cells and the 133 and 22D8 cells is 1 in 1000. CAT activity was not induced in cells transfected with the pCMV-MIG-AP1 construct, indicating that the integrity of the AP-1 site in the inserted oligonucleotide was essential. Thus, Nef-mediated inhibition of AP-1 DNA-binding activity prevented AP-1-mediated transcriptional activation.

What role AP-1 plays with respect to HIV-1 regulation is unclear. Nef could inhibit AP-1-mediated activation of HIV-1 directly, by preventing the interaction of AP-1 with the intragenic enhancer in the pol gene. In addition, by inhibiting AP-1 induction during T-cell activation, Nef may affect the regulation of AP-1 activated cellular genes. Effects on such cellular genes may alter the cellular environment, positively or negatively, which may indirectly affect HIV-1 replication. For example, the finding that c-Fos and c-Jun are early response mediators of T-cell activation, coupled with the observation that HIV-1 cannot replicate in resting, unactivated T-cells, presents a scenario for indirect effects of Nef on HIV-1 expression.

In addition to mediating the suppression of AP-1 induction, we found that Nef also inhibited the mitogen-mediated induction of NF-kB. NF-kB, like AP-1, is an early response effector of T-cell activation and has been shown to be an important activation of HIV-1 replication in stimulated T-cells. Thus, Nef mediated inhibition of recruitment of both AP-1 and NF-kB may intensify the negative effects on HIV-1 replication in T-cells. By inhibiting virus replication directly, and/or by blocking T-cell activation, Nef may provide a reservoir of persistently infected cells which may ultimately contribute to HIV-1 clinical latency, HIV-1 mediated T-cell depletion, and AIDS.

The mechanism underlying Nef's negative transcriptional effects on viral transcription may be its ability to inhibit NF-kB activity in T-cells and NF-kB-like activity in non-lymphoid cells, such as COS cells. This conclusion is based upon results obtained from Jurkat and COS cells transfected with HIV-1-LTR CAT constructs containing mutations in both of the NF-kB sites. Basal CAT activity in these cells was significantly lower compared to cells transfected with constructs bearing intact NF-kB sites. The effect of Nef on the NF-kB-like factors present in COS cells may be identified by incubating nuclear extracts from Nef-expressing and parental COS cells with an NF-kB oligonucleotide probe. In these experiments, we will cotransfect a plasmid expressing a chimeric version of NF-kB fusing the p65 and p50 subunits. It is expected that this assay system will have a low background, and thus will be amenable to a transient transfection assay for Nef. This will provide a useful system for analyzing the effect of various mutations in Nef.
Activity of Nef

extracellular space

cytoplasm

nucleus

Antigen Presenting Cell

Nef

HIV (+)

IL-2 (+)

NF-κB

IκB

MHC II

CD4

TCR

CD3

fyn

Ick

G

jun fos

(-)

(-)

(-)

P.I.: Lee Ratner, M.D., Ph.D.

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Basic
That Nef inhibits the recruitment of NF-κB in response to the T-cell mitogen, PHA, which acts presumably through the T-cell receptor (TCR) complex, coupled with the results of Luria and colleagues, which indicate that Nef inhibits IL-2 mRNA induction, following treatment with anti-TCR and anti-CD3 antibodies suggests that Nef inhibits signals emanating from the TCR complex. However, since Nef also inhibits, although to a less extent, NF-κB and AP-1 induction by PMA together with ionomycin, which bypasses the TCR, Nef may also affect signalling that occurs downstream of the TCR complex or through alternative pathways (Fig. 22).

To further support the notion that Nef suppresses NF-κB induction primarily by disrupting TCR derived signals, we will determine whether Nef blocks NF-κB induction mediated by anti-TCR and anti-CD3 antibodies. To assess whether Nef disturbs other pathways leading to NF-κB activation, will assess Nef's ability to inhibit NF-κB recruitment by the Tax protein of HTLV-1, which binds directly to the NF-κB precursor, and by tumor necrosis factor alpha (TNF-alpha). We will also determine whether Nef affects the state of phosphorylation of the cytoplasmic inhibitor of NF-κB, IKK. It is postulated that phosphorylation of IKK causes the release of the p65 subunit of NF-κB, allowing NF-κB to migrate to the nucleus.

That NF-κB induction occurs within 30 min. following stimulation, whereas, significant induction of AP-1 requires a period of approximately 2 hrs, suggests that NF-κB may be responsible for activating c-fos and c-jun expression. If this is true, then Nef mediated inhibition of NF-κB induction underlies the inhibition of AP-1. In addition to the temporal activation of NF-κB and AP-1, results from other experiments support this relationship between NF-κB and AP-1. For example, the Tax protein of HTLV-1 activates NF-κB activity by directly binding to the NF-κB precursor. Tax expressing cells also exhibit elevated levels of c-fos expression. In other experiments, treatment of T-cells with the protein synthesis inhibitor, cycloheximide, enhances NF-κB translocation to the nucleus, presumably by inhibiting the synthesis of IKK or another negative regulatory co-factor. Cycloheximide treatment of T-cells also sharply increases the level of c-fos mRNA. Furthermore, NF-κB induction does not require new protein synthesis, whereas treatment of cells with cycloheximide does inhibit AP-1 induction. This shows that de novo protein synthesis is required for AP-1 induction and that a cycloheximide-insensitive transcription factor is involved in that induction. Additionally, it was recently reported that protein kinase C (PKC) activity is required for efficient induction of both NF-κB and AP-1 in T-cells. This finding is consistent with the notion that the activation of both factors occurs through the same pathway.

Finally, there is an NF-κB recognition sequence in the c-fos gene, and it is located approximately 150 nucleotides upstream of the RNA initiation site. Therefore, in order to test the hypothesis that NF-κB is directly responsible for AP-1 induction in T-cells, one could mutate the NF-κB sequences in the c-fos promoter by site-directed mutagenesis. Both wild-type and mutated promoter sequences could then be cloned upstream of a convenient reporter gene, such as the CAT gene. One could then compare inducible CAT activity in T-cells transfected with either the wild-type of NF-κB mutant construct.
c) To characterize the mechanism of transcriptional suppression by Nef

i) Gel retardation experiments

These experiments are discussed in depth in the preceding section. In addition, to the experiments to be performed with COS cell nuclear extracts, we will also perform assays with a variety of different NF-κB binding sites, in light of recent data of significant differences in the binding ability of different related proteins to each oligonucleotide sequence.

ii) DNA footprinting studies

DNA footprinting studies are planned to precisely map the binding sites affected by Nef.

iii) In vitro transcription studies

We will initiate studies using in vitro transcription to study the mechanism of Nef effects on NF-κB and AP-1.

d) To determine the role of phosphorylation, GTP binding, GTPase activity, and myristoylation acceptor activity on Nef activity

We have constructed a myristoylation acceptor mutant of Nef in a full proviral clone and a Nef expression vector. Studies are underway with this mutant, to examine its effect on virus replication in lymphoid cell lines and primary lymphocytes.

We have also expressed parental and myristoylated acceptor mutant of Nef in E. coli, co-transfected with the yeast N-myristoyl transferase gene. We have established binding assays with lymphoid membranes and cytoskeleton and found myristoyl-dependent binding of Nef to cytoskeleton only. No effect of myristoylation was found for binding of gag p55 to cytoskeleton, demonstrating the specificity of the effects on Nef. The binding of myristoylated Nef to cytoskeleton was disrupted by SDS or heat treatment.

The binding of myristoylated Nef to cytoskeleton appears to be saturable, suggesting a definite number of receptors. Competition studies with unlabeled Nef proteins and N-terminal Nef peptides are underway. We are particularly interested in determining whether there is a specific cellular protein that binds Nef. In this light, we will also perform cross-linking experiments with E. coli Nef and lymphoid cytoskeleton to define whether there is a specific cytoskeleton protein that binds Nef. Particular interest will be paid to vinculin, as a candidate binding protein.

Oligonucleotides for the other mutations described in the original contract application have been synthesized. In addition, the C-terminal deletion mutants of Nef have also been constructed.
e) To determine effects of Nef on cellular proteins including those which may modulate HIV-1 infectivity or replication

We have screened several lambda gt11 expression libraries for Nef binding proteins without success. However, we are looking for Nef binding proteins by other techniques. Studies aimed at examining Nef-binding cytoskeletal proteins were described in the previous section.

We are also examining whether Nef binds to or modulates CD4 expression. Though Garcia and Miller have shown that Nef down-regulates the cell-surface expression of CD4, but not the intracellular concentration or processing of CD4, the mechanism of this effect remains to be described. One possibility is that Nef binds directly to the intracellular tail of CD4, causing internalization. Interestingly, the amino acid sequences in the CD4 intracellular tail that are required for binding to myristoylated p56^[1] appear to overlap with the sequences required by Nef to down-modulate cell-surface expression. However, a direct association of Nef and CD4 has yet to be reported. However, our preliminary immunogold electron microscopy experiments in which we simultaneously labeled CD4 and Nef at the cell surface demonstrate co-localization (Fig. 23).

Clearly, an important experiment to determine if a Nef-CD4 complex exists is to co-express high levels of CD4 and Nef and attempt to co-immunoprecipitate a CD4-Nef complex with both anti-CD4 and anti-Nef antibodies. This experiment is currently underway in the laboratory. This approach was successful in demonstrating the existence of a CD4-p56^[1] complex. It is possible that the association of Nef and CD4 is weak, thus, any co-immunoprecipitation experiment would necessitate the use of very mild detergents, such as digitonin or NP-40, to solubilize intact cells. Another approach would be to add cross-linking reagents to a cell-lysate to stabilize a Nef-CD4 complex. Following cross-linking, attempts to co-immunoprecipitate may be more successful. Finally, there is a possibility that Nef binds to a protein which binds directly to CD4, such as p56^[1] or the G-protein associated with CD4. Such a ternary complex may be even more difficult to co-immunoprecipitate due to increased lability and antibody accessibility.

An alternative explanation for Nef mediated down-regulation of CD4 is that Nef affects the architecture at the cell-surface, such that the proper expression of CD4 and the TCR, may be affected. This could possibly occur if Nef affected the cytoskeletal organization of the plasma membrane. As was discussed, Nef appears to associate with cytoskeletal components of both unstimulated and stimulated T-cells.

Finally, Nef could affect the rate of endocytosis of CD4 into intracellular vesicles. The expression of individual CD4 molecules at the cell surface is normally transient because they are repeatedly endocytosed into vesicles and recycled to the surface. Perhaps Nef increases the rate of cycling or prevents the internalized CD4 from returning to the cell-surface.
A final possibility is that Nef causes a post-translational modification, such as phosphorylation to occur on the CD4 intracytoplasmic tail. PKC phosphorylation of three serine residues causes CD4 internalization. Although Nef does not require these three serine residues to mediate down-modulation, it is possible that Nef mediates internalization by causing the phosphorylation of other residues, including a threonine residue which is present in the intracytoplasmic tail.

To help distinguish between the numerous possibilities, we are determining the cellular location of the majority of CD4 molecules in Nef expressing cells. This is being done by immunoelectron microscopy and by subcellular fractionation. Finally, a clue to elucidating the mechanism of CD4 down-modulation, currently underway, is to block the transport of CD4 to the cell-surface, or alternatively, mediate increased internalization. One straightforward experiment to address these two possibilities will be to surface label intact cells with $^{125}$I. Significant labeling of CD4 would favor the internalization model versus the cytoplasmic-retention model. Additionally, the rate of internalization of $^{125}$I labeled CD4 in Nef-expressing versus control cells will be measured. This experiment will lend further support to the internalization model if the results indicate that the rate of CD4 internalization is higher in the Nef-expressing compared to control cells.

f) To determine the role of Nef in HIV-2 and SIV replication

Experiments on the replication of SIVmac102 and SIVmac239 Nef+ and Nef- clones have been completed. Studies of HIV-2 nef are underway.

g) To determine the role of Nef in vivo with animal model systems

The SIVmac239 clones used for our analysis of Nef have already been studied by Desrosiers and colleagues in rhesus macaques, and these investigators demonstrated that Nef was critical for pathogenicity. Our studies in vitro with those virus strains and with the HIV-2 strains should provide important information in explaining this finding. One possibility is that Nef is required for establishment of a state of "latency" in vivo, and that in the absence of Nef, infected cells are rapidly lost due to lysis or immune clearance.
Studies in scid-hu mice with the HIV-1 clones will be performed after the initial studies outlined in 1.i. are completed. We expect that these experiments will be initiated within the next 6 mos.

h) To determine the role of Nef in modulating manifestations of HIV-1 infection

We have used PBMCs from over the full course of disease of individuals described in 1.j. We have obtained the first 20 nef clones and preliminary sequence analysis has been initiated. Interestingly deleted and rearranged forms of nef were commonly found late in disease but not early in disease. We will complete sequence analysis of about 100 clones in the next 6 mos.

We have also used the amplified product to examine possible functional consequences of LTR variation in these same sequences. Five CAT vectors have been constructed and transfection studies in COS and Jurkat cells have been initiated to examine if there are variations in constitutive transcriptional activity of LTRs obtained from early versus late disease stages. We will also examine tat and NF-kB activated transcription and nef mediated suppression.

i) To determine the therapeutic role of Nef in down-regulating HIV-1

Since Nef has been shown to be a pathogenic factor in SIVmac in rhesus macaques, our goal in this regard is altered. Nevertheless, we are in the midst of constructing the retroviral expression clones for Nef that would be useful for a wide range of studies, including down-regulation of HIV-1.
(7) CONCLUSIONS

The studies of Vpr have shown that this protein is critically important for productive infection in monocytes. In addition, Vpu may functionally complement the activity of Vpr. The mechanism of action of Vpr and the domains critical for this activity are under study. In addition, we have preliminary evidence to suggest that Vpr is a nuclear protein, potentially providing a clue to its mechanism of action.

The studies of Vpx have focused on the mechanism of packaging of Vpx into virus particles. This has led to the very interesting observation of an association of Vpx with gag p24. Associations with other gag proteins or RNA can not be excluded by these results, but will be addressed in the upcoming year. This is likely to provide specific ideas about the mechanism of Vpx activity.

The studies of Nef have clearly demonstrated an effect on NF-kB and AP1 activity. The effects are shown to be specific by the lack effect on other transcriptional factors, including SP1, URS, and USF. Moreover, we have found that these effects are functionally important with regards to HIV-1 transcription and IL2 expression. It is likely that a common mediator in signal transduction is affected by Nef.

We have also found that Nef interacts with the cellular cytoskeleton and that this interaction depends upon Nef myristoylation. This may provide specific clues to the way Nef interrupts signal transduction and down-regulates cell surface CD4 expression.

Studies of nef sequences from patients at different stages of disease have shown no major alterations in the gene early during HIV-1 infection, but frequent insertions, deletions, or rearrangements at late stages of disease. This may have important implications concerning the role of Nef in disease progression.
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(9) APPENDIX

Manuscripts and Abstracts
(* indicates new listing during this period, x indicates provided in Appendix)

Cumulative List of Manuscripts Submitted to Peer-Review Journals Supported by this Contract:


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Dual Regulation of Silent and Productive Infection in Monocytes by Distinct Human Immunodeficiency Virus Type 1 Determinants

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The regulation of human immunodeficiency virus type 1 infection and replication in primary monocytes was investigated by mutagenesis of recombinant proviral clones containing an env determinant required for the infectivity of monocytes. Virus replication was assayed by determination of reverse transcriptase activity in culture fluids and by recovery of virus from monocytes following cocultivation with uninfected peripheral blood mononuclear cells. Three virus replication phenotypes were observed in monocytes: productive infection, silent infection, and no infection. Incorporation of the monocytotropic env determinant in a full-length clone incapable of infection or replication in primary monocytes (no infection) conferred the capacity for highly efficient virus replication in monocytes (productive infection). Clones with the env determinant but lacking either functional vpr or vpu genes generated lower replication levels in monocytes. Mutation of both vpr and vpu, however, resulted in nearly complete attenuation of virus replication in monocytes, despite subsequent virus recovery from infected monocytes by cocultivation with uninfected peripheral blood mononuclear cells (silent infection). These findings indicate a central role for the "accessory" genes vpu and vpr in productive human immunodeficiency virus type 1 replication in monocytes and indicate that vpu and vpr may be capable of functional complementation.

Human immunodeficiency virus type 1 (HIV-1) infection of macrophages has been demonstrated in brain, spinal cord, lung, lymph node, and skin during subclinical infection and disease and is postulated to underlie important clinical manifestations of HIV-1 infection, including disease latency and development of a spectrum of AIDS-related central nervous system disorders (2, 4, 11, 14, 15, 19, 33, 37). However, detailed molecular analysis of virus-host cell interactions involving monocytes was limited until recently by the restricted tropism of the earliest and most widely studied HIV-1 genetic clones for primary monocytes cultured in vitro (14, 15). Previously, we and others have demonstrated that a discrete env determinant, including the V3 loop but not the CD4-binding domain, is necessary and sufficient for HIV-1 infection of monocytes (23, 29, 36). Additionally, we have identified three virus replication phenotypes in monocytes in vitro, using molecularly defined proviral clones (35). These include productive infection, with the generation of high virus replication levels; silent infection, with low to undetectable virus replication in monocytes, despite ultimate virus recovery from infected monocytes following cocultivation with uninfected, phytohemagglutinin-stimulated peripheral blood mononuclear cells (PBMCs [lymphoblasts]); and no infection, with neither virus replication in nor virus recovery from monocytes observed. In the present study, we investigated the roles of the HIV-1 "accessory" genes vpr and vpu, which are dispensable for virus replication in primary and immortalized CD4+ T lymphocytes. We demonstrate that vpr and vpu are central to the regulation of virus replication in primary monocytes and together mediate the expression of silent versus productive infection.

To study viral regulation of monocyte infection, we utilized a panel of chimeric HIV-1 clones, constructed from the nonmonocytotropic clone HXB2 and the monocytotropic clone ADA, as previously described (16, 26, 35, 36). To correct a vpr defect in each of these clones, the result of a single base insertion in HXB2, 2.7-kb SalI-BamHI HXADA DNA fragments (nucleotides 5785 to 8474) were subcloned into the full-length proviral clone NL4-3, in which the vpr open reading frame is intact (1). The resultant NLHXADA clones contained the ADA-derived env' determinant previously localized to nucleotides 7040 to 7323, flanked by additional ADA- or HXB2-derived sequences encoding other portions of env and vpu and small portions of tat and rev. A clone in which the entire 5785-to-8474 sequence was HXB2 derived (thus lacking a monocytotropic env' determinant) was used as a negative control for these experiments. Because HXB2 lacks a vpu initiator methionine codon, clones in which vpu was HXB2 derived were defective for that product, in contrast to clones with an ADA-encoded vpu. Finally, a vpr mutant corresponding to each NLHXADA clone was generated by introducing a frameshift mutation at codon 63.

Virions from the recombinant clones, generated by transfection, were assayed for their ability to infect and replicate in primary monocytes by the presence of reverse transcriptase (RT) activity in culture supernatants (25) and by the ultimate recovery of virus following cocultivation of monocytes with uninfected PBMCs. The results are summarized in Fig. 1. All clones containing the ADA-derived env' determinants and an intact vpr gene generated high virus replication levels in monocytes. Inactivation of vpr in these
FIG. 1. Replication of recombinant HIV-1 clones with both wild-type and mutant vpr genes. (A) The panel of recombinant NLHXA clones is represented diagrammatically. The region of the genome corresponding to the HXADA fragments (nucleotides 5785 to 8474) expanded to highlight the relative positions of HXB2- and ADA-derived sequences. The open reading frames in this portion of the genome are represented above. Recombinant clones were generated by reciprocal DNA fragment exchanges of ADA- and HXB2-derived sequences into a SalI-BamHI fragment (5785 to 8474) from HXB2 subcloned into an intermediate shuttle vector, utilizing the restriction enzyme sites indicated on top. The resultant chimeric SalI-BamHI fragments were then subcloned into the clone NL4-3 to gen...
clones, however, generated divergent results, depending upon the derivation of nucleotide sequences 5999 to 6345 (SK fragment). Clones in which this portion of the genome was ADA derived generated lower (but readily detectable) virus replication levels than did their wild-type: vpr counterparts. However, vpr mutants in which SK was HXB2 derived typically failed to generate virus replication levels detectable above background in monocytes, despite subsequent virus recovery from these cultures onto uninfected PBMCs. The negative control clone, which carried a wild-type vpr but lacked the monocytotropic env determinant, generated virions which neither replicated in nor were recovered from monocytes, as previously demonstrated. No significant differences were seen in the replication of each virus strain on PBMCs obtained from several different donors.

Monocytes were infected with recombinant HIV-1 clones containing a functional vpr gene, stained with toluidine blue, and examined by light microscopy (1-μm-thick plastic sections). Cultures infected with a nonmonocytotropic virus, NLHXADA-SK, which contains a functional vpu gene, were indistinguishable from uninfected cells, with rare, small multinucleated cells (Fig. 2A). Cultures productively infected with virus containing the monocytotropic env determinant and a functional (NLHXADA-SM [Fig. 2B]) or nonfunctional (NLHXADA-GG [Fig. 2C]) vpu gene showed characteristic cytopathic effects (15). These consisted of the formation of multinucleated giant cells, often containing 10 or more nuclei per cell, and cell lysis. The frequencies and sizes of these cells were comparable in the NLHXADA-SM- and NLHXADA-GG-infected monocyte cultures. Virus production and cellular degeneration and necrosis were primarily confined to the multinucleated cells. Transmission electron microscopy examination demonstrated typical budding and mature virions in intracellular vacuoles that were associated with the plasma membrane, in both the presence and absence of vpu, but not in the NLHXADA-SK-infected cells (Fig. 2D). Freeze fracture scanning electron microscopy demonstrated budding of virion particles from the plasma membrane of monocytes infected with virus which lacked a functional vpu (Fig. 2E). No virus could be detected in monocytes infected with recombinant clones lacking both vpr and vpu (data not shown).

The SK fragment encodes the entire vpu gene product, 14 amino acids at the C terminus of both the tat and the rev first exons, and the N-terminal 41 amino acids of env (Fig. 3). Although the absence of a vpu initiator methionine codon in HXB2 is the most obvious difference between the SK portions of HXB2 and ADA, a role for tat, rev, or env could not be ruled out. The env sequences differ at 7 of 41 predicted amino acid positions, not including the nonaligned insertion of 3 residues and deletion of 4 residues in ADA. All but three of these differences are confined to the signal peptide, which varies by up to 30% between different clones (20). Furthermore, tat and rev both differ at 3 of 14 amino acid positions between the ADA and HXB2 SK fragments, with four of these six changes being conservative in nature. Therefore, it is unlikely that these alterations in env, tat, or rev alter their function. However, to formally determine the specific requirement for vpu during HIV-1 infection of monocytes, the vpu initiator methionine codon of the silent infection clone NLHXADA-GG (vpr mutant) was restored by site-directed mutagenesis. The resultant clone was found to generate virus capable of productive infection of monocytes (data not shown).

HIV-1 and related lentiviruses are distinct from most other retroviruses in that besides the structural gag, pol, and env genes common to all retroviruses, they also encode a number of genes whose functions have been shown or are speculated to be regulatory in nature. In HIV-1, these genes include tat, rev, vif, nef, vpu, and vpr (6–8, 26, 32, 38). While tat, rev, and vif are essential for viral gene expression or virion infectivity, the precise role and overall importance of vpu, vpr, and nef are unclear, since these genes are dispensable for virus infection and replication in CD4+ lymphocytes in vitro (8–10, 12, 13, 22, 24, 30, 32). The availability of molecular HIV-1 clones which infect and replicate in monocytes at levels comparable to those observed with many monocytotropic virus isolates has facilitated investigation of the role that these viral genes may play in regulating the virus life cycle in monocytes. In the present study, we observed moderately decreased levels of virus replication in the absence of either vpr or vpu, whereas in the absence of both genes, virus replication in monocytes dropped to levels barely at or below the level of detection by the RT assay. Such infection of these cells usually could be detected only by virus rescue onto PBMCs.

The vpr open reading frame encodes a protein of 96 amino acids in most HIV-1 clones and is conserved in other lentiviruses, including visna-maedi virus (20, 31). Previous studies have shown that vpr is not required for HIV-1 infection or replication in CD4+ lymphocytic cell lines in vitro, although its inactivation led to slower replication kinetics and delayed cytopathogenicity in these cells (6, 10, 24). A recent study involving HIV type 2 (HIV-2) has shown that vpr is likewise dispensable during infection of PBMCs and T-cell lines but essential for productive infection of monocytes (17). The vpr protein has been demonstrated by radioimmunoprecipitation to be virion associated, and thus it is speculated to function either late in the virus life cycle, during particle assembly or maturation, or early, during the initial stages of infection (6). The vpu gene encodes an 80-to-82-amino-acid protein. It has not been reported whether the vpu protein is found in virion particles. vpu has been shown to augment virion particle release from infected cells without affecting levels of viral RNA or protein synthesis (8, 32). In the absence of vpu, a higher ratio of immature to mature particles has been seen, with a shift in capsid
FIG. 2. Light, transmission, and freeze fracture scanning electron microscopy of infected monocytes. Light micrographs of toluidine blue-stained semithin plastic sections showing typical fields of primary monocytes infected by nonmonocytotropic clone NLHXADA-SK (35) (A) and monocytotropic clones NLHXADA-SM (B) and NLHXADA-GG (C) are shown (15). The multinucleated giant cells were fewer and smaller in panel A than in panels B and C. Magnification, ×480. Infected adherent cultured cells were carefully washed twice with phosphate-buffered saline (PBS), fixed in situ with 2% glutaraldehyde (pH 7.2) in PBS, scraped free with a rubber policeman, transferred to a 15-ml plastic conical tube, and pelleted for 10 min at 600 × g centrifugation. The cells were mixed with warm agar, repelleted in the Microfuge for 1 min, and refrigerated overnight to form a firm agar block. The cell block was divided into small pieces and processed into Spur's plastic, after osmification and block uranyl acetate staining (15). Sections (1 μm thick) were stained with toluidine blue for light microscopy, while thin sections (600 Å [60.0 nm]) were stained with uranyl acetate and lead citrate for transmission electron microscopy. (D) Transmission electron micrograph of a small portion of a multinucleated cell from NLHXADA-GG-infected monocytes showing a cytoplasmic vacuole (lower left) containing immature and mature virions and numerous typical mature particles associated with a stretch of plasma membrane. Magnification, ×34,000. (E) Transmission electron microscopy view of NLHXADA-GG-infected monocytes, stabilized by formaldehyde fixation before quick-freezing, freeze-drying, and platinum replication (18). Budding from the convoluted surface are several 50-nm-diameter brightly outlined spherical virus particles. At higher magnification (not shown), these display characteristic surface coats of gp120 "pegs."*

formation from the plasma membrane to intracellular membranes (8). In monocytes, however, particle assembly and release occur both at the plasma membrane and in intracellular vacuoles in the presence or absence of vpu, as shown in Fig. 2D.

It is intriguing that HIV-2 and simian immunodeficiency virus lack a vpu open reading frame but instead carry a gene designated vpr, which encodes a protein of 114 to 118 amino acids in these viruses (20). vpu and vpr occupy similar positions in their respective viral genomes, between pol and env, but have only distant amino acid homology. Recently, it has been suggested that vpr and vpu arose by duplication from a common progenitor in HIV-2 and simian immunodeficiency virus, on the basis of predicted amino acid sequence homology between the genes (34). To investigate the possibility of a similar link between vpr and vpu in HIV-1, the predicted amino acid sequences of both vpu and vpr were aligned with that of vpr (Fig. 4). Although less compelling that the homology between vpr and vpu, a 38% identity was observed between vpr and vpu over a 24-residue overlap at the C terminus of vpu and the N terminus of vpr. These sequences were particularly rich in acidic residues. Similarity in the hydrophilicity profiles of these portions of the vpu, vpr, and vpx products was also noted. The striking effect on virus replication levels in monocytes observed only when both genes were defective suggests that their gene products may perform similar roles and thus provide partial functional complementation. Alternatively, since lower replication levels were observed in the absence of either gene, the nearly complete attenuation observed in the absence of both may result from a compound effect of the loss of two relatively important but functionally unrelated genes. More detailed studies to determine the precise mechanisms of action of the vpr and vpu gene products will be required to address these alternatives. In either case, our data indicate that together, vpr and a second determinant, vpu, are more important for efficient HIV-1 infection and replication in primary monocytes than was observed previously in lymphocytes. These observations provide a rationale for designing potential antiviral therapies to block the action of these gene products during HIV-1 infection of monocytes.

Persistent infection of tissue macrophages plays an important role in the pathogenic effects of other lentiviruses, including equine infectious anemia virus, visna-maedi virus, and caprine arthritis-encephalitis virus, providing a sanctuary for continuous virus replication in the face of a vigorous host immune response (15, 21). The onset of increased virus replication has been correlated with the onset of clinical disease manifestations, such as encephalitis, pneumonitis, arthritis, and hemolytic anemia. Similarly, HIV-1 infection of macrophages generates a reservoir of virus which is present throughout the course of subclinical infection and clinical disease. The existence of poorly replicative HIV-1 variants may be essential for establishment of persistent macrophage infection during the early, asymptomatic stage of disease. Several studies have suggested a relationship

![Diagram](image-url)
between the in vitro replicative properties of HIV-1 isolates in T lymphocytes and clinical-disease stage, with earlier isolates tending to replicate more slowly and to lower levels ("slow, low") than isolates from later stages of disease ("rapid, high") (3, 5, 27). Nonessential regulatory genes are ideally suited to act as "molecular switches" for control of replication phenotypes by their activation or inactivation, particularly in viruses such as HIV-1, which characteristically generate high levels of sequence diversity. We demonstrate here that discrete genetic alterations in such accessory genes result in profoundly different replication rates in monocytes in vitro, which suggests a mechanism for transition from subclinical to clinical disease in vivo. These findings thus provide a rationale for addressing on a wider scale whether functional status of vpr and/or vpu correlates with disease stage or serves as a potential prognostic indicator of disease progression and outcome.

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This work was also supported by NIH grant AI 24745, DAMD contract 17-90C-0125, the Washington University Medical Scientist Training Program (P.W.), and training grant 5T32AI07172 (P.W.). Immunodeficiency syndrome patients with myelopathy: expression of Applied Retroviral Research for continued support. Identities are indicated within boxes. Hydrophilicity profiles for the corresponding segments of each protein are shown at the bottom.

FIG. 4. Predicted amino acid homology between vpr, vpu, and vpx. The predicted amino acid sequence of the NL4-3-derived vpr gene is aligned with homologous regions of the ADA-derived vpu gene and the vpx gene encoded by the HIV-2EcoR clone, with single-letter amino acid codings. Identical residues are indicated within boxes. Hydrophilicity profiles for the corresponding segments of each protein are shown at the bottom.

