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The adenovirus E4 region-encoded open reading frame 1 (9ORF1) transforming protein is the major oncogenic determinant of human adenovirus type 9 (Ad9), a virus that generates exclusively estrogen-dependent mammary tumors in rats. Our previous studies demonstrated that 9ORF1 possesses a PDZ domain-binding motif and that disruption of this motif abolishes 9ORF1 transforming activity. PDZ domains are protein-protein interaction modules found in certain cell signaling proteins that localize to specialized membrane sites in cells. Consistent with these observations, we have now identified four different cellular PDZ-domain proteins that complex with 9ORF1 in cells. The fact that three of four 9ORF1-associated PDZ-domain proteins are common targets for high-risk papillomavirus E6 oncoproteins provides further support for the idea that interaction of 9ORF1 with these cellular factors contributes to transformation. Finally, we present results showing that 9ORF1 transforms cells, in part, by activating the phosphoinositide 3-kinase (PI 3-K)/protein kinase B (PKB) signaling pathway. Significantly, our results with 9ORF1 mutant proteins indicate that this important activity depends on the ability of 9ORF1 to complex with the cellular PDZ-domain proteins. It is hoped that these studies will reveal new mechanisms involved in the development of some types of breast tumors in women.

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INTRODUCTION

One of my research career goals is to decipher a novel cellular pathway which, when perturbed, leads to the development of breast neoplasms. The proposed research focuses on a unique viral oncogenic determinant encoded by human adenovirus type 9 (Ad9), a virus that elicits exclusively mammary tumors in rats. Following infection of newborn rats with Ad9, female animals develop estrogen-dependent mammary tumors (primarily fibroadenomas) within multiple mammary glands after a three-month latency period, whereas no tumors of any type form in infected male rats. In contrast to other adenoviruses, Ad9's major oncogenic determinant is its E4 region-encoded open reading frame 1 (9ORF1) transforming protein. Results obtained to date suggest than the abilities of 9ORF1 to bind a select group of cellular proteins and to activate the phosphoinositide 3-kinase (PI 3-K)/protein kinase B (PKB) signaling pathway correlate well with its transforming function. Therefore, studies of this model system may reveal a completely new route for breast cancer development. The main goal of this project is to elucidate a detailed molecular model of 9ORF1 oncoprotein function. The two objectives of the proposed work are: (1) To identify the 9ORF1-associated cellular proteins and (2) To reveal the mechanism whereby 9ORF1 activates the PI 3-K/PKB signaling pathway in cells (revised objective).

BODY OF ANNUAL SUMMARY

OBJECTIVE 1: Identify the 9ORF1-associated cellular proteins.

1A. The transforming activity of 9ORF1 is needed for Ad9 to generate mammary tumors in rats.

We previously showed that Ad9 mutant viruses unable to express the 9ORF1 protein fail to generate mammary tumors in rats. In addition, we subsequently reported the isolation and characterization of six stably-expressed 9ORF1 mutant genes (IA, IIA, IIB, IIIA, IIIC, and IIID) which are defective for transformation of CREF cells, an established rat embryo fibroblast cell line. The mutations within these six 9ORF1 mutant genes have defined three separate protein regions (Region I, II, and III) required for 9ORF1 transforming activity. Region III at the extreme C-terminus of 9ORF1 represents a functional PDZ domain-binding motif.

In an attempt to provide a direct link between 9ORF1 *in vitro* transforming activity and the ability of Ad9 to induce mammary tumors, we recently introduced each of these six 9ORF1 mutant genes into Ad9 viruses and tested them for an ability to induce mammary tumors in rats. The fact that all six mutant viruses coding for transformation-defective 9ORF1 genes failed to elicit tumors whereas virus Ad9/9ORF1-T108 coding for a transformation-proficient 9ORF1 mutant gene succeeded in producing tumors (Table 1) provides strong evidence that 9ORF1 transforming activity *per se* is required for Ad9 to promote mammary tumors in animals.

Table 1. Ad9 mutant viruses coding for transformation-defective 9ORF1 genes fail to induce mammary tumors in rats.

	No. of rats which developed tumors/ No. of rats infected with virus	
Virus	Females	Males
Ad9	3/3	0/2
Ad9/9ORF1-T108S	4/4	0/3
Region I mutant		
Ad9/9ORF1-IA	0/5	0/3
Region II mutants		
Ad9/9ORF1-IIA	0/4	0/3
Ad9/9ORF1-IIB	0/5	0/4
Region III mutants		
Ad9/9ORF1-IIIA	0/7	0/5
Ad9/9ORF1-IIIC	0/5	0/3
Ad9/9ORF1-IIID	0/5	0/4

1B. Evidence that 9ORF1 is the major oncogenic determinant of Ad9.

i. E1 region transforming functions are dispensable for Ad9-induced mammary tumorigenesis. The major determinants of tumorigenesis by prototype human adenoviruses are their E1 region-encoded E1A and E1B oncoproteins. Although the E4 region-encoded 9ORF1 oncoprotein is essential for mammary tumorigenesis by Ad9, it had not been determined whether Ad9 E1 region functions also contribute to this process. To test this possibility, we engineered large deletions within both the E1A and E1B genes of Ad9. Although such deletions abolished the transforming activity of an Ad9 E1 region-containing plasmid, Ad9 mutant viruses carrying identical deletions retained the ability to generate mammary tumors in rats (see attached paper by Thomas *et al.*). This finding provides strong evidence that 9ORF1 is indeed the major oncogenic determinant of Ad9.

ii. A 9ORF1-expressing Ad5 virus vector generates exclusively mammary tumors in rats. In an attempt to confirm that 9ORF1 is the major oncogenic determinant of Ad9, we generated the adenovirus vector shown in Fig. 1. Virus vector Ad5/CMV-9ORF1 consists of a non-tumorigenic human adenovirus type 5 (Ad5) genome having its E1 region (E1A and E1B genes) replaced by a CMV-9ORF1 expression cassette.

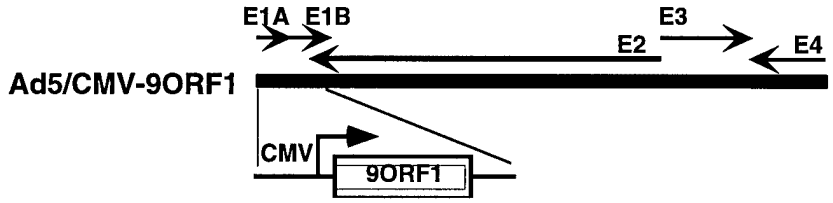

Fig. 1. Genome structure of the E1 region-deficient Ad5/CMV-9ORF1 virus vector.

In addition to it being tumorigenic following injection into rats, the Ad5/CMV-9ORF1 virus vector also elicits exclusively mammary tumors (Table 2), which are identical to those induced by Ad9 (data not shown). Expression of 9ORF1 is responsible for production of these tumors because a control Ad5 virus vector having its E1 region replaced by a CMV-LacZ expression cassette (Ad5/CMV-LacZ) fails to generate tumors of any type in animals (Table 2). This finding is significant because it suggests that, in addition to 9ORF1 representing the major oncogenic determinant of Ad9, this viral oncoprotein also participates in targeting tumorigenesis of Ad9 specifically within the rat mammary gland.

Table 2. An E1 region-deficient Ad5 virus vector carrying a CMV-9ORF1 expression-cassette induces mammary tumors in rats.

	no. of rats which developed tumors/ no. of rats infected with virus	
virus	females	males
Ad5/CMV-LacZ	0/5	0/3
Ad5/CMV-9ORF1	7/7	0/4

1C. The 9ORF1 oncoprotein complexes with four different cellular PDZ-domain proteins.

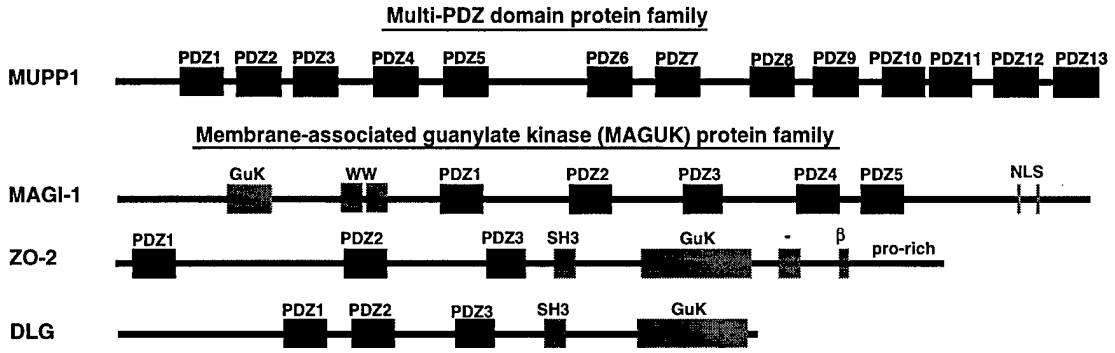

Fig. 2. The four identified 9ORF1-associated cellular PDZ domain proteins. Three of these polypeptides are MAGUK family proteins, which are characterized by having, in addition to PDZ domains, an SH3 or WW domain and a yeast guanylate kinase-homology domain.

We previously showed that Region III at the extreme C-terminus of 9ORF1 is critical for 9ORF1 to transform cells and to bind to four different cellular proteins (p220, p180, p160, and p140/p130). In my

last Annual Summary, I reported our findings that 9ORF1 Region III is a functional PDZ domain-binding motif and, in addition, that 9ORF1-associated proteins p220 and p140/p130 are the multi-PDZ domain protein MUPP1 and the membrane-associated guanylate kinase (MAGUK) protein DLG, respectively (Fig. 2). It is noteworthy that DLG is functionally homologous to the *Drosophila* tumor suppressor protein dlg and, in addition, similarly complexes with high-risk but not low-risk human papillomavirus (HPV) E6 oncoproteins. Like 9ORF1, high-risk HPV E6 oncoproteins possess a PDZ domain-binding motif at their C-termini and disruption of this motif abolishes their transforming activity.

By screening known cellular PDZ-domain proteins having sizes similar to 9ORF1–associated proteins p180 and p160, we have identified these cellular factors as DLG-related MAGUK proteins MAGI-1 and ZO-2 (Fig. 2). Similar to MUPP1 and DLG, MAGI-1 and ZO-2 also bind to wild-type 9ORF1 in both GST pulldown and co-immunoprecipitation assays, whereas they fail to bind 9ORF1 mutants having a disrupted PDZ domain-binding motif (*e.g.*, 9ORF1 mutant IIIA; data not shown). Results from protein blotting assays further indicate that 9ORF1 interacts specifically and directly with MAGI-1 PDZ domains 1 and 3 and ZO-2 PDZ domain 1 (data not shown).

We have therefore now succeeded in identifying all of the major 9ORF1-associated cellular PDZ-domain proteins. Although the functions of these cellular factors are not yet known, their domain structures suggest a role in signal transduction. In addition, the fact that DLG is functionally homologous to a *Drosophila* tumor suppressor protein leads us to hypothesize that all of the 9ORF1-associated PDZ-domain proteins may function to negatively regulate cell proliferation and that 9ORF1 inactivates their activity.

1D. The 9ORF1 oncoprotein aberrantly sequesters the PDZ-domain proteins in the cytoplasm of cells.

In my last Annual Summary, I showed results suggesting that 9ORF1 aberrantly sequesters DLG in the cytoplasm of cells. To assess whether 9ORF1 similarly sequesters the other 9ORF1-associated PDZ-domain proteins in cells, we performed indirect immunofluorescence assays to determine the subcellular localization of endogenous MUPP1 in normal CREF cells and CREF cells stably expressing wild-type or mutant 9ORF1 proteins. Similar to our results with DLG, we found that MUPP1 primarily localizes diffusely within the cytoplasm of normal CREF cells, whereas it becomes aberrantly sequestered within punctate bodies in the cytoplasm of CREF cells expressing wild-type 9ORF1 (data not shown). In addition, the 9ORF1 and MUPP1 proteins also co-localized within these cytoplasmic bodies (data not shown). The aberrant sequestration of MUPP1 by wild-type 9ORF1 in CREF cells was specific because CREF cells expressing 9ORF1 mutants IIIA and IIIC, which do not bind MUPP1, showed no sequestration of MUPP1, whereas CREF cells expressing 9ORF1 mutant IIID, which binds weakly to MUPP1, showed some reduced sequestration of MUPP1 (data not shown). We have obtained similar results for both MAGI-1 and ZO-2 in these CREF cell lines (data not shown). The functional consequences of 9ORF1 sequestering these cellular PDZ-domain proteins in the cytoplasm of cells is not yet known, although one intriguing possibility is that this effect leads to inactivation of these cellular factors.

1E. High-risk HPV E6 oncoproteins bind to three of the four 9ORF1-associated PDZ-domain proteins and may target them for degradation in cells.

The fact that DLG is also a cellular target for high-risk HPV E6 oncoproteins prompted us to determine whether these viral proteins similarly complex with other 9ORF1-associated PDZ-domain proteins. We found that, in GST pulldown assays, the wild-type HPV type 18 (HPV-18) E6 oncoprotein also binds to MUPP1 and MAGI-1, but not to ZO-2 (Fig. 3A). This binding was specific because the HPV-18 E6 mutant V158A, which has a disrupted PDZ domain-binding motif, failed to complex with these cellular factors. Therefore, three (MUPP1, MAGI-1 and DLG) of the four 9ORF1-associated PDZ-domain proteins are common cellular targets for the high-risk HPV-18 E6 oncoprotein.

Because high-risk HPV E6 oncoproteins target the tumor suppressor protein p53 for degradation in cells, we next examined whether similar effects could be detected for the 9ORF1-associated PDZ-domain proteins. This idea was tested by either expressing one of the 9ORF1-associated PDZ-domain proteins alone or together with HPV-18 E6 in COS7 cells. Significantly, compared to their levels of expression alone, we observed significantly reduced steady-state protein levels for MUPP1, MAGI-1, and DLG

when each was co-expressed with the HPV-18 E6 oncoprotein (Fig. 3B). Again, this effect was specific because the HPV-18 E6(V158A) mutant failed to reduce the protein levels of these PDZ-domain proteins. In addition, no reduction in ZO-2 protein levels was observed when ZO-2 was co-expressed with wild-type HPV-18 E6, consistent with our failure to detect complex formation between these proteins. The findings for DLG have been accepted for publication in *Oncogene* (see attached paper by Gardiol *et al.*).

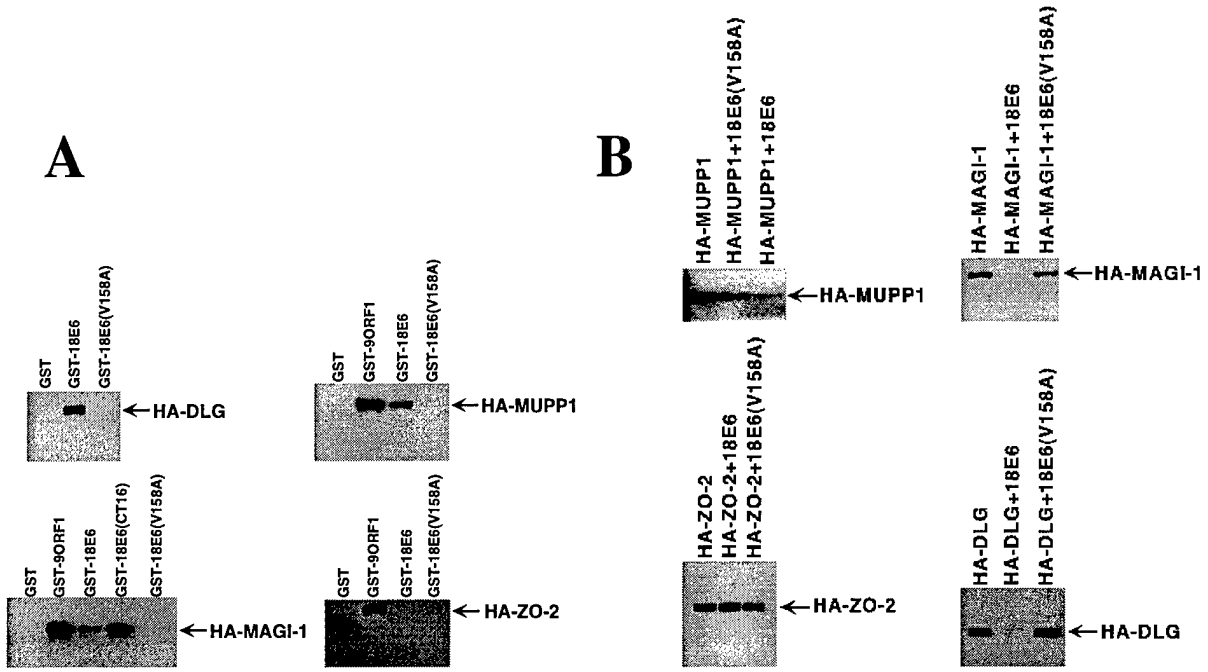

Fig. 3. **(A)** The HPV-18 E6 protein binds to DLG, MUPP1, and MAG1-1, but not to ZO-2. COS7 cells were transfected with a plasmid encoding an HA-tagged version of either DLG, MUPP1, MAGI-1, or ZO-2. At 48h post-transfection, the cells were lysed in RIPA buffer, and cell lysates were subjected to pulldown assays with the indicated GST fusion protein. Bound proteins were subjected to SDS-PAGE. Proteins were transferred to a PVDF membrane and then subjected to immunoblot analysis with the 12CA5 anti-HA monoclonal antibody. **(B)** The HPV-18 E6 protein reduced the steady-state levels of MUPP1, MAGI-1 and DLG in cells. COS7 cells either expressing the indicated HA-tagged PDZ-domain protein alone or together with wild-type or mutant 18E6 were harvested and lysed in 1X sample buffer. Similar amounts of cell lysate were subjected to SDS-PAGE. Proteins were subjected to immunoblot analysis with the 12CA5 monoclonal antibody as described above in (A). 18E6, wt HPV-18 E6; 18E6(V158A), mutant 18E6 with mutation in the PDZ domain-binding motif; 18E6(CT16), the 18E6 C-terminal 16 amino-acid residues containing the PDZ domain-binding motif.