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Human Immunodeficiency Virus Type 1 Nef Protein Inhibits NF-κB Induction in Human T Cells

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Human immunodeficiency virus type 1 (HIV-1) can establish a latent infection in CD4+ T cells (14, 29). Production of HIV-1 from latently infected cells requires host cell stimulation by T-cell mitogens (9, 34). Stimulation of T cells by T-cell-specific stimuli (e.g., antigen or antibody to CD2 or CD3) or nonspecific mitogens (e.g., phytohemagglutinin [PHA] and phorbol 12-myristate 13-acetate [PMA]) results in the induction of the DNA-binding activity of the host transcription factor NF-κB (14). The NF-κB family of proteins normally regulates the expression of genes involved in T-cell activation and proliferation, such as interleukin 2 (IL-2) and the alpha subunit of the IL-2 receptor (14). The HIV-1 promoter possesses two adjacent NF-κB-binding sites which allow the virus to subvert the normal activity of NF-κB to enhance its own replication (23).

Previous work suggests that the HIV-1-encoded Nef protein is a negative regulator of HIV-1 replication (1, 7, 20, 25, 31). Furthermore, we and others have found that Nef may suppress both HIV-1 and IL-2 transcription (1, 21, 25). To investigate whether Nef affects the DNA binding activity of NF-κB or other transcription factors implicated in HIV-1 replication, we used human T-cell lines stably transfected with the nef gene. Jurkat (J25) human T-cell clone 133 constitutively expresses the NLA3 nef gene. 22F6 cells represent another antibody-resistant clone of J25 cells; however, these cells do not contain nef sequences and do not express Nef (21). Additionally, we used oligoclonal Jurkat E61 and HPB-ALL cells expressing the SF2 nef gene either in the correct orientation (Jurkat/LnefSN and HPB-ALL/LnefSNSI cells) or in the reverse orientation (Jurkat/LnefSN and HPB-ALL/LnefSN cells) with respect to the Moloney murine leukemia virus promoter (10). These cells represent a mixed population of cells expressing Nef to various degrees and were used to exclude the possibility that clonal selection accounts for Nef effects observed in the J25 clones.

To determine the impact of T-cell activation on the expression of Nef, the human T-cell lines were stimulated with PHA and PMA. Cells were maintained in logarithmic growth in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM glutamine.


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perazime-N'-2-ethanesulfonic acid; pH 7.9), 20 mM KCl, 1 mM MgCl₂, 2 mM dithiothreitol, and 17% glycerol (33) with the addition of 10 mM NaF, 0.1 mM sodium vanadate, and 50 mM beta-glycerophosphate. Cytoplasmic extracts consisted of the supernatant resulting from the lysis of cells in hypotonic lysis solution, Dounce homogenization, and low-speed centrifugation to pellet nuclei. Binding reaction mixtures contained 2 µl (2 µg) of nuclear extract (Fig. 2a through d) or 6 µl (7 µg) of cytoplasmic extract (Fig. 2e), 2 µg of poly(dI-dC) (Pharmacia), 100-fold molar excess of unlabeled NF-kB mutant oligonucleotide (ACAACTCATTTCGCT GTCACTTTCGAGGA), and 20,000 cpm of end-labeled oligonucleotide probe, in DNA binding buffer (27), in a final volume of 22 µl. Reactions were performed at 30°C for 25 min, immediately loaded on a 5.0% Tris-borate-EDTA, and run at 200 V. Oligonucleotides used were as follows: NF-kB, ACAAGGACTTTCGCG TGGGACTTTCGAGGA; SP-1, CAGGGAGGCGTGGCC TGGGCGGGACTGGGGAGTGGCGTCC. All DNA probes were gel purified and end labeled with [γ-32P]ATP. The intensity of the indicated bands was determined by laser densitometry and by measuring the radioactivity of excised bands in a liquid scintillation counter. There was a linear relationship between the amount of extract used and DNA-binding activity (data not shown). There was no NF-kB DNA-binding activity with the cytoplasmic extracts in the absence of deoxycholic acid (data not shown). Protein concentration was determined with the Bradford reagent (14) as adapted by NF-kB activity in nuclei from unstimulated cells was 3-fold higher than in the 22F6 cells, which inhibited the Bradford reagent (Bio-Rad) with bovine serum albumin as a standard. Nuclear extract preparations and binding reactions were repeated on three separate occasions with similar results.

The induction of NF-kB activity in stimulated 133 cells was suppressed five-fold compared with that in the 22F6 cells. This inhibition was evident 40 min poststimulation and was sustained throughout the 4-h stimulation period (Fig. 2a). J25 clone 22D8 cells represent a distinct clonal cell line which, like the 133 cells, also stably express Nef (21). NF-kB induction was suppressed four- to fivefold in the 22D8 cells compared with that in the 22F6 cells (Fig. 2b). NF-kB suppression was more profound in the 133 cells than in the 22D8 cells, which correlates with the observation that Nef expression was higher in the 133 cells (21). Similarly to the Nef-expressing J25 clones, Nef inhibited NF-kB induction three- to fourfold in the Jurkat/LnefSN and HPB-ALL/LnefSNS1 cells compared with their non-Nef-expressing counterparts (Fig. 2c and d). NF-mediated NF-kB suppression was more profound in the Jurkat/LnefSN cells than in the HPB-ALL/LnefSNS1 cells, even though the HPB-ALL/LnefSNS1 cells expressed several fold higher levels of Nef. This result is likely due to the biological differences that exist between the two cell lines. That is, Jurkat cells may be more sensitive to the effects of Nef than HPB-ALL cells because of differential expression of proteins involved in signal transduction. That Nef-mediated NF-kB suppression in the 133 and 22D8 cells was greater than in the Jurkat/LnefSN and HPB-ALL/LnefSNS1 cells may be due to the expression of a different nef allele in the 133 and 22D8 cells. Alternatively, this result could be due to the fact that every cell in the culture of 133 and 22D8 cells produced a relatively high level of Nef, whereas the Jurkat/LnefSN and HPB-ALL/LnefSNS1 cells represented a mixed population of cells expressing low and high levels of Nef or no Nef at all.

NF-kB activity in nuclei from unstimulated cells was extremely low but detectable, and no differences between the Nef-expressing and control cells were observed (data not shown). Additionally, when cytoplasmic extracts from un-
stimulated cells were treated with deoxycholic acid (which releases NF-κB from its cytoplasmic inhibitor, IκB [3]), they exhibited NF-κB activity independent of Nef expression (Fig. 2e). Finally, that Nef suppressed the level of NF-κB induction after only 40 min of stimulation suggests that Nef does not suppress p110 or p65 NF-κB mRNA expression. These observations indicate that Nef affects the recruitment and not the cytoplasmic concentration of NF-κB. The binding of SP-1 was independent of Nef expression and stimulation, and the amount of SP-1 probe used in these gel shift assays was not limiting (Fig. 2a through d). In addition, no differences in binding to NF-AT-, USF-, and URS-specific probes between the 22F6 and 133 cells were observed (data not shown). These data suggest that Nef specifically inhibited the induction of NF-κB activity.

To further demonstrate Nef’s suppressive effect on NF-κB recruitment, 22F6 cells were transiently transfected with DNA plasmids expressing Nef from the SV40 early pro-
Fig. 3. Gel shift analysis of nuclear extracts prepared from 22F6 cells (a) transiently transfected with the indicated DNA plasmids that were not stimulated (0) or were stimulated for 4 h (4) with PHA-P and PMA and 22F6 and 133 cells (b) stimulated for 4 h with PHA-P (H), PMA (M), or ionomycin (I) (2 μM) or combinations of any two mitogens. N, U, S, and P, NF-κB-specific binding, USF-specific binding, SP-1-specific binding, and free probe, respectively. (c) Nuclear extract 22F6 H+M from panel b was preincubated with the specified antiserum for 15 min before the NF-κB DNA probe was added. NF-κB p65-p50 heterodimer- and p50-p50 homodimer-DNA complexes and supershifted heterodimer- and homodimer-DNA-antibody complexes are indicated at the right. Data represent at least three independent experiments.
Similarly, CAT activity induction was suppressed twofold in nucleotides -453 to -156 of the HIV-1 long terminal repeat that was fivefold less than that of 22F6 cells (Fig. 4a). Element deletion clone, 8NRE-HIV-1-CAT, which lacks that 133 cells exhibited a capacity to induce CAT activity with both wild-type HIV-1-CAT and the negative regulatory element sequences present in C15-CAT. The Jurkat/LnefSN cells compared with that in the Jurkat/LnefSN cells (Fig. 4b). This inhibition was demonstrated with both wild-type HIV-1-CAT and the negative regulatory element deletion clone, 8NRE-HIV-1-CAT, which lacks nucleotides -453 to -156 of the HIV-1 long terminal repeat.
expression of viral gene products in infected cells, thereby allowing the cells to evade clearance by the cellular and humoral arms of the immune response. This model for Nef-mediated viral persistence in vivo may be consistent with the results of Kestler et al., which demonstrated that the presence of an intact nef gene was required to prolong simian immunodeficiency virus infection and induce pathogenesis in infected macaques (18). Furthermore, we and others demonstrated that simian immunodeficiency virus Nef inhibited simian immunodeficiency virus replication in vitro in a way that was analogous to the way in which HIV-1 Nef inhibited HIV-1 (4, 24). It is possible that high-level Nef expression early after infection (28) is sufficient to maintain HIV-1 in a relatively latent state, which may be critical for establishing a reservoir of HIV-1-infected cells and the eventual development of AIDS.

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HIV-1 Nef protein inhibits the induction of AP-1 DNA-binding activity in human T-cells

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Abstract

The human immunodeficiency virus type 1 long terminal repeat, HIV-1-LTR, contains binding sites for several cellular transcription factors which contribute to HIV-1 gene expression. Our previous studies on the function of the HIV-1 encoded Nef protein suggested that Nef may be an inhibitor HIV-1 transcription. To determine whether Nef affects the binding of cellular factors implicated in HIV-1 regulation, $^{32}$P-labeled oligonucleotides corresponding to the binding sites were incubated with nuclear extracts prepared from Nef-expressing T-cell lines that were not stimulated or were stimulated with T-cell mitogens. We found that Nef inhibited the mitogen-mediated induction of AP-1 DNA-binding activity in human T-cells. Additionally, Nef expressing cells were transiently transfected with a plasmid in which HIV-1 AP-1 DNA recognition sequences were cloned downstream of the chloramphenicol acetyltransferase (CAT) gene. Mitogen-mediated transcriptional activation of the CAT gene in this construct was inhibited in Nef expressing cells but not in control cells. These studies suggest that, by inhibiting AP-1 induction, Nef may play a role in regulating HIV-1 gene expression in infected T-cells.
The human immunodeficiency virus type 1 long terminal repeat, HIV-1-LTR, contains two adjacent AP-1 binding sites and three intragenic AP-1 sites are located within the **pol** gene.\(^1,2\) The AP-1 DNA-binding complex is composed of homo- and heterodimers of the c-\textit{fos} and c-\textit{jun} family of transcription factors\(^3\) which dimerize by interdigitation of hydrophobic alpha-helices, called leucine-zippers.\(^4\) The consensus DNA recognition site for AP-1 is TGACTCA\(^5\)\(^-\)\(^7\) and this sequence has also been shown to confer phorbol ester inducibility.\(^8\) c-\textit{fos} and c-\textit{jun} mRNAs are induced in T-cells by the lectin phytohemagglutinin\(^9\) (PHA), the calcium ionophore A23187\(^10\), and the phorbol ester, phorbol-12-myristate-13-acetate, (PMA).\(^8\) AP-1 can serve as both a positive and a negative regulator with respect to the expression of a variety genes under different conditions.\(^11\)\(^-\)\(^15\)

It has been reported that the product of the \textit{nef} gene of HIV-1 and SIV could function as a negative regulator of virus replication.\(^16\)\(^-\)\(^22\) Furthermore, we found that Nef could function as a viral transcriptional inhibitor.\(^16\)\(^,\)\(^17\) This result was confirmed by other investigators\(^18\)\(^,\)\(^19\) although, not by all investigators.\(^23\)\(^,\)\(^24\) Recently, Luria et al. showed that Nef, stably expressed in Jurkat human T-cell clones, prevented the transcriptional activation of the interleukin-2 (IL-2) gene.\(^25\) IL-2 is a critical T-cell proliferation factor and serves as a marker for T-cell activation. Interestingly, the HIV-1-LTR and the IL-2 promoter contain the T-cell activation-associated
transcription factors, AP-1 and NF-kB.

In order to elucidate the mechanism underlying Nef's negative effects on HIV-1 and IL-2 transcription in T-cells, we examined the binding of cellular transcription factors with recognition sites in the HIV-1-LTR in the presence, and absence, of Nef. In these studies, we used the Jurkat (J25) human T-cell clone 133, which stably expresses the Nef protein derived from the HIV-1 isolate NL-43. As a control, we used a G418 resistant, Jurkat 25 clone, 22F6, which does not contain any HIV-1 sequences and does not express Nef. Additionally, we used oligoclonal HPB-ALL human T-cells stably transduced with a recombinant retrovirus expressing the nef gene, derived from the HIV-1 isolate SF-2, either in the correct orientation, HPB-ALL/LnefSNS1 cells, or the reverse orientation, HPB-ALL/LfenSN cells, with respect to the Moloney murine leukemia virus promoter. Nef was expressed to a high degree in the Jurkat 133 cells and the HPB-ALL/LnefSNS1 cells, but was not produced in the Jurkat 22F6 cells or the HPB-ALL/LfenSN cells as determined by Western blot and immunoprecipitation analysis (data not shown). The HPB-ALL cells, in contrast to the Jurkat cells, represent a mixed population of cells expressing Nef, and thus, do not suffer from the potential limitation that Nef mediated effects observed in the Jurkat cells are a result of cloning. However, the advantage of the clonal Jurkat 133 cells is that Nef is expressed in every cell, thereby magnifying the effects exerted by Nef. It is noteworthy that
the doubling times for the Nef expressing and the control cells were indistinguishable and no gross morphologic differences between the cells were noted either prior to or post-stimulation.

Gel shift analyses were performed with nuclear extracts prepared from cells that were not stimulated, or were stimulated, with the T-cell mitogen, phytohemagglutinin (PHA-P) and the protein kinase C (PKC) activator, PMA. Nuclear extracts were incubated with a $^{32}$P-labeled oligonucleotide corresponding to the HIV-1 AP-1 DNA recognition sites. In the parental 22F6 Jurkat cells, an induced AP-1/DNA complex, which was not present in unstimulated cells, was detected between 1 and 2 hr. post-stimulation and was abundant 4 hr. post-stimulation (Figure 1). In contrast, the recruitment of the same AP-1/DNA complex was inhibited 5-fold at 2 hr. and 9-fold at 4 hr. in the Nef expressing 133 cells compared to the 22F6 cells (Figure 1). Addition of 100-fold molar excess of unlabeled AP-1 specific oligonucleotide inhibited the appearance of the major inducible complex (Figure 1). However, an oligonucleotide with three nucleotide substitutions in the AP-1 recognition site did not compete away the inducible complex, and we included a 100-fold excess of the unlabeled mutant AP-1 oligonucleotide in all binding reactions as a non-specific inhibitor.

The presence of the constitutive AP-1/DNA complex (the slowest migrating complex in Figure 1) was minimally, if at all, affected by Nef and may be due to the constant presence of serum

-5-
in the cell growth media. Moreover, this complex was not inducible (Figure 3b). In addition, the constitutively active transcription factor SP-1 was not affected by the presence or absence of Nef, and was used as a control for extract quality (Figure 1). Therefore, Nef inhibited the inducible AP-1/DNA complexes, specifically.

Gel shift analysis with extracts prepared from stimulated and unstimulated HPB-ALL cells afforded results similar to those obtained with the Jurkat cells (Figure 2). However, in contrast to the Jurkat cells, the mitogen-inducible AP-1/DNA complexes were present in unstimulated cells as well as the stimulated cells. Whereas the stimulatable AP-1/DNA complexes in the HPB-ALL/LfenSN cells were induced approximately 5-fold after 4 hr. of PHA and PMA treatment, there was no significant induction of these complexes in the HPB-ALL/LniefSNS1 cells (Figure 2). In this experiment, the amount of AP-1 activity in unstimulated HPB-ALL/LfenSN cells was lower than the HPB-ALL/LniefSNS1 cells, however, this was not a consistent finding (unpublished results). In this experiment, we included another Nef non-responsive transcription factor, USF, to demonstrate the specificity of Nef action and the integrity of the extract. The apparent difference in migration of the major inducible AP-1/DNA complexes between the HPB-ALL and the Jurkat cells probably reflects differences that exist between the different T-cell lines.
Previous studies indicated that c-fos expression is induced by PHA, the calcium ionophore A23187, and PMA. In order to determine the signalling pathway required to induce AP-1 DNA-binding activity, we assessed the role of PHA, PMA, and the calcium ionophore, ionomycin, alone or in combination (Figure 3a). Interestingly, the induction of AP-1 activity was maximal with PHA treatment alone and addition of PMA did not significantly increase AP-1/DNA complex formation. The level of inducible AP-1 activity was 18-fold higher in the 22F6 cells compared to the Nef expressing 133 cells with PHA alone (Figure 3a). PMA alone only slightly induced AP-1 activity in the 22F6 cells, however, no detectable AP-1 activity was observed in the 133 cells treated with PMA alone (Figure 3a). Ionomycin alone was not sufficient to elicit AP-1 recruitment in either cell line (Figure 3a).

T-cell activation is mediated by increased Ca\textsuperscript{2+} influx and PKC activation which both occur as a consequence of phospholipase-C activation by the T-cell receptor (TCR) complex. Treatment of the Jurkat cells with a combination of ionomycin and PMA, which both bypass the TCR complex, led to significant induction of AP-1 activity, albeit 2.5-fold less efficiently than PHA alone. Whereas there was an 18-fold higher level of induced AP-1 DNA-binding activity in the 22F6 cells compared to the 133 cells using PHA alone, there was only a 3-fold difference using the combination of ionomycin and PMA.
Since PHA mimics the normal activation signal (i.e. antigen binding to the TCR) of T-cells, it appeared that Nef exerted its effects primarily (although not exclusively) on TCR initiated signalling, as has been suggested previously.

To determine whether the induction of AP-1 activity required the activation of pre-existing complexes or new protein synthesis, cycloheximide was added 30 min. before mitogen treatment. That cycloheximide treatment inhibited the recruitment of AP-1 activity, suggests that de novo protein synthesis must be involved (Figure 3b). This result was consistent with the observation that two hours of stimulation were required before significant induction of AP-1 DNA-binding activity (Figure 1).

To identify the polypeptides present in the inducible AP-1 complex, we incubated nuclear extracts derived from the 22F6 cells with anti-c-Fos and anti-c-Jun antibodies, prior to the addition of labeled oligonucleotides (Figure 3c). Antisera to both c-Fos and c-Jun inhibited complex formation approximately 3-fold, suggesting the presence of c-Fos and c-Jun in the complex. However, these antibodies did not cause a super-shift, presumably because antibody binding to c-Fos and c-Jun caused conformational changes which are not permissive for DNA binding activity. In these experiments, normal rabbit serum and anti-Nef antibodies were used as negative controls.

Previous studies indicated that the binding of AP-1 to the HIV-1-LTR AP-1 recognition sites play little, if any, role in
affecting transcriptional activity.\textsuperscript{2,30} Zeichner and coworkers generated several HIV-1-LTR-CAT linker-scanning mutants in the region of the AP-1 recognition sites and transfected the mutant plasmids into Jurkat cells.\textsuperscript{30} There were no significant differences in CAT activity between the wild-type HIV-1-LTR-CAT plasmid and the AP-1 mutant plasmids in cells that were or were not stimulated with PHA and PMA.\textsuperscript{30}

However, the intragenic AP-1 recognition sites were capable of mediating transcriptional activation following phorbol ester treatment.\textsuperscript{2} Therefore, we cloned a synthetic oligonucleotide, corresponding to the two adjacent AP-1 sites within the \textit{pol} gene,\textsuperscript{2} or an oligonucleotide which contained three nucleotide substitutions in these AP-1 consenus sites, into the polylinker of the enhancer-less pCAT promoter plasmid (Promega). These plasmids were called pCAT-IG-API and pCAT-MIG-API, respectively. The pCAT promoter construct, in the absence of the AP-1 sites, contains the SV40 core promoter, afforded low basal chloramphenicol acetyltransferase (CAT) activity in T-cells, and was not inducible in T-cells following treatment with T-cell mitogens (data not shown).

The pCAT-IG-API and pCAT-MIG-API constructs were transiently transfected into the Jurkat 22F6 and 133 cells, as well as Jurkat 25 clone 22D8 cells. The 22D8 cells represent a distinct clonal cell line which, like the 133 cells, also stably express the nef gene from HIV-1 isolate NL-43.\textsuperscript{25} Transiently transfected
cells were either not stimulated or were stimulated with PHA and PMA for 18 hr. and CAT activity was then measured. CAT activity in transfected cells was relatively low, between 1-3% conversion to acetylated products. However, we found an average fold induction in CAT activity of 3.6 ± 0.4 in the 22F6 cells transfected with the pCAT-IG-AP1 plasmid, compared to an average fold induction of 1.4 ± 0.2 in the 133 cells and no induction in the 22D8 cells (Figure 4). Transfection efficiencies were higher in the Nef expressing cells and were determined by parallel transfections with non-Nef responsive promoters including Rous sarcoma virus-CAT, cytomegalovirus-CAT, and simian polyoma virus 40-CAT (data not shown). These determinations were statistically significant, with 95% confidence intervals, with respect to fold-induction, of 2.8-4.4 for the 22F6 cells, 1.0-1.8 for the 133 cells, and 0.9-1.1 for the 22D8 cells. Using a Mann-Whitney U test analysis, the probability that there is no difference in the fold induction between the 22F6 cells and the 133 and 22D8 cells is 1 in a 1000. CAT activity was not induced in cells transfected with the pCAT-MIG-AP1 construct, indicating that the integrity of the AP-1 site in the inserted oligonucleotide was essential. Thus, Nef-mediated inhibition of AP-1 DNA-binding activity prevented AP-1-mediated transcriptional activation.

What role AP-1 plays with respect to HIV-1 regulation is unclear. Nef could inhibit AP-1-mediated activation of HIV-1 directly, by preventing the interaction of AP-1 with the
intragenic enhancer in the pol gene. In addition, by inhibiting AP-1 induction during T-cell activation, Nef may affect the regulation of AP-1 activated cellular genes. Effects on such cellular genes may alter the cellular environment, positively or negatively, which may indirectly affect HIV-1 replication. For example, the finding that c-Fos and c-Jun are early response mediators of T-cell activation,\textsuperscript{9} coupled with the observation that HIV-1 cannot replicate in resting, unactivated T-cells,\textsuperscript{31,32} presents a scenario for indirect effects of Nef on HIV-1 expression.

In addition to mediating the suppression of AP-1 induction, we found that Nef also inhibited the mitogen-mediated induction of NF-kB.\textsuperscript{33} NF-kB, like AP-1, is an early response effector of T-cell activation,\textsuperscript{34} and has been shown to be an important activator of HIV-1 replication in stimulated T-cells.\textsuperscript{35} Thus, Nef mediated inhibition of recruitment of both AP-1 and NF-kB may intensify the negative effects on HIV-1 replication in T-cells. By inhibiting virus replication directly, and/or by blocking T-cell activation, Nef may provide a reservoir of persistently infected cells which may ultimately contribute to HIV-1 clinical latency, HIV-1 mediated CD4 T-cell depletion, and AIDS.
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Figure 1. Gel shift analysis of AP-1 DNA binding activity in extracts prepared from Jurkat 25 cells. Cells were stimulated with PHA-P (13 µg/mL, [SIGMA]) and PMA (75 ng/mL, [SIGMA]) for 0, 40, 80, 120, or 240 min. Oligonucleotide probes used for binding are specified on the top of each panel. "A" indicates the inducible AP-1/DNA complexes. S and P represent SP-1 specific binding and free probe respectively. SP-1 binding served as a control for extract quality and specificity of Nef mediated effects. Cold indicates that 100-fold molar excess of unlabeled DNA was added for competition.

Methods. Cells were maintained in logarithmic growth in RPMI 1640 medium supplemented with 10% fetal calf serum and 2 mM glutamine. Nuclear extracts were prepared from 5 X 10^7 cells using a modified version of the method of Dignam et al. as adapted by Montminy and Bilezikjian. Following ammonium sulfate precipitation, nuclear proteins were resuspended in 100 µl of 20 mM Hepes (pH 7.9), 20 mM KCl, 1 mM MgCl₂, 2 mM DTT, and 17% glycerol with the addition of 10 mM NaF, 0.1 mM sodium vanadate, and 50 mM beta-glycerol-phosphate. Binding reactions contained 2 µl (2 µg) of nuclear extract, 2 µg poly dI-dC (Pharmacia), 100-fold molar excess of unlabeled intragenic AP-1 mutant oligonucleotide (GATCTCAAAGCGGATATCAGCTGTTAATCAAAT-AAT), and 20-40,000 cpm of end-labeled oligonucleotide probe,
in DNA binding buffer, in a final volume of 22 μL. Reactions were performed at 30°C for 30 min, immediately loaded onto a 4.5% polyacrylamide gel using 0.5 X TBE, and run at 200 V. Oligonucleotides used were as follows: AP-1, CAGGGCCAGGAGTCAGATATCCACTGACCTTTGGATGGTGCT; SP-1, CAGGGAGGCGTGGCCTGGCGGGACTGGGGAGTGGCGTCC. All DNA probes were gel purified and end-labeled with [γ-32P]-ATP. The intensity of indicated bands was determined by laser densitometry scanning. There was a linear relationship between the amount of extract used and the DNA binding activity. Nuclear extract preparations and binding reactions were repeated on 3 separate occasions with similar results.
Figure 2. Gel shift analysis of nuclear extracts prepared from HPB-ALL cells that were not stimulated (-) or were stimulated with (+) with PHA (13 μg/mL) and PMA (75 ng/mL) for 4 hr. The labeled oligonucleotide probe used is indicated above each panel. The "A"s indicate inducible AP-1/DNA complexes and P represents free probe.

Methods. Nuclear extract preparations and DNA-binding reactions were performed as described in Figure 1. For the USF probe, we used an oligonucleotide corresponding to nucleotides -159 to -173 of the HIV-1 LTR\textsuperscript{40}, GCCGCTAGCATTTCCATCAGTGGCCCGAGAGCTGC. Experiments were repeated 3 times with similar results.
Figure 3. a. Gel shift analysis of nuclear extracts prepared from Jurkat 25 22F6 and 133 cells stimulated for 4 hr. with either PHA-P (H), PMA (M), or ionomycin, 2 μM [SIGMA], (I), or combinations of any two mitogens. A, S, and P indicate AP-1 specific binding, SP-1 specific binding, and free probe, respectively. b. Jurkat 22F6 cells were (+) or were not treated (-) with cycloheximide (20 μg/mL, SIGMA) for 30 min. prior to stimulation for 4 hr. with PHA-P and PMA. The labeled oligonucleotide used is indicated on the side of each panel. A_C, A_I, USF, and n.s. indicate constitutive AP-1/DNA complex, inducible AP-1/DNA complex, USF specific complex, and not specific, respectively. The "cold DNA" indicates that 100-fold molar excess of unlabeled oligonucleotide (the same oligonucleotide used as the probe) was used as a non-specific competitor. c. Nuclear extract 22F^- (H) from part a. was pre-incubated with 2μl of the specified antisera for 15 min. before the AP-1 DNA probe was added. The A's indicate the major inducible AP-1/DNA complexes.

Methods. Nuclear extract preparations and DNA binding reactions were described in Figure 1. c. Anti-Fos-1, anti-Fos-2, anti-Jun-1, and anti-Jun-2 correspond to antibodies anti-c-fos-Ab-1, anti-c-fos-Ab-2, anti-c-jun-Ab-1, and anti-c-jun-Ab-2, respectively (Oncogene Science, Inc.). The anti-Nef sera was from a rabbit and was used as a negative control antibody. This gel was run for twice as long as the other
gels and the two bands indicated in this gel appears as a single band on the other gels.
Figure 4. Chloramphenicol acetyltransferase (CAT) assays of extracts prepared from Jurkat 25 cells transfected with the pCAT-IG-API and pCAT-MIG-API plasmids. The data is presented as the mean ratio of the level of CAT activity present in cells stimulated for 18 hr. with PHA-P and PMA compared to the level present in unstimulated cells. The error bars represent the standard deviation of the mean.

Methods. Jurkat cells were transfected with 40 μg of the indicated plasmid using DEAE-dextran. Briefly, 10⁷ cells were incubated with plasmid DNA suspended in 10 mL of serum-free RPMI 1640, 0.25M Tris (pH 7.3), and 125 μg/mL DEAE-dextran (SIGMA) at 37°C for 40 min. Following centrifugation at 2,000 X g, for 7 min., cells were maintained in growth media containing 10% fetal calf serum for 24 hr. Cells were or were not treated with PHA-P (13 μg/mL) and PMA (75 ng/mL) and incubated an additional 18-24 hr. Cell extracts were prepared and CAT activity was measured by standard methods. Extract equivalent to 3 X 10⁶ cells, as determined by Bradford reagent analysis (Bio-rad), was used for each 18 hr. reaction. CAT activity was in the linear range of analysis with respect to extract amount and incubation time. The amount of CAT activity was quantitated by excising the spots corresponding to the unacetylated and acetylated forms of ¹⁴C-chloramphenicol and measuring radioactivity in a scintillation counter. The plasmid pCAT-IG-API was generated by cloning a double-stranded oligonucleotide corresponding to the two adjacent intragenic AP-1 recognition sites and containing a
Bam HI sticky end on the 5' end, GATCTCAAAGTGAATCAGAGTTAGTCAA-
TCAAATAAT, and a Sal I sticky end on the 5' end of the 
complementary oligonucleotide, TCGAATTATTTGATTTGACTAACCGTGTCAA-
CTTTGA, into the Bam HI and Sal I sites in the enhancer-less 
pCAT-promoter plasmid (Promega). Plasmid pCAT-MIG-AP1 was made in 
the same way except that the AP-1 recognition sites in the 
oligonucleotide used for cloning were disrupted by substitutions 
at the following, underlined positions; GATCTCAAAGCGATATCAGC-
TTATAATCAAATAAT. Cells were transfected 4 to 6 times with each 
plasmid, a mean fold induction was calculated, and the standard 
deviation of the mean was determined. The average fold induction 
and standard deviations were 3.6 ± 0.4, 1.4 ± 0.2, and 0.9 ± 0.1 
for the 22F6, 133, and 22D8 cells, respectively.
ON THE HIV nef GENE PRODUCT

3rd FORUM
IN VIROLOGY
Instructions to Forum organizers and participants

Forums are meant to promote the exchange of opinions and divergent ideas on important topics in the field. From a practical point of view, the procedure for organizing these Forums is as follows. After approval, by the Editorial Board, of the selected topic and of the Organizer/Chairman, the latter contacts international specialists on the chosen subject. Each of these participants sends his/her views which are dispatched to the other contributors, allowing them to comment on and discuss their colleagues’ opinions. These views and commentaries are grouped together to build the Forum. This approach has the advantage of avoiding the natural limitations of a general review written by only one author. Furthermore, it emphasizes hard facts, point of controversy, conflicting hypotheses, etc.

1. The organizer contacts 7-14 specialists on the subject. Those who agree to participate are given about 6 weeks to write their text.