<u>OBJECTIVE 2</u>: To reveal the mechanism whereby 9ORF1 activates the phosphoinositide 3-kinase/protein kinase B signaling pathway in cells (revised).

2A. Inability to recover soluble 9ORF1 protein for oligomerization experiments. The original Objective 2 was to determine the molecular-weights of purified wild-type 9ORF1 and IIIA mutant 9ORF1 homo-oligomers. As indicated in my previous Annual Report, 9ORF1 proteins expressed in *E. coli* were completely insoluble, even after addition of 0.2% SDS or 1% sarkosyl. Subsequent attempts to obtain soluble 9ORF1 protein from baculovirus-infected insect cells have similarly failed. Therefore, due to these unanticipated problems, we have abandoned the proposed oligomerization studies with the 9ORF1 protein. As we have recently discovered that 9ORF1 activates the phosphoinositide 3-kinase (PI 3-K)/protein kinase B (PKB) signaling pathway in cells (see below), we will now refocus the efforts of this objective toward revealing the mechanism for this important 9ORF1 activity.

2B. Wild-type but not transformation-defective mutant 9ORF1 proteins activate protein kinase B (PKB) in cells.

Our finding that 9ORF1 binds and sequesters cellular PDZ-domain proteins, which are predicted to function in signal transduction, suggests that 9ORF1 perturbs one or more signaling pathways in cells. In this regard, we have found that PKB is highly activated in CREF cells stably expressing wild-type 9ORF1 (Fig. 4). Similar results are obtained after transient transfection of CREF cells with a 9ORF1 expression plasmid (data not shown). In addition, activation of PKB by 9ORF1 is highly selective as no alterations were detected for multiple other cell signaling pathways in these cells (data not shown). Significantly, activation of PKB was also tightly linked to transformation by 9ORF1 because severely transformation-defective 9ORF1 mutants (IA, IIA, IIB, and IIIA) fail to activate PKB whereas weakly transforming 9ORF1 mutants (IIIC and IIID) retain a reduced capacity to activate this enzyme in CREF cells. Moreover, interaction of 9ORF1 with the cellular PDZ-domain proteins is likely to be important for this activity because 9ORF1 mutant IIIA, which lacks an ability to bind cellular PDZ-domain proteins, also fails to activate PKB in cells.

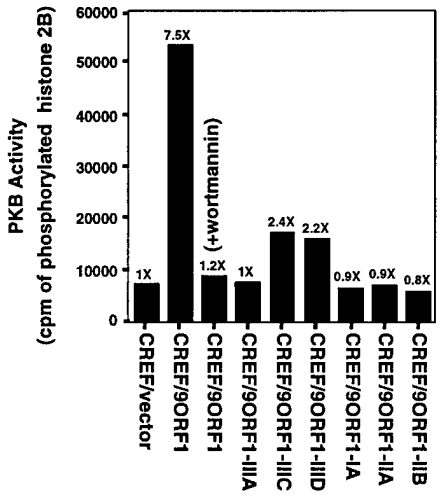

Fig. 4. Activation of protein kinase B (PKB) in CREF cells stably expressing wild-type but not transformation-defective 9ORF1 proteins. PKB protein was immunoprecipitated from lysates of serum-starved CREF cells and subjected to an *in vitro* kinase assay using histone 2B as a substrate. The amount of radioactive ^{32}P-phosphate incorporated into histone 2B was quantitated with a phophorimager. The indicated 9ORF1-expressing CREF cells were treated with 100nM wortmannin 15 min prior to harvesting.

In cells, PKB is normally activated by phosphorylated lipid second messengers produced by PI 3-K. The fact that the drug wortmannin, which specifically and irreversibly inhibits the enzymatic activity of PI 3-K in cells, blocks the ability of 9ORF1 to activate PKB (Fig. 4) suggests that 9ORF1 acts at or upstream of PI 3-K in cells.

APPENDICES

1) Bulletted list of key research accomplishments

- **The transforming activity of 9ORF1 is needed for Ad9 to generate mammary tumors in rats**
- **9ORF1 is the major oncogenic determinant of Ad9**
- **The 9ORF1 oncoprotein complexes with four different cellular PDZ-domain proteins**
- **The 9ORF1 oncoprotein aberrantly sequesters these PDZ-domain proteins in the cytoplasm of cells**
- **High-risk HPV E6 oncoproteins bind to three of the four 9ORF1-associated PDZ-domain proteins and may target them for degradation in cells**
- **Wild-type but not transformation-defective mutant 9ORF1 proteins activate protein kinase B (PKB) in cells**

2) List of reportable outcomes

Manuscripts

Thomas, D.L., Shin, S., Jiang, B.H., Vogel, H., Ross, M.A., Kaplitt, M., Shenk, T.E., and Javier, R.T. (1999) Early Region 1 Transforming Functions Are Dispensable for Mammary Tumorigenesis by Human Adenovirus Type 9. *J. Virology* 73:3071-3079.

D. Gardiol, C. Kuhn, B. Glaunsinger, S. Lee, R. Javier, L. Banks. Oncogenic HPV E6 proteins target hDLG for proteasome-mediated dedradation. *Oncogene*, in press.

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3) See attached manuscripts

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Early Region 1 Transforming Functions Are Dispensable for Mammary Tumorigenesis by Human Adenovirus Type 9

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Some human adenoviruses are tumorigenic in rodents. Subgroup A and B human adenoviruses generally induce sarcomas in both male and female animals, and the gene products encoded within viral early region 1 (E1 region) are both necessary and sufficient for this tumorigenicity. In contrast, subgroup D human adenovirus type 9 (Ad9) induces estrogen-dependent mammary tumors in female rats and requires the E4 region-encoded ORF1 oncoprotein for its tumorigenicity. Considering the established importance of the viral E1 region for tumorigenesis by adenoviruses, we investigated whether this viral transcription unit is also necessary for Ad9 to generate mammary tumors. The nucleotide sequence of the Ad9 E1 region indicated that the gene organization and predicted E1A and E1B polypeptides of Ad9 are closely related to those of other human adenovirus E1 regions. In addition, an Ad9 E1 region plasmid demonstrated focus-forming activity in both low-passage-number and established rat embryo fibroblasts, whereas a large deletion within either the E1A or E1B gene of this plasmid diminished transforming activity. Surprisingly, we found that introducing the same transformation-inactivating E1A and E1B deletions into Ad9 results in mutant viruses that retain the ability to elicit mammary tumors in rats. These results are novel in showing that Ad9 represents a unique oncogenic adenovirus in which the E4 region, rather than the E1 region, encodes the major oncogenic determinant in the rat.

Human adenoviruses cause primarily respiratory, gastrointestinal, and eye infections in people and are divided into six subgroups (A to F) based upon several physical characteristics (25, 48). In rodents, however, the subgroup A and B adenoviruses are tumorigenic, eliciting undifferentiated sarcomas at the site of viral inoculation in both male and female animals (22, 54). Although subgroup D adenoviruses are nononcogenic in hamsters (54), subgroup D human adenovirus type 9 (Ad9) elicits mammary tumors in rats (3, 4, 29). Three months after subcutaneous injection with Ad9, female rats develop exclusively estrogen-dependent mammary tumors, while male rats fail to develop tumors of any kind. Tumors that form in the female rats are predominantly mammary fibroadenomas, the most common type of benign breast tumor found in young women (29, 44).

For the subgroup A and B adenoviruses, the E1A and E1B gene products encoded within the viral early region 1 (E1 region) are both necessary and sufficient for oncogenic transformation of primary rodent cell cultures (22, 49, 51). Individually, E1A is capable of immortalizing cells (26), whereas E1B displays no transforming potential (55). Together, however, these viral genes cooperate to produce transformed cells (22). The mechanism by which E1 region gene products transform cells can be attributed, in part, to their ability to inactivate the cellular tumor suppressor proteins pRB and p53 (48).

Unlike subgroup A and B adenoviruses, subgroup D Ad9 requires the E4 region ORF1 oncoprotein to generate tumors (30, 32). Nevertheless, the facts that (i) E1A mRNA is expressed in Ad9-induced mammary tumors (29) and (ii) the Ad9 E1 and E4 regions together cooperate to induce focus formation in CREF cells (30) suggest that the viral E1 region may also be required for Ad9-induced mammary tumorigenesis. To address this possibility, we constructed Ad9 mutant viruses containing transformation-defective E1A and E1B genes. Despite the critical role of the viral E1 region in oncogenesis by subgroup A and B adenoviruses, we present results here indicating that E1 region transforming functions are dispensable for Ad9 to induce mammary tumors in rats.

MATERIALS AND METHODS

Cell lines. Rat embryo fibroblasts (REFs) were cultured from 16-day Fisher rat embryos (Harlan Sprague-Dawley, Indianapolis, Ind.) by using standard methods (20). REF cultures, rat CREF (19) and 3Y1 cell lines (37), and human A549 and 293 cell lines (2, 23) were maintained in culture medium (Dulbecco's modified Eagle medium supplemented with 20 μg of gentamicin per ml and 6 or 10% fetal bovine serum) under a 5% CO_2 atmosphere at 37°C.

Nucleotide sequence analyses and plasmid construction. Plasmids pUC19-Ad9[0-7.5] and pSP72-Ad9[7.5-12.5] containing Ad9 DNA sequences from 0 to 7.5 and 7.5 to 12.5 map units (m.u.), respectively, were used to determine the nucleotide sequence of the Ad9 E1 region.

A DNA fragment (0 to 12.5 m.u.) containing the Ad9 E1 region was inserted into the *Kpn*I and *Bgl*II sites of plasmid pSP72 (Promega) to make pAd9E1. Deletions within the Ad9 E1A and E1B genes were first introduced into pUC19-Ad9[0-7.5] by removing the Ad9 *Sac*I-*Bsp*EI fragment (nucleotides [nt] 542 to 1049) and the Ad9 *Nae*I-*Cla*I fragment (nt 1609 to 2495), respectively. These two deletion mutations were subsequently transferred to pAd9E1 within the Ad9 *Bam*HI-*Eco*RI fragment (0 to 7.5 m.u.), resulting in pAd9E1(ΔE1A) and pAd9E1(ΔE1B), respectively. The presence of the correct deletion in each mutant plasmid was verified by restriction enzyme and limited sequence analyses.

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101   AGAAGACGATGCAAATGACGTCACGACTGCACGGCTAACGGTCGCCGCGGAGGCGTGGCCTAGCCCGGAAGCAAGTCGCGGGGCTGATGACGTATAAAAA
201   AGCGGACTTTAGACCCGGAAACGGCCGATTTTCCCGCGCCACGCCCGGATATGAGGTAATTCTGGGCGGATGCAAGTGAAATTAGGTCATTTTGGCGCGA
301   AAACTGAATGAGGAAGTGAAAAGCGAAAAATACCGGTCCCTCCCAGGGCGGAATATTTACCGAGGGCCGAGAGACTTTGACCGATTACGTGGGGGTTTCG
                                                                                                E1A TATA
401   ATTGCGGTGTTTTTTTCGCGAATTTCCGCGTCCGTGTCAAAGTCCGGTGTTTATGTCACAGATCAGCTGATCCGCAGGTATTTAAACCAGTCGAGTCCGT
                                                SacI                    Start E1A protein
501   CAAGAGGCCACTCTTGAGTGCCAGCGAGTAGAGATTTCTCTGAGCTCCGCTCCCAGAGACCGAGAAAAATGAGACACCTGCGCCTCCTGCCTTCAACTGT
          SD1                                                                   M  R  H  L  R  L  L  P  S  T
601   GCCCGGTGAGCTGGCTGTGCTTATGCTGGAGGACTTTGTGGATACAGTATTGGAGGACGAACTGCATCCAAGTCCGTTCGAGCTGGGACCCACACTTCAG
      V  P  G  E  L  A  V  L  M  L  E  D  F  V  D  T  V  L  E  D  E  L  H  P  S  P  F  E  L  G  P  T  L  Q
                                                                                          SA1
701   GATCTCTATGATCTGGAGGTAGATGCCCATGATGACGACCCTAACGAGGAGGCTGTGAATTTAATATTTCCAGAATCTATGATTCTTCAGGCTGACATAG
       D  L  Y  D  L  E  V  D  A  H  D  D  D  P  N  E  E  A  V  N  L  I  F  P  E  S  M  I  L  Q  A  D  I
801   CCAACGAATCTACTCCACTTCATACACCGACTCTGTCACCCATACCTGAATTGGAAGAGGAGGACGAACTAGACCTCCGGTGTTATGAGGAAGGTTTTCC
       A  N  E  S  T  P  L  H  T  P  T  L  S  P  I  P  E  L  E  E  E  D  E  L  D  L  R  C  Y  E  E  G  F
                                SD2
901   TCCCAGCGATTCAGAGGATGAACGGGGTGAGCAGACCATGGCTCTGATCTCAGACTATGCTTGTGTGATTGTGGAGGAGCAAGATGTGATTGAAAAATCT
      P  P  S  D  S  E  D  E  R  G  E  Q  T  M  A  L  I  S  D  Y  A  C  V  I  V  E  E  Q  D  V  I  E  K  S
                                                                BspEI
1001  ACTGAGCCAGTACAAGGCTGTAGGAACTGCCAGTACCACCGGGATAAGTCCGGAGATGTGAACGCCTCCTGCGCTTTGTGCTATATGAAACAGACTTTCA
       T  E  P  V  Q  G  C  R  N  C  Q  Y  H  R  D  K  S  G  D  V  N  A  S  C  A  L  C  Y  M  K  Q  T  F
                 SD3
1101  GCTTTATTTACAGTAAGTGGAGTGAATGTGAGAGAGGCTGAGTGCTTAACACATAACTGTAATGCTTGAACAGCTGTGCTAAGTGTGGTTTATTTTGTTA
       S  F  I  Y  S
       SA2
1201  CTAGGTCCGGTGTCAGAGGATGAGTTATCACCCTCAGAAGAAGACCACCCGTCTCCCCCTGAGCTGTCAGGCGAAACGCCCCTGCAAGTGTTCAGACCCA
                P  V  S  E  D  E  L  S  P  S  E  E  D  H  P  S  P  P  E  L  S  G  E  T  P  L  Q  V  F  R  P
1301  CCCCAGTCAGACCCAGTGGCGAGAGGCGAGCGGCTGTTGACAAAATTGAGGACTTGTTGCAGGACATGGGTGGGGATGAACCTTTGGACCTGAGCTTGAA
       T  P  V  R  P  S  G  E  R  R  A  A  V  D  K  I  E  D  L  L  Q  D  M  G  G  D  E  P  L  D  L  S  L
                   stop E1A protein                                poly A E1A
1401  ACGCCCCAGGAACTAGACGCACGTGCGCTTAGTCATGTGTAAATAAAGTTGTACAATAAAAGTCTATGTGACGCATGCAAGGTGTGGTTTATGACTCATG
      K  R  P  R  N  *
                   E1B TATA                                          start E1B 19K protein
1501  GGCGGGGCTTAGTCCTATATAAGTGGCAACACCTGGGCACTTGGGCACAGACCTTCAGGGAGTTCCTGATGGATGTGTGGACTATCCTTGCAGACTTTAG
                NaeI                                                      M  D  V  W  T  I  L  A  D  F
1601  CAAGACACGCCGGCTTGTAGAGGATAGTTCAGACGGGTGCTCCGGGTTCTGGAGACACTGGTTTGGAACTCCTCTATCTCGCCTGGTGTACACAGTTAAG
      S  K  T  R  R  L  V  E  D  S  S  D  G  C  S  G  F  W  R  H  W  F  G  T  P  L  S  R  L  V  Y  T  V  K
1701  AAGGATTATAAAGAGGAATTTGAAAATATTTTTGCTGACTGCTCTGGCCTGCTAGATTCTCTGAATCTTGGCCACCAGTCCCTTTTCCAGGAAAGGGTAC
       K  D  Y  K  E  E  F  E  N  I  F  A  D  C  S  G  L  L  D  S  L  N  L  G  H  Q  S  L  F  Q  E  R  V
                                                                                  start E1B 55K protein
1801  TCCACAGCCTTGATTTTTCCAGCCCAGGGCGCACTACAGCCGGGGTTGCTTTTGTGGTTTTTCTGGTTGACAAATGGAGCCAGGACACCCAACTGAGCAG
       L  H  S  L  D  F  S  S  P  G  R  T  T  A  G  V  A  F  V  V  F  L  V  D  K  W  S  Q  D  T  Q  L  S
                                                                                     M  E  P  G  H  P  T  E  Q
1901  GGGCTACATCCTGGACTTCGCAGCCATGCACCTGTGGAGGGCCTGGATCAGGCAGCGGGGACAGAGAATCTTGAATTACTGGCTTCTACAGCCAGCAGCT
      R  G  Y  I  L  D  F  A  A  M  H  L  W  R  A  W  I  R  Q  R  G  Q  R  I  L  N  Y  W  L  L  Q  P  A  A
       G  L  H  P  G  L  R  S  H  A  P  V  E  G  L  D  Q  A  A  G  T  E  N  L  E  L  L  A  S  T  A  S  S
2001  CCGGGTCTTCTTCGTCTACACAGACAAACATCCATGTTGGAGGAAGAAATGAGGCAGGCCATGGACGAGAACCCGAGGAGCGGCCTGGACCCTCCGTCGG
       P  G  L  L  R  L  H  R  Q  T  S  M  L  E  E  E  M  R  Q  A  M  D  E  N  P  R  S  G  L  D  P  P  S
       S  G  S  S  S  S  T  Q  T  N  I  H  V  G  G  R  N  E  A  G  H  G  R  E  P  E  E  R  P  G  P  S  V
      stop E1B 19K protein  SD4A                          SD4B
2101  AAGAGGAGCTGGATTGAATCAGGTATCCAGCCTGTACCCAGAGCTTAGCAAGGTGCTGACATCCATGGCCAGGGGAGTTAAGAGGGAGAGGAGCGATGGG
       E  E  E  L  D  *
      G  R  G  A  G  L  N  Q  V  S  S  L  Y  P  E  L  S  K  V  L  T  S  M  A  R  G  V  K  R  E  R  S  D  G
2201  GGTAATACCGGGATGATGACCGAGCTGACGGCCAGCCTGATGAATCGGAAACGCCCAGAGCGCCTTACCTGGTACGAGCTACAGCAGGAGTGCAGGGATG
       G  N  T  G  M  M  T  E  L  T  A  S  L  M  N  R  K  R  P  E  R  L  T  W  Y  E  L  Q  Q  E  C  R  D
2301  AGTTGGGCCTGATGCAGGATAAATATGGCCTGGAGCAGATAAAAACCCATTGGTTGAACCCAGATGAGGATTGGGAGGAGGCTATTAAGAAGTATGCCAA
       E  L  G  L  M  Q  D  K  Y  G  L  E  Q  I  K  T  H  W  L  N  P  D  E  D  W  E  E  A  I  K  K  Y  A
                                                                                                    ClaI
2401  GATAGCCCTGCGCCCAGATTGCAAGTACATAGTGACCAAGACCGTGAATATCAGACATGCCTGCTACATCTCGGGGAACGGGGCAGAGGTGGTCATCGAT
      K  I  A  L  R  P  D  C  K  Y  I  V  T  K  T  V  N  I  R  H  A  C  Y  I  S  G  N  G  A  E  V  V  I  D
                                                                     EcoRI
2501  ACCCTGGACAAGGCCGCCTTCAGGTGTTGCATGATGGGAATGAGAGCAGGAGTGATGAATATGAATTCCATGATCTTCATGAACATGAAGTTCAATGGAG
       T  L  D  K  A  A  F  R  C  C  M  M  G  M  R  A  G  V  M  N  M  N  S  M  I  F  M  N  M  K  F  N  G
2601  AGAAGTTTAATGGGGTGCTGTTCATGGCCAACAGCCAGATGACCCTGCATGGCTGCAGTTTCTTCGGCTTCAACAATATGTGCGCAGAGGTCTGGGGCGC
       E  K  F  N  G  V  L  F  M  A  N  S  Q  M  T  L  H  G  C  S  F  F  G  F  N  N  M  C  A  E  V  W  G
2701  TTCCAAGATCAGGGGATGTAAGTTTTATGGCTGCTGGATGGGCGTGGTCGGAAGACCAGAGAGCGAGATGTCTGTGAAGCAGTGTGTGTTTGAGAAATGC
      A  S  K  I  R  G  C  K  F  Y  G  C  W  M  G  V  V  G  R  P  E  S  E  M  S  V  K  Q  C  V  F  E  K  C
2801  TACCTGGGAGTCTCTACCGAGGGCAATGCTAGAGTGAGACACTGCTCTTCCCTGGAGACGGGCTGCTTCTGCCTGGTGAAGGGCACAGCCTCTCTGAAGC
       Y  L  G  V  S  T  E  G  N  A  R  V  R  H  C  S  S  L  E  T  G  C  F  C  L  V  K  G  T  A  S  L  K
2901  ATAACATGGTGAAGGGCTGCACGGATGAGCGCATGTACAACATGCTGACCTGCGATTCGGGGGTCTGCCATATCCTGAAGAACATCCATGTGACCTCCCA
       H  N  M  V  K  G  C  T  D  E  R  M  Y  N  M  L  T  C  D  S  G  V  C  H  I  L  K  N  I  H  V  T  S