2. Each participant submits a text of 2 to 8 pages, in triple-spaced typescript, to the organizer. References in the text should be by author’s last name and year.

3. The organizer sends all texts to all participants, giving them 3-4 weeks to reply.

4. Based upon reading of all texts, each participant submits a discussion/comment/opinion statement of 2-4 pages, triple-spaced, to the organizer.

5. The organizer collates and organizes texts and discussion statements, together with an introduction and, if necessary, a conclusion, and submits this to the Editorial office. At this point, the organizer should indicate the running title and order in which papers are to appear.

6. To speed up publication, galley proofs will be read only by the Chairman.
On the HIV *nef* gene product

Organized by A.G. Hovanessian

INTRODUCTION

The human immunodeficiency viruses 1 and 2 (HIV-1 and HIV-2) and their simian counterparts, simian immunodeficiency viruses (SIV), contain an open reading frame (ORF) that overlaps with the 3' long terminal repeat (LTR) sequence of the viral genome (for a recent review, see Cullen, 1991). This gene was designated as 3' *orf*, and also as F, *orf B* or E', and finally as *nef* standing for negative factor. In preliminary reports, the product of the *nef* gene, Nef, was reported to mediate downregulation of virus replication through transcriptional inhibition of the viral LTR (Terwilliger *et al.*, 1986; Luciw *et al.*, 1987; Ahmed and Venkatesan, 1988; Cheng-Mayer *et al.*, 1989; Niederman *et al.*, 1989, 1991). On the other hand, other groups have found no action of *nef* on HIV LTR nor on overall HIV replication (Hammes *et al.*, 1989; Kim *et al.*, 1989; Bachelieri *et al.*, 1990; Schwartz *et al.*, 1991). A logical compromise to these controversial reports might be the possibility that the functioning of Nef is sometimes hindered according to the cell-type studied. The existence of nuclear factors interacting with Nef and the negative regulatory element (NRE) of the viral LTR (Guy *et al.*, 1990a), in addition to the structural similarities between Nef and leucine zipper-like sequences characteristic of transcriptional factors (Samuel *et al.*, 1991), suggest that Nef could act as a potential signal-transducing protein.

A high degree of polymorphism of the *nef* gene exists between different HIV isolates (Ratner *et al.*, 1985). Nef is presumably produced early in infection, since its mRNA is detectable along with those of the two regulatory proteins, Tat and Rev (Schwartz *et al.*, 1990). In contrast to Tat and Rev which are nuclear proteins, Nef is cytoplasmic and partly associated with membranes (Franchini *et al.*, 1986; Laurent *et al.*, 1990). Two translation products, of 27 and 25 kDa, might be generated from the *nef* gene (Guy *et al.*, 1990b; Kaminchik *et al.*, 1991).

The 27-kDa Nef is myristylated in accordance with the presence of the myristylation motif GXXXS, following the initiator methionine residue. The 25-kDa Nef is the product of internal initiation of the *nef* gene mRNA, and consequently it is not myristylated. The 25-kDa product is not always detectable in HIV-infected cells (Laurent *et al.*, 1990). Myristylation of Nef probably allows its interaction with cell membranes and might be critical for its activity.

Nef can be phosphorylated by protein kinase C at threonine residue 15 and also appears to have autophosphorylation activity toward serine residues (Guy *et al.*, 1987, 1990b; Nebreda *et al.*, 1991). The significance of these phosphorylations is not yet clear. However, it is interesting to note that threonine-15 becomes mutated both in vivo, in patients over a few years (Delassus *et al.*, 1991), and in vitro by prolonged passaging of the virus (Laurent *et al.*, 1990). For the moment, there are two examples of cellular proteins, CD4 and interleukin-2 (IL2), which seem to be regulated by Nef (Guy *et al.*, 1987; Luria *et al.*, 1991). Interestingly, these proteins are downregulated by two different mechanisms: Nef regulates IL2 production at the transcriptional level (Luria *et al.*, 1991), whereas it causes downregulation of cell-surface expression of CD4 by a post-translational mechanism (Garcia and Miller, 1991). The relevance of the effect of Nef on CD4 and IL2 in the pathogenesis of AIDS is not yet clear. Such effects could be used as convenient tools to illustrate the biological activity of Nef and consequently shed some light on its function.

Because of the earlier reports of its inhibitory effect on HIV LTR and virus replication in cell cultures, Nef has been considered to be involved in the establishment of viral latency (Haseltine, 1988). In
accordance with this hypothesis, it has been reported that antibodies against Nef appear prior to the antibodies against HIV structural proteins, and therefore anti-Nef antibodies could contribute to early diagnosis of infection (Ranki et al., 1987; Ameisen et al., 1989; Gombert et al., 1990). These studies, however, have been refuted by other reports which failed to show either a high prevalence of Nef-specific antibodies prior to seroconversion, or their presence in HIV-seronegative but exposed individuals (De Ronde et al., 1988; Reiss et al., 1989; Cheingsong-Popov et al., 1990; Kirchhoff et al., 1991). Nef is relatively antigenic in that 70% of HIV-seropositive individuals seem to produce anti-Nef antibodies (Sabatier et al., 1989; Schneider et al., 1991). In addition, specific cytotoxic T lymphocytes have been reported in HIV-seropositive individuals (Culmann et al., 1989; Rivière et al., 1989; Walker and Plata, 1990; Koenig et al., 1990). These host immune responses against Nef could be considered of importance during HIV infection in view of a recent report demonstrating the possible function of Nef in efficient viral replication and in the development of AIDS in monkeys (Kestler et al., 1991).

Desrosiers and collaborators experimentally infected rhesus monkeys with 3 types of SIV provirus preparations which contained either the wild-type nef gene (nef-open), a deletion in the nef gene (nef-deleted) or a nef gene with a premature stop signal (nef-stop). Intriguingly, the provirus with the nef-stop gene was found to replicate in monkeys like the wild-type counterpart due to the removal of the stop signal. This indicates that in vivo there could be a strong selective pressure to maintain an open nef gene in order to produce "functional forms" of Nef.

Provirus with the nef-deleted gene was found to have a decreased pathogenic potential in monkeys. It should be emphasized that in contrast to these in vivo observations, no significant differences in the replication of the 3 types of proviruses were observed in cell lines or in primary rhesus monkey peripheral blood lymphocytes (Kestler et al., 1991). These results therefore indicate that Nef might be totally unnecessary for virus replication in vitro, although in vivo it seems to play an important role in virus infection and pathogenesis.

The numerous published reports on Nef provide somewhat conflicting results which clearly illustrate one of the aspects of this complex retrovirus and the disease which it causes. In view of the recently established putative positive function of Nef in monkeys, it will be important to develop cell system models in order to clarify the conflicting results observed in vitro. Realization of the latter, however, is probably difficult since different cell lines may have specific factors which interact directly or indirectly with Nef, thus interfering with its function.

Contributions from several researchers in this "Forum" point to some of the important features of Nef and its potential biological function(s). No doubt, Nef has a significant potential to be classified among the regulatory proteins of HIV. Once its precise biological functions become clarified, then it will be possible to change the name of Nef from "negative factor" to a much more appropriate one.

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ON THE HIV nef GENE PRODUCT


The HIV Nef protein: facts and hypotheses

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Four years ago, in our first study of the Nef protein (Guy et al., 1987), we wrote in the conclusion of our paper: "We have shown that the F (Nef) protein of HIV is a myristylated GTP-binding phosphoprotein with features similar to the cellular src and ras oncogene products [...]. We have shown that F may participate with env in downregulation of T4 lymphocytes [...]. Latent infection of lymphocytes is important in the pathophysiology of AIDS, and we speculate that F protein, by intervening in cellular regulatory pathways, may play a key role in the establishment or maintenance of latent HIV infection."

A considerable amount of work has been done on Nef since 1987 in many laboratories including ours, generating a large amount of information about this protein. In the light of these in vitro and in vivo results, what can be maintained from our initial conclusions on the structure and the role of the nef gene product? This Forum is a good opportunity to try to answer this question.

Nef and nucleotides

Based on its in vitro activities (GTP binding, GTPase activity and autophosphorylation in the presence of GTP), we surmised that Nef was a "ras-like" protein.

Some authors have reported results which are to some extent similar to ours (Cheng-Mayer et al., 1989), but other groups have failed to observe such properties for Nef (Kaminchik et al., 1990; Matsuura et al., 1991). On repeating our GTP-binding experiments on purified Nef numerous times, we have found this activity very reproducible, although its stoichiometry is low. The autophosphorylation of Nef in the presence of GTP is also always easily detectable in our hands, which signifies that Nef is at least capable of binding and hydrolyzing GTP. Moreover, we have recently observed a similar autophosphorylation for the HIV2ROO Nef protein, purified by a completely different protocol from baculovirus-infected cells (unpublished results). The autophosphorylation results have been confirmed by others (Nebreda et al., 1991), although ATP was also shown in this study to be a substrate. Nevertheless, we found GTP to be a more specific substrate.

We have mapped on Nef at least three conserved and important domains (Guy et al., 1990a) related to the consensus domains of GTP-binding proteins (KGGLEG, GXXXD and WR/KFD). The sequence of these domains is degenerate compared to G-protein canonic sequences, but it is of note that the critical "G" domain of the bacterial GTP-binding protein hDPα (Römsisch et al., 1989) is highly homologous to the related domain present in SIV Nef (IVLTKFDT in hDPα and ILVWKFDP in SIV Nef). Moreover, it is worth mentioning that in all HIV and SIV Nef the first amino acid of the critical tetrapeptide (WR/KFD) is a tryptophan (W), and that such a conserved domain (domain IX; DXW) is present in all but one kinase using ATP (Hanks et al., 1988).
Taken together, these observations may explain the greater specificity of Nef, capable of using both purine nucleotides with a preference for GTP. However, we are in complete agreement that Nef is different from the ras or aG-related proteins, and our initial speculation on this point may have been too ambitious.

**Nef and phosphorylation**

The ability of Nef to autophosphorylate in vitro led us to examine more closely its potential in vivo phosphorylation capacity using recombinant vaccinia viruses. We had observed that Thr15 was a site for protein kinase (PKC) phosphorylation (Guy et al., 1987) and although the biological significance of this phosphorylation is still unknown, it is important to point out that a recent study (Delassus et al., 1991) comparing the sequences of different nef genes from HIV isolated over a 4-year period from patients (in vivo), or after they had been passed in culture (in vitro), revealed a significant rate of mutation at this site (T → A) during the course of the disease, and between the in vivo and in vitro situations (Delassus et al., 1991; Laurent et al., 1990). This is in agreement with a possible regulation of Nef activity by phosphorylation of Thr15 by PKC, as is the case, for instance, for the aGI proteins (Katada et al., 1985).

A recent study of the regulation of IL2 expression by Nef (Luria et al., 1991 and see, below) also supports such a hypothesis. In this work, the only Nef allele showing this effect when expressed constitutively bears an Ala at position 15, and is thus not phosphorylatable by PKC. In our studies using recombinant vaccinia viruses, we have not observed biological differences between Thr and Ala15 mutants with respect to CD4 downregulation (see below), but in such a system, nef is only expressed during a short term-period and subsequent regulation might thus be different.

Moreover, we have observed the existence of more phosphorylation sites (Guy et al., 1990a). One of them may be related to in vitro phosphorylation (likely on a serine residue as it is alkali-sensitive). We have already pointed out the potential importance of the conserved Ser88 in this phosphorylation, but mutations at this site do not completely abolish the alkali-sensitive phosphorylation of Nef (Guy et al., 1990a). We have further observed that the presence of His89, situated within a sequence environment highly similar to the corresponding sequences surrounding the phosphorylated histidine residues of prokaryotic transducing proteins (for a review, see Stock et al., 1990), is critical for an additional phosphorylation of Nef. Moreover, this phosphorylation is alkali-resistant, abolished by a His89 → Gin mutation and easily detectable only on a truncated non-myristylated variant of Nef which is unable to interact with mem-

brane proteins (Guy et al., 1990a; Kaminchik et al., 1991). Although we have not been able to ascertain the precise identity of the phosphorylated amino acid, we surmise that a situation similar to that observed in prokaryotic transducing proteins may occur, i.e. the rapid transfer of the phosphate group to another protein, resulting in the regulation of some cellular pathway (for a review, see Sailer et al., 1990).

These results demonstrate that Nef is involved in phosphorylation-dephosphorylation activities and, at this point, we wish to examine the validity of our main hypothesis which is that Nef is involved in the regulation (through phosphorylation events?) of cellular pathways.

**Nef and signal transduction**

What are the elements allowing us to put forward such a hypothesis? Nef is a myristylated protein, and myristylation enables Nef interaction with cell membranes and therefore membrane proteins (Kaminchik et al., 1991), an interaction which is critical for its activity (Guy et al., 1990a); Nef has a structure enabling its interaction with purine nucleotides (Guy et al., 1987, 1990a; Nebrada et al., 1991) and in particular, guanine nucleotides, although with a low affinity, at least in vitro; Nef is phosphorylated, and a phosphotransferase and/or nucleotidase activity is associated with Nef (Guy et al., 1987, 1990a, and in preparation). Taken separately, none of these elements is sufficient to allow us to conclude that Nef is involved in signal transduction. However, taken together, these results make the latter hypothesis attractive.

Thus, Nef may play a role at the beginning of a cascade including other membrane-associated proteins. In favour of the existence of such a mechanism, cross-linking experiments in T cells infected with vaccinia viruses expressing Nef have enabled us to visualize a 36–38-kDa protein or protein complex interacting with Nef (unpublished).

Since all these data are suggestive enough to justify the hypothesis that Nef may regulate some viral or cellular pathway, we have tried to identify protein or nucleotide targets for Nef.

**Nef and viral and/or cellular gene regulation**

The first results demonstrating a role for Nef as a regulatory protein gave its name to this factor (negative factor) (Terwilliger et al., 1986; Luciw et al., 1987) Nef has indeed been demonstrated to act at a transcriptional level, possibly on the negative regulatory element (NRE) of the viral LTR (Ahmad and Venkatesan, 1988; Niederman et al., 1989) although some controversy has been raised concern-
We have undertaken to examine the possible effect of Nef on HIV LTR by an indirect method, i.e., gel retardation showing the interaction of nuclear factors with promoter elements. We have identified such factors (called R and A1, or A family), with the A1 factor associated with antigen and IL2-dependent T-cell activation, and regulated by Nef (Guy et al., 1990b). A critical point of our study was that the presence of the A factors and their regulation by Nef were dependent on the cell context, and that we could obtain opposite results in different cells. Collaboration with Dr. M. Chuah Lay Kihm in Belgium has been initiated in order to define the functional role of the domains that we have mapped on the NRE, and preliminary results support our hypothesis that these domains are important for regulation ofLTR-promoter activity (M. Chuah Lay Kihm et al., in preparation). In the conclusion of the study of the nuclear factors regulated by Nef (Guy et al., 1990b), several hypotheses were considered. Regulation of the binding of A1 (activation-dependent nuclear factor) to the LTR by Nef may involve direct interaction between Nef and A1, or Nef activity may involve an inhibitor molecule neutralizing A1 in a manner such as IκB, which has been shown to modulate NFκB. Alternatively, if A1 is a complex, it is possible that Nef impedes its formation. It has been shown, for instance, in the case of the nuclear factor CREB or the complex IκB/NFκB, that the formation of DNA-protein or protein-protein complexes was dependent on phosphorylation-dephosphorylation. As we have shown that the nef gene product is involved in phosphorylation activities, it is tempting to speculate that Nef might alter the formation of an A1-related complex by a phosphorylation-dephosphorylation event. These different points are to some extent supported by structural similarities between Nef and leucine zipper transcriptional activation factors (Samuel et al., 1991).

Interestingly, and this makes a link between viral and cellular gene regulation, we have discovered (Guy et al., 1990b) that an oligonucleotide corresponding to a region of the IL2 promoter could efficiently compete for the binding of the A factor on the HIV LTR. This finding raised the possibility that Nef might regulate the expression of IL2; indeed a recent publication (Luria et al., 1991) has demonstrated that Nef downregulates IL2 production at a transcriptional level, thus demonstrating the influence of Nef on the physiology of the infected cell.

Moreover, we have shown (Guy et al., 1987), and this has been recently confirmed (Garcia and Miller, 1991), that Nef is able to regulate cell surface expression of the CD4 antigen. These results show at least two examples of cellular proteins (IL2 and CD4) regulated by Nef, and this is in good agreement with our hypothesis on the interaction between Nef and some cellular pathways. In addition, the CD4 and IL2 molecules are directly involved in T-cell activation and HIV infection, and this allows Nef to play a general role in HIV infection in vivo. In this respect, it is important to mention the study published by Kestler et al. (1991) which demonstrates the critical importance of the SIV nef gene for the maintenance of a high virus load and the development of AIDS in rhesus monkeys. It demonstrates for the first time the absolute necessity of this previously termed “non-essential” gene in the development of AIDS. We had previously asked the question: “if Nef is a Nef, why does HIV need a Nef?” (Guy et al., 1989). Results of research on SIV and the existence of regulation by Nef of important T-cell activation-associated molecules such as IL2 or CD4 have begun to provide an answer to such a question.

Conclusion

Looking back to our 1987 conclusions, our central claim that Nef was acting on cell regulation still holds. Our speculation on the role of Nef in latent infections has not yet been confirmed, at least in vivo, and the precise identification of Nef with well-established signal-transducing G proteins has not been demonstrated. However, we do believe that Nef is involved in signal transduction and is capable of binding guanine nucleotides, although we do not know if the latter property is essential to its function. However, it seems likely that the phosphorylation-dephosphorylation events associated with Nef are important for its activity. Finally, in the title of our first paper on Nef (Guy et al., 1987), we mentioned the word “oncogene”. This was probably not the best choice, and “cellular gene product” would have been more appropriate, since in the same superfamilly of oncogenes like ras, we may find factors both activating and repressing cell growth (Kitamura et al., 1989). We are presently looking for a cellular equivalent of Nef, and hope to be able to clone a Nef-like cellular gene.

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ON THE HIV nef GENE PRODUCT

...cy virus (HIV) nef protein in human astrocytes does not influence basal or induced HIV long terminal repeat activity. J. Virol., 64, 3059-3062.


Virological and cellular physiological roles of HIV Nef protein

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For the optimal execution of its life cycle, human immunodeficiency virus (HIV) requires the function of two small virus-encoded regulatory proteins Tat and Rev in association with cellular helper factors which may be tissue- or species-specific. Tat and Rev are expressed from a class of multiply spliced early viral mRNA (Muesing et al., 1985; Schwartz et al., 1990) which also encode the 27-kDa Nef protein. Unlike the Tat and Rev nuclear proteins, Nef is a 27-kDa membrane-associated myristoylated protein (Allan et al., 1985; Franchini et al., 1986) with no demonstrated nucleic-acid-binding activity and is dispensable for viral replication. Consistent with this observation, Nef proteins of HIV1 isolates exhibit significant divergence at the primary structure level and in some isolates, the Nef protein is prematurely terminated (Delassius et al., 1991). Although there is a consensus for the positive regulatory functions of Tat and Rev in viral replication, the functional roles for Nef are not agreed upon. However, several roles have been assigned to Nef, including (a) negative regulation of virus replication, (b) repression of LTR transcription, (c) GTP binding and GTPase activities, (d) modulation of cytoplasmic signalling via the protein kinase C pathway, (e) cellular gene regulation, and (f) a role in the maintenance of high virus loads in vivo.

Negative effects on virus replication

In view of the long period of latency before the onset of HIV1 disease in humans (Hoxie et al., 1985) and the restricted tropism of HIV (Klatzmann et al., 1984), the discovery of a virus-encoded negative factor suggested that Nef may be one of the determinants of viral latency in vivo. The earlier studies which showed that Nef-defective proviruses acquired accelerated replication potential over their wild-type counterparts (Terwilliger et al., 1986; Luciw et al., 1987) have been confirmed by other transient expression studies with HIV and SIV proviruses mutated in the nef gene (Ahmad and Venkatesan, 1988; Niederman et al., 1989; Niederman et al., 1991). Using stable Nef-expressing Jurkat lymphocytes and HeLa cell lines, up to ten-fold repression of HIV replication was demonstrated under transient expression conditions (Cheng-Mayer et al., 1989; Maitra et al., 1991). Since HeLa cells cannot be reinfected with progeny virus (unlike Nef+ CD4+ Jurkat cells), the reduction in virus production resulted from Nef effect on provirus expression.

Nef+ viruses replicated better than their otherwise isogenic Nef+ counterparts during acute infection of selected T lymphoid cell lines (Terwilliger et al., 1986; Luciw et al., 1987; Ahmad and Venkatesan, 1988; Niederman et al., 1989; Niederman et al., 1991). These observations have been challenged by other investigators (Kim et al., 1989). Infectivity of isogenic HIV strains which were Nef+ or Nef− was variable depending on the host cells and multiplicity of infection (m.o.i.). In A3.01 and H9 lymphocytes, at low m.o.i., Nef− viruses replicated better than Nef+ viruses. At higher m.o.i. there was no appreciable difference in the replication of Nef− and Nef+ viruses. Reconciling the repressive effects of Nef in the context of transient expression with the observed variability in the context of virus infection remains problematic. These differences may reflect cell-line-specific factors that act on Nef directly or that are involved in subsequent Nef effects.

Negative effects on viral transcription

We and others (Ahmad and Venkatesan, 1988; Niederman et al., 1989; Ahmad et al., 1989) have shown that Nef repressed viral replication through transcriptional inhibition of the HIV1 LTR. A far upstream cis element in the LTR, referred to as the negative regulatory element (NRE; Rosen et al., 1985) was identified to be the putative target of Nef

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GTP-binding and GTPase activities and oncogenic potential

Imperfect amino acid sequence homologies with the GTP-binding and GTPase domains of the known G proteins and HIV Nef have been demonstrated (Dever et al., 1987; Samuel et al., 1987). Nef expressed in E. coli was shown to be both a GTP-binding and a GTPase protein (Guy et al., 1987). Recent biochemical studies, however, have failed to substantiate the GTP-binding and GTPase activities of Nef (Kaminchik et al., 1990; Matsura et al., 1991; Nebreda et al., 1991). We expressed two different p21 ras proteins and four different Nef (1: TB Nef, Nef from the NL4-3 strain; 2: TB Nef01, a TB Nef mutant with THR in the place of Ala at position 15; 3: HB102 Nef, Nef from the BH102 strain; and 4: BruNef of the LAV strain) in E. coli and purified the proteins to near homogeneity. In contrast to ras, Nef-protein preparations had no detectable GTP binding. However, Nef proteins were phosphorylated when incubated in the presence of either GTP or ATP. The autokinase activity of Nef proteins with threonine at position 15 was higher than those with Ala at this site. Two different Nef proteins also failed to induce oncogenic transformation of permanently transfected NIH 3T3 cells under conditions that led to oncogenic transformation using activated ras genes. Unlike ras, Nef failed to induce meiotic maturation when injected into fully-grown Xenopus oocytes (Nebreda et al., 1991).

The lack of ras-like GTP binding or biological activity for Nef proteins is not surprising when the primary structures of these proteins are compared. The guanine nucleotide-binding ability of ras and other GTP-binding proteins like EF-Tu is contingent upon a distinct three-dimensional spacing of four consensus amino acid sequence motifs (Nebreda et al., 1991). Two of these motifs, GXGGXGK and DXSAX, are recognized by the phosphate groups of the guanine nucleotides, while the NKXD and EXSAX domains form the pocket that the guanine base fits in (Barbacid, 1987; Deshpande et al., 1987; Samuel et al., 1987; Pai et al., 1989; Milburn et al., 1990). The glycine-rich KEKGGLEG motif of Nef, designated "P" (Guy et al., 1990b), somewhat resembles the glycine-rich GXGGXGK motif in the ATP-binding site of proteins with kinase activity like src (Samuel et al., 1987) rather than the GXGGXGK motif of ras proteins. The glycine-rich regions of ATP-binding proteins show marked variability, unlike the GTP-binding proteins which show an absolute conservation of the GLY-rich motif. Although the NKGE motif in Nef (a.a. 156-160) is somewhat similar to the NKXD motif found in G proteins, mutating the D of the NKGD motif drastically reduces the affinity of ras proteins for GTP (Der et al., 1986; Feig et al., 1986; Sigal et al., 1986; Feig et al., 1988).

Nef has no sequence homology with the other two motifs, DXSAX and EXSAX that are critical for GTP binding of ras proteins. On the basis of these observations, it is felt that Nef may have a potential for interaction with the gamma phosphate of nucleoside triphosphates, in a manner analogous to ATP-binding kinases, but has no ability to specifically bind the guanine base of GTP. It is possible that phosphorylation of Nef, either by protein kinase C or through its autokinase activity, may be functionally significant in vivo.

Modulation of cytoplasmic signalling and cellular gene regulation

Purified Nef protein expressed in E. coli was phosphorylated by crude preparations of protein kinase C, predominantly at a Thr residue at position 15 (Guy et al., 1987) and the phosphorylation status of Thr at position 15 is presumed to influence the metabolic half-life of Nef protein expressed in HIV-infected T4 lymphocytes (Guy et al., 1990a; Laurent et al., 1990). Nef expressed from a vaccinia virus was shown to be phosphorylated at the same THR residue if Nef was first myristoylated (Guy et al., 1990). Substituting the Gly at position 2 prevent-
ed myristoylation of Nef, its cellular localization and metabolic turn-over (Guy et al., 1990a; Kaminchik et al., 1991). These observations lend credence to the premise that Nef may modulate membrane signalling and secondarily influence the expression of receptors such as CD4 and unidentified transcriptional factors which recognize sequences in the HIV1 LTR (Guy et al., 1987 and 1990b; Garcia and Miller, 1991). Although a protein-kinase-C-mediated mechanism was initially proposed for the above Nef effects (Guy et al., 1987), the downregulation of CD4 may not use this pathway (Garcia and Miller, 1991). A recent report (Luria and Berg, 1991) demonstrated that Nef expression in Jurkat T lymphocytes inhibited the transcriptional activation of both the endogenous IL2 gene and that of an exogenous IL2 promoter by signals from T-cell receptor-binding. Interestingly, only Nef protein with alanine at 15 was capable of this effect. Nef with Thr at 15 had no effect on IL2 transcription. The above observations reinforce the need to explore the potential role(s) of the phosphorylation status of Nef and, more specifically, the importance of the individual SER and THR residues of Nef in cellular physiology.

Pathogenic potential of HIV1 Nef

We have generated transgenic mice carrying HIV1 Nef linked to HIV1 LTR- or MMTV LTR-linked nef gene. Three of the six HIV1 LTR-linked nef transgenic lines expressed Nef exclusively in the skin. A significant fraction of the F1 and F3 progeny of these founders (30-70%) spontaneously developed persistent papillomas without other signs of pathology. Nef expression in the skin was localized to the basal cell layer of the epidermis and was confined to the diseased areas of the skin. Limited cutaneous expression of Nef may have reflected the permissivity of HIV1 LTR transcription in the CD4-positive Langerhan’s macrophages of the skin. In HIV1-infected humans, epidermal Langerhan’s cells may be a reservoir for the virus and, because they are antigen-presenting cells, their dysfunction may be germane to the many cutaneous manifestations of AIDS. Nef expression in the Langerhan’s macrophages of transgenic mice may have modulated the expression or release of cytokine(s) that control epidermal cell proliferation. Four of seven MMTV (mouse mammary tumour virus) LTR-linked nef transgenic lines expressed Nef predominantly in the mammary gland, salivary gland and seminal vesicle with no apparent pathological effect. A small number of the offspring of the three MMTV-Nef transgenic lines developed mild skin disease, and in the female mice the severity of the disease coincided with pregnancy. Nef expression was visualized in the affected regions of the skin of the MMTV-LTR-Nef transgenic mice (Dickie and Venkatesan, unpub. observ.).

Maintenance of in vivo virus load

A recent report demonstrated that although the cloned SIVmac viruses with a prematurely terminated nef replicated as well as their wt counterparts in tissue culture systems, they were unable to maintain high virus loads in monkeys and quickly reverted to wt Nef phenotype (Kestler et al., 1991). This has raised the possibility that important functional role(s) of Nef may operate in vivo to maintain virus growth and pathogenicity. Although these fascinating observations raise many questions, they are not necessarily incompatible with the in vitro Nef effects. If Nef moderates viral expression in vivo, it may actually contribute to the establishment of viral persistence which, over a period of time, may lead to progressive disease. On the other hand, Nef-defective viruses may replicate unchecked and be readily cleared by an alert immune surveillance system (Niederman et al., 1991). Alternatively, Nef may function as a superantigen in a manner analogous to the 3′ ORF in the MMTV LTR. For MMTV, it has been shown that the presence of superantigen may lead to immunosuppression (ablation of specific Vβ14 class of T cells) which may indirectly facilitate virus propagation (Choi et al., 1991; Marrack et al., 1991).

Future prospects

The basal transcription of the HIV genome is governed by the concerted interactions of several cis elements in the HIV LTR with numerous cellular transcriptional factors such as TFIIID, TFIIIB, SP1 and NFκB etc. The NRE region of the LTR, a presumptive target for Nef effects, has numerous cis elements including Apl, NFATI, NFATII, etc. Transcriptional regulation under homeotic conditions may be mediated by enhancer or enhancer accessory protein binding to these targets, and Nef may indirectly influence these interactions. A laudable effort to identify the Nef-responsive transcriptional factors has been reported (Guy et al., 1990b). These efforts may be expanded by the use of nuclear extracts of Nef-expressing cell lines to evaluate the DNA-binding potential of known cellular transcriptional factors. Specific targets of PKC, PKA, cAMP-dependent protein kinase and SRS pathways, may be examined in the context of Nef+ cell lines. Alternatively, specific sites within NRE may be mutated and the effect of Nef on the transcriptional potential of these mutants examined both under transient assay conditions and in the context of Nef+ cell lines.

Although it is unlikely that Nef protein is GTP-binding, phosphorylation of Nef either by itself or
by PKC, PKA or cyclic-AMP-dependent kinase may modulate a broad range of cellular physiological processes. Two potential cellular targets of Nef, namely CD4 and IL2, appear to be regulated by different mechanisms. Three Thr and two Ser residues in Nef may be phosphorylated by different kinases (Guy et al., 1990; Venkatesan, unpub. data) and since they may modulate different cellular and viral functions, careful analysis of mutations at these sites is in order. Apropos of the Nef effect on LTR transcription, Nef mutants which abolish myristoylation or force initiation from the second MET codon are markedly devoid of LTR effects (Yu and Felsted, 1991; Venkatesan, unpub. data) and are therefore likely candidates to explore the other role(s) of Nef in viral and cellular physiology. The internal —KEKKLEG— and the —NKENT— sequence in Nef, somewhat analogous to the —GXXGXK— and the —NNKXD— sequence found in ras proteins are also attractive mutagenic target to examine the possibility that Nef may have evolutionarily drifted from an ancestral viral “G-protein” with oncogenic potential. Finally, the potential superantigen-like behaviour of Nef may be evaluated using Nef-expressing murine or human B-cell targets with defined TCR phenotypes.