3001  CCCCAGAAAGAAGTGGCCAGTGTTTGAGAATAACCTGCTGATCAAGTGCCATATGCACCTGGGAGCCAGAAGGGGCACCTTCCAGCCGTACCAGTGCAAC
      H  P  R  K  K  W  P  V  F  E  N  N  L  L  I  K  C  H  M  H  L  G  A  R  R  G  T  F  Q  P  Y  Q  C  N
3101  TTTAGCCAGACCAAGCTGCTGTTGGAGAACGATGCCTTCTCCAGGGTGAACCTGAACGGCATCTTTGACATGGATGTCTCGGTGTACAAGATCCTGAGAT
       F  S  Q  T  K  L  L  L  E  N  D  A  F  S  R  V  N  L  N  G  I  F  D  M  D  V  S  V  Y  K  I  L  R
3201  ACGATGAGACCAAGTCCAGGGTGCGCGCTTGCGAGTGCGGGGGCAGACACACCAGGATGCAGCCAGTGGCCCTGGATGTGACCGAGGAGCTGAGACCAGA
       Y  D  E  T  K  S  R  V  R  A  C  E  C  G  G  R  H  T  R  M  Q  P  V  A  L  D  V  T  E  E  L  R  P
                                                stop E1B 55K protein  SD5
3301  CCACCTGGTGATGGCCTGTACCGGGACCGAGTTCAGCTCCAGTGGGGAGGACACAGATTAGAGGTAGGTCGAGTGAGTAGTGGGCGTGGCTAAGGTGACT
      D  H  L  V  M  A  C  T  G  T  E  F  S  S  S  G  E  D  T  D  *
      Protein IX TATA                           SA3    start protein IX
3401  ATAAAGGCGGGTGTCTTACGAGGGTCTTTTTGCTTTTCTGCAGACATCATGAACGGGACGGGCGGGGCCTTCGAAGGGGCGCTTTTTAGCCCTTATTTGA
                                                         M  N  G  T  G  G  A  F  E  G  A  L  F  S  P  Y  L
3501  CAACCCGCCTGCCGGGATGGGCCGGAGTTCGTCAGAATGTGATGGGATCGACGGTGGATGGGCGCCCAGTGCTTCCAGCAAATTCCTCGACCATGACCTA
       T  T  R  L  P  G  W  A  G  V  R  Q  N  V  M  G  S  T  V  D  G  R  P  V  L  P  A  N  S  S  T  M  T
3601  CGCGACCGTGGGGAGCTCGTCGCTCGACAGCACCGCCGCAGCCGCGGCAGCCGCAGCTGCCATGACAGCGACGAGACTGGCCTCGAGCTACATGCCCAGC
      Y  A  T  V  G  S  S  S  L  D  S  T  A  A  A  A  A  A  A  A  A  M  T  A  T  R  L  A  S  S  Y  M  P  S
3701  AGCGGCAGCAGCCCCTCTGTGCCCAGTTCCATCATCGCCGAGGAGAAACTGCTGGCCCTGCTGGCCGAGCTGGAAGCCCTGAGCCGCCAGCTGGCCGCCC
       S  G  S  S  P  S  V  P  S  S  I  I  A  E  E  K  L  L  A  L  L  A  E  L  E  A  L  S  R  Q  L  A  A
                                                stop protein IX   poly A E1B                  poly A protein IX
3801  TGACCCAGCAGGTGTCCGACGTCCGCGAGCAGCAACAGCAGCAAAATAAATGATTCAATAAACACAGATTCTGATTCAAACAGCAAAGCATCTTTATTAT
       L  T  Q  Q  V  S  D  V  R  E  Q  Q  Q  Q  Q  N  K  *
3901  TATTTATTTTTTCGCGCGCGGTAGGCCCTGGTCCACCTCTCCCGATCATTGAGAGTGCGGTGGATTTTTTCCAGGACCCGGTAGAGGTGGGATTGGATGT
4001  TGAGGT
```

B

```
                                                       |-------------- CR1 ---------------------|
13S  MRHLRLLPST VPGELAVLML EDFVDTVLED ELHPSPFELG PTLQDLYDLE VDAHDDDPNE EAVNLIFPES MILQADIANE STPLHTPTLS PIPELEEEDE
12S  MRHLRLLPST VPGELAVLML EDFVDTVLED ELHPSPFELG PTLQDLYDLE VDAHDDDPNE EAVNLIFPES MILQADIANE STPLHTPTLS PIPELEEEDE
10S  MRHLRLLPST VP-------- ---------- ---------- ---------- ---------- --------ES MILQADIANE STPLHTPTLS PIPELEEEDE

     |---- CR2 ------------| |--SPACER--||-------------------- CR3 -----------------------------|
13S  LDLRCYEEGF PPSDSEDERG EQTMALISDY ACVIVEEQDV IEKSTEPVQG CRNCQYHRDK SGDVNASCAL CYMKQTFSFI YSPVSEDELS PSEEDHPSPP
12S  LDLRCYEEGF PPSDSEDERG ---------- ---------- ---------- ---------- ---------- ---------- --PVSEDELS PSEEDHPSPP
10S  LDLRCYEEGF PPSDSEDERG ---------- ---------- ---------- ---------- ---------- ---------- --PVSEDELS PSEEDHPSPP