References


ON THE HIV nef GENE PRODUCT


ON THE HIV nef GENE PRODUCT

Functional analysis of HIV1 and SIV Nef proteins

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The conservation of the nef gene within the human and simian lentiviruses, HIV and SIV, suggests that its protein product, Nef, affords these viruses a survival advantage. Nef was found to be a negative regulatory factor with respect to virus replication in vitro, hence its name. What survival advantage could an inhibitory factor confer upon these pathogenic retroviruses? The relationship between Nef's suppressive effects in vitro and its biological role in vivo will be the ultimate goal of this review.

Nef is a transcriptional silencer

Initial progress towards the understanding of HIV1 Nef function in vitro suggested that virus replication was inversely related to Nef expression (Terwilliger et al., 1986; Luciw et al., 1987). In these studies, Nef- or Nef+ proviral DNA were transfected into lymphoid cell lines, and virus replication was monitored by cell-free, reverse transcriptase activity. In an attempt to identify the step in the virus life cycle affected by Nef, we transfected CD4+ and CD4- cells with either Nef+ or Nef- proviral DNA. Northern blot analysis of total RNA isolated from transfected cells indicated that the level of all viral mRNA species was 3- to 5-fold lower in the cells transfected with the Nef+ clone compared to the cells transfected with the Nef- clone (Niederman et al., 1989). Nuclear run-on analysis of transiently transfected cells revealed that Nef inhibited the rate of viral mRNA synthesis 2- to 3-fold. The suppressed levels of viral mRNA correlated with decreased levels of p24 gag core antigen and reverse transcriptase activity. Based on these results, we concluded that the primary function of Nef in vitro was to suppress virus expression at the RNA level. This conclusion was reached independently by other investigators (Ahmad and Venkatesan, 1988).

We then focused our studies on the function of SIV Nef. Although HIV1 and SIV Nef share only 38 % amino acid sequence homology, we found that SIV Nef was also a transcriptional silencer. In these studies, CD4- COS cells were transiently transfected with SIV Nef- or Nef+ proviral DNA. Northern blot and SI protection analyses demonstrated that viral mRNA levels were suppressed 2- to 6-fold in the cells transfected with the Nef+ clone compared to cells transfected with the Nef- clone (Niederman et al., 1991). Nuclear run-on analysis revealed that the rate of viral mRNA synthesis was inhibited 2- to 3-fold. Additionally, Northern blot analysis with RNA isolated from cells treated with the transcriptional inhibitor actinomycin indicated that Nef did not alter the turnover of viral mRNA. Again, we concluded that Nef's function was primarily to inhibit virus replication at the transcriptional level. Recently, others have also demonstrated negative regulation mediated by SIV Nef (Binninger et al., 1991).

Does Nef mediate transcriptional repression through specific elements present in the long terminal repeat (LTR)?

To determine whether the transcriptional effects of Nef were mediated through LTR sequences, we co-transfected COS cells with an HIV1 Nef-expression vector, pSVF, and a plasmid expressing the chloramphenicol acetyltransferase (CAT) gene driven by the HIV1-LTR. We demonstrated a dose-dependent suppression of CAT activity as the concentration of co-transfected pSVF was increased (Niederman et al., 1989). Other investigators have obtained similar results (Ahmad and Venkatesan, 1988; Cheng-Mayer et al., 1989; Maitra et al., 1991; Gama-Sosa et al., 1991). However, questions have arisen with respect to the validity of our conclusion that Nef

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was responsible for the decreased CAT activity (Hammes et al., 1989; Bachelerie et al., 1990; Luria et al., 1991). The objection raised was that LTR enhancer elements present in the nef expression plasmids, immediately downstream of the nef gene, were competing with the HIV-LTR-CAT plasmid for stimulatory transcription factors, thereby causing the dose-dependent inhibition of CAT activity. To address this possibility, we co-transfected the HIV-LTR-CAT plasmid with either increasing concentrations of pSVF or a plasmid containing only the HIV-LTR, pC15. We found that pC15 decreased CAT activity although not to the same extent as similar concentrations of pSVF (unpublished observation). This objection was addressed similarly by others (Maitra et al., 1991).

Does the multiplicity of infection (MOI) affect the ability of Nef to mediate transcriptional suppression?

Our initial efforts to compare the replication of HIV Nef- and Nef+ viruses in lymphoid cells indicated that the Nef+ virus afforded a 20-50-fold lower level of reverse transcriptase activity compared to its Nef- counterpart (Niederman et al., 1989). In that study, we generated virus by transfecting COS-1 cells with proviral DNA and then co-cultivating the COS cells with CD4+ Jurkat cells. Under these conditions, there is a relatively low titre of virus from COS cells and the majority of the reverse transcriptase activity arises from virus amplification through Jurkat cells. The details of this system are important to consider when comparing our results to those of others who have not observed HIV Nef suppressive effects in lymphoid cells (Kim et al., 1989). We believe that a difference in the initial MOI between our approach and that of Kim et al. may explain the disparate results.

It is reasonable to assess the relevance of the MOI, considering the relatively moderate effects of Nef compared to the effects of Tat or Rev. The hypothesis is that at relatively high MOI, subtle effects of Nef may be masked due to an overload of viral DNA templates. That is, Nef may require a limited pool of cellular factors in order to maintain transcriptional suppression. Once a cascade of virus replication has occurred, and high-titre virus results, it may be impossible for Nef to reverse or even halt the cascade.

To assess the effect of the MOI on Nef's suppressive capacity, we compared the replication of Nef+ versus Nef- viruses in lymphoid cells under conditions of varied MOI. We found that at relatively high MOI, replication of the Nef+ and Nef- viruses was indistinguishable as measured by reverse transcriptase activity. However, upon serial 10-fold dilutions of the initial virus inoculum, the replication of the Nef- virus was significantly inhibited (up to 25-fold) in several T-cell lines compared to its Nef counterpart (unpublished observation). In addition to generating lower levels of reverse transcriptase activity, the Nef+ viruses lagged by 4 to 8 days in comparison to the Nef- virus with regards to detection of reverse transcriptase activity (unpublished observation). This lag period may represent an in vitro form of latency. Recently, other investigators compared the replicative capacities of SIV Nef+ versus Nef- viruses. They also found Nef-mediated suppression to be dependent upon low MOI conditions and also observed a lag period for viral growth in the presence of Nef (Binninger et al., 1991). Finally, there are other examples demonstrating the relevance of the MOI when determining the function of other HIV-gene products including Vpr (Ogawa et al., 1989) and Vpx (Hu et al., 1989; Guyader et al., 1989; Kappes et al., 1991).

Other factors contributing to the differences observed between our laboratory and that of Kim and coworkers may involve different modes of virus transmission. In our system, Jurkat cells were co-cultivated with COS cells which were constantly shedding extremely low concentrations of virus, too low to be detected by reverse transcriptase activity. Viral transmission may be either through cell-to-cell contact or via cell-free virus. In contrast, Kim et al. (1989) used virus derived from lymphocytes and adjusted the level of the virus inocula based on measurable reverse transcriptase activity; additionally, there are several amino acid differences between our nef allele and that of Kim et al., and it is interesting to note that the Nef proteins differed with respect to their mobilities on SDS polyacrylamide gels.

Which form of nef is active?

Although the nef gene is present in all species of human and simian lentiviruses, there is significant sequence heterogeneity at this locus, second only to the surface envelope glycoprotein, gp120. There is up to 17% amino acid diversity on comparing the nef genes of different HIV isolates, and 62% amino acid differences on comparing HIV Nef to SIVmac Nef. It is possible that only some nef alleles encode functional Nef proteins while other alleles generate non-functional or impaired gene products. It is interesting to note that, by SDS-PAGE analysis, the nef gene used in our studies, HXB2/3 (Niederman et al., 1989), encodes a protein which migrates with a different apparent molecular weight than the Nef protein encoded by the virus used by Kim et al. (1989) even though the predicted size of the two proteins is the same. The observation that different Nef proteins of the same amino acid length migrate substantially differently from one another has been reported by others (Kaminchik et al., 1990). In addition, it is
unknown whether or not the propagation of viral isolates in vitro contributes to the rise of non-functional forms of Nef proteins.

What is the role of nef in vivo?

The ability of Nef to mediate transcriptional suppression in vitro may facilitate our understanding of the importance of Nef in vivo. It has been demonstrated that the doubly spliced viral transcripts are the first to appear in infected cells and 80% of those transcripts are Nef-specific (Robert-Guroff et al., 1990). Therefore, Nef expression is a major early event during infection. Nef expression early in infection may prevent Tat-mediated virus activation by inhibiting Tat's effects on the LTR and by inhibiting the further synthesis of Tat mRNA.

Nef may also inhibit activation of the infected lymphocytes. Virus replication has recently been shown to activate the positive transcription factor NF-kB (Bachelerie et al., 1991) and therefore, inhibition of replication would inhibit this positive feedback loop as well. Additionally, Luria et al. (1991) have shown that Nef suppresses the activation of the IL2 gene by external stimuli which lends further support for the contention that Nef may block primary lymphocyte activation. Activation of lymphocytes has been shown to be important in promoting accelerated virus replication in vitro (Nabel and Baltimore, 1987).

The end result of the Nef-mediated transcriptional suppression would be that a number of cells would remain latently infected and may provide a reservoir of infected cells that could become activated at a later time. This proposed consequence of Nef expression in vivo is analogous to the lysogenic phenotype of lambda phage in Escherichia coli. Conversely, viruses containing non-functional Nef proteins may replicate unchecked in infected cells, causing lysis of the cell and the inability to maintain a virus reservoir. This scenario resembles the lytic phenotype of lambda phage.

By restricting the amount of virus expressed in and released from infected cells, Nef may also contribute to the host's inability to mount effective humoral and cellular immune responses. Viruses expressing non-functional Nef proteins may be abundant soon after infection due to accelerated replication; however, the higher levels of virus may induce a heightened and more effective immune response compared to viruses expressing functional Nef. In the case of Nef-defective viruses, a heightened humoral immune response may clear cell-free virions from the host while heavily infected cells may be cleared more effectively by the cellular arm of the immune response. In either case, it may be that Nef-defective viruses are unable to establish a virus reservoir in the host, whereas Nef-expressing viruses may successfully evade clearance from the host by maintaining the level of virus expression in check. Therefore, Nef may be critical in maintaining a persistently infected state in the host.

A significant demonstration of the biological relevance of Nef in vivo was recently reported by Kestler et al. (1991). This work clearly demonstrated that there was selective pressure to maintain a functional Nef protein in rhesus macaques infected with SIV nef mutants containing a premature stop codon. In each case, the premature termination codon was replaced by a coding codon. Additionally, Kestler et al. (1991) concluded that Nef is important in maintaining persistent viral infection, with associated pathogenicity, and suggest that viruses lacking a functional Nef protein are slowly cleared from the host. The mechanism by which Nef mediates pathogenicity in vivo is not clear. However, we feel that our proposed role for Nef function in vivo may help to explain the results obtained by Kestler et al. (1991).

Conclusion

We and others have demonstrated that Nef is a negative regulator of HIV and SIV replication. Moreover, this regulation occurs at the level of transcription. That Nef is a negative regulator of virus expression suggests that Nef may play a key role in establishing and maintaining viral latency in vivo which may be critical for pathogenicity. Future experiments which may shed light on Nef's role in vivo will involve studies of the effects of Nef on immune responses, cell-free virus load and concentrations of infected cells. Understanding Nef activity and its ramifications in vivo may provide insight into the design of novel therapeutic agents.

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Points to ponder on the function of Nef

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Introduction

Although the subject of numerous studies, the function of the HIV nef gene (formerly 3'-orf, E', F' and orfB) in the life cycle of the virus still remains to be elucidated. Nef was first identified as a single open reading frame overlapping the 3' LTR of the HIV genome (Allan et al., 1985; Wain-Hobson et al., 1985; Arya and Gallo, 1986; Franchini et al., 1986a). The product of the nef gene is an immunogenic M, 27,000 polypeptide recognized by sera from patients infected with HIV (Allan et al., 1985; Franchini et al., 1986a, 1987). The mature polypeptide is myristoylated and localized to the extranuclear regions of infected lymphocytes, probably associated with the internal region of the plasma membrane by virtue of its myristoyl anchor (Allan et al., 1985; Kaminchik et al., 1990).

Early reports indicated that nef was not obligatory for virus growth in vitro and that mutations in its coding sequences resulted in more efficient virus replication (Terwilliger et al., 1986; Luciw et al., 1987). As a consequence of these and further studies, nef was believed to encode a negative factor (from which the name “nef” was derived) which exerts a suppressive effect on viral replication in trans through a negative regulatory target with the 5' LTR of the integrated provirus (Luciw et al., 1987; Ahmad and Venkatesan, 1988; Niederman et al., 1989; Garcia et al., 1987).

How, then, could Nef affect an integrated provirus when, unlike Tat and Rev, and as mentioned above, it is not demonstrably present in the nucleus? A possible mechanism was suggested by reports that Nef was both phosphorylated and capable of GTPase activity, functions shared by the oncogene products v-Ha-Ras and pp60-Src, the latter with which nef was reported to share some sequence similarities (Guy et al., 1987; Samuel et al., 1987). Therefore, it was proposed that Nef exerted a negative regulation on HIV replication through intermediary molecules from the host cell, and it was further suggested that Nef may be responsible for the apparent latent period between infection with HIV and progression to AIDS.

More recent reports, however, have contradicted the earlier findings. Hammes et al. (1989) reported that Nef expression does not have a negative effect on HIV replication, and both Kim et al. (1989) and Bachelerie et al. (1990) reported that there were no negative effects on HIV LTR-driven transcription attributable to Nef in either lymphoblastoid or myelomonocytic cells. The possibility of Nef exhibiting the enzymic activity of a G protein has also been questioned (Franchini et al., 1987).

In the following, we will briefly discuss some of the approaches we have taken, the reasons why we have taken them and the relevance of our findings to future attempts at understanding the biological function(s) of nef in the life cycle of HIV and, ultimately, the pathology of AIDS.

Expression of Nef mRNA in infected cells

We have used polymerase chain reaction (PCR) amplification of reverse transcripts of viral mRNA in infected cells to determine the relative abundance of the different transcripts which have undergone multiple splicing events. By using amplification primers which bind outside the splice donor and acceptor sites, and analysing Southern blots of the PCR products with oligomers representing all the possible exons, we were able to estimate the relative amounts of Tat, Rev and Nef mRNA (Robert-Guroff et al., 1990). Nef was by far the most abundant transcript in H9 cells chronically infected with HIV (HTLV-IIIIB). Of the multiply spliced mRNA detected, Nef comprised about 80%, Rev about 18% and Tat only about 2%. Approximately the same ratios were found in acutely infected (two days) PHA-stimulated normal human lymphocytes using the same strain of virus, and in macrophages infected for two weeks with the highly monocyte-tropic HIV (BA-L). The relative abundance of Nef transcripts in a variety of situations suggests that Nef has an important function.

We also observed that there were several forms of Nef which differed by the number of exons. Alternate splicing resulted in Nef transcripts with from
two to five exons. The most common form was the	hree-exon form, but some transcripts contained one
or two extra exons derived from upstream of the mid-
dle exon and may have derived from further splic-
ing of Vif and Vpr transcripts. In the infected
macrophages, the most abundant: Nef transcripts
contained only two exons, which could explained by
either single or triple splicing.

Conservation of nef with time in infected individuals

We have analysed the DNA sequences of the env
and nef genes from viruses isolated over periods of
five to eight years from several individuals (Guo
et al., manuscript in preparation). We found that
the rate of drift in the nef gene was approximately half
that of the env gene. The same general results have
been reported by Delassus et al. (1991) looking at se-
quences obtained directly from blood by PCR ampli-
fication. This conservation, like the high ex-
pression of Nef transcripts in infected cells, sug-
gest that Nef plays some critical role in the viral life
cycle.

What does Nef do at the plasma membrane?

Since the Nef polypeptide is not detectable as a
packaged product in mature HIV1 virions, it is prob-
able that it is not involved in the initial events of viral
attachment, fusion or entry into lymphocytes (Fran-
chini et al., 1986b). Localization to the inside of the
infected lymphocyte membrane (Allan et al., 1985;
Kaminchik et al., 1990; Franchini et al., 1986b) sug-
gests a possible role in post-transcriptional events
such as viral packaging and budding. Alternatively,
since Nef appears to be phosphorylated at
threonine-15 (Guy et al., 1987), it is possible that it
is involved in a cellular second-signal pathway (Guy
et al., 1987; Laurent et al., 1990). Therefore, we con-
cluded that it seemed reasonable to attempt to iden-
tify interactions between Nef, the cellular membrane
and/or membrane-associated proteins.

Cross-linking studies

A recent report by Resh and Ling (1990) indicat-
ed that a synthetic myristoylated N-terminal pp60-Src
polypeptide could be specifically cross-linked to a
M, 32,000-protein from vole fibroblast plasma
membranes. The sequence similarities between Nef
and pp60-Src mentioned earlier suggested that a
similar approach might be useful in identifying any
specific interactions between Nef and lympho-
cyte plasma-membrane-associated proteins (Guy et
al., 1987; Samuel et al., 1987; Bachelerie et al.,
1990).

The first question to address was which N-ter-
nal Nef sequence would be most useful for this
study. Both nef and env are subject to greater in-
terolate sequence diversity than are other HIV1
genes, and at the amino acid level this can be as high
as 17% non-identities (Rainer et al., 1985: Myers
et al., 1991). We also know that a high proportion
of integrated HIV1 proviruses are defective (as many
as 15% are defective for tat alone) (Meyerhans et
al., 1989). Consequently, many if not all of the
reported nef sequences could represent non-
functional genes.

After comparison of the known nef gene se-
quences (Myers et al., 1991), we chose a peptide that
represented the most conserved N-terminal residues,
including a threonine at position 15. The sequence
GGKWSKRSGVWGPTVRERMY (the C-terminal
 tyrosine residue was added to facilitate iodina-
tion) was synthesized as an N-terminal myristyl peptide (to
more nearly approximate the native molecule). The
myristyl peptide was radiiodinated and incubated
with a number of different human peripheral blood
 mononuclear cell (PBMC) membrane preparations
from both PHA-stimulated and unstimulated
PBMC. The membrane/peptide solution was coval-
ently cross-linked using an N-hydroxysuccinimide es-
ter (BS3) and analysed by SDS-PAGE and
autoradiography.

To date, we have not detected any specific inter-
actions between the N-terminal myristyl Nef peptide
and the plasma membrane preparations, although a
number of possible explanations present themselves
(in addition to the null hypothesis that there is no
interaction). Our peptide may not represent a func-
tional Nef sequence and may therefore be unable to
bind to its normal target. The peptide may be too
short to assume a proper configuration for binding.
Alternatively, the membrane may not contain the
proper target. Finally, Nef may be dependent on the
presence of other HIV1 proteins, necessitating the use
of membrane/cellular fractions from HIV1-infected
PBMC. We are pursuing this work further in an at-
tem to address these possibilities.

Conclusions

Although nef is not obligatory for HIV1 repli-
cation in vitro, a growing number of reports suggest
an important role in vivo. Kestler et al. (1991) have
studied nef using cloned simian immunodeficiency
virus (SIV) to infect rhesus monkeys. They demon-
strated that there is strong selective pressure in vivo
to maintain the full coding region for Nef by infe-
tion with SIV containing a premature stop codon.
In all cases examined, the stop codon was changed
in vivo to give a complete reading frame. They fur-
ther showed that SIV nef was necessary for both a
high viral load and full pathological effects. Much, however, remains to be determined about the function of nef in the life cycle of these viruses.

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Molecular characterization of HIV1 Nef protein

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Introduction

The involvement of nef gene in the life cycle and pathogenicity of HIV has been investigated in recent years by several groups. Considerable progress in the characterization of the gene and its products has been achieved. What is still lacking is a clear understanding of nef's biological role in the regulation of HIV replication in vivo. Here we review our studies on the expression of the nef gene, biochemical characterization of its products, and insights into Nef's cellular localization.

As the nef genes of different virus isolates show extensive sequence polymorphism — particularly among laboratory-propagated strains vis-à-vis patients' isolates — the choice of virus strain in the studies of nef gene function(s) is important. Indeed, some of the conflicting reports on the function of Nef may be attributed to strain specificity. To minimize this problem, we have compared throughout this work the nef genes of 3 virus isolates: BH10 (Ratner et al., 1985), LAV (Wain-Hobson et al., 1985) and JRcf (Cann et al., 1990). For BH10 nef, the internal termination codon was reconstituted based on LAV-nef primary sequence (Kaminchik et al., 1990).

HIV1 nef gene codes for two polypeptides

To analyse the pattern of nef-gene expression in mammalian cells, the gene was expressed in COS cells. Immunoprecipitation with specific antibodies revealed two Nef-related polypeptides with an apparent molecular weight of 27 and 25 kDa (designated Nef-p27 and Nef-p25). Nef-p27 initiates at the first AUG of the open reading frame, which is immediately followed by a sequence coding for myristoylation signal, NH2-Gly-Lys-Trp-Ser. Indeed, we have demonstrated myristoylation of Nef-p27 with 3H-myristic acid both in vitro and in cell culture (Kaminchik et al., 1991). The second gene product, Nef-p25, is initiated at an internal AUG codon 60 nucleotides downstream of the first AUG codon. This was demonstrated by expressing several deletion and substitution nef mutants and analysing the protein products. The conclusion that Nef-p25 is a primary translation product was confirmed by following the kinetics of Nef-p27 and Nef-p25 synthesis; no precursor-product relationships was observed in 35S-methionine pulse-chase experiments. Moreover, Nef-p25 was not modified by myristic acid, consistent with lack of myristoylation signal downstream of the second methionine codon in nef transnational frame.

Alternative translation initiation of the two Nef proteins was observed with BH10 and LAV nef genes. Although this mode of genetic control is rather rare in mammalian cells, translation of hepatitis B virus (HBS) surface antigens is also controlled by alternative translation initiation (Tiollais et al., 1985).

Cellular distribution of Nef

To further understand the function of the two Nef proteins in HIV1 biology, cellular localization of Nef was investigated. Cellular fractionation experiments have indicated that 30-50% of Nef-p27 is associated with an insoluble fraction of the cytoplasm, while the rest is found as a soluble component in the cytosol. In contrast, Nef-p25 is found only in the cytosol. Labelling experiments with 3H-myristic acid have indicated that myristoylated Nef-p27 was present both in the insoluble and the soluble fractions of the cytoplasm. Other experiments with a nef-gene mutant deficient in the myristoylation signal led us to conclude that myristoylation of Nef-p27 is required, but not sufficient, for its association with the particulate fraction of the cytoplasm. Internal Nef
domains, specifically amino acids 73-89, are critical for this specific interaction. Thus, it is conceivable that polymorphic sequences in nef genes derived from different HIV isolates account for differences in Nef's cellular distribution, as reported by several laboratories (Franchini et al., 1986; Hammes et al., 1989).

When the particulate cytoplasmic material was treated with nonionic detergent, most of Nef-p27 remained within the insoluble fraction. This unexpected observation prompted us to investigate the possibility that Nef-p27 is, in fact, associated with the cytoskeleton. Our preliminary results indicate that part of Nef-p27 co-fractionates with the cytoskeleton fraction of the cell. Subsequent genetic analysis has confirmed these observations and demonstrates that two determinants of Nef-p27 are important for interaction with the cytoskeleton — the myristic acid moiety and a domain encompassing amino acids 73-89. The cellular localization of Nef-p27 is reminiscent of that of Src pp60; in both instances, N-myristoylation and internal amino acid signalling are important for association with the insoluble fraction of the cytoplasm. The exact cellular component with which Nef-p27 interacts remains to be determined.

Biochemical characterization of Nef

To study biochemical activities of Nef, nef genes of 3 HIV isolates, BH10, LAV and JRcsf, were expressed in bacterial cells and purified to homogeneity. The respective recombinant Nef proteins were all soluble and found in a monomeric form. Expression levels are estimated at 10-30% of total bacterial proteins. Purification of Nef to apparent homogeneity was achieved by two column chromatographies (Kaminchik et al., 1990).

Previous studies have suggested that Nef expressed by Escherichia coli retains properties of a G protein, namely, binding and hydrolysis of GTP (Guy et al., 1987). We elaborated on these studies with recombinant Nef of the three HIV isolates (BH10, LAV-1, JRcsf). As a positive control of G protein, Ras p21 was included in a side-by-side experiment. The levels of GTP binding in extracts of bacteria expressing Nef were similar to that in cells lacking the nef gene. In contrast, extracts of bacteria expressing Ras p21 bound 10 times more GTP than control bacterial cells. Because of high endogenous GTPase activity, Nef-specific activity could not be measured in crude extracts. However, when GTPase activity, as well as GTP binding, were monitored during the purification of Nef, these activities did not co-purify with Nef. In our hands, purified recombinant Nef of the three HIV isolates (BH10, LAV-1 and JRcsf) exhibited no G protein-like activity.

It should also be noted that although certain domains in Nef show limited similarities to the three consensus elements of G proteins (Dever et al., 1987), the spacing of these sequences deviate substantially from that of G protein consensus elements (Samuel et al., 1987). Moreover, the first putative G-protein-like consensus sequence in Nef overlaps the AUG initiation codon and, thus, is partially upstream of nef coding sequence. Several explanations may be invoked to account for the apparent discrepancy between our observations and the results of Guy et al. (1987). First, bacterial protein impurities co-purified with recombinant Nef preparations may give rise to G-protein-like activities associated with endogenous bacterial proteins. Second, different purification procedures may affect G protein activities. To address this possibility, the recombinant Nef proteins were also purified according to the protocol reported before (Guy et al., 1987). However, as we originally observed, no G-protein-like activity was detected in any of the recombinant Nef (Guy et al., 1987).

Third, changes introduced during cloning of the gene may lead to translation initiation at a cryptic start codon(s) other than the first AUG (position 8,374). The protein expressed in our laboratory has the expected authentic sequence NH2-Gly-Gly-Lys-Trp-Ser. Fourth, subtle variations in the primary sequences of LAV nef used by the two laboratories may have also led to different observations. As some properties of Nef are reminiscent of Src pp60, namely myristic acid modification and cellular localization, recombinant Nef proteins were also tested for protein kinase activity. However, neither kinase nor ATPase activity was detected in any of the three Nef preparations.

In summary, data presented here demonstrate that nef codes for two proteins, Nef-p27 and Nef-p25, by a mechanism of alternative transnational initiation. Nef-p27 is a myristoylated protein, partially associated with various components of the cytoplasmic particulate fraction. Nef-p25 is a soluble monomeric protein found exclusively in the cytosol. The association of Nef-p27 with the cytoskeletal infrastructure may be related to its biological role in vivo, an area under intense investigation. No G-protein-like activity was detected in purified recombinant Nef coded by nef genes of three different HIV isolates.

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References

Downregulation of cell surface CD4 by nef

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Nef is a 27-kDa protein encoded by a single ORF (open reading frame) that overlaps with the 3' LTR in HIV (Ratner et al., 1985a). Nef is myristylated at the amino terminus and is found associated with the intracellular portion of the cell membrane (Franchini et al., 1986). The name "Nef" is an acronym that stands for negative factor. The name was given to this protein based on the observation by some investigators that Nef had a negative effect on the HIV LTR as well as on HIV replication (Ahmad and Venkataraman, 1988; Cheng-Mayer et al., 1989). However, this observation has been disputed by others (Kim et al., 1989; Hammes et al., 1989).

Guy et al. (1987) observed a marked decrease in CD4 cell surface levels in a human T-cell line (CEM) expressing nef (Bru isolate) via a vaccinia vector. Cheng-Mayer et al. (1989), on the other hand, concluded that expression of nef (SF2 isolate) by using plasmid vectors had no effect on CD4-cell surface levels. There is no precedent in any other retrovirus for downregulation of its receptor by a retroviral protein other than the virus envelope. Because CD4 downregulation of HIV-infected cells might in principle play an important role during HIV infection in vivo, we developed a retroviral vector for nef expression (SF2 isolate) based on the Moloney murine leukaemia virus. This retroviral vector, LnejSN, has been used to express nef in human and murine cells to determine the effect of Nef on CD4 cell surface levels. Some of our observations are described in the following paragraphs.
Human CD4 downregulation by nef is specific and cell-type-independent

Retroviral-mediated transfer of the nef gene into human cell lines of T, B and monocyte/macrophage origin (HPBALL, AA2 and U937) expressing CD4 resulted in a significant decrease in cell surface CD4 levels (Garcia and Miller, 1991). In all cases, cell surface CD4 expression was found to be inversely related to nef expression. Transduction of nef into HeLa-CD4⁺ cells also resulted in lower CD4 cell surface levels (Garcia and Miller, 1991). More recently, we have introduced the nef gene into the Jurkat and Hut-78 cells described by Cheng-Mayer et al. (1989) and found that, under our experimental conditions (Garcia and Miller, 1991), CD4 is downregulated in these two cell lines as well (Garcia and Miller, unpublished observations). These results indicate that the effect of Nef on CD4 cell surface levels is not restricted to a specific cell type. Therefore, it might occur in all cell types infected by HIV in vivo. Under our experimental conditions, nef expression had no significant effect on cell surface levels of human CD8 or the human transferrin receptor (Garcia and Miller, 1991). These results indicate that nef is relatively specific for CD4 and that CD4 downregulation is not a result of a non-specific effect. HIV LTR sequences overlapping with the nef ORF and present in LnefSN might compete for transcription factors and have an effect on CD4 cell surface levels. Such a possibility was tested by using the retroviral vector LfenSN (Garcia and Miller, 1991).

In LfenSN the same DNA fragment containing the nef gene was cloned in the reverse orientation. No downregulation of CD4 cell surface levels was observed in cells transduced with LfenSN (Garcia and Miller, 1991). This result indicates that the HIV-LTR sequences present in LnefSN do not play a significant role in CD4 downregulation. There is an apparent discrepancy between our conclusions and those of Cheng-Mayer et al. (1989). However, our interpretation of the data presented by Cheng-Mayer et al. (1989) is that indeed CD4 is downregulated by nef. Thus, CD4 downregulation by nef is relatively specific and cell-type-independent.

CD4 is “trapped” inside cells expressing nef

Comparison of the steady-state levels of CD4 mRNA between control cells and cells expressing nef by Northern blot analysis indicated that Nef does not alter CD4 RNA levels (Garcia and Miller, 1991). This result suggests that nef does not act on the CD4 promoter, this is consistent with the observed downregulation by nef of CD4 in HeLa-CD4⁺ cells in which the CD4 RNA was transcribed from a heterologous (CMV) promoter (Kestler et al., 1991). Therefore, it is unlikely that the downregulation of cell-surface CD4 by nef is caused by a negative effect of Nef on CD4 transcription. Because Nef might act at the level of translation, steady-state levels of CD4 protein and its stability were determined. No significant differences were observed on the steady-state levels or the rate of degradation of the CD4 protein (Garcia and Miller, 1991). Those results indicated that Nef does not alter CD4 biosynthesis. Further analysis of CD4 in HPBALL cells expressing nef by using immunofluorescence and confocal microscopy displayed almost all fluorescence within the cell (Garcia and Miller, 1991). The use of the HPBALL T-cell line with a large nucleus and a small cytoplasm for the above experiments precluded the identification of the precise location of the intracellular CD4. However, we could rule out a nuclear localization. The fact that CD4 could be found inside the cells expressing nef was in complete agreement with the lack of cell surface CD4 determined by FAC5 analysis as well as with no significant effect on CD4 biosynthesis by Nef.

CD4 downregulation by nef is independent of serine phosphorylation

CD4 is downregulated from the cell surface by phorbol esters (Hoxie et al., 1986; Acres et al., 1986). Phorbol esters induce protein kinase C (PKC), which in turn phosphorylates CD4 at 3 intracellular serine residues (positions 408, 415 and 431). Mutation of all 3 serine residues to alanine prevents the phorbol-ester-induced downregulation of CD4 (Shin et al., 1990). CD4 downregulation by nef resembles that induced by phorbol esters. To test whether these serine residues are necessary for -Nef-mediated downregulation of CD4, HeLa cells expressing this mutant were transduced with LnefSN. Nef expression in these cells resulted in a significant downregulation of the cell surface levels of the mutant CD4. Therefore, Nef-induced downregulation of cell-surface CD4 expression is independent of these three phosphorylation sites, suggesting that it occurs by a protein-kinase-C-independent mechanism.

Nef itself has been shown to be phosphorylated (Guy et al., 1987), and phosphorylation of Nef at the Thr residue in position 15 has been postulated as important for nef function (Guy et al., 1987). The nef gene from ARV-2 has an alanine residue at position 15 and yet is fully functional as determined by its ability to downregulate CD4 (Garcia and Miller, 1991). Therefore, it seems unlikely that phosphorylation by PKC at Thr-15 plays a significant role in CD4 downregulation by nef.
Why downregulate CD4 from the cell surface?

In humans infected with HIV, a decline in CD4+ T cells usually marks the onset of progressive immunological disease (Gallo, 1990). Our results and those of Guy et al. (1987; Garcia and Miller, 1991) indicate that expression of the nef gene of HIV1 results in CD4 downregulation. Because the number of HIV-infected cells in vivo is only a small fraction of the total number of CD4+ cells, it is unlikely that this downregulation of CD4 from the cell surface is responsible for the loss of CD4+ T cells in HIV-infected individuals. However, downregulation of cell surface CD4 by nef could be advantageous for the survival and spread of HIV in vivo. By removing the CD4 molecule from the cell surface, the virus may (1) impair a pathway of T-cell activation, (2) minimize the toxic effects of envelope expression as well as cell death syncitia formation, and (3) lower the chance of superinfection which might result in cell death. Thus, nef might play a role in HIV persistence in vivo.

The HIV-infected CD4+ cell paradox

If HIV infection results in nef expression, why is it that in samples from HIV-infected individuals only CD4+ cells seem to harbour HIV? This apparent paradox is the subject of study in several laboratories including ours, but it is a difficult question to address. All experiments reported to date dealing with CD4 downregulation by nef have been restrictive in that only one of the HIV genes (e.g. nef) is being expressed. Such experiments circumvent the obvious problems associated with HIV-envelope expression in CD4+ cells. One explanation could be that CD4 downregulation by nef is a temporal effect that occurs only early after infection during which no structural genes are expressed. Another is that CD4 downregulation might occur in cells harbouring HIV which are destined to be "dormant" or not expressing HIV structural genes.

It should be noted that most if not all the studies mentioned above have only examined the presence of HIV in peripheral blood lymphocytes. However, alterations in the expression of cell surface antigens might result in a redistribution of T cells in the organism and, to our knowledge, similar studies have not been carried out in other organs or tissues known to harbour T cells.

At least two important questions remain to be addressed with respect to CD4 downregulation by HIV: first, in the context of HIV provirus, does nef expression result in CD4 downregulation, and second, what is the effect that expression of other HIV genes, especially regulatory genes such as tat, rev and vpu, has on CD4 downregulation by nef? Experiments geared to address these two questions could result in information that might clarify the role of nef in HIV infection.

Does the nef from primary isolates of HIV downregulate CD4?

There is significant variation at the amino acid level between the nef genes from different HIV isolates (Ratner et al., 1985b). At present, only two have been determined to be functional by their ability to downregulate CD4 (SF2 and Bru). To determine which is the "real" nef and which is its "real" function, an effort has to be made to determine the structure of a variety of nef primary isolates (from HIV1 and 2 and SIV) never passed in culture and therefore representing the "true" nef gene(s) found in vivo. Analysis of their sequences, their ability to downregulate CD4, their effects on the HIV LTR etc., should clarify the "true" function of nef.

What is the relevance of CD4 downregulation by nef in vivo?

Recently, Kestler et al. (1991) reported that SIV-nef expression is required for maintenance of a high virus load and progression to AIDS in vivo. These results indicate that nef plays an important role during infection in vivo, yet nef is totally dispensable during infection in vitro. It is clear that nef is a good target for antiviral drug development. Even though at present it is not obvious what the role of CD4 downregulation by nef in vivo might be, CD4 downregulation by nef represents an assay for nef function in vitro. Therefore, CD4 downregulation by nef might be used to evaluate antiviral drugs targeted to nef in vitro.

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References


ON THE HIV nef GENE PRODUCT


The nef gene products:
biochemical properties and effects on host cell functions

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Of all non-essential genes conserved in HIV and SIV retroviruses of primates, nef is probably the most controversial, as completely opposite views (negative, neutral or positive regulatory element) on its biological role have been reported (summarized in Haseltine and Levy, 1991; Venkatesan et al., 1991). Although it has been suggested that different nef alleles may show different and even opposite effects on viral replication, the variety of conflicting in vivo and in vitro results reported suggest to us also that the cellular context in which nef is studied may well determine the different biological effects observed. Therefore, rather than concentrating on the role of nef on the viral cycle, it may prove more useful at this time to study the primary biochemical and genetic properties of nef that underly the so far contradicting biological behaviour. Here, we will discuss work from our lab and others dealing with the study of (1) the direct biochemical properties of purified Nef proteins as well as (2) the effect of exogenous nef expression on host cell functions in a variety of biological systems.

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Structural properties

The Nef mRNA (about 2 kb in size) originates from an open reading frame (ORF) located at the 3' end of the viral genome, overlapping the U3 region of the LTR. The nef translation product(s) are in fact synthesized in vivo and may play an early role in the infectious process since antibodies against Nef appear early in infection and may even precede the occurrence of antibodies against structural proteins (Gombert et al., 1990).

The full-length, mature form of Nef protein is a 27-kDa protein (207 amino acids in length) possessing a consensus myristoylation sequence at the NH2 terminus. There appears to exist significant divergence in the primary sequence between different strains (Delassus et al., 1991). This sequence divergence appears to be the cause of differences in electrophoretic mobility that we and others have observed, for example, between LAV Nef and other (BH10, V102, NL43) Nef strains (Kaminchik et al., 1991; Nebreda et al., 1991).

It seems firmly established now that two Nef-related peptides of 27 and 25 kDa can be the translation products of the single nef gene. Both proteins exhibit different turnover rates and cellular localization and may play different roles, although this is unknown at this time. The higher molecular weight peptide is myristoylated and localizes to the plasma membrane. The 25-kDa protein appears to originate by initiation at an ATG located 57 bp downstream of the Nef initiation site, and is localized cytoplasmically; it also appears that the mature, myristoylated form has a significantly longer half-life than the shorter, cytoplasmic 25-kDa form (Kaminchik et al., 1991). It is interesting to note that certain mutations (Ala-Thr15) that appear to shorten the half-life of the 27-kDa form (Laurent et al., 1990) also seem to alter the biochemical properties (Nebreda et al., 1991) and the biological properties of Nef (Laurent et al., 1990; Luria et al., 1991) (see below).

Biochemical properties

The nef gene product has been reported to possess partial amino acid sequence homology with certain oncogene products like src and ras (Guy et al., 1987; Samuel et al., 1987). Of particular interest were the reports that Bru Nef proteins exhibit GTP binding and GTPase activities in a manner similar to ras proteins (Guy et al., 1987, 1990). On the basis of these homologies, Nef was suggested to act as an oncogene or a G-like protein capable of modulating second-messenger signalling pathways of cellular activation. We tested this hypothesis by expressing cDNA clones of Nef and ras using the same bacterial and eukaryotic expression vectors, and comparing the biochemical and biological properties of their respective expressed gene products.

Nef is not a GTP-binding protein

We have tested the Nef proteins of 3 different HIV strains (BH102, Bru and NL43) expressed in bacteria for their ability to bind guanine nucleotides, in comparison to ras proteins used as controls. Two different assays (reactivity with α-32P-GTP after immobilization of these proteins on nitrocellulose filters following SDS/PAGE, or quantitative filter-binding assays of soluble proteins) were used in order to obtain definite unequivocal results. In all cases, the GTP-binding levels of Nef corresponded to background binding obtained with a ras mutants lacking affinity for guanine nucleotides (Nebreda et al., 1991). Similar negative results were also obtained under a variety of binding conditions and irrespective of whether non-hydrolysable nucleotide γ-32S-GTP or the hydrolysable nucleotide α-32P-GTP were used. Our results are essentially consistent with recent reports from other laboratories (Kaminchik et al., 1990; Matsuura et al., 1991) and allow us to conclude unequivocally that the Nef proteins lack a genuine GTP-binding activity comparable to that of ras and other G proteins. The discrepancy between these results and those of Guy et al. (1987) concerning GTP binding are more than likely explained by the presence of bacterial protein impurities in the partially purified Nef preparations used in that initial report.

The lack of ras-like GTP-binding activity in the Nef proteins is not surprising when the primary structure of these proteins is compared (Nebreda et al., 1991). The nucleotide-binding ability of ras and most other GTP-binding proteins is absolutely dependent on the distinct three-dimensional spacing of 4 consensus sequence motifs present in their molecules (Dever et al., 1987; Santos and Nebreda, 1989). Two of these sequence motifs, GXXXXGK and DXGX, are involved in interaction with the phosphate groups of the bound guanine nucleotides, while the other two motifs, NKXD and EXSAX, form the pocket that the guanine base fits in. Interestingly, the first motif, GXXXXGK, is a glycine-rich sequence (GXGXXGK in ras proteins) and is similar to a glycine-rich consensus motif present in the ATP-binding site of proteins with kinase activity like src (Samuel et al., 1987). However, the glycine-rich regions of ATP-binding proteins show marked variability in the sequence unlike the GTP-binding proteins which require an absolute conservation of this motif (Dever et al., 1987).

The analysis of the amino acid sequence of Nef proteins reveals the presence of a glycine-rich motif (a.a. 94-99) similar to that present in ATP-binding
proteins. The linear sequence of this motif showed (1) an imperfect match (one less X residue) and (2) an inverted orientation with respect to the GXXXXGK sequence of GTP-binding ras proteins. Furthermore, although an NKGE motif in Nef (a.a. 156-160) could be loosely interpreted as a degenerate relative of the NKXD motif found in G proteins, Nef has no sequence counterparts for the other 2 motifs, DXXG and EXSAX, that are critical for GTP binding of ras proteins.

This analysis of the amino acid sequence and our experimental observations led us to propose that Nef has a potential for interaction with the gamma phosphate of nucleoside triphosphates, in a manner analogous to ATP-binding kinases, but not an ability to specifically bind the guanine base of GTP.

**Nef autophosphorylation**

Autophosphorylation in the presence of GTP was also initially described as a property of Bru Nef protein (Guy et al., 1987). Since cellular ras proteins lacking Thr at position 59 are not autophosphorylated by bound GTP (Santos and Nebreda, 1989), we evaluated the autokinase activity of various purified Nef proteins using ras proteins as negative controls. Thus, in contrast to control ras proteins, purified Nef proteins from 3 different HIV strains showed autophosphorylation activity when incubated in the presence of hydrolyzable $^{32}$P-GTP. Surprisingly however, Nef autophosphorylation was not specific for GTP as previously suggested (Guy et al., 1987). We observed that $^{32}$P-ATP could readily substitute as a phosphate donor in our reaction mixtures (Nebreda et al., 1991).

We also observed that almost similar levels of phosphorylation of the Nef proteins were obtained when the assays were performed at 0°C or at 37°C, strongly suggesting that the observed phosphorylation is an intramolecular (autophosphorylation) rather than an intermolecular reaction. This makes the possibility very unlikely that a separate, contaminant kinase activity may be responsible for the observed Nef phosphorylation. The fact that the BH102 and Bru Nef proteins, purified by completely different procedures than our PTBNef proteins, showed the same phosphorylation activity in presence of GTP and ATP, further supports that contention. Analysis of the phosphorylated Nef proteins showed also that the autophosphorylation label is acid-stable but is removed in the presence of hot alkali, indicating the involvement of serine and/or threonine residues. In fact, our results indicate an important participation of Thr15 in the process of autophosphorylation: when the Ala15 of pTNBNef was changed to Thr (pTBNef01), the resulting Nef exhibited significantly higher levels of autophosphorylation. Thr15 has also been reported to be phosphorylated by PKC (Guy et al., 1987) and to influence CD4 downregulation as well as the phosphorylation state and metabolic half-life of Nef proteins expressed in HIV-infected T4 lymphocytes (Laurent et al., 1990; Guy et al., 1990). However, other residues beside Thr15 must also be important for Nef autophosphorylation since BH102 Nef, which exhibits higher levels of autophosphorylation than pTBNef, has Ala instead of Thr at that position. The Ser residue at position 163 in BH102 Nef (substituted for Arg in pTBNef) may be responsible for this differential phosphorylation.

In agreement with the earlier report (Guy et al., 1987), the autophosphorylation detected in our assays (Nebreda et al., 1991) appeared to be rather inefficient, with a calculated stoichiometry equal to or lower than 0.01 moles $^{32}$P per 100 moles of Nef protein. Because of this low stoichiometry, it remains to be determined whether the phosphorylated Nef forms reflect a genuine autokinase activity of the proteins or are merely catalytic intermediates in the transfer of the phosphate group.

All the evidence discussed above indicate that autophosphorylation is most likely an intrinsic property of Nef proteins reflecting their ability to have a broad interaction with the triphosphate moiety of a variety of nucleotides rather than specifically recognize the guanine base of GTP, like ras and other G proteins do. The phosphorylation of Nef, either by protein kinase C or through its autokinase activity, may be functionally significant in vivo.

**Interaction of nef gene products with host cell functions**

The introduction by various means of Nef into adequate cells may offer an instrument to study Nef function and compare it to ras and other oncogenes. So far Nef has been introduced in a variety of cell types ranging from amphibian oocytes to cultured mammalian fibroblastic, epithelial or haemopoietic cells.

The expression of nef in *Xenopus* oocytes and in transfected NIH 3T3 fibroblasts (Nebreda et al., 1991) allowed us to conclude that Nef differs from ras not only at the biochemical level but also in its biological properties. Thus, microinjection of 3 different bacterially expressed Nef proteins (or their *in vitro* generated mRNA transcripts, to facilitate co- and post-translational modifications) in the cytoplasm of fully grown oocytes, failed to induce any significant meiotic maturation under conditions where ras proteins or mRNA-induced 100 % germinal vesicle breakdown (GVBD). On the other hand, expression of Nef in NIH-3T3 fibroblasts does not result in any of the morphological changes associat-
ed with transformation nor in anchorage-independent growth (Nebreda et al., 1991). Similarly, constitutive heterologous expression of transfected Nef in HeLa or Jurkat cells (Maitra et al., 1991), or transient expression in COS cells (Kaminchik et al., 1991), did not result in any obvious new morphological phenotype.

We noticed however, that when nef was transfected along with a neo-selectable marker into NIH3T3 cells the number of Nef-expressing clones were rarer than expected among the neo' colonies obtained. A similar observation has been described (Luria et al., 1991) in transfected T cells, raising the possibility that high levels of Nef might be cytotoxic.

One of the most interesting phenotypes described in association with exogenous nef expression is the downregulation of CD4 in those cells expressing this molecule. Although the initial report (Guy et al., 1987), based on using a recombinant vaccinia virus carrying Nef was challenged (Cheng-Mayer et al., 1989), recent studies using a retroviral vector as the means of expression in lymphocytic cell lines (Garcia and Miller, 1991) seem to confirm that in all cases cell surface expression of CD4 is inversely related to Nef expression. The mechanism for this remains unknown although it appears not to involve a PKC-dependent pathway.

Of special interest is also the recent report that expression of transfected nef in Jurkat T cells interferes with signals emanating from the T-cell receptor (TCR) complex that induces IL2-gene transcription (Luria et al., 1991). Strikingly, only nef alleles carrying Thr15 (plus Arg29 and Ala33) are able to interfere with TCR signals and prevent antigen-receptor-mediated induction of IL2 mRNA (Luria et al., 1991). It is pertinent to note that nef alleles lacking Thr at position 15 lack the ability to downregulate CD4 (Laurent et al., 1990; Guy et al., 1990). This points to a likely important role of Thr15, and its possible in vivo phosphorylation, in a variety of functional processes affected by Nef.

Both the downregulation of CD4 and the blockade of IL2-mRNA production in response to TCR stimulation may have direct significance to the pathogenesis of AIDS. Possible mechanisms have been put forward in both cases (Garcia and Miller, 1991; Luria et al., 1991) to explain how these phenomena may contribute and lead ultimately to the T-cell population demise.

Highly suggestive in the line of thinking that links Nef expression to immunological decay of host cells, is the demonstration that a superantigen is encoded in the open reading frame of the 3'-terminal repeat of the retroviral MMTV sequence (Marrack et al., 1991). Furthermore, the presence of that superantigen has been linked to immunosuppression (disappearance of the specific target VB14-bearing T cells) occurring in the infected animals (Choi et al., 1991). These observations are highly reminiscent of the location of the nef ORF in HIV and SIIV genomes, and of the depletion of the CD4+ cells in HIV infection. Both the location of the ORF at the 3' LTR and the possible involvement in T-cell depletion are tantalizing analogies between superantigens and Nef that, although yet highly speculative, may be worth testing in the near future.

Conclusions and perspectives

The still unknown mechanism of action of Nef proteins is certainly not similar to that of ras or G-like oncogenes. The Nef proteins lack the transforming and GVBD-inducing activity of ras proteins and, as suggested by the absence of all the required consensus sequences, they also lack any GTP-binding or GTPase activity. However, Nef proteins appear to be able to interact with the gamma phosphate of GTP and ATP and become autophosphorylated. A threonine residue at position 15 appears to be involved in the auto-phosphorylation process and has also been suggested to be a target for PKC in vivo. nef alleles affected in this residue show differences in phosphorylation and stability of the protein product, and confer specific phenotypes to host cells regarding CD4 downregulation or T-cell receptor complex signaling. A possible role of nef in the progressive immunological deterioration typical of AIDS should be explored.

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Characterization of the Nef protein in HIV1-infected CEM cells

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Introduction

The function of the Nef protein still remains highly controversial, although earlier reports from several groups have defined it as a negative factor on the basis of its capacity to downregulate HIV replication (see Cullen and Greene, 1989, for references). In collaboration with Marie-Paule Kienv and Bruno Guy of "Transgene" (Strasbourg), we investigated the synthesis of the Nef protein in CEM cells infected with a highly cytopathic HIV1 stock prepared from the HIV1 Bru isolate (Laurent et al., 1990).

Our biochemical and biological studies indicated that the nef gene of this cytopathic virus had two important mutations affecting phosphorylation by protein kinase C and the putative GTP-binding site, which is responsible for the proper folding of the Nef protein. As a consequence of these mutations, the
Nef protein was not phosphorylated and its half-life was reduced. In addition, the mutated Nef protein was unable to down-regulate the expression of CD4 on the surface of CEM cells. These results suggest that some naturally acquired mutations of the nef gene produce a biologically inactive product. By such a strategy, latent HIV might become virulent in cell cultures.

**Virus infection**

For these studies, the HIV1 Bru isolate now referred to as HIV1 Lai (Wain-Hobson et al., 1991) was passaged approximately 10 times in the human lymphoid CEM-T4 cell line, generating a highly infectious virus stock (HIV1 Hi). A virus preparation characterized by slow kinetics of infection (HIV1 Lo), obtained from the HIV1 Lai isolate at an early passage, and an infectious molecular clone p-BRU2 (K. Peden, manuscript in preparation) were also used for comparative purposes. Infection of CEM cells with HIV1 Hi was monitored by the cytopathic effect, involving vacuolization of cells and appearance of small syncitia. At different times after infection, cells were labelled metabolically with $^{35}$S-methionine and $^{35}$S-cysteine to investigate the synthesis of viral proteins.

**The detection of the Nef protein**

The synthesis of the Nef protein in infected cells is studied by metabolic labelling of cells and immunoprecipitation of extracts with HIV1-positive patient serum. The metabolic labelling should be for a short period such as 45 to 90 min. Longer periods of labelling result in the accumulation of comparatively large quantities of p25 produced by the cleavage of the Gag precursor. Since the level of the Nef protein is low, its detection then becomes masked by the large quantities of p25. The labelled Nef protein can be characterized by electrophoresis in polyacrylamide-SDS gels or analysed by two-dimensional gel isoelectric focusing. Under both experimental conditions, the low level of p25 following the short periods of labelling can be used as a convenient marker to localize the Nef protein which migrates slightly slower than p25.

**The Nef protein of HIV1 Hi has an unusually short half-life**

Following PAGE analysis, the Nef protein of HIV1 Hi migrated as a single band of approximately 27 kDa which corresponded to the fastest of the two species described previously in HIV1-infected human T cells (Franchini et al., 1986) as well as in hamster BHK21 cells infected with the vaccinia virus recombinant expressing the nef gene (Guy et al., 1987). On two-dimensional gel isoelectric focusing, the Nef protein was resolved as a single spot with an isoelectric point (pl) of 6.2. This result indicated that the product of the nef gene of HIV1 Hi is homogenous and is probably not phosphorylated, since a covalently bound phosphate moiety would cause a slight shift in its pl. Consequently, in the case of phosphorylation, it is usual to find two spots with related pl values corresponding to the phosphorylated and unphosphorylated forms. Interestingly, the Nef protein synthesized in cells infected with the original low-replicating virus HIV1 Lo or with the molecular clone p-Bru2 was resolved as two spots, the second spot having a slightly more basic pl of 6.4.

As reported previously (Franchini et al., 1986), the Nef protein was found to be cytoplasmic. The Nef protein was not detected in the nuclei of infected cells nor in their culture medium. Pulse-chase experiments indicated that the Nef protein of HIV1 Hi had a very short half-life of approximately 30 min. In contrast, the half-life of the Nef protein of HIV1 Lo and that of p-Bru2 was estimated to be around 4 h. The reason for the unusually reduced half-life of the HIV1 Hi Nef protein is most probably its rapid degradation due to specific mutations of the nef gene (see below).

The Nef protein is synthesized late in infection, concomitantly with the gag- and env-gene products

Infection of CEM cells with the HIV1 Hi stock results in kinetics of infection which last 4 days, i.e., 80-90 % of cells become producers of HIV1 proteins (detected by immunoenzymatic staining assay) at 3 days post infection (p.i.) and the cells start to die from the fourth day onwards. During this infection, the Nef protein becomes clearly detectable on the third day p.i., in parallel with the envelope and Gag precursors and their cleaved products. The late synthesis of the nef protein is not specific to the HIV1 Hi preparation since similar results are also observed in the case of HIV1 Lo and p-Bru2. For example, in the case of HIV1 Lo infection, about 90 % of cells become positive for the synthesis of viral proteins 6 days p.i., and cell death occurs on the seventh day p.i. In this latter infection, synthesis of the Nef protein occurs 4-5 days p.i. in parallel with envelope and Gag precursors. The reason for the late synthesis of the Nef protein is not clear. It might be an artifact of the cell culture conditions. It might also be possible that the Nef protein is required at late stages in HIV replication.
The Nef protein of HIV-1 Hi is myristylated but not phosphorylated

Myristylation and phosphorylation are 2 post-translational modifications assigned to the Nef protein (Allan et al., 1986; Franchini et al., 1986; Guy et al., 1987). As expected, the Nef protein of HIV-1 Hi was found to be myristylated along with the Gag precursor p55 and the matrix protein p18 (derived from the N-terminal cleavage of p55). In order to investigate phosphorylation of the Nef protein, infected cells were labelled with $^{32}$PO$_4$ at the peak of viral protein production. Under these conditions, the gag gene products p55, p18 and p25 were phosphorylated as described earlier (Veronese et al., 1988; Laurent et al., 1989). However, Nef protein was not phosphorylated. This was later shown to be due to specific mutations of the nef gene (see below).

The HIV-1 Hi nef gene has mutations at critical sites

The HIV-1 Hi nef gene was isolated using PCR techniques in order to verify its sequence compared to that of the original isolate, HIV-1 Lai. At the nucleic acid level, 10 mutations were identified along the length of the nef gene, except for a cluster of 4 mutations within 20 bp. The 4 mutations have no incidence at the amino acid level, but are located within the binding site of cellular factors that may be regulated by the Nef protein (between nucleotide 414 and 465) (Guy et al., 1990a).

At the amino acid level, two important mutations were observed. Firstly, threonine, at amino acid number 15, the site of phosphorylation by protein kinase C, was changed into an alanine. Secondly, the aspartic acid of the tetrapeptide Trp-Arg-Phe-Asp (WRFD) situated in the carboxyl-terminal region of the protein, was changed into an asparagine. This tetrapeptide might be implicated in GTP binding and in the correct folding of the protein (Guy et al., 1990b).

The reduced half-life of the Nef protein of HIV-1 Hi, therefore, is most likely due to the aspartate to asparagine mutation.

The mutated nef gene of HIV-1 does not mediate downregulation of CD4 expression

It has been demonstrated that expression of the Nef protein results in downregulation of CD4 expression (Guy et al., 1987). To investigate this effect, vaccinia virus recombinants were constructed, expressing the wild-type and the mutated nef gene, and were employed to infect CEM cells (Laurent et al., 1990). By fluorescence-activated cell sorting (FACS) analysis it was then possible to demonstrate that cell surface expression of the CD4 antigen was significantly reduced by the wild-type Nef protein, whereas no modification occurred with the mutated nef gene product. These results therefore demonstrated that the mutated Nef protein was devoid of a specific biological activity. Furthermore, these results indicated that FACS analysis of CD4 expression could serve as a convenient test to verify the biological activity of the Nef protein.

Conclusion

Several modifications were observed for the Nef protein of the highly cytopathic HIV-1 Hi preparation: 1) a reduced half-life; 2) a lack of phosphorylation; 3) an inability to downregulate CD4 expression. These modifications were found to be due to two specific mutations of the nef gene which had probably occurred during long-term passaging of the original less cytopathic virus. One mutation affected the phosphorylation site by the protein kinase C, whereas the other mutation affected the putative GTP-binding site which is responsible for the proper folding of the Nef protein (Laurent et al., 1990).

The mutation of Thr15 into Ala at the phosphorylation site was previously observed in other HIV isolates (Ratner et al., 1985; Alizon et al., 1986; Spire et al., 1989). Mutant viruses with deletions in the 5' region of the nef gene were shown to be highly cytopathic with a replication rate 5- to 10-fold higher than the wild-type virus (Luciw et al., 1987). Other observations have suggested that the development of disease is correlated with the emergence of HIV1 variants more cytopathic in vitro (Cheng-Mayer et al., 1988) and perhaps this effect might be due in part to mutations in the nef gene.

As we have demonstrated to be the case for cell cultures infected with HIV1, consecutive culturing might favour the selection of highly cytopathic clones resulting from specific mutations in the nef gene. By such a selection, highly cytopathic viruses might be generated, a phenomenon which is in favour of a negative role of the Nef protein. However, since several parameters are involved in the regulation of HIV infection (Cullen et Greene, 1989), it should be emphasized that mutations of the nef gene could represent one of the mechanisms by which HIV infection could be controlled.

On the other hand, it is also possible that cell culture conditions do not precisely reflect virus production in the host and that nef suffers counter-selection during viral propagation in vitro. Very recent data in the SIV-infected rhesus monkey demonstrates a critical role for Nef protein in the development of full pathogenicity (Kestler et al., 1991).

Finally, our studies demonstrate the importance
of a detailed characterization of the Nef protein in any study concerning its function.

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References


HIV1 3' ORF is open in pathological tissue and accelerates viral replication in primary lymphocytes

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The 3' ORF gene of HIV1 encodes a 26-27-kDa protein whose function is the subject of current controversy (Cullen, 1991). Initial studies suggested that this protein downregulated virus replication by decreasing transcription from the viral LTR, and was thus important for viral latency (Ahmad and Venkatesan, 1988; Cheng-Mayer et al., 1989; Luciw et al., 1987; Niederman et al., 1987). Although the 3' ORF protein was renamed Nef (negative factor) based on these initial results, several groups of investigators later found that it had little or no effect on replication (Hammes et al., 1989; Kim et al., 1989). All of the functional studies on 3' ORF to date were performed in cultured T-cell lines with HIV1 strains passaged over many generations in vitro. Much of these data may not be valid in vivo, as some of the differences in the regulatory effects have been attributed to the cell type or HIV1 isolate studied (Cheng-Mayer et al., 1989; Hammes et al., 1989; Kim et al., 1989). Analysis of 3' ORF function is further complicated by the fact that the protein is multivalent: a large fraction is myristoylated in vivo and thus may have membrane-associated activities, and it has been shown to have structural and functional homology to G proteins (Guy et al., 1987). The true role of 3' ORF remains undetermined.

Recently, Kestler et al. (1991) have shown in rhesus monkeys that SIV 3' ORF mutants containing a premature stop codon rapidly revert to a full open reading frame and are pathogenic, while SIV mutants with deleted 3' ORF genes do not produce clinical disease or pathological changes. This in vivo evidence demonstrates that SIV 3' ORF protein is functionally important in pathogenesis, and suggests that HIV1 3' ORF may similarly play an important role in AIDS. In order to determine the function of HIV1 3' ORF in the context of the natural infection, our laboratories have studied the 3' ORF gene in vivo, in pathogenic AIDS tissue, and its effects in primary human lymphocytes in vitro. We find that the 3' ORF is open in tissues from HIV1-infected individuals and that naturally occurring HIV1 3' ORF genes accelerate virus production in primary human lymphocytes, but not in T-cell lines.

In the first line of study, to determine the status of the 3' ORF in pathologic tissue, we cloned the gene directly from DNA extracted from brain and spleen tissues of 4 children who had died of AIDS, using nested primer-targeted PCR amplification and the "Invitrogen T/A" cloning vector to avoid loss of sequence information. To date, we have obtained 43 HIV1 3' ORF gene sequences. In 40 of these sequences the reading frame was fully open, in contrast to 3' ORF genes from cultured HIV1 (and SLV) isolates which often terminate prematurely. All our clones differed from each other at several positions, but unique mutations at two positions characterized isolates from 2 patients ("signature sequences").

This was similar to the situation we observed in sequences of the hypervariable V3 domain of the HIV1 env gene, which we interpreted as "tissue-specific evolution within host-determined quasispecies" (Epstein et al., 1991). Also, brain-derived clones showed fewer unique mutations than spleen-derived clones, suggesting that HIV1 may evolve more slowly in brain than in peripheral tissues, perhaps because of brain-specific constraints on evolution. This differ-

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ence predicts that the brain-derived sequences are more closely related to the original inoculum HIV1 strain.