13S  ELSGETPLQV FRPTPVRPSG ERRAAVDKIE DLLQDMGGDE PLDLSLKRPR N 251
12S  ELSGETPLQV FRPTPVRPSG ERRAAVDKIE DLLQDMGGDE PLDLSLKRPR N 189
10S  ELSGETPLQV FRPTPVRPSG ERRAAVDKIE DLLQDMGGDE PLDLSLKRPR N 133
```

FIG. 1. (A) Nucleotide sequence of the Ad9 E1 region. The locations of E1A, E1B, and IX gene promoter TATA boxes, known and putative splice donor (SD) and splice acceptor (SA) sites, and poly(A) signal sequences are shown. Relevant restriction enzyme sites are indicated (underlined) on the nucleotide sequence. E1A and E1B mRNA splice variants result from the use of the following SD and SA sites: 10S E1A, SD1 and SA1 plus SD2 and SA2; 12S E1A, SD2 and SA2; 13S E1A, SD3 and SA2; 13S E1B, SD4A or SD4B and SA3; and 22S E1B, SD5 and SA3. The predicted amino acid sequences of the 13S E1A, 19K and 55K E1B, and pIX polypeptides are shown beneath their coding sequences. (B) Comparison of the Ad9 13S, 12S, and 10S E1A polypeptide sequences. CR1, CR2, spacer region, and CR3 are indicated (22, 33, 48, 53).

Construction of adenovirus mutants. Ad9 mutant viruses having the same E1A and E1B gene deletions described above for plasmids pAd9E1(ΔE1A) and pAd9E1(ΔE1B) were generated. Briefly, the full-length Ad9 genome (0 to 100 m.u.) consists of three *Eco*RI fragments: A (7.5 to 95 m.u.), B (0 to 7.5 m.u.), and C (95 to 100 m.u.). Deletions were first introduced into the Ad9 *Eco*RI B fragment of a plasmid, pAd9-EcoRI(B+C), which contains properly oriented terminal Ad9 *Eco*RI B and C fragments but lacks the intervening Ad9 *Eco*RI A fragment. Full-length mutant Ad9 genomes were subsequently assembled by inserting a virion-derived Ad9 *Eco*RI A fragment in the correct orientation at the unique *Eco*RI site of mutant pAd9-EcoRI(B+C) plasmids. The resulting infectious pAd9-EcoRI(A+B+C) plasmids were digested with *Spe*I to release intact linear viral genomes, which were transfected into 293 cells to complement expected E1 region deficiencies of the mutant viruses (2, 23). Recovered viruses were amplified and titrated in 293 cells (31, 48).

Isolation of RNA and Northern blot analyses. Total RNA was isolated from mock-infected or Ad9-infected A549 cells (multiplicity of infection of 10; 9 h postinfection). Cells were washed with ice-cold phosphate-buffered saline (4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl) and lysed in guanidinium solution (4 M guanidinium isothiocyanate, 20 mM sodium acetate [pH 5.2], 0.1 mM dithiothreitol, and 0.5% [wt/vol] Sarkosyl) (12). The resulting lysate was drawn through a 20-gauge needle to shear cellular DNA, layered onto a 5.7 M CsCl cushion, and centrifuged at 150,000 × *g* for 18 h. The RNA pellet was dissolved in TES buffer (1 mM Tris-HCl [pH 7.5], 2.5 mM EDTA, 1% [wt/vol] sodium dodecyl sulfate [SDS]), precipitated with ethanol, and resuspended in water.

For Northern blot analyses, total RNA was separated on a formaldehyde agarose gel and transferred to a nitrocellulose membrane (13). The membrane was preincubated in hybridization buffer (0.5 M Na_2HPO_4 [pH 7.2], 1 mM EDTA, 7% [wt/vol] SDS) at 65°C for 4 h and then incubated in hybridization buffer containing a radiolabeled DNA probe (4.3×10^6 cpm/ml) at 65°C for 16 h. E1A and E1B probes, derived from Ad9 E1 region DNA fragments *Sac*I-*Sph*I (nt 542 to 1473) and *Nae*I-*Eco*RI (nt 1609 to 2563), respectively, were radiolabeled by the random priming method (17) and purified by gel filtration on NICK columns (Pharmacia). Probed membranes were washed in SSC wash buffer (45 mM NaCl, 4.5 mM sodium citrate, 0.1% [wt/vol] SDS) at 65°C.

Isolation of virion and cellular DNA. For isolation of adenovirus virion DNA, 293 cells were infected at a multiplicity of infection of 10 and, at 72 h postinfection, were harvested and lysed in lysis buffer (55 mM Tris-HCl [pH 9.0], 0.5 mM EDTA, 0.2% [wt/vol] sodium deoxycholate, 10% [vol/vol] ethanol, 0.5 mM spermine-HCl). Cell lysates were cleared by centrifugation, treated with proteinase K solution (0.75% [wt/vol] SDS, 12.5 mM EDTA, 2.5 mg of proteinase K per ml) at 37°C for 1 h, and extracted with phenol and chloroform. Virion DNA was precipitated with ethanol and resuspended in water.

For isolation of cellular DNA, 400 mg of frozen tumor tissue was ground in a liquid nitrogen-chilled mortar and pestle. The resulting frozen tumor powder was suspended in 4.8 ml of digestion buffer (10 mM Tris-HCl [pH 8.0], 100 mM NaCl, 25 mM EDTA, 0.5% [wt/vol] SDS, 0.1 mg of proteinase K per ml), incubated at 50°C for 16 h, and extracted with phenol (52). Cellular DNA was precipitated with ethanol and resuspended in TE buffer (10 mM Tris-HCl [pH 7.4], 1 mM EDTA).

PCR analyses. For PCR amplification of cDNAs (reverse transcription-PCR analysis), 2 μg of total RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase, using random hexamers, as suggested by the manufacturer (Gibco-BRL). Ad9 E1A cDNAs were PCR amplified with *Taq* polymerase (Promega) by using E1A primers 1 (nt 551 to 570; 5′ CTC CTG CAG TCC CAG AGA CCG AGA AAA AT 3′) and 2 (nt 1430 to 1411; 5′ CTC AAG CTT AAG CGC ACG TGC GTC TAG TT 3′). *Pst*I and *Hin*dIII sites (underlined) engineered within the E1A oligonucleotides allowed PCR products to be inserted at the same sites of plasmid ds56rII6HI (1) for sequencing. Portions of the Ad9 E1A and E1B genes and the entire Ad9 E4 ORF1 gene were PCR amplified from tumor DNAs, using the following oligonucleotide pairs: E1A primers *a* (nt 487 to 513; 5′ CCA GTC GAG TCC GTC AAG AGG CCA CTC 3′) and *b* (nt 1487 to 1461; 5′ CCA CAC CTT GCA TGC GTC ACA TAG AC 3′); E1B primers *c* (nt 1584 to 1609; 5′ ATC CTT GCA GAC TTT AGC AAG ACA CG 3′) and *d* (nt 2651 to 2628; 5′ CAT GCA GGG TCA TCT GGC TGT TGG 3′); and Ad9 E4 ORF1 primers 1 (5′ ATG GCT GAA TCT CTG TAT GCT TTC 3′) and 2 (5′-CAT GGT TAG TAG AGA TGA GAG TCT GAA 3′). For E1A and E1B nested PCRs, DNA products derived from each of the first PCR amplifications described above were extracted with phenol, precipitated with ethanol, and resuspended in water. One-twentieth of each sample was subjected to a second round of PCR amplification using the following oligonucleotide pairs: E1A primers *e* (nt 726 to 746; 5′ CCC ATG ATG ACG ACC CTA ACG 3′) and *b*; and E1B primers *c* and *f* (nt 2116 to 2094; 5′ CAA TCC AGC TCC TCT TCC GAC GG 3′).

Immunoprecipitation and immunoblot analyses. Immunoprecipitations and immunoblot analyses were performed as described previously (32). Briefly, frozen tumor powder, generated as described above for the isolation of cellular DNA, was suspended in ice-cold radioimmunoprecipitation assay buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% [wt/vol] SDS, 1% [vol/vol] Nonidet P-40, 0.5% [wt/vol] deoxycholate) containing protease inhibitors (2 μg of aprotinin, 2 μg of leupeptin, and 100 μg of phenylmethylsulfonyl fluoride per ml), sonicated briefly, and cleared by centrifugation (16,000 × *g*, 10 min). The protein concentration of tumor lysates was determined by the method of Bradford (9). Three milligrams of protein from tumor lysates was subjected to immunoprecipitation with 15 μl of Ad9 E4 ORF1 antiserum prebound to 30 μl of protein A-Sepharose beads (Pharmacia) (32). Beads were washed with ice-cold radioimmunoprecipitation assay buffer and boiled in 2× sample buffer (0.13 M Tris-HCl [pH 6.8], 4% [wt/vol] SDS, 20% [vol/vol] glycerol, 2% [vol/vol] β-mercaptoethanol, 0.003% [wt/vol] bromophenol blue). Proteins were separated by SDS-polyacrylamide gel electrophoresis (40) and electrophoretically transferred to a polyvinylidene difluoride membrane, which was blocked in TBST (50 mM Tris-HCl [pH 7.5], 200 mM NaCl, 0.1% [vol/vol] Tween 20) containing 5% (wt/vol) both nonfat dry milk and bovine serum albumin. In these assays, Ad9 E4 ORF1 antiserum (1:5,000 in TBST) (32) and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:5,000 in TBST; Southern Biotechnology Associates) were used as primary and secondary antibodies, respectively. After extensive washing with TBST, the membrane was developed by enhanced chemiluminescence (Pierce).