Although the 3' ORF was usually open in pathologic tissues from children with AIDS, in 2 spleen clones, we observed truncated ORF due to a two-base insertion and a one-base deletion mutation. One clone had a premature stop due to a point mutation. The finding of insertion/deletion mutations among our 3' ORF clones is unlikely to represent errors due to Taq polymerase, and is in sharp contrast to our experience with over 80 V3-domain clones, from the same tissues, where no reading frame dislocations or stop codon point mutations were noted. This contrast suggests that, unlike the V3 domain, the 3' ORF is not under strong immune selection pressure.

In the second line of study, to mimic natural HIV1 infection as closely as possible in vitro, we isolated 3' ORF genes directly from peripheral blood lymphocyte DNA of two HIV1-infected individuals and studied their effects on virus production in primary human lymphocytes. As the carboxy-terminal part of the HIV1 3' ORF gene overlaps the 3' LTR, we directly cloned both the 3' ORF and its potential target, the LTR, from peripheral blood lymphocytes of HIV1-infected individuals (de Wolf et al., 1988). A 900-bp 3' ORF plus LTR fragment was amplified by nested PCR using primers within env and at the 3' end of the LTR, and cloned into the background of the HIV1 molecular clone HXB-2 (Shaw et al., 1984), replacing the corresponding HXB-2 sequences. By DNA sequencing, the 3' ORF was found to be open in all but one clone. To distinguish between 3' ORF and LTR-derived viral properties, each of the cloned 3' ORF genes was further modified within the amino-terminal coding region by insertion of a linker containing stop codons in all 3 reading frames.

The chimeric HIV1 clones were transfected into the Jurkat and SupT1 T-cell lines, and into primary human peripheral blood lymphocytes. Virus replication was assayed by p24 core antigen production. No significant difference in replication rate was observed between virus containing stopped or open 3' ORF genes in the Jurkat or SupT1 T-cell lines. The phenotype of the chimeric viruses appeared to be fast replicating, similar to the parental HXB-2 strain (the parental HXB-2 3' ORF gene contains a premature stop codon).

In human primary lymphocytes, the replication of both the open and stopped 3' ORF HXB-2 virus appeared to be slow compared to replication in Jurkat cells. In contrast, the onset of detectable replication of the viruses containing a naturally occurring open 3' ORF appeared much earlier than for those viruses with stopped or HXB-2 3' ORF. The eventual level of p24 antigen production was also higher for viruses containing a natural open 3' ORF. Immunoblot analysis of transfected human lymphocytes and Jurkat cells showed that all viruses with open 3' ORF genes synthesized a detectable 3' ORF protein. Thus, expression of natural 3' ORF proteins is correlated with accelerated virus replication in primary human lymphocytes.

Taken together, these complementary lines of evidence show that the naturally occurring 3' ORF gene found in HIV1 pathologic lesions is open, and that the 3' ORF protein acts as a positive regulator of replication in primary human lymphocytes. We tentatively conclude that the HIV1 3' ORF exerts a positive regulatory role in vivo. Our results are in agreement with the observations of Hammers et al. (1989) and Kim et al. (1989), and emphasize that only in the natural context of primary human infection is the true role of the HIV1 3' ORF revealed.

It had previously been found that mutation of the SIV env gene leading to truncation of the transmembrane protein occurs as a result of adaptation for growth in human T-cell lines (Hirsch et al., 1989; Kodama et al., 1989). When inoculated into rhesus monkeys, SIV strains with truncated env and also truncated 3' ORF genes rapidly revert to viruses with complete open reading frames (Kestler et al., 1991; Hirsch et al., 1989; Kodama et al., 1989). The fact that almost all natural 3' ORF genes are open, suggests that the intricate regulatory environment evolved by HIV and SIV for survival within their respective hosts exerts strong functional pressure on the 3' ORF.

Kestler et al. (1991) speculate that the open SIV 3' ORF allows an increased viral load in the host, leading to pathology. As HIV and SIV are lentiviruses, whose hallmark is early invasion of the central nervous system, followed by viral persistence and slow pathogenesis, the 3' ORF protein in the central nervous system may be a critical determinant of the course of disease. The largest amounts of HIV (and SIV) are present in the brain (Shaw et al., 1985), mainly in productively infected multinucleated giant cells, activated microglial cells and pleiomorphic inflammatory cell infiltrates (Sharer et al., 1988), and children often succumb to primary HIV1 brain infection. However, little is known regarding 3' ORF activity in monocyte/macrophage-tropic HIV1 strains thought to predominate in brain tissue (Gendelman et al., 1990).

While the 3' ORF may have a positive regulatory role on viral replication in peripheral lymphocytes, leading to increased viral load, through its G-protein character it could also exert either sparing or toxic effects on neural cell functions, leading to persistency or encephalopathy. It is thus important to determine the nature of the functional pressure on the 3' ORF, as the multivalent character of the 3' ORF protein may produce paradoxical effects in different tissues.
ON THE HIV nef GENE PRODUCT

References


The negative (?) factor of HIV and SIV

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Unlike other retroviruses, the human immunodeficiency viruses 1 and 2 (HIV1 and HIV2) and the related simian immunodeficiency viruses (SIV) have very complex genomic structures (for review see Cullen, 1991). In addition to the characteristic retroviral structural genes, gag, pol and env, their genomes contain the essential regulatory genes tat and rev, the vif gene, which is important for efficient viral replication, and 4 short open reading frames (ORF), coding for several small proteins, termed vpr, vpx, vpu and nef. These latter proteins are dispensable for virus replication in vitro and have been named "nonessential" viral genes. Vpr, vpx and vpu are not present in all primate lentivirus genomes. In contrast, the nef open reading frame, located near the 3' end of the viral genomes, is generally conserved, indicating its importance for the virus life cycle in vivo.

The products of the HIV1 nef genes are myristylated proteins of 25 or 27 kDa, which are often phosphorylated by protein kinase C (PKC) on Thr15 and a second site (Allan et al., 1985; Franchini et al., 1986; Guy et al., 1987; Zweig et al., 1990). Nef is expressed early in the virus life cycle (Schwartz et al., 1990) and is localized in the cytoplasm associated with intracellular cell membranes (Franchini et al., 1986). Certain sequence homologies of nef to oncogene products like src and ras have been reported (Guy et al., 1987) and partially purified recombinant nef was found to share some biochemical properties, such as GTP binding. GTPase and autophosphorylation activity with the ras protooncogene (Guy et al., 1987, 1990a). However, other investigators were unable to detect substantial GTP-binding or GTPase activity (Kaminchik et al., 1990, 1991; Nebreda et al., 1991). Moreover, nef does not cause oncogenic transformation (Nebreda et al., 1991), so that the biological relevance of these limited homologies has yet to be defined.

Interesting features of certain Nef proteins are their ability to downregulate the cell-surface expression of CD4 (Guy et al., 1987; Garcia and Miller, 1991) and to prevent the antigen-receptor-mediated induction of IL2 mRNA (Luria et al., 1991). Both functions may be important for the development of immunological disorders. However, Cheng-Mayer et al. (1989) reported, that they found no downregulation of CD4 by Nef, and the data of Luria et al. (1991), who found the abovementioned effect only for one certain form of Nef, have to be confirmed by other investigators.

Is Nef a negative regulatory factor?

While it is clear that nef is dispensable for efficient viral replication in the currently used in vitro systems, it is highly controversial whether nef is a repressor of viral replication, acting at the transcriptional level on a negative regulatory element (NRE) in the LTR region. Since Nef itself is attached to membranes and not found in the nucleus (Franchini et al., 1986; Guy et al., 1987; Guy et al., 1990a), it has been proposed that Nef is acting as a signal-transducing protein, by the downregulation of cellular activation-associated factors which bind to the NRE region (Guy et al., 1990b). The claimed degrees of downregulation of viral replication or RNA transcription by HIV1 nef varied from about 2-fold up to 50-fold (Terwilliger et al., 1986; Luciw et al., 1987; Ahmad and Venkatesan, 1988; Niederman et al., 1989; Maitra et al., 1991). However, other investigators were unable to find a negative effect on viral replication or LTR activity even with HIV1 isolates used in previous studies (Kim et al., 1989; Hammes et al., 1989; Bachelier et al., 1990; Luria et al., 1991).

Similar to the results of Kestler et al. (1991), who reported that Nef has no significant influence on the

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In vitro replication rates of SIVMAC, we found that nef-deficient mutants of the HIV-2BEN MK6 clone (Kirchhoff et al., 1990a,b) replicated with comparable efficiencies in the human T-cell lines HUT78, CEM-T4 and Jurkat, as well as in human peripheral blood lymphocytes. In contrast, others have reported a negative influence of Nef on HIV2 and SIV replication (Cheng-Mayer et al., 1989; Niederman et al., 1991). Interestingly, in our hands, a Nef-deficient clone of HIV-2BEN replicated with a 2.5-fold lower efficiency and with a considerably delayed multiplication in Molt-4 clone-8 cells compared to an otherwise isogenic Nef-positive clone. A similar slight positive effect in certain cell lines was also described for some HIV1 isolates (Kim et al., 1989).

Like the data concerning the action of Nef in cell culture systems, the serological results about Nef are also conflicting. Following the first reports about the role of nef as a suppressor of HIV replication in vitro, it has been proposed, that nef is important in the establishment of viral latency (Ranki et al., 1987; Gaines et al., 1987; Haseltine, 1988; Ameisen et al., 1989).

In accordance with these hypotheses, it has been reported that Nef-specific antibodies, indicative for Nef expression, often appear in otherwise seronegative HIV1-exposed individuals and before full seroconversion to the structural viral proteins, and are thus important for the early diagnosis of infection (Ranki et al., 1987; Ameisen et al., 1989). In support of this model of viral latency, Cheng-Mayer et al. (1989) reported that the LTR regions of fast-replicating and highly cytopathic (rapid/high) isolates of HIV, derived from patients with advanced disease, are not affected by nef. Moreover, Laurent et al. (1990) described the selection of an inactive form of Nef in vitro and proposed that such inactive products are also important for disease progression in natural infection.

In our opinion, there is strong evidence against the role of Nef as a negative factor in vivo. Recent serological studies showed that the appearance of Nef-specific antibodies before full seroconversion and in otherwise seronegative HIV-exposed individuals is a rare event (deRonde et al., 1988; Reiss et al., 1989; Cheingsong-Popov et al., 1990). In agreement with these results, we found that the extent and appearance of Nef-specific antibodies does not precede seroconversion to structural viral proteins in rhesus monkeys infected with HIV2 or SIVMAC (Kirchhoff et al., 1991). In contrast to the results of Cheng-Mayer et al. (1989), Delassus et al. (1991) found only small differences among LTR sequences of slow/low and rapid/high viruses derived from one patient with progressing AIDS. Moreover, these authors further described that in vitro culture led to the selection of minor forms of Nef and LTR sequences present in vivo. The differential effect of nef on the HIV1 strains SF2 and SF33 reported by Cheng-Mayer et al. (1989) could not be confirmed by Maitra et al. (1991). Moreover, an efficient selection for Thr15 instead of Ala in the latter stage of infection has been described in a long-term evolutionary study on Nef sequences from HIV1 of an infected patient with progressing AIDS (Delassus et al., 1991). This Thr15 represents the site of phosphorylation by PKC (Guy et al., 1987, 1990a) and is probably important for Nef function (Laurent et al., 1990).

Thus, contradictory to suggestions of Laurent et al. (1990), it is possible that more biologically active forms of Nef are selected during the course of infection. Interestingly, a Thr residue is also present at positions 23 to 29 of most SIV, but not at the N terminus of Nef from the non-pathogenic SIVMAC142 clone and most HIV2 clones (Myers et al., 1990), which are to our knowledge also non-infectious or non-pathogenic in the macaque animal model.

The strongest evidence against the role of nef as a negative regulator comes from results obtained after infection of rhesus macaques infected with cloned SIVMAC. Since this animal model closely parallels the course of HIV1 infection in humans (for review see Desrosiers and Ringler, 1989), it is apparently suitable for studies on viral determinants of HIV-induced disease. As mentioned above, Kestler et al. (1991) found no significant differences in the in vitro replication rates of Nef-deficient SIV clones and those expressing an intact Nef using a variety of standard cell culture conditions. Dramatic differences were observed in vivo, however. A strong selection for the open form of the nef gene in infected rhesus monkeys and at least 100-fold lower virus loads in animals infected with nef-deletion virus were observed. Moreover, only SIVMAC containing an intact nef gene caused AIDS-like symptoms and death in infected animals, whereas animals infected with nef-deleted virus are so far without any clinical signs. Despite the fact that similar to reports about HIV1-infected individuals (Reiss et al., 1989), we were unable to find a correlation between Nef-specific antibodies and disease in infected monkeys (Kirchhoff et al., 1991), this does not exclude an important role for Nef in the pathogenic potential of primate lentiviruses.

Conclusions and future experiments

Regarding the highly controversial and not always reproducible results of the in vitro experiments about Nef function, it must be questioned if most currently used in vitro systems are suitable for monitoring monitor Nef function. It seems that Nef may have the opposite effect on the course of SIV or HIV infection than that predicted from the initial reports. The data of Kestler et al. (1991) strongly suggest that
Nef positively influences virus replication, leading to high virus loads and disease. Despite the fact that the nef genes of SIVMAC are larger than those of HIV-1 and that only about 35 to 40% of its amino acid sequences are conserved among these proteins, this variability is mainly restricted to the amino- and carboxy-terminal regions of Nef. The SIV nef genes have the same genomic location as the HIV nef gene, and large domains within the central part of nef, including 2 serine residues and a putative "P site" (Guy et al., 1987, 1990b), are relatively conserved among the 4 main groups of primate lentiviruses, HIV-1/SIVCPZ, HIV-1/SIVMAC/SIVSM, SIVAGM and SIVMND (Kirchhoff et al., 1990a). As described (Desrosiers and Ringer, 1989), the biological properties of SIV and HIV are very similar. It seems highly likely that, whatever the function of Nef is, it is similar in SIV and HIV and that Nef is important for their pathogenic potential. Even though the function of Nef has yet to be defined, some properties of Nef, such as the downregulation of cell-surface expression of CD4 (Guy et al., 1987; Garcia and Miller, 1991) and its ability to interfere with signal transduction pathways in HIV-1-infected T cells (Luria et al., 1991), may give helpful hints toward how Nef expression supports the development of immunological disorders.

The data of Kestler et al. (1991) are based on experiments with the SIVMAC239 clone. However, similar results were obtained with env variants of this clone (Burns and Desrosiers, unpublished results), different SIV clones have to be used in further animal studies to prove their results. Additional experiments have to be performed to clarify even the functional role of the "negative" factor. It is clearly not in the interest of a virus to kill its host, and to our knowledge several SIV such as SIVAGM, SIVCPZ and SIVMND do not cause disease in their natural hosts.

Does Nef also lead to higher virus loads and more efficient replication in these species? Are there indications for an important role of Nef for the pathogenic potential of HIV-1? Is it possible to establish in vitro systems which more closely mimic the in vivo situation to investigate Nef function? Is a phosphorylation site near the N terminus of Nef a prerequisite for the development of disease? Which domains, e.g. conserved Ser residues, putative GTP-binding or phosphorylation sites, are important for Nef function in vivo? We feel that these are some of the important questions for continued investigation in the immediate future.

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References


B-cell epitopes of the Nef protein

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One of the reasons for carrying out a detailed epitope mapping of the Nef protein was the potential induction of anti-Nef antibodies before seroconversion (Ranki et al., 1987; Ameisen et al., 1989). Individual fine specificities of the antibodies of HIV-infected patients which recognize recombinant HIV Nef protein (r-Nef) (Ranki et al., 1987, 1990; Ameisen et al., 1989; Gombert et al., 1990; Schneider et al., 1991; Sabatier et al., 1989; Reiss et al., 1989) and questions concerning the state of latency and cross-reactivity with antibodies against unknown proteins of healthy donors, are major problems to be solved. In particular, the order of appearance of each of the epitopes in individuals during the course of the HIV infection are of interest, because anti-Nef antibodies have been found long before (Ranki et al., 1987; Ameisen et al., 1989) or almost simultaneously with the induction of antibodies against structural proteins. One investigation using immunoblots revealed a high percentage of anti-Nef antibodies in all stages of disease (Wieland et al., 1990). However, in one study only 30% of HIV-positive sera were found to react with Nef (Kienzle et al., 1991) and in another study a high prevalence of anti-Nef antibodies prior to seroconversion could not be found (Cheingsong-Popov et al., 1990).

The sequence variability of Nef proteins of different HIV isolates with respect to antibody binding is a further problem which needs to be considered.

Our group is trying to elaborate an experimentally supported structural model of the Nef protein using defined sets of overlapping 15- and 20-mer peptides for conformational mapping by circular dichroism. The folding pattern obtained should enable the construction of a spatial model taking into account all information from epitope fine mapping of patient sera and monoclonal antibodies (mAb) raised against Nef protein and against synthetic lipopeptide vaccines (Wiesmüller et al., 1989; Deres et al., 1989) containing partial Nef sequences. The feasibility of such an approach was recently demonstrated by modelling the structure of thymidine kinase of Herpes simplex virus (Zimmermann et al., 1991).

In the following, we summarize the few publications (Gombert et al., 1990; Schneider et al., 1991; Ovod et al., 1991; Tähtinen et al., 1991) which have appeared so far on the determination of defined B-cell epitopes of the Nef protein. Particular emphasis is given to our results obtained in cooperation with several immunological laboratories and clinics.

Epitopes of Nef protein from human sera

Methods of multiple peptide synthesis have been shown to be a valuable tool for creating sets of overlapping peptides used as antigens in ELISA. In third generation ELISA for HIV infection, antigen mixtures of chemically synthesized peptides representing the most relevant viral B-cell epitopes were used, which raised the specificity to more than 99.5%. In our first approach to define Nef epitopes, 3 sets of overlapping nonapeptides were synthesized on polyethylene pin plates fitting onto microtitre plates, which represented a total of 3 x 200 peptides of the Nef sequence of the HIV isolates Bru, Mal and SF2 (Gombert et al., 1990).

Sera from randomly selected HIV-positive patients (recognizing r-Nef) reacted with the following regions of the protein (only sequence positions for Bru isolate are given): 8-16 (epitope 1), 52-60 (2), 80-90 (3), 98-101 (4), 115-127 (5), 136-147 (6), 158-169 (7), 180-190 (8), 197-206 (9). The HIV-positive sera (Western blot) recognized several of these peptides; some sera, however, reacted only with one or two of these peptides. HIV-negative controls showed no reactivity. With the exception of epitope 7 (not found in Mal isolate), 5 to 9 of the ELISA-positive regions 1-9 were detected in all 3 isolates.

The experimental results of this investigation only confirm B-cell epitope predictions 50-60%. However, the identified epitopes (Gombert et al., 1990) were in good agreement with findings of Sabatier et al. (1989) and Schneider et al. (1991). Four immunogenic regions for 7 HIVI (Bru) sera were found using 200 overlapping decapeptides: 26-44, 51-67, 122-135 and 143-176 (Schneider et al., 1991). Most
interesting was the finding that sera of several individuals of a high-risk group showed solitary anti-Nef antibodies in identical epitope regions, indicating possible latent HIV infection (Gombert et al., 1990). A recent communication by Tähönen et al. (1991) describes the fine specificity of B-cell epitopes (minimization) recognized in HIV1 Nef by 10 human sera in a Pin-ELISA.

Murine B-cell epitopes

The HIV Nef protein is a highly immunogenic molecule also in mice (Schneider et al., 1991; Ovod et al.; Gombert et al., 1991), chimpanzees (Bahraoui et al., 1990) and macaques (Kirchhoff et al., 1991) inducing a humoral immune response as well as cell-mediated immunity. In order to investigate the specific B-cell response against Nef in mice, epitopes recognized by mAb and polyclonal mouse sera against the Nef protein were characterized (Schneider et al., 1991; Ovod et al.; Gombert et al., 1991). mAb directed against different parts of the Nef protein are useful tools for further investigations about the function and the molecular characterization of the Nef protein.

L. Shi in the group of P. Wernet (University of Düsseldorf) produced more than 70 mAb by immunization with the recombinant Nef protein (Bru isolate). We have undertaken the epitope characterization of these and other mAb (Ovod et al., 1991) by using more than 600 synthetic peptides and lipopeptides in ELISA and “Pepscan” ELISA. The peptides were between 2 and 21 amino acids long (overlapping 20mers, 6mers and truncated peptides for minimizations and replacement analysis). All mapped mAb were screened for recognition of the recombinant Nef protein. Eight out of more than 80 mAb did not recognize any peptide even when they were up to 20 amino acids long. It was surprising that less than 10% of the antibodies produced recognized discontinuous epitopes; more than 90% had sequential epitopes.

By mapping this large number of mAb we identified 8 binding regions within the Nef protein. Three of the regions were very strong immunogenically as most mAb (more than 40) were directed against them. These major epitopes were located between amino acids 31-39, 170-190 and in the C-terminal part from 200 to 206. Fine mapping of these regions showed that nearly every antibody recognized its special sequence pattern within one binding region.

Antibodies which bound in the region from amino acid 170 to 190 recognized either the sequence LHGM (170-173) or longer peptides with the sequence from 175-190. For the exact characterization of the LHGM epitope, we carried out total replacement analysis of the sequence SLHGMDD. Other binding regions were found from amino acids 56-60, 64-73, 90-97 and 153-157.

Schneider et al. (1991) characterized 8 murine mAb raised against a recombinant Nef protein fragment of HIV1 strain BH10 (1-123) by Pepscan ELISA using overlapping decapeptides. The antibodies recognized epitopes in the regions of amino acids 11-24, 28-43, 60-73 and 82-103. These findings were in good agreement with our results and revealed two additional epitopes in the N-terminal part of the protein and in the region between 82 and 103.

The reactivity of 5 polyclonal mouse sera with 24 synthetic lipopeptides spanning the whole HIV1 Nef sequence in ELISA showed several epitopes in the regions 21-91 and 151-206 (Ovod et al., 1991) which are the same regions where epitopes of the mAb are located.

As expected, the human and murine antibodies did not always recognize identical binding regions. In the human system, a strong B-cell response against the middle region of Nef, amino acids 90-150, was found which was completely non-reactive with mouse anti-Nef sera. Furthermore, there were no mAb which recognized this region. On the other hand, there were many mouse mAb which bound in the region 31-39, which was not very reactive with human sera. Only Schneider et al. (1991) found antibodies in human sera which reacted with the region 26-44.

In order to obtain mAb to regions which are not immunogenic in the Nef protein, L. Shi immunized mice with a series of lipopeptide vaccines synthesized in our lab. These immunization revealed, on one hand, a number of mAb against the lipopeptides which recognized the used immunogen as well as the Nef protein. On the other hand, some of the mAb recognized only the lipopeptide used as immunogen. The question is whether these regions are hidden inside the recombinant protein molecule or does the conformation of these regions differ in comparison to the conformation of the lipopeptides.

In order to overcome the high variability of the Nef sequences, Gombert et al. (1991) constructed a consensus sequence of the C-terminal part of the protein by sequence alignment representing the most frequent amino acid sequence of the HIV isolates known to date. We synthesized a 13-amino-acid-long lipopeptide which was used to immunize BALB/c mice. Eleven mAb with different epitopes were induced which recognized the recombinant Nef protein (Bru isolate). Some of these mAb recognized peptides corresponding to sequences of all other isolates. Therefore, these experiments demonstrate the possibility of overcoming the variability of sequences by immunization with a consensus peptide.
Conclusion

It can be concluded that probably during each HIV infection, antibodies against the Nef protein do occur. However, for diagnostic purposes the present data do not permit an exact correlation between the occurrence of the antibodies and disease progress.

The results of the epitope mapping with mAb are very promising. We obtained a representative series of mAb with well-defined binding regions in different parts of the protein, which can be used in experimental systems for functional characterization of this protein. These mAb would also enable characterization of the structural surface areas.

References


DISCUSSION

B. Guy, R. Bruce Acres, Y. Riviere and M.-P. Kieny

It is surprising to notice how little controversy is raised by the present Forum on Nef. Indeed, most of the observations related are not contradictory and can be accommodated in the same theory of Nef function. The different important points can be analyzed as follows.

1) Effect of Nef on HIV replication

S. Venkatesan and Niederman and Ratner have demonstrated that Nef of HIV-1 and SIV may act as negative regulators of viral replication. On the contrary, Blumberg et al. and Kirchhoff and Hunsmann have shown that HIV1 and HIV2 replication is up-regulated by Nef. We have evidence in the SIV model that Nef slows down viral regulation in human PBL (unpublished experiments).

Actually, it seems that the effect of Nef on the transcription level from the LTR, and thus on viral expression, is variable according to the nef allele and to the nature of the infected cell line. Relevant to this point is that Niederman and Ratner have shown that the apparent molecular weight of their nef-gene product on an SDS gel is different from that of the Nef protein of Kim et al. (1989, Proc. nat. Acad. Sci. (Wash.), 86, 9544-9548). All these differences may lead to opposite effects in the variety of cells infected in vivo and explain in part the silent expression of the lentiviruses in certain tissues.

2) G-protein activity

Although most of the data presented at this Forum disagree with our observation that Nef is capable of binding GTP, several authors (S. Venkatesan and Nebreda et al.) have confirmed the autophosphorylation activity of Nef in the presence of purine nucleotides and agree with the fact that Nef has the potential to interact with the gamma phosphate of nucleoside triphosphates.

3) Interactions of Nef with the cellular membrane

We have surmised that Nef might interfere with membrane proteins and have evidence for the interaction of Nef with a non-identified 38-kDa protein. Kaminchick et al. also describe that Nef interacts directly with membrane proteins and implies, in addition, the involvement of the N-terminal myristic acid and the participation of a domain spanning amino acids 73 to 89.

Although Smythe and Reitz have not been able to show the direct interaction of the myristylated peptide corresponding to the 19 N-terminal amino acids of Nef with the membrane of PBMC, it does not mean that such an interaction does not exist for the complete Nef.

4) Regulation of the expression of cellular genes

We have demonstrated that Nef is capable of downregulating CD4-surface expression but not CD8 expression. These results have been confirmed by Garcia and Miller, who have also shown that expression of transferrin is not downregulated. Moreover, this disappearance of CD4 from the cell surface does not result from a transcription downregulation, but rather from a sequestration of the protein in the cytoplasm. We also agree with Garcia and Miller on the fact that PKC phosphorylation of Nef on Thr15 is not important for this function.

Lucia et al. (1991, Proc. Nat. Acad. Sci. (Wash.), 88, 5326-5330) have demonstrated that Nef (Ala15) is capable of downregulating transcription of IL2 following TCR stimulation, and it is of importance to stress that this result might have been suspected from our experiments in trying to identify nuclear factors binding to the NRE portion of the HIV promoter. We have indeed identified a sequence on the IL2 promoter as a potential binding site for factors responsive to Nef.

Nebreda et al. have shown that nef does not induce the transformation of cultured cells, but mention that nef transfectants were unusually rare, implying that some toxicity is generated by nef expression. Consistent with this is the fact that we have never been able (unpublished observations) to generate transgenic mice expressing nef ubiquitously and very early in the embryonic stage. The animals harboring the nef gene were always chimera and did not pass the transgene to their offspring.

Antibody response to Nef

In conclusion, it seems that everybody agrees on the potential importance of Nef in HIV infection, possibly on HIV latency, and Kestler et al. (1981, Cell, 65, 651-662) have definitively shown that the classification of nef within the "non-essential" genes has to be revised. A lot of work is still to be per-
formed on nef, as understanding the variety of Nef activities and their in vivo implications may provide insight into the design of novel AIDS therapies.

S. Venkatesan:

This Forum on Nef highlights the sharp disagreement about almost every aspect of Nef function. It is particularly unfortunate that neither the Baltimore nor the Greene group has contributed to this Forum. Since their refutation of the negative influence of Nef on virus replication and LTR transcription, several papers have been published supporting the negative effect of Nef, and it is of interest to the HIV scientific community to learn how they could reconcile the recent findings. The Forum also suffers from the lack of participation by the Desrosiers group who have made perhaps the most fascinating observations pertaining to the in vivo role(s) of Nef.

The lack of consensus for the results of current in vitro assays measuring virus replication and transcription necessitates a shift in the focus from the virological to the cellular physiological roles of Nef. Effects of Nef on two cellular targets, namely the CD4 and the IL2 gene, are the most promising avenues of research. Nef-induced CD4 downregulation appears to be due to an aberrant posttranslational trafficking/processing phenomenon. This mechanism appears to be distinct from the usual mechanism of CD4 internalization consequent to serine phosphorylation (by protein kinase C). However, the relevance of the Nef effect on CD4 during the virus life cycle remains to be determined. During HIV infection, Env gp160/120 also leads to a loss of CD4 expression; therefore, proviruses mutated in the CD4-binding domain of gp120 may be used to measure the effect of other regulatory and accessory genes (viz. vpu) on CD4. As pointed out by Garcia and Miller, the CD4 effect may be a temporary phenomenon or may be restricted to cell types that are "dormant" for HIV expression. In either case, the CD4 effect may be coupled to the repressive effect on the LTR, or the two effects may be mutually exclusive. There may be a predominant CD4 effect at low Nef expression which disappears with increasing Nef when the LTR repression becomes dominant. Such a dual repressive switch can maintain virus latency in certain cell types.

Nef-mediated IL2-gene repression is apparently transcriptional. The highly inducible IL2 promoter, rich in regulatory sequences is an excellent choice for unravelling the effects of Nef on transcriptional enhancers. IL2 promoter has many cis elements which are homologous to sequences in the NRE region of the HIV LTR, and if Nef-sensitive IL2 promoter elements and their effectors are discovered then these studies may be extended to the HIV LTR. These efforts may be expanded by the use of nuclear extracts of Nef-expressing cell lines to evaluate the DNA-binding potential of known cellular transcriptional factors. Specific targets of PKC, PKA, cAMP-dependent protein kinase, SRS pathways, may be examined in the context of Nef” cell lines.

Although the Nef-induced downregulation of CD4 and IL2 is mediated by different mechanisms, they may be initiated by a common cellular signalling or activation trigger. In this regard, the intracellular localization and phosphorylation status of Nef may be the crucial determinants. Non-myristoylated Nef protein is distributed diffusely in the cytoplasm and is devoid of repressive effects on the LTR. Whether the loss of myristoylation also eliminates the effect on CD4 and IL2 and whether the internally initiated Nef has any role(s) remains to be determined. Since there appears to be reasonable agreement that Nef has a measurable autokinase activity and may in turn be phosphorylated by cellular protein kinases, mutational analysis of the different Ser and Thr residues of Nef is in order. Protein cross-linking studies of the type suggested by Smythe and Reitz may help identify some of the interactions of Nef with plasma-membrane-associated proteins.