Focus assays. Plasmid DNA purified by CsCl density gradient centrifugation was transfected onto 50% confluent tertiary REF cultures or CREF cells on 100-mm-diameter dishes, using the calcium phosphate precipitation method with a glycerol shock (38). At 72 h posttransfection, REF and CREF cells were passaged 1:3 and maintained in culture medium containing 10 and 6% filtered fetal bovine serum, respectively. Four to six weeks posttransfection, cells were fixed in methanol and stained with Giemsa to quantify transformed foci (32).

Mammary tumorigenicity of viruses in rats. Female rats with 1- or 2-day-old litters were obtained from Harlan Sprague-Dawley; 12 to 24 h after arrival, newborn rats were injected subcutaneously with 0.4 ml of virus solution on their anterior flanks, using a 26-gauge needle. Beginning 2 months postinfection, animals were examined weekly by palpation for the presence of tumors, until the experiment was terminated at 8 months postinfection. At this time, animals were euthanized, and portions of tumors were removed and either fixed in 10% formalin for histological examination or frozen at −80°C for isolation of DNA or

TABLE 1. Amino acid sequence identities between subgroups A to D and F adenovirus E1A, E1B, and pIX proteins[a]

Virus	E1A 13S					E1B 19K					E1B 55K					pIX				
	Ad12	Ad7	Ad5	Ad9	Ad40	Ad12	Ad7	Ad5	Ad9	Ad40	Ad12	Ad7	Ad5	Ad9	Ad40	Ad12	Ad7	Ad5	Ad9	Ad40
Ad12	100					100					100					100				
Ad7	41.9	100				41.6	100				47.9	100				53.7	100			
Ad5	39.9	37.5	100			43.2	48.1	100			48.6	53.8	100			49.3	49.6	100		
Ad9	**39.3**	*43.1*	**38.1**	100		**43.2**	*52.4*	**47.6**	100		**45.3**	*56.2*	**52.2**	100		**50.0**	*58.2*	**46.5**	100	
Ad40	38.4	38.5	33.7	**40.1**	100	48.5	45.5	43.6	**41.8**	100	55.9	48.3	48.6	**44.5**	100	62.5	51.7	49.3	**49.6**	100

[a] Sequence identities were determined with the full-length sequence of each polypeptide by using the ALIGN program (50). Ad12, subgroup A; Ad7, subgroup B; Ad5, subgroup C; Ad9, subgroup D; Ad40, subgroup F. Boldface values indicate amino acid sequence comparisons between Ad9 E1 region polypeptides and other E1 region polypeptides; values in italics show that all Ad9 E1 region polypeptides are most closely related to those of subgroup B virus Ad7.

protein. Animals were cared for and handled according to institutional guidelines.

Protein sequence alignments. Sequences of Ad12, Ad7, Ad5, and Ad40 E1 region polypeptides were obtained from GenBank. Alignments were made by using the Pairwise Sequence Alignment program (ALIGN) of the BCM Search Launcher (50).

Nucleotide sequence accession number. The nucleotide and polypeptide sequences reported in this paper were submitted to GenBank (accession no. AF099665).

RESULTS

Gene organization and predicted polypeptides of the Ad9 E1 region. To initiate our characterization of the subgroup D Ad9 E1 region, we determined the sequence of the left 4006 nt of the Ad9 genome. From this analysis, we found that the gene organization of the Ad9 E1 region closely resembles that of other human adenovirus E1 regions (Fig. 1A) (48). In addition, the predicted Ad9 13S E1A, 19K and 55K E1B, and pIX proteins displayed significant sequence similarity with the corresponding proteins from other human adenoviruses, although they were most closely related to the E1 region polypeptides of subgroup B adenoviruses (Table 1).

Northern blot analyses of total cellular RNA isolated from Ad9-infected A549 cells were also performed to detect Ad9 E1A and E1B mRNAs. In these assays, a diffuse E1A mRNA band migrating at approximately 1 kb and distinct 1.2- and 2.2-kb E1B mRNA bands were observed (Fig. 2). The size of the E1A mRNA band was consistent with that predicted for the 12S and 13S transcripts (see below), and the two E1B mRNAs corresponded well with the sizes predicted for 13S and 22S transcripts (47).

Because E1A but not E1B mRNA is detected in Ad9-induced rat mammary tumors (29), we determined the structures of Ad9 E1A transcripts by using reverse transcription-PCR techniques on total RNA from Ad9-infected A549 cells. Sequencing of PCR products obtained from these analyses revealed three Ad9 E1A splice-variant transcripts resembling 13S, 12S, and 10S mRNAs from other human adenoviruses (Fig. 1A) (47). Ad9 E1A mRNA having a size consistent with that expected for the 10S mRNA was not detected by Northern blot analyses (Fig. 2), presumably due to its low abundance at early times after infection. The 13S, 12S, and 10S Ad9 E1A cDNAs are predicted to encode 251-, 189-, and 133-amino-acid-residue polypeptides, respectively (Fig. 1B). Between conserved regions 2 (CR2) and 3 (CR3), the 13S E1A protein of subgroup A virus Ad12 possesses an alanine-rich spacer region which is, in part, responsible for the highly oncogenic phenotype of this virus (33, 53). In contrast, the Ad9 13S E1A protein was found to contain a non-alanine-rich spacer region similar to the one present in 13S E1A proteins of weakly oncogenic subgroup B adenoviruses (Fig. 1B).

A large deletion within the E1A or E1B gene abolishes focus-forming activity by the Ad9 E1 region. To investigate the transforming potential of the Ad9 E1 region, we constructed an Ad9 E1 region (0 to 12.5 m.u.) plasmid, pAd9E1, and examined its ability to induce transformed foci on low-passage-number REF cultures. Unlike other adenovirus E1 regions, the Ad9 E1 region is unable to transform primary REF or baby rat kidney cell cultures (28). Consistent with these previous findings, pAd9E1 alone failed to generate transformed foci on REFs (Table 2). Nevertheless, whereas an activated *ras* plasmid alone also lacked detectable focus-forming activity on REFs, pAd9E1 and the activated *ras* plasmid together cooperated to produce transformed foci on these cells (Table 2). To determine whether Ad9 E1A and E1B gene functions were required for this cooperation, we introduced a large deletion into each of these genes within pAd9E1. A segment of the E1A gene coding for the initiation codon, conserved region 1 (CR1), CR2, and half of CR3 (48) was removed in plasmid pAd9E1(ΔE1A), and E1B gene coding sequences downstream of E1B-19K amino acid residue 14, as well as the first 208 amino acid residues of the 495-residue E1B-55K protein, were removed in plasmid pAd9E1(ΔE1B) (Fig. 3). Each deletion would be anticipated to inactivate the transforming potential of the relevant gene (21, 22, 57). When cotransfected with the activated *ras* plasmid, pAd9E1(ΔE1A) failed to generate any foci on REFs, whereas pAd9E1(ΔE1B) retained significant focus-forming activity, albeit at a reduced efficiency compared to wild-type pAd9E1 (Table 2). These results are concordant with previous results showing that activated *ras* cooperates with the Ad5 E1A but not the E1B gene (18). Therefore, our findings provided evidence that the transforming potential of the E1A gene is inactivated in pAd9E1(ΔE1A); however, it was un-

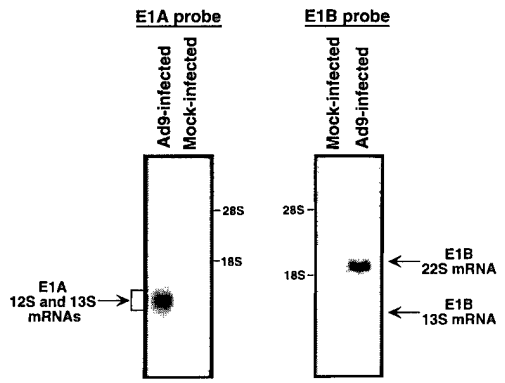

FIG. 2. Northern blot analyses of Ad9 E1A and E1B mRNAs. Total RNA (23 μg), isolated from Ad9-infected (9 h postinfection) or mock-infected A549 cells, was separated on a formaldehyde agarose gel, transferred to a nitrocellulose membrane, and hybridized to either an E1A or E1B ^{32}P-labeled DNA probe. RNA bands were visualized by autoradiography. Locations of 28S and 18S rRNAs are indicated. The indicated Ad9 mRNA species are predicted from their sizes.