At present no molecular or immunological mechanisms explain adequately the in vivo observation that strong selectional pressure maintains a functional Nef during natural infection. Until an in vitro tissue culture system mimicking the behaviour of Nef+ viruses in monkeys is developed, the in vivo Nef effects may be correlated with the myristoylation status, the presence or absence of internal initiation, conserved residues such as Thr at 15, other Ser or Thr targets of protein phosphorylation. If the in vivo phenomenon can be correlated with discrete molecular changes in Nef, they may then be compared with their effect on cellular targets such as CD4 and IL2.

T.M.J. Niederman and L. Ratner:

The elucidation of Nef function in vitro has been enigmatic, and no consensus opinion is apparent. The data and opinions presented in this Forum suggest that Nef may have pleiotropic effects, depending on the allele, host cell type and specific experimental conditions.

We and others (Niederman and Ratner, 1991; Venkatessan, 1991) have presented data supporting the claim that Nef is a transcriptional silencer of the HIV and SIV promoter in cultured cells. This transcriptional suppression correlated with decreased levels of virus production in vitro. Furthermore, we and
others (Niederman and Ratner, 1991; Venkatessan, 1991; Binninger et al., 1991) have found that the multiplicity of infection (MOI) had a significant impact on the apparent activity of Nef in cultured cells. Experiments performed under conditions of relatively high MOI demonstrated no effect of Nef on virus replication, whereas, at low MOI, Nef exerted suppressive effects.

In this Forum, Blumberg et al., 1991, suggested that Nef cloned from fresh tissue exerted no effect on virus replication in cultured T-cell lines. However, no data were provided concerning the MOI used in the experiments nor the magnitude of the effects in peripheral blood lymphocytes. The approach utilized by Blumberg et al., 1991, namely, the study of nef genes cloned from fresh tissue, is both refreshing and intriguing. The hypothesis suggested by these authors is that nef genes cloned from fresh tissue will provide a more accurate representation of a "true" Nef protein. However, as they report, 40 out of 40 cloned nef genes displayed different amino acid sequences. Thus, we are left with the question, "which Nef is the true Nef?".

Kirchhoff and Hunsmann (this Forum) suggest that Nef is not a negative factor. These authors cited studies indicating that Nef-specific antibodies are rarely detected before full seroconversion in infected individuals. However, these findings are not relevant to the function of Nef.

We cannot rule out the possibility that Nef exerts effects other than transcriptional silencing in vivo. Other effects may be manifest in tissues not yet studied in our laboratory, i.e. primary lymphocytes and monocytes. Additionally, there are many parameters which may affect Nef function in vivo, which are not represented with in vitro systems, such as immune responses, cofactors etc. Finally, as described in our manuscript in this Forum, it remains to be determined how transcriptional suppression is related to Nef activity in vivo.

Our previous studies revealed the role of Nef as a transcriptional silencer. The focus of our present research is directed towards elucidating the mechanism by which Nef exerts this effect. A detailed understanding of this effect may explain the divergent and often contradictory results reported in the literature, and will provide specific hypotheses to test appropriate in vivo models.

References


J.A. Smythe and M.S. Reitz:

The biological function of the nef gene has been the subject of much controversy, and the accord of this Forum appears to be that the enigma remains. For example, one of the earliest functions ascribed to Nef was that of a negative regulator of HIV gene expression. The accompanying reports describe experiments testing this assertion and come to the conclusions that Nef is (a) a negative regulator of virus replication (responsible for viral latency), (b) a positive regulator of virus replication (leading to high virus load and ultimate pathology) or (c) that Nef has absolutely no effect on virus replication or gene expression. There is similar discord regarding whether Nef is, or is not a GTP-binding protein, although the weight of evidence seems to favor the negative. These conflicting observations and conclusions serve to underscore the difficulties associated with attempting to define the function of a protein that demonstrates such a high degree of sequence diversity, and is apparently dispensable for viral growth in vitro. Nonetheless, a number of important conclusions can be drawn from these reports, along with several excellent suggestions for future experimentation.

The lack of agreement about some of the proposed Nef functions (such as it being a negative regulator) may in part reflect the wide variety of nef genotypes. Obviously since we do not know what Nef does, it is difficult to be sure that the genes studied are functional. The use of different cell types in many of the studies may also contribute to the problem since we cannot be sure that nef will be able to interact with the necessary factors in each cell type. Also, as Niederman and Ratner described, Nef function may vary with the multiplicity of infection used in the assay. Although nef does not appear necessary for viral growth in vitro, the in vivo data does point to its importance in pathogenesis and maintenance of high viral load in the infected animals.

We found the demonstration by Kaminchik et al. of an association between Nef-p27 and the cellular cytoskeletal infrastructure of particular interest. This interaction is dependent on both the N-terminal myristic acid moiety and a domain encompassing amino acids 73-89 of Nef. The necessity for the amino acid domain may explain why we were unable to detect any interactions between our N-terminal myristyl Nef peptide and human PBMC membrane preparations, and will undoubtedly be the subject of further scrutiny. A better understanding of the interactions between Nef and the infected cell membrane may ultimately be relevant to the downregulation of IL2-gene expression and surface localization of CD4 apparently attributable to Nef expression in lymphocytes.

The findings of Kaminchik et al. also raise questions about the role of Nef protein that does not as-
associate with the cellular cytoskeleton. This soluble Nef consists of a fraction of the myristylated Nef-p27 and all of the Nef-p25 that results from alternative initiation of translation at an internal AUG codon (it should be noted that this second AUG does not exist in all nef genes). Bearing in mind the results of Nebraska et al. it would be interesting to see what, if any, effects exogenous soluble Nef-p25 may have on uninfected cells since both the HIV-1 Tat protein (Ensoli et al., 1990) and more recently the HTLV-1 Tax protein (Gartenhaus et al., this Forum) can be released as biological active molecules, and have profound stimulatory effects when taken up by uninfected cells in vitro.

Finally, as discussed by Nebraska et al., there have been a growing number of reports recently indicating that both viruses and bacteria produce proteins that can act as superantigens. The current paper by Imberti et al. (1991) provides strong evidence for the presence of an HIV-encoded superantigen (s) that may ultimately be responsible for the devastating pathogenic effects of the virus. As Nebraska et al. point out, although still highly speculative, the analogies between Nef and the known superantigens suggest that this may be an important avenue of future study.

References


J. Kaminchik, N. Sarver, M. Gorecki and A. Panet:

In contrast to some other nonstructural HIV genes (vpr, vpu, vpx), nef is conserved among lentiviruses of human and primate origin, suggesting that it has provided these viruses with some evolutionary advantage. An insight into the role of Nef was recently provided by the work of Kestler et al. (Cell, 1991, 65, 651), in which Nef expression was found to be crucial for viral replication and pathogenicity in SIV-infected primates. However, it is not possible to reveal the molecular mechanism by which Nef functions in such studies because of the inherent complexity of the animal system. It is our belief that carefully crafted cellular systems along with the animal models will ultimately unveil Nef's mode of action.

The functions currently ascribed to Nef are puzzling. Studies on the effect of Nef during virus infection indicate a decline in viral replication by downregulation of transcription from the LTR (possibly by interfering with a specific transcription factor entering the nucleus). On the cellular level, Nef was found to interfere with two processes, transport of CD4 to the plasma membrane and IL2 production. In contrast to its effect on viral and IL2 transcription, the block on CD4 is post-translational. The questions now are whether and how a single protein directly impairs diverse functions such as transport to the nucleus (of transcription factors) versus transport of proteins to the plasma membrane. We are attempting to resolve these issues by studying the molecular and cellular biology of Nef.

When expressed in mammalian cells both BH10 and LAV nef genes encode two polypeptides, Nef-p27 and Nef-p25. Nef-p27 is myristoylated and partially associated with a particulate fraction of the cytoplasm. The less abundant, Nef-p25, initiates from an internal ATG and is a soluble non-myristoylated protein with a relatively high turnover rate. Myristoylation of Nef is essential for its association with the insoluble fraction of the cytoplasm. We found that abolishing myristoylation of Nef-p27 by genetic manipulation renders the protein soluble, while myristoylation of Nef-p25 causes the protein to be partially associated with the insoluble fraction.

In an attempt to identify the cellular binding site of Nef, we treated crude membranal preparations with non-ionic detergents. To our surprise part of the Nef was recovered with the detergent-resistant fraction, a fraction which appears to represent the cytoskeleton. Moreover, we found that non-myristoylated Nef-p27 or Nef-p27 mutant, from which amino acids 73-89 were deleted, fail to bind to the cytoskeleton fraction. The cellular distribution of Nef, as emerges from our work, is more complex than previously predicted. The pattern of Nef's distribution in the cell might eventually explain the multiple activities ascribed to Nef.

J.V. Garcia and D.D. Miller:

The human immunodeficiency virus (HIV) is the aetiologic agent of AIDS. Because HIV is a very complex retrovirus, analysis of the function of individual genes has been useful in understanding HIV biology and pathogenesis. The divide and conquer approach has illustrated very clearly the role of Tat and Rev during HIV replication; however, these two genes are required for replication both in vivo and in vitro. On the contrary, the same approach has not yielded such clear cut results for Nef. This is mainly due to the fact that in vitro Nef is not required for replication. The recent in vivo experiments which indicate that Nef might be important for disease...
progression are encouraging but have to be carefully interpreted until more data are available and the original results are confirmed by other investigators. The points of view presented by the authors of this collection of articles indicate very clearly that the role of Nef is a complex one and that Nef might not only affect HIV replication but that it might also affect cell function in several ways.

So far all of the published studies on Nef have used in vitro isolates. Because of the great deal of diversity found in nef sequences it will be particularly important to confirm earlier results by using the nef genes of primary isolates. Such nef genes should be used for future biochemical and molecular characterizations. Future biochemical characterizations should be done with Nef isolated under non-denaturing conditions from mammalian cells in which Nef is adequately processed and where it has been shown to be functional. Such Nef should also be used for immunological studies.

It is clear from the different contributions presented that the commitment to study nef is serious and that there is a sincere interest in resolving previous disagreements. On the contrary, the absence of contributions from certain well-known laboratories indicates their lack of interest and commitment to resolve conflicting issues. The recently published data on nef from both in vivo and in vitro experiments clearly indicates that nef might have several different effects on cell proliferation and function as well as in virus spread and persistence. Therefore, nef represents an interesting subject both for basic scientific studies as well as for the development of drugs to combat AIDS.

A.R. Nebreda, F. Segade and E. Santos:

The conflicting reports on practically every single aspect of Nef studied has turned this field of study into a confusing area of work. Different papers presented in this Forum appear, however, to start providing some badly needed common ground which may help in making sense of many previous conflicting reports on Nef. Regarding the study of the role of Nef on viral replication and the viral infection cycle, there is the emerging suspicion that perhaps the true role of Nef can only be revealed in the natural context of in vivo primary infection. The remarkable contrast between the in vivo studies of Kestler et al. (Cell, 1991, 65, 651) and the bewildering array of previous conflicting results arising from in vitro culture systems casts significant doubts on whether in vitro culture systems are suitable for monitoring Nef function.

The paper by Niederman and Ratner is rather illuminating in attempting to explain why the conflicting in vitro results were obtained. In retrospective, it seems reasonable that not only (1) the sequence differences in the Nef products assayed and (2) the different cellular contexts in which they were assayed may be a reason for obtaining the different results. Also simple differences in experimental design (as, for example, multiplicity of infection) may well be responsible for the very different outcomes reported when apparently similar experimental designs were carried out in different laboratories. Therefore, if in vitro culture systems are to be used in future Nef studies, it is crucial to define and control all experimental parameters in much greater detail, in order to be able to interpret the results meaningfully.

There are also now converging lines of thinking regarding the biochemical properties of Nef proteins and the phenotypes caused in host cells. First and foremost, it is now clearly established that Nef proteins are not ras-like GTP-binding proteins, and therefore a search for the biochemical function of Nef should not be biased by such putative oncogene similarities (Nebreda et al., Kaminchik et al., Guy et al., this Forum).

On the other hand, it appears that Nef proteins show in vitro autokinase activity in the presence of purine nucleotides (Nebreda et al., Guy et al., this Forum). The analysis of this kinase activity and the participation of specific amino acid residues of the molecule in that process are open and important fields of study at this time. In particular, it remains to be seen whether this activity is functionally significant in vivo and whether the phosphorylated Nef forms reflect a genuine autokinase activity or just catalytic intermediate forms in the transfer of phosphate groups.

A role of Nef as a possible in vivo substrate of PKC should also be studied and clarified, due to its potential functional implications. Thr15, a potential target for autophosphorylation and PKC action, is a conspicuous candidate for site-directed mutagenesis studies, particularly in light of reports of variable biochemical and biological properties associated with mutations at that position.

The studies of Garcia and Miller appear to confirm the earlier report by Guy et al. that introduction of Nef in cultured cells causes CD4 downregulation. Here again, as with the studies on viral replication, a clear need exists now to extend those in vitro cell culture observations to studies where Nef is introduced in the context of the whole virus, and to the process of in vivo infection.

Finally, since the in vivo cellular context is likely to be an important determinant of the biological effects of Nef, cross-linking studies of the type discussed by Smythe and Reitz may be important to identify cellular factors interacting with Nef, mostly if it holds true that Nef proteins associate with the cyto-klepton (Kaminchik et al., this Forum).
A. Laurent-Crawford and A.G. Hovanessian:

There is a high degree of polymorphism of the nef gene between different HIV strains (Ratner et al., 1985). In spite of this, the nef gene is conserved among all HIV and SIV isolates. Such conservation favours the hypothesis that the product of the nef gene, Nef, is probably an important factor for the viral life cycle. Once the recent report on the positive role of Nef in monkeys by Kestler et al. (1991) becomes reproduced by other groups, then it will be confirmed that Nef is an essential element in vivo for HIV infection and AIDS pathogenesis. On the other hand, the contradictory results on the function of nef obtained in vitro in cell cultures should be considered seriously since each report in itself is convincing, the variability being most probably due to the pleitropic action of Nef in vitro in cell cultures. In fact, Nef seems to act as a transcriptional silencer (Niederman and Ratner, this Forum), or a putative activator (Blumberg et al., this Forum) in addition to its capacity to regulate the expression of cellular proteins by different mechanisms (Garcia and Miller, 1991; Guy et al., this Forum).

We believe that any study of the nef gene should also include the characterization of its protein product synthesized during infection of cells with HIV. In a recent study (Laurent et al., 1990), we demonstrated that prolonged passaging of an HIV preparation leads to the generation of highly cytopathic and replicative virus with two important mutations in the nef gene: the first one affecting the phosphorylation site of protein kinase C and the second one affecting the putative GTP-binding site which is responsible for the proper folding of Nef. As a consequence of these mutations, Nef was not phosphorylated and its half-life was reduced. In addition, the mutated Nef was unable to downregulate the expression of CD4 on the surface of CEM cells. These results indicate that some naturally acquired mutations of the nef gene produce a biologically inactive product. Recently, Spire et al. (1989) have described a highly cytopathic strain of HIV-1 (HIV-1NDK) which interestingly contains exactly the same amino acid substitutions in Nef as our in vitro modified virus preparation.

The significance of the protein-kinase-C-mediated phosphorylation of the threonine-15 in Nef is not known. Whatever is the case, there seems to be selective pressure both in vivo and in vitro for substitution of this Thr by Ala. A long term evolutionary study of the nef gene in the course of disease progression have indicated a significant rate of mutation Thr15 to Ala14 (Delassus et al., 1991). Nevertheless, this mutation does not seem to be critical for the CD4 downregulation (Guy et al., 1987; Garcia and Miller, 1991) nor for the inhibition of IL2 mRNA transcription (Luria et al., 1991).

Guy et al. (1987) have designated the sequence WRFD (amino acids 183 to 186) as the putative GTP-binding site in Nef. The significance of this sequence as GTP-binding site is not very clear. However, it should be emphasized that this site is of great importance in mediating the proper folding and stability of Nef. Accordingly, mutation of WRFD to WRFN results in the production of a Nef-mutant with a reduced half-life. Using HIV1 preparations or vaccinia virus recombinants expressing either the wild-type or the mutant Nef, we demonstrated that the half-life of the Nef mutant is 30 min compared to the Nef wild-type which is more than 4 h. Therefore, this type of mutation should be very critical, since it will lead to the production of a functionally inactive and unstable Nef.

The production of Nef mRNA has been detected early in the infection along with the mRNA of the regulatory proteins Tat and Rev (Schwartz et al., 1990). However, in our in vitro cultures infected with HIV1 expressing either the Nef wild-type or the Nef mutant, the synthesis of Nef was observed concomitantly with the structural Gag and Env polyproteins. These results suggest that Nef might be required for later stages of the virus cycle and furthermore emphasize the importance of parallel characterization of Nef mRNA production and its translation. For precise characterization of Nef and its function, future studies will hopefully clarify the mechanism of action of Nef on the HIV LTR, on CD4 downregulation and inhibition of IL2 mRNA transcription.

References


A. de Ronde, B. Blumberg, L. Epstein and J. Goudsmit:

NEF is a positive regulator of viral production

In vivo HIV-1 Nef is an essential factor which functions as a positive regulator for viral production. This hypothesis is based on the observations of Kestler et al. (1991) that SIV Nef upregulates virus production in monkeys. Our own data show that the open reading frame of HIV-1 Nef is intact in over 90% of Nef sequences isolated from HIV-1-infected individuals. When replaced in an HXB-2 viral background, the naturally occurring HIV-1 Nef functions as a positive regulator of viral production in primary human blood lymphocytes, but not in human T-cell lines. Also in primary human lymphocytes, the difference in phenotype between a NEF+ and a NEF− virus is lost upon high-dose infection. This again demonstrates that the in vitro system used determines the outcome of the experiments. We should attempt to develop our assays in in vitro systems mimicking as closely as possible the in vivo situation.

One model explaining the positive regulatory effect of Nef is that Nef functions as a nucleotide-binding molecular switch which directs the transport of Env and possibly Gag as well to the cytoplasmic membrane. This highly speculative model is based on the following observations and assumptions:

1) a substantial portion of Env is transported to and degraded in the lysosomes (Willey et al., 1988);

2) the location of Nef is in the cytoplasmic membrane, in the lysosomal membrane (our own preliminary observations) and in the cytoskeleton (Kaminchik et al., this Forum);

3) Nef is a protein, which like Gag and Env, is expressed late in infection (Laurent-Crawford and Hovanessian, this Forum), and probably early as well (Schwartz et al., 1990);

4) Nef binds nucleotides and can function as a molecular switch (Guy et al., this Forum). Many molecular switches are involved in directing the transport of vesicles containing specific proteins (Balch, 1990);

5) the downregulation of CD4 cytoplasmic membrane expression by Nef (Garcia and Miller, this Forum) is caused by altering the route of transport of the CD4 molecule.

The Nef enigma is a clear example of conflicts that may arise from the use of in vitro models to study a protein whose function is absolutely required in vivo. Our model does not attempt to explain all available data on Nef; rather, it makes selective use of the conflicting data. For example, the effects of Nef vary widely according to the cell type used, and Nef protein has been reported to be located at various sites within the cytoplasm. All data indicating that Nef is a negative factor have been excluded from the model, based on our observations that Nef acts as a positive regulator in vivo. Also, the accumulation early in infection of mRNA encoding Nef (Schwartz et al., 1990; note that Nef protein detection was not performed) has been disregarded in our model.

In our model, late synthesis of Nef protein would result in redirection of nascent Env proteins to the cytoplasmic membrane instead of to the lysosome, leading to higher production of virions. Redirection of Env proteins directly to the cytoplasmic membrane would in turn lead to attenuation of the production of degraded peptides which can be presented in the context of the MHC class I molecule as a target for cytotoxic T lymphocytes. In vivo, these effects would be synergistic, as both viral production would be up-regulated, and the HIV-1-infected cells would be rendered less susceptible to attack by the immune system.

References


F. Kirchhoff and G. Hunsmann:

The papers in this Forum show that after the initial reports describing Nef as a moderate negative regulator on viral replication, numerous data about the biochemical properties of Nef and its ability to interfere with cellular regulatory pathways were obtained. It has become clear that investigations about the multivalent functions of nef are complicated, since many of the reported nef sequences may represent non-functional genes and since different Nef proteins vary in their biochemical and biological properties. Most important, recent data provide strong evidence that most currently used in vitro assays are not suitable to reproduce Nef function in vivo (Kestler et al., 1991; Delassus et al., 1991; Blumberg et al., this Forum).

Kestler et al. (1991) have demonstrated that Nef is important for the development of AIDS in SIVMAC-infected rhesus monkeys. They also found a strong selective pressure for an intact nef gene in vivo. In agreement with their results, only a few defective Nef sequences were detected in tissues and peripheral blood mononuclear cells obtained from HIVI-infected individuals (Delassus et al., 1991; Blumberg et al., this Forum).

To explain the importance of Nef for viral pathogenicity, some contributors to this Forum propose that Nef is a negative regulator in vivo, that nef-deficient viruses may replicate faster ("unchecked") in their hosts, may be more efficiently cleared by the immune response and therefore be unable to maintain a reservoir of latent infected cells (Niederman and Ratner; Venkatesan; this Forum). We think that some of the recent experimental findings argue against this hypothesis.

1) Nef did not downregulate replication of the SIVMAC239 clone used by Kestler et al. (1991) under various cell culture conditions, even after infection of rhesus monkey PBL cultures at low multiplicity of infection.

2) During the initial weeks following infection, before the host immune response was fully established, significant amounts of viral antigen were detected in animals that received virus with intact nef, but not in those which received virus with a deletion in nef. These findings do not support the hypothesis of fast and unchecked replication of the nef-defective clone during the initial phase of infection.

3) In support of the experimental data of Kestler et al. (1991), Blumberg et al. (this Forum) found that the HIV1 nef genes in pathological tissues are intact and that the HIV1 nef is most likely to be a positive regulator in vivo.

The experimental data of Blumberg et al. support our hypothesis that Nef function is similar in HIV and SIVMAC and very important for the pathogenic potential of both groups of primate lentiviruses. However, antibody formation against Nef does not seem to be a useful diagnostic marker for disease progression in the course of HIV1 and SIVMAC infection (Kirchhoff et al., 1991; Spohn, this Forum). The replication of nef-defective SIVMAC is already decreased before the host immune response is fully established (Kirchhoff et al., 1991) and functional nef accelerated HIV1 replication in primary human lymphocytes (Blumberg et al., this Forum). Thus, nef is most likely positively influencing viral gene expression in vivo. Nef is apparently not acting like an oncoprotein or G-like protein (Venkatesan; Nebreda et al.; Kaminich et al.; this Forum) and it still remains to be elucidated which of the multivalent properties of Nef are important for the positive regulatory effect during the initial phase of viral infection. As already mentioned by the other contributors to this Forum, the down-regulation of cell surface CD4 (Garcia and Miller, this Forum) or the prevention of antigen-receptor-mediated induction of IL2 mRNA (Luria et al., 1991) by Nef can contribute to the later development of immunological disorders.

It is interesting that our results also indicate that Nef is positively influencing replication of the HIV2-BEN clone in Molt-4 clone 8 cells. Perhaps, for unknown reasons, some cell culture systems are more suitable indicators of Nef functions in vivo.

Despite at least 100-fold lower virus loads, rhesus monkeys infected with nef-defective SIVMAC showed a strong antibody response. If an intact nef gene is indeed obligatory for the development of disease in SIV-infected monkeys and HIV-infected humans, it presents an excellent target for the development of an attenuated live vaccine.

References


J. Brünjes, R. Spohn and G. Jung:

The physiological function of the Nef protein remains enigmatic and no consensus can be reached with respect to its biochemical or physiological activity. Currently, we would like to focus on the question of the GTP-binding and GTPase activity of the Nef protein.

Initially proposed by B. Guy et al. (1987), GTP-
binding and GTPase activity had been assigned to HIV Nef. Therefore, in order to suggest a 3-D model of Nef using molecular modelling based on structurally known proteins of the GTPase superfamily, we tried some time ago to find sequential — and structural — homologies between Nef and proteins with GTPase activity.

Nearly all members of the GTPase superfamily have the characteristic major sequence motif GXXXXXGK(S,T) (see also Venkatesan, this Forum) which seems to be essential for the ability to bind guanine nucleotides. The motif is preceded by a β-strand and, in general, is followed by an α-helical structure. This can be seen e.g. in the case of thymidine kinase (TK) of Herpes simplex viruses HSV1 and HSV2 and related viruses. These well-characterized proteins have different lengths, but nevertheless a similar overall 3-dimensional folding structure is known. Also, the G-loop region is highly conserved, and furthermore, both the preceding hydrophobic β-strand and the following α-helix are found.

Alternatively, Nef does not fulfil this requirement and no such motif can be found. It is therefore, in our opinion, not a member of the GTPase superfamily.

However, we would like to emphasize that in contrast to Venkatesan's findings, there are very rare exceptions (e.g. the thymidine kinase of fish lymphocystis disease virus and some procaryotic elongation factors, EC EF-G, SR EF-G and AN EF-G) where the sequence motif GXXXXXGK(S,T) is not absolutely necessary for GTP-binding. The reason for this is that the binding sites can be characterized by dependent interaction of steric and electrostatic interaction and induction of di- and multipoles in a dynamic manner. We suppose that switching states of H-bond networks could be responsible for substrate entry and non-covalent binding. Therefore, it is not out of the question that functional equivalence could be realized by a different amino acid pattern, especially within this dynamic aspect.

Lastly, it should be mentioned that the patterns of consensus like GXXXXXG suggest arbitrary degrees of freedom in non-conserved amino acid positions. However, their dependency with respect to protein dynamics has not so far been investigated. The experimental results are very controversial and, in comparison to Ras p21, only very low effects are detectable (Kaminchik et al., this Forum). In our view (and as mentioned by other authors), Nef is completely different from Ras and the G-protein superfamily. Therefore, it appears that no rational approaches for molecule modelling of Nef can be based on structurally known proteins of the GTPase superfamily.

"Some similarity" of the ATP-binding motif GXGGXGK may be seen for KEKGGLEG. Indeed, this is one of the most conserved motifs within all Nef sequences. However, as mentioned by Venkatesan, this motif is significantly less characteristic than the G-loop motif since some substitutions occur at hydrophilic positions 2, 3 and 7. Also, no substitution to lysine at the last position can be detected within the whole set of sequences. Furthermore, the spacing between position 5 and 8 is different.

The middle region of Nef is comparatively well conserved within the two sequence groups represented by HIV1 and HIV2. Functional or structural importance can be suggested, but significant localization of a probable ATP-binding site seems to be purely speculative at the moment. A possible reason for the conserved region of Nef-p27 (position 73-89) is association to an insoluble fraction of the cytoplasm as discussed by Kaminchik et al. Furthermore the possible surface association is supported by experimental results. A proline cluster in this region as well as significant amino acid exchanges with the fourth proline residue (which is next to a positively charged lysine) indicate both possible chain reversal and surface association. Therefore, we do not agree with the assumption of Smythe and Reitz, that many if not all Nef sequences represent non-functional proteins. Numerous globin sequences are functional despite having wide sequence space. The proposed binding of guanine nucleotides suggests a functional role in signal transduction and cellular regulation. Possible autophosphorylation or phosphorylation of protein kinases (including protein kinase C, as suggested by Laurent-Crawford) points to a similar function. It must be stressed, however, that the proposed phosphorylation site at position 15 is not well conserved within the highly homologous group of HIV1, HIV2 and related virus isolates since Ala and even Lys can occupy this position.

Because the cellular localization, extracellular occurrence and functional importance are not clear, the use of sequence-characterized and highly Nef-specific monoclonal antibodies to study the localization and functional characteristics of Nef in vitro and in vivo is an exciting prospect. Furthermore, results of epitope fine mapping, replacement analysis and CD measurements taken on overlapping peptides should give hints of some structural aspects of Nef protein.

Key-words: HIV, AIDS, Nef; Genetic regulation, Protein, Functions, Relationships; Forum.
During the past decade an enormous amount has been learned about the human immunodeficiency virus. Its structure, life cycle and regulation have been dissected and elucidated. Although much remains to be learned, the progress to date has been unprecedented. Fundamental to understanding the pathogenesis of HIV and to the strategies for treatment and prevention is an appreciation of its biology and molecular biology. Marty Bryant and Lee Ratner offer a succinct and extraordinary well-informed review. This is a paper worth reading—and rereading. The result will be a better appreciation about the range of problems and potential solutions that pertain to acquired immunodeficiency syndrome.

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Biology and molecular biology of human immunodeficiency virus

MARTIN L. BRYANT, MD, PHD AND LEE RATNER, MD, PHD

Our ability to control the spread of acquired immunodeficiency syndrome (AIDS) in the human population depends on a basic understanding of the biology and molecular biology of the human immunodeficiency virus (HIV). What we learn about the replication and pathogenesis of HIV will determine our approaches to the treatment and prevention of HIV disease. This information will also influence the outcomes of future encounters with other human retroviruses. To date what is known about HIV is remarkable, given the short period of time since its discovery as the causative agent of AIDS. Clinicians and epidemiologists have carefully described the rate of spread of HIV in specific population groups and defined multiple risk factors for infection and disease. Biologists have determined the mechanism of transmission and discovered that certain cellular and environmental cofactors, including other pathogens, may modify the progression and pathogenesis of HIV. Molecular biologists have dissected the genome of the virus and identified functionally significant segments that determine virulence, tissue tropism and sensitivity to specific antibodies or antiviral agents. However, major gaps in our understanding of the molecular details of HIV infection and pathogenesis of disease remain unanswered. How does sequence heterogeneity relate to biologic heterogeneity? What are the genetic determinants that confer cytopathicity and what is their clinical significance? What is the nature of the "latent" state of infection that is presumed to occur in vivo, and what immune or nonimmune host factors and viral factors play a role in this process? How do viral and cellular factors interact to mediate or influence the virus life cycle? The active pursuit of knowledge in these areas is crucial not only to understanding HIV disease but also to the development of new therapeutic modalities and vaccine strategies against infection by any human retrovirus, known or unknown.
BIOLOGY OF HUMAN IMMUNODEFICIENCY VIRUSES

Since the initial identification of HIV as the etiologic agent of AIDS, two related but distinct subtypes, HIV-1 and HIV-2, have been described. The original isolates of HIV-1, lymphadenopathy-associated virus, human T-lymphotropic virus type III (HTLV-III), and AIDS-associated retrovirus, as well as the more recently isolated strains, share significant molecular and biologic properties. HIV-1 and its variants are associated with the AIDS epidemic in central Africa, Haiti, western Europe and the United States. The second subtype, HIV-2, is much less prevalent than HIV-1 and currently is endemic in western Africa. It has also been found sporadically in the United Kingdom, several European countries, Brazil and recently in the United States. The genetic similarity between HIV-1 and HIV-2 is markedly less (40 to 50% nucleotide similarity) than that among different HIV-1 isolates (85 to 97% nucleotide identity). However, serologic testing can identify cross-reactive antibodies to HIV-1 and HIV-2. Despite speculation that infection with HIV-2 is less pathogenic or causes a slower progression toward disease than HIV-1 infection, recent evidence clearly demonstrates that both HIV subtypes are associated with AIDS and related clinical syndromes.

Theories on the origin of HIV and evolutionary relationships between HIV-1, HIV-2 and the simian immunodeficiency viruses (SIV) are based on seroepidemiologic data and computer-assisted sequence analysis of conserved and divergent regions of the viral genomes. In general, these types of comparisons indicate that the SIV are very similar to their human counterparts (HIV). However, SIVsmin, SIVmac and HIV-2 appear to form a subgroup of more closely related viruses (80 to 100% similar) that are divergent (50% dissimilar) from HIV-1. SIVmnd is equally related to HIV-1, HIV-2 and SIVmac. A retrovirus isolate recently isolated from a chimpanzee caught in the wild appears to be more similar to HIV-1 than to HIV-2, but further characterization is needed. Perhaps the most consistent interpretation of the existing data is that the transmission of a primate lentivirus (SIV) to humans (HIV-2) was followed by its rapid evolution (HIV-1) and explosive escape from an isolated human population. This and other theories that attempt to describe the origin of HIV-1 and HIV-2 will be continually tested as additional seroepidemiologic and molecular data become available.

Understanding the biologic heterogeneity among isolates of HIV-1 and the mechanism of evolution, or selection of isolates with altered properties in the host over time, remains the greatest challenge in the development of antiviral and vaccine strategies. The specific clinical condition and rate of progression of AIDS have been associated with the prevalence of certain HIV variants that differ in replicative rate, cell host range or tropism and syncytia-forming ability. In addition, virus persistence and induction of latency, as well as sensitivity to neutralizing or enhancing antibodies, vary significantly between virus isolates from different individuals and those from the same patient at different stages of HIV disease. The term "quasispecies" has been used to describe HIV isolates in terms of populations of closely related but divergent viruses present simultaneously in a single patient. The genetic evolution and selection of the "quasispecies" of HIV may contribute to progression of HIV-associated disease. HIV isolated from asymptomatic carriers tends to grow more slowly and to lower titers in cell culture (slow/low virus strains). In contrast isolates from AIDS patients more frequently grow rapidly and to higher titers (rapid/high strains), establish continuous viral replication in CD4+ lymphoid and mononucleated cell lines and often induce syncytia. The replicative capacity of certain HIV-1 isolates in primary human monocytes has recently been found to correlate with a discrete tropism determined in vivo.

The variability in the time between HIV infection and overt clinical disease (AIDS) among different individuals can be partially explained by the size of the virus inoculum, by the influence of the particular genetic background governing the host's immune response and by the cell cycle-dependent expression of regulatory factors. However, another possible explanation might be the influence of certain exogenous cofactors in determining progression of HIV-related disease. Infection by an immunosuppressive pathogen like cytomegalovirus, in addition to HIV, or the use of immunomodulating drugs could compromise normal defenses so that an individual cannot resist HIV infection. It is also possible that regulatory proteins specified by other viruses could influence the replication of HIV. Superinfection of cells carrying the HIV genome with DNA viruses from the herpes and papova groups can result in activation of HIV expression. Also infection of B cells with Epstein-Barr virus or infection of other cell types with HTLV-I increases their susceptibility to the infection, replication and cytopathicity of HIV-1.

The CD4-bearing helper-inducer T lymphocyte (T4 cell) was initially identified as the specific target of HIV infection because of its selective depletion in vivo. When the CD4 gene is inserted and expressed in certain CD4-negative human cells, normally resistant to HIV, they become susceptible to infection and form multinucleated giant cells. Antibodies directed against the amino-terminal region of the CD4 molecule block HIV-1 binding and syncytia formation and prevent
the spread of infection. The CD4 molecule is present primarily on the surface of certain T lymphocytes, bone marrow progenitor cells and monocytes and macrophages. In addition the expression of low levels of CD4 on the surface of a variety of nonhematopoietic cells, including epidermal Langerhans cells, follicular dendritic cells of the lymph nodes and certain cells of the central nervous system appears to explain their susceptibility to HIV infection. However, infection of fetal brain cells, glial cell lines and colorectal cells, which do not express detectable surface CD4, suggests that another mechanism could also be responsible for viral entry.

The extent of virus replication also depends on the specific host interaction with the virus. HIV strains differ not only in their ability to productively infect various human cell types but also in the level of virus production once infection has taken place. Established T cell lines expressing similar amounts of CD4 antigen show varying degrees of virus production, indicating that intracellular mechanisms influence the ability of different HIV strains to replicate. In addition a virus that grows in one established T cell line will replicate in other T cell lines but not necessarily in B cells or macrophages. However, other virus isolates, particularly those isolated from the brain, grow best in macrophages and not in established human T cell lines. Moreover the blood isolates are often cytotoxic in peripheral blood mononuclear cells, unlike most brain isolates.

The susceptibility of astrocytes and oligodendrocytes to HIV infection demonstrates the neurotropism of HIV and its similarity to the lentiviruses suggests a mechanism by which HIV can directly cause neurologic damage. In addition de novo synthesis of immunoglobulin directed against HIV present in the cerebrospinal fluid confirms that certain cells within the central nervous system (CNS) are infected early after the initial infection. Despite these observations little is known concerning HIV neurotropism and how the virus actually gains access to the CNS in infected individuals. However, brain isolates replicate more efficiently in cells of the macrophage-monocyte lineage, indicating that neurotropism of HIV may be coupled with macrophage tropism. It is generally believed that HIV crosses a compromised blood-brain barrier within infected macrophages. Alternatively extracellular virus may reach the cerebrospinal fluid after replication in cells of the choroid plexus. In the developing nervous system virus shed from the infected mother may cross the placenta and directly infect partially differentiated precursor cells. HIV infection of human glial cell lines is not blocked by treatment with monoclonal antibody to the gp120-binding epitope of CD4 or by a soluble form of CD4, suggesting that infection of certain cells of the CNS may proceed by a CD4 receptor-independent mechanism.

The most consistent feature of the pathogenesis of HIV infection is the gradual decrease of the CD4+ T lymphocyte from the circulation. Extensive virus replication and cytopathic require allogeneic lymphocyte stimulation or activation. The gradual loss of CD4+ cells could be explained by activation of only a subset of cells at any one time or by the requirement of multiple stimuli to activate different lymphocyte subsets, leading to their destruction by HIV. Depletion of CD4+ lymphocytes by HIV may be caused by single cell killing or the formation of multinucleated giant cells. The process of HIV cytopathicity involves disruption of the integrity of the cell and interference with normal cellular metabolic processes. The major cytotoxic effect observed in vitro under some experimental conditions is the formation of multinucleated giant cells (syncytia), a result of the interaction of the virus envelope glycoproteins in gp120 and gp41, and the CD4 molecule on the surface of susceptible cells. The multinucleated giant cells undergo ballooning degeneration, which is probably caused by a disruption in the activity of membrane-associated ion pumps (Na+, K+ATPase and K+ channels). Alternatively the cytopathic mechanism may involve an increase in membrane permeability to cations, particularly Ca2+ which alters the plasma membrane electrical potential, depresses synthesis of phosphatidylincholine and diacylglycerol and interferes with second messenger activity. However, cell fusion is only one of several explanations for cell loss after acute infection with HIV. The release of virus particles may directly disrupt membrane integrity, leading to the change in permeability described above. In addition there is a direct correlation of cytopathicity with the accumulation of unintegrated viral DNA in infected cells similar to that observed with the other lentiviruses.

The loss of T helper cells disrupts normal cellular immune function and eventually leads to loss of the control of fungal, protozoal and other viral opportunistic infections or unopposed proliferation of virus-transformed and malignant cells. In addition the role of T helper lymphocytes is impaired in HIV-infected individuals in the regulation of B cell responses to soluble antigens. Polyclonal activation of B cells results in hypergammaglobulinemia and an impaired ability to initiate a new antibody response. CD8+ lymphocytes can suppress HIV replication without killing the infected cells. One mechanism responsible for this antiviral effect is the production by CD8+ cells of a cytokine with anti-HIV activity. Because the level of CD8+ lymphocytes remains relatively elevated during the course of infection, the extent of antiviral activity would depend on the function of a subgroup of the CD8+ population.
HIV infection of monocytes and macrophages may disturb antigen presentation and local immune phenomena. Such a loss of immunosurveillance in the lung could predispose a child to Pneumocystis carinii pneumonia and contribute to the development of lymphoid interstitial pneumonitis. Persistent and latent infection of tissue-specific macrophages may also play a role in the chronicity and progressive nature of HIV infection. One important biologic characteristic of retroviruses in general, and lentiviruses in particular, is their ability to establish a persistent or latent infection throughout the life of the host. This is accomplished by integration of a DNA copy of the viral genome into the host chromosomal DNA. Very little cell-free virus is found in infected persons, and fewer than 1% of circulating lymphocytes harbor an HIV provirus or express detectable HIV messenger RNA. Therefore systemic spread of HIV during the acute infection must be followed by restricted virus replication imposed by dominant viral or cell-specific factors. HIV infection in macrophages isolated throughout the body, but particularly those isolated from the CNS, are thought to contribute to HIV persistence because of their refractoriness to the cytopathic effect of HIV. In addition the infected monocyte or macrophage in the CNS may be protected from immunologic mediated clearance. In certain cases a small percentage of infected T lymphocytes may survive the cytopathic effects of HIV and lead to a chronic low level production of virus. The persistence of HIV can be demonstrated in vitro by activation of virus replication with halogenated pyrimidines or by mitogens (pokeweed mitogen), certain growth factors or monokines (tumor necrosis factor alpha and granulocyte or monocyte colony-stimulating factor) or concomitant infection with other viruses such as Epstein-Barr virus, HTLV-I, cytomegalovirus, hepatitis B virus, human herpesvirus type 6 or herpes simplex virus. In addition latency may result from the down-regulation of HIV expression by specific virus-encoded proteins or the influence of cell type-specific factors.

The immunologic response of the host may also contribute to the observed immune abnormalities. Autoantibodies produced against the specific fusion complex between the HIV envelope protein (gp120) and cell surface receptor (CD4 molecule) have been detected. In addition autoantibodies to neoantigens, induced or modified by the virus infection of lymphocytes, may contribute to the immunologic disorder. Cytotoxic T cells that attack both infected and uninfected CD4+ cells and activation of suppressor T cells have also been implicated. Finally antibodies that enhance viral infection through a complement-dependent mechanism have been detected. Binding free gp120 in the circulation to the surface of uninfected CD4+ cells may sensitize these cells to destruction by several immunologic mechanisms. Additional indirect mechanisms may also play an important role in HIV-1 cytopathicity, particularly in AIDS dementia. Certain neurotropic strains of HIV-1 that lack direct lymphocytotoxic effects can infect macrophages, which may release toxic cytokines. Disease induction in the brain could involve specific inhibition or interference of neuronal growth by these cytokines or directly by the viral envelope glycoprotein.

**STRUCTURE AND LIFE CYCLE OF HIV**

The structure of HIV typifies the morphologic composition of retroviruses in general and lentiviruses in particular (Fig. 1). HIV has a cylindrical eccentric core, or nucleoid, containing the diploid RNA genome associated with a basic nucleic acid-binding protein p9 (Mr, 9000) and the reverse transcriptase (RT). The capsid antigen p24 encloses the nucleoid components, completing the nucleocapsid structure. The matrix antigen p17 surrounds the core of the virus and lines the inner surface of the envelope of HIV. The surface of the virion displays external knoblike structures formed by the envelope glycoprotein gp120. The transmembrane protein gp41 has both external and internal domains. It anchors the external gp120 to the viral envelope. The lipid bilayer is derived from the host cell plasma membrane.

HIV attaches to cells through a specific interaction of the viral envelope glycoprotein (gp120) and the cell surface-associated CD4 molecule (Fig. 2). The transmembrane component gp41 of the envelope gene product noncovalently interacts with gp120 and plays a role in virus-cell and cell-cell fusion events. Together these surface components of HIV are responsible for virion binding of CD4-bearing cells and for syncytia formation between infected and uninfected cells. Penetration of the virus capsid into the cell probably occurs by fusion of the viral and cellular membranes. Species-specific cellular factors are important for this event, because murine cells with the human CD4 antigen bind but do not take up HIV. However, nothing is currently known about the characteristics of such cellular infectivity factors.

Once the virus core is internalized and partially...
uncoated, a virus-specific polymerase (reverse transcriptase) converts the single stranded viral RNA into double stranded linear DNA. This activity is characteristic of all retroviruses and is the basis for their name. The DNA product is translocated from the cytoplasm to the nucleus. Although the direct precursor for the integrated proviral DNA has not been identified, recent findings implicate the linear molecules as the critical substrate for this reaction.22 Processing by the virus-specific integrase is required for insertion into the host's chromosome.

HIV gene expression proceeds via synthesis of viral transcripts (messenger RNA) with the use of host cell RNA polymerase II and other cellular and viral transcriptional factors. Full length transcripts comprise the messenger RNAs (mRNAs) for gag and gag-pol precursors and for the viral genomes found in the mature virion. The singly spliced transcript encodes the envelope components. Multiply spliced transcripts are translated into regulatory proteins that are not generally found in the viral particle but that have a significant effect on virus expression.23 The proportion of each type of viral mRNA reaching the cytoplasm is determined by another virus-specific protein encoded by the rev gene of HIV.

Morphogenesis involves formation of a ribonucleoprotein core consisting of a dimer of identical molecules complexed with gag and pol gene products. This core structure buds from the cellular membrane, acquiring a coat of virus envelope glycoprotein and a cellular lipid bilayer. During this process specific cleavage of the internal core components by the viral protease completes the maturation process before the next cycle of infection. Cell-to-cell spread of HIV, independent of virus release, can also occur through the fusion or syncytia formation of an infected cell and an uninfected cell.

The various laboratory methods available for the diagnosis of HIV-1 infection and isolation are listed in Table 1. Clinically useful tests such as the enzyme-linked immunosorbent assay and Western blot assay measure antibodies to the major structural proteins of the virus or the antigens themselves. The enzymatic assay for RT is generally used to detect infectious virus in cell culture after cocultivation of patient peripheral blood mononuclear cells, plasma or cerebrospinal fluid with susceptible cells.

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**TABLE 1. Methods of HIV detection**

<table>
<thead>
<tr>
<th>Detection of antiviral antibody</th>
<th>Target</th>
<th>Sensitivity (Specificity)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA</td>
<td>Viral lysate</td>
<td>99.3-99.7% (population dependent)</td>
<td>Licensed clinical assay</td>
</tr>
<tr>
<td></td>
<td>Recombinant protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Synthetic peptide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immunoblot (WB)</td>
<td>Proteins of disrupted virus</td>
<td>Similar to ELISA (WB specificity 99.4-100%)</td>
<td></td>
</tr>
<tr>
<td>IFA</td>
<td>Inactivated HIV-infected cells</td>
<td>Similar to ELISA</td>
<td></td>
</tr>
<tr>
<td>RIPA</td>
<td>Metabolically or chemically radiolabeled viral protein</td>
<td>Similar to ELISA</td>
<td></td>
</tr>
<tr>
<td>Particle agglutination</td>
<td>Antigen-coated latex beads</td>
<td>Similar to ELISA</td>
<td></td>
</tr>
<tr>
<td>Detection of virus or antigen</td>
<td>Fixed red blood cells</td>
<td>Similar to ELISA</td>
<td></td>
</tr>
<tr>
<td>Whole virus-cell culture (lymphoid cells and plasma)</td>
<td>Isolation of infectious virus</td>
<td>70-98% (laboratory dependent)</td>
<td></td>
</tr>
<tr>
<td>Protein-ELISA, Western blot, IFA, RIPA</td>
<td>Monoclonal or polyclonal antiviral antibody Antibody bound to solid support</td>
<td>50-100 pg/ml for p24</td>
<td></td>
</tr>
</tbody>
</table>

**Detection of nucleic acid**

<table>
<thead>
<tr>
<th>Direct</th>
<th>Sensitivity</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Southern blot</td>
<td>Proviral DNA</td>
<td>1 copy/10^6 cells</td>
</tr>
<tr>
<td>Northern blot</td>
<td>Viral or mRNA</td>
<td>1 copy/10^6 cells</td>
</tr>
<tr>
<td>Indirect PCR</td>
<td>Proviral DNA or cDNA copy of virion RNA</td>
<td>1 copy/10^6 cells</td>
</tr>
</tbody>
</table>

ELISA, enzyme-linked immunosorbent assay; WB, Western blot; IFA, immunofluorescent assay; RIPA, radioimmunoprecipitation assay; cDNA, complementary DNA.
In certain patients infection with HIV-1 may be difficult to determine by antibody or antigen enzyme-linked immunosorbent assay or virus isolation techniques because of latent infection or the presence of high-titered, passively acquired antibody (e.g. neonates). In these circumstances it is possible to detect HIV-1 DNA and RNA directly from cells of the patient by the polymerase chain reaction (PCR). This technique is used to amplify a specific HIV gene sequence present in very low copy number. The amplified segment of the HIV genome is identified by molecular hybridization techniques with a synthetic complementary DNA probe. PCR is capable of detecting a single virus-positive cell in 10⁶ cells, early in virus infection and before seroconversion. Because of its sensitivity PCR could be adapted to the routine screening of asymptomatic high risk individuals, such as infants born to seropositive mothers, or for monitoring the effect of antiviral therapy. If the sensitivity and specificity of PCR for detection of HIV-1 proviral sequences can be confirmed, this technique should prove to be an important diagnostic and prognostic tool.

**GENOMIC ORGANIZATION AND DIVERSITY**

The basic organization of the HIV genome is similar to the genetic structure of all other retroviruses (Fig. 3). Essential regulatory sequences, called long terminal repeats (LTRs), are present at both ends of the provirus. The retroviral LTRs contain control elements that direct and regulate expression of the viral genome. Four major regions have been identified in the HIV-1 LTR (Fig. 4): (1) the promoter, where RNA synthesis is initiated by binding of a cellular RNA polymerase; (2) an enhancer element subject to viral and cellular controls; (3) a negative regulatory element; and (4) the trans-acting (TAT) response region (TAR) that is affected by the tat gene product also encoded by the virus. Additional regulatory elements include a TATA box and multiple Sp1 binding sequences to which cellular factors can bind and possibly influence transcription. The 3' LTR specifies the addition of a polyadenylate tail to RNA transcripts, which influences their processing and export from the nucleus into the cytoplasm of the infected cell. Between the two LTR sequences are the gag and env genes, encoding the viral structural proteins, and the pol gene, which specifies the replicative enzymes of the virus. Each of these genes encodes a polyprotein precursor that is specifically processed into the different structural proteins and replicative enzymes found in the mature infectious virion. In addition, the HIV genome contains unique genes that encode nonviral proteins that function within the cell to control virus replication (vpr, tat, rev, tev, nef) and genes involved in virus transmission (vpu) and infectivity (vif). The genomic organization of all HIVs is very similar except that the vpu gene in HIV-1 is replaced by alternative gene, vpx, in HIV-2 and in all SIVs except SIVmnd and SIVcpz.

Considerable genetic variation exists among the different isolates of HIV-1 from different individuals and between sequential isolates from the same individual. Most isolates that have been sequenced show a nucleotide divergence of 6 to 10% over the entire genome. However, variation in the envelope protein among HIVs can be as much as 26%. In general, viroteintroduction into a new host undergoes more molecular change than virus maintained in the same host.
ditionally molecular variants isolated from a single individual clearly evolved from one another or from a common precursor virus and do not represent concomitant infection by independent or unrelated viruses. The rate of evolution of HIV-1 in persistently infected patients has been estimated to be at least $10^{-3}$ nucleotide/site/year in env and $10^{-4}$ in gag. Furthermore the changes seen in env are usually larger mutations involving deletions, insertions or duplications, whereas in gag and pol most are point mutations.

HIV replication includes three steps at which mutations are likely to be introduced into the genome. The viral DNA polymerase is error-prone and lacks a correcting mechanism usually associated with cellular polymerases. Also errors can be compounded during subsequent second strand synthesis, which follows the conversion of virus RNA into a single stranded DNA intermediate. Finally the cellular RNA polymerase that forms the new viral genome from the DNA provirus which is also incapable of error correction. Together these effects have been estimated to introduce one mutation per virus replication cycle. Many of these mutations are either lethal to the virus and eliminated, or occur in a part of a virus protein that has a limited functional role. However, certain genotypes may arise by these mechanisms or through genetic recombination that significantly affects important biologic properties, including tissue and cell type specificity, virulence and clinical spectrum of disease, immune responsiveness and resistance or sensitivity to antiviral agents.

**REPLICATION OF HIV**

The envelope gene (env) codes for a polyprotein (p85), which is glycosylated (gp160) and processed to form the surface virion envelope glycoprotein (gp120 or SU) and a noncovalently associated transmembrane glycoprotein (gp41 or TM). The gp120 molecule contains specific amino acid domains responsible for CD4 binding and virus-host interactions (Fig. 5). Other domains have been mapped in gp120 that are important for interaction with gp41. The gp41 molecule contains stretches of amino acids analogous to the fusogenic domains of paramyxoviruses and plays an important role in syncytia formation and perhaps in cell-to-cell spread of HIV. Fusion, mediated by gp41, is probably also necessary for penetration of the viral core into the interior of the cell. Another domain of gp41 spans the membrane and anchors the envelope molecule (gp120) onto the infected cell or virus particle. Additional domains of gp41 are critical for cell killing and for replication in cells of a particular species.

Glycosylation involving asparagine linkage of complex carbohydrates and specific proteolytic cleavage of the envelope protein of HIV-1 to SU and TM is host cell-mediated. The high mannose forms of the envelope protein are trimmed by cellular glycosidases and mannosidases to yield the gp160 protein. About 85% of this protein is targeted to lysosomes and degraded, while further processing in the Golgi apparatus. This includes proteolytic processing by a cellular protease, addition of fucose and sialic acid residues, oligomerization to trimers or tetramers and transport to the plasma membrane. At this point the envelope protein may undergo one of the three fates: (1) the SU-TM complex may interact with the gag-pol-RNA complex to assemble into a virus particle; (2) the SU-TM complex on the infected cell may interact with CD4 on uninfected lymphocytes, leading to cell-cell fusion; interaction of the SU-TM complex with CD4 on the same cell may also occur and contribute to cytotoxicity; (3) SU may be released from TM and the free SU molecule may interact with uninfected CD4+ lymphocytes. Mutations that affect virus attachment (gp120) or virus-mediated cell fusion (gp41) or drugs which alter glycosylation patterns disrupt the normal infectivity of HIV (Fig. 6).

The gag gene encodes a polyprotein precursor molecular (Pr55gag) that is cleaved into the major structural components of the virus capsid (p17 and p24) and core components (p15, further processed to p9 and p6) surrounding the virion RNA. During synthesis of Pr55gag the fatty acid myristate (C14:0) is cotranslationally attached to the amino-terminal end (p17) of the precursor molecule. Myristoylation of Pr55gag is required for production of infectious virions. The change in hydrophobicity of the protein conferred by addition of the fatty acid moiety effects virion assembly by directing the spatial aggregation and processing of the polyprotein precursor at the membrane necessary for maturation and budding of the virus from the cell. Protein-N-myristoylation requires the cellular enzyme N-myristoyl transferase and a conserved recognition sequence, Gly-X-X-X-Ser/Tyr, at the amino terminus of the capsid precursor protein. Substitution or deletion of the amino-terminal glycine (Gly) residue prevents myristoylation and virus replication. Although virus structural proteins can be detected by immunologic techniques, no virus particles are identified by biochemical or electron microscopic methods. The two virion proteins, p9 and p6, are derived from p15, which is processed from the carboxy terminus of Pr55gag. The p9 molecule contains multiple cysteine residues that probably help to form specific secondary structures similar to other metallo-binding proteins (Zn$^{2+}$ fingers) involved in nucleic acid binding. The proline-rich virion core-associated protein, p6, is involved in the release of the binding particle.

A second polyprotein precursor, Pr160gag-pol, contains the gag, protease, polymerase and integrase gene products translated from the same genomic RNA message as the gag polyprotein Pr55gag. However, the pol components are produced by ribosomal frame-shifting...
FIG. 5. The gp120 molecule contains specific amino acid domains responsible for CD4 binding and virus-host interactions. Reproduced from Reference 10 with permission.

FIG. 6. Schematic representation of the synthesis and processing of the HIV envelope glycoprotein.

along the mRNA during translation that brings the overlapping, out-of-phase, gag and pol genes into translational phase. HIV frame-shifting is mediated by short, linear, homopolymeric sequences and possibly a downstream stem-loop structure in the viral RNA. In the mature virion the gag and pol products are present in a ratio of about 20:1. The pol part of the message actually encodes three proteins that are cleaved from the larger precursor molecule (Pr160gag-pol). These include the protease, RT and integrase. Self-dimerization of the gag-pol precursor at the cell membrane is thought to lead to the autocatalytic activation of the virus-specific protease, which is required for further processing of Pr160gag-pol as well as Pr55gag. The viral protease is an aspartyl proteinase that is necessary for normal morphogenesis and infectious virus particle production. In the absence of protease activity resulting from mutation, deletion or
inhibitory drugs, noninfectious and morphologically aberrant virus particles lacking electron-dense cores are formed. Myristoylation of Pr160gag-pol has also been described and is postulated to affect the phosphorylation and processing of the polyprotein through an influence on localization and orientation of the molecule at the cell membrane (Fig. 7).

Cleavage of the gag-pol precursor by a functional protease releases the DNA-dependent RNA polymerase (RT) and integrase. The virus RT has an associated ribonuclease H activity, which forms a replication complex responsible for synthesis of a DNA copy (provirus) of the viral RNA genome during early stages of HIV infection. Initiation of DNA synthesis begins with the specific primer, tRNA, bound by the polymerase. This is followed by the addition of deoxynucleotides to the 3' end of the transfer RNA primer. Elongation proceeds through the further addition of nucleotides to the 3' end of the growing chain in an order complementary to the viral RNA. This elongation reaction is inhibited by dideoxynucleoside analogs such as azidothymidine, dideoxycytidine and dideoxyinosine. Conversion to the active triphosphate form by cellular enzymes is required, however, before these exogenously added analogs can terminate DNA elongation. Once incorporated, they terminate this process because they lack the 3-hydroxyl group needed for the addition of the next nucleotide. Isolates of HIV with reduced sensitivity to azidothymidine or dideoxyinosine have been isolated from patients after prolonged therapy. In some cases azidothymidine (or dideoxyinosine) resistance is caused by mutations in the reverse transcriptase. However, additional studies are required to determine the clinical significance of drug resistance.

After initial DNA synthesis has occurred at the 5' end of the genome, the polymerase jumps to an identical sequence (LTR) repeated at the 3' end of a second genome. The partially formed DNA strand continues to elongate until a complete DNA copy of the RNA is removed or degraded during the synthesis of the second DNA strand by the ribonuclease H activity. The completed provirus is now double stranded DNA containing duplicated sequences at both ends (LTR). It moves by an unknown mechanism to the nucleus of the infected cell, where integration into the host chromosome takes place. The viral integrase (M, 31,000 protein) cleaved from the carboxy terminus of the gag-pol precursor functions to remove the two terminal nucleotides of the provirus in preparation for integration. Integrase-defective mutants of HIV fail to replicate efficiently in T lymphocytes. There are conflicting data as to the necessity for integration for subsequent steps in the virus life cycle.

REGULATION OF REPLICATION

The control of HIV replication is modulated through the interplay of HIV-encoded regulatory gene products (tat, rev, vpu, vpr and nef) and specific sequences present in the viral RNA or provirus. It is possible that activation or repression of virus life cycle-specific events are influenced by cellular factors or proteins produced by other pathogens. Each regulatory protein reacts either directly with a specific nucleotide sequence present in the genome or virus-specific RNA transcript, or directly via additional factors present in the cell. The regulatory protein is said to act in trans if it produces an effect at a distance (e.g., through a protein product). A response sequence that affects adjacent genes is said to be cis-acting. Individually or in concert these regulatory functions influence the rate of viral replication and therefore determine the development of a cytopathic infection or viral persistence and latency.

The level of HIV replication is profoundly affected by activation of a resting lymphocyte. Resting T cells are nonpermissive for the replication of HIV-1 despite efficient binding of the virus to the CD4 receptor molecule displayed at the surface of the cell. T cell activation is important for virus penetration. It is also speculated that the infecting virus is maintained in an unintegrated form before activation of the resting lymphocyte. Once the host cell is activated, inducible host transcription factors stimulate a low level of early HIV-1 gene expression.

The initial mRNA molecules that reach the cytoplasm are exclusively multiply spliced (~2-kilobase) messages that encode the HIV-1-regulatory gene products. Once the tat protein is produced it affects a positive feedback of HIV gene expression. tat acts by enhancing the rate of transcription of all sequences linked to the HIV LTR. The cis-acting target sequence, designated TAR, is a hairpin loop structure formed within the HIV-1 LTR from nucleotides 1 to 45. The TAR sequence may also play a role in translation regulation. Interesting recent data suggest that tat may be exported from infected cells and taken up by uninfected cells. tat may also have effects on cellular gene expression and has been implicated as an immune suppressor molecule or a paracrine growth factor important for the development...
of Kaposi's sarcoma. By interacting with the TAR-containing RNA, tat can facilitate transcription at an initiation or elongation step.

In contrast to the positive effect of the regulatory protein tat on HIV replication, the nef gene product has been reported to down-regulate transcription from the HIV-1 LTR by interfering with cellular signaling or by activating factors that interact with a negative regulatory sequence in the LTR. nef binds and hydrolyzes guanosine triphosphate, suggesting that it may belong to the family of signal-transducing proteins. Mutations in the nef gene produce viruses that replicate to higher titers in tissue culture than the wild-type virus under at least some experimental conditions. However, the role of nef in vivo remains to be determined.

The transition from early regulatory gene expression to HIV structural gene expression is determined by the effect of the rev gene product. It is a 19-kilobase phosphorylated protein that is localized in the nucleus of virus-expressing cells. Expression of the rev protein induces the appearance of cytoplasmic unspliced (gag-pol) and singly spliced (env) viral mRNAs and decreases the expression of multiply spliced RNAs that encode the viral regulatory proteins. rev appears to function by activating the nuclear export of a constitutively expressed pool of incompletely spliced viral RNAs that are normally excluded from the cytoplasm of the cell. It is unclear whether this is a result of an interaction with the RNA splicing or transport machinery or whether these processes are in fact separable. Each of the mRNAs for the structural genes contains negative cis-acting sequences that act to mark the particular RNA species for splicing, or perhaps degradation, in the absence of rev. rev can interact directly with a complex secondary structure designated the rev response element, which is located within the HIV-1 env gene. Viral mRNAs are then displaced into the cytoplasm from the nucleus and from the carboxyl terminal tail of the TM envelope protein by the effect of the rev gene product. It is a 19-kilobase RNA virus that has budded from one cell to infect another cell. However, rev is not required for transmissor virus via cell-cell fusion. HIV strains with mutati on that carry a full complement of RNA, enzymatic, and structural proteins but infect cells 100-fold more efficiently. Recently it has been suggested that rev may encode a protease that cleaves a small peptide from the carboxyl terminal tail of the TM envelope glycoprotein.

An additional protein with potential regulatory activity has been designated viral protein T (vpt). It is presumed to be synthesized by a ribosomal frame shifting event. However, its function remains obscure.

REFERENCES