TABLE 2. Focus formation by wild-type and mutant Ad9 E1 region plasmids on low-passage-number REF cultures and the CREF cell line[a]

Plasmid(s)	No. of transformed foci/ 2 100-mm-diam dishes					
	REF cultures[b]				CREF cell line[c]	
	−ras		*+ras*			
	Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
pSP72	0	0	0	0	0	1
pAd9E1	0	0	24	53	71	116
pAd9E1(ΔE1A)	0	0	0	0	2	3
pAd9E1(ΔE1B)	0	0	12	14	0	0
pAd9E1(ΔE1A) + pAd9E1(ΔE1B)	ND[d]	ND	ND	ND	36	45

[a] 50% confluent tertiary REF or CREF cells on 100-mm-diameter dishes were transfected with the indicated plasmid(s). At 72 h posttransfection, cells were passaged 1:3 and then maintained in culture medium. REF and CREF cells were fixed in methanol and stained with Giemsa at 4 and 6 weeks posttransfection, respectively, to quantify the number of transformed foci.

[b] 15 μg of the indicated Ad9 E1 region plasmid plus 5 μg of empty pSP72 (*−ras*) or 5 μg of pSP72-*ras* (*+ras*) plasmid (18) were transfected into REF cells.

[c] 10 μg of the indicated Ad9 E1 region plasmid plus 10 μg of empty pSP72 plasmid were transfected into CREF cells. For the pAd9E1(ΔE1A)-plus-pAd9E1(ΔE1B) cotransfection, 10 μg of each plasmid was used.

[d] ND, not determined.

clear from these REF assays whether the deletion in pAd9E1 (ΔE1B) similarly affects the transforming potential of the E1B gene.

In an attempt to reveal more striking transforming deficiencies for pAd9E1(ΔE1B), we next performed focus assays in the established REF cell line CREF (19). Contrary to results obtained in REFs, transfection of pAd9E1 alone into CREF cells led to the formation of numerous transformed foci (Table 2). The fact that a plasmid containing Ad9 sequences from 0 to 17.5 m.u. exhibits weaker transforming activity in CREF cells (30) may indicate that Ad9 sequences from 12.5 to 17.5 m.u. interfere with focus formation in these cells. More important, when transfected individually into CREF cells, both pAd9E1 (ΔE1A) and pAd9E1(ΔE1B) displayed significantly impaired focus-forming activity compared to wild-type pAd9E1 (Table 2). Cotransfection of pAd9E1(ΔE1A) and pAd9E1(ΔE1B) into CREF cells, however, resulted in a moderate number of transformed foci, revealing cooperation between the functional E1A and E1B genes retained collectively in the two plasmids. Taken together, the results obtained for low-passage-number REFs and the cell line CREF showed that the deletions within pAd9E1(ΔE1A) and pAd9E1(ΔE1B) greatly diminish the transforming activity of the Ad9 E1A and E1B genes, respectively.

Isolation of Ad9 E1A or E1B deletion mutant viruses. The importance of the Ad9 E1 region in mammary oncogenesis was assessed by introducing the same E1A and E1B deletion mutations of pAd9E1(ΔE1A) and pAd9E1(ΔE1B) into infectious Ad9 plasmids for recovery of mutant viruses. To complement their E1 region deficiencies, we transfected each of the mutant viral DNAs into human 293 cells, which stably express Ad5 E1 region proteins (2, 23). In 293 cells, the E1A mutant virus Ad9ΔE1A replicated to titers comparable to those of wild-type Ad9, whereas the E1B mutant virus Ad9ΔE1B replicated to titers approximately 10-fold lower. Because wild-type Ad9 fails to complement the replication defects of the Ad5 E1B-55K mutant *dl*252 (28), the reduced replication of Ad9ΔE1B conversely may be due to it being poorly complemented by Ad5 E1B proteins expressed in 293 cells. Restriction enzyme analyses of virion DNA verified that Ad9ΔE1A and Ad9ΔE1B contained the expected deletions and further showed that these viruses had not acquired Ad5 E1 region sequences from the 293 cells (Fig. 4).

Ad9 E1A and E1B mutant viruses retain the ability to elicit mammary tumors in rats. We next tested the ability of mutant viruses Ad9ΔE1A and Ad9ΔE1B to generate mammary tumors in Wistar-Furth rats. In accordance with our previous results (29, 30), wild-type Ad9 elicited mammary tumors in all of the female rats but none of the male rats, whereas subgroup D Ad26 failed to elicit tumors in any animals (Table 3). Significantly, we found that both Ad9ΔE1A and Ad9ΔE1B retained the ability to generate mammary tumors in female rats, despite the fact that Ad9ΔE1B-infected animals received a ninefold-lower dose of virus than did animals infected with either wild-type Ad9 or Ad9ΔE1A (Table 3). The tumorigenic phenotype of Ad9ΔE1B may not be surprising, considering that, unlike E1A mRNA, E1B mRNA is not detected in Ad9-induced mammary tumors (29). Furthermore, although mammary tumors elicited by all of the viruses were histologically identical (Table 4), the tumors produced by Ad9ΔE1A were generally smaller than those induced by either wild-type Ad9 or Ad9ΔE1B, both of which generated tumors of similar size (data not shown).

Mutant Ad9 virus-induced tumors do not contain wild-type Ad9 E1 region sequences. Because retention of tumorigenicity by both Ad9ΔE1A and Ad9ΔE1B was unanticipated, it was important to demonstrate that the mammary tumors caused by these viruses do not contain wild-type Ad9 DNA. For this

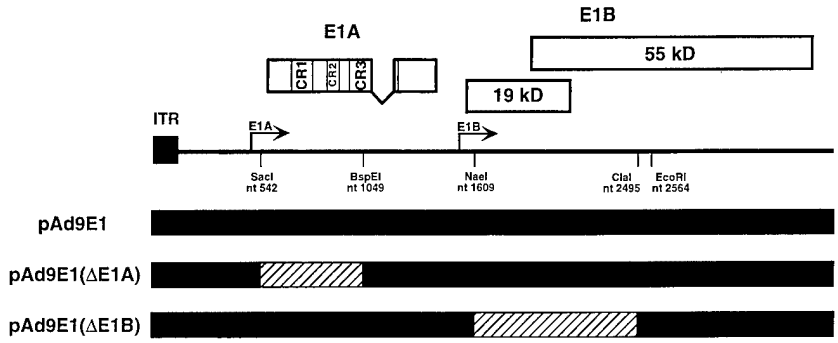

FIG. 3. Illustration of E1A and E1B deletion mutations introduced into the Ad9 E1 region of plasmid pAd9E1. Wild-type Ad9 sequences are represented by a black line; deleted sequences are represented by a hatched line. The restriction enzyme sites used to generate the deletions are shown. The locations of E1A CR1, CR2, and CR3 (22, 48) are also indicated. ITR, inverted terminal repeat.

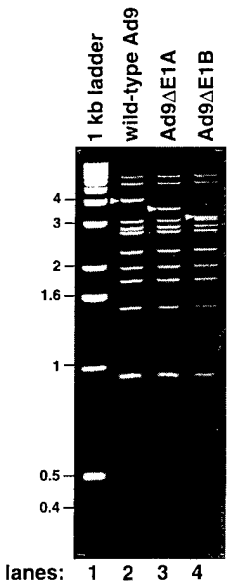

FIG. 4. *Sma*I digestion pattern of wild-type and E1A and E1B mutant Ad9 virion DNAs. The wild-type Ad9 E1 region-containing *Sma*I DNA band migrating at approximately 4 kb, as well as the corresponding faster-migrating DNA bands of the Ad9 E1A mutant virus Ad9ΔE1A and of the Ad9 E1B mutant virus Ad9ΔE1B, are indicated with arrows. A 1-kb ladder (Gibco-BRL) was used as a DNA size marker (lane 1).

analysis, we subjected tumor DNAs to a two-step nested PCR procedure (Fig. 5). In the first step, DNAs were PCR amplified with E1A primers (*a* plus *b*) or E1B primers (*c* plus *d*) flanking the deleted regions (Fig. 5A). From these reactions, wild-type Ad9-induced tumors yielded the expected 1,001-bp E1A product and 1,068-bp E1B product, but Ad9ΔE1A-induced tumors and Ad9ΔE1B-induced tumors yielded only the expected smaller 550-bp E1A product and 180-bp E1B product, respectively (Fig. 5B). To rule out the possibility of low-level contamination by wild-type Ad9 genomes in these mutant virus-induced tumors, we next used a nested set of E1A primers (*e* plus *b*) or E1B primers (*c* plus *f*) to subject the DNA products of the first PCRs described above to a second PCR (Fig. 5A). Using these nested primers in control PCRs, we were able to amplify the expected 762-bp E1A and 533-bp E1B products directly from the DNA of a wild-type Ad9-induced tumor (Fig. 5C). In contrast, we failed to amplify any such wild-type Ad9 DNA products from the first E1A and E1B PCRs of mutant virus-induced tumor DNAs (Fig. 5C). These results indicated that wild-type Ad9 E1 region sequences are absent from the mutant virus-induced mammary tumors and, consequently, that Ad9ΔE1A and Ad9ΔE1B are able to produce mammary tumors in rats.

TABLE 3. Tumorigenicities of wild-type and mutant Ad9 viruses in Wistar-Furth rats[a]

Virus	No. of rats that developed tumors/ no. infected with virus	
	Females	Males
Ad9	3/3	0/2
Ad9ΔE1A	8/8	0/2
Ad9ΔE1B[b]	3/3	0/3
Ad26[c]	0/3	0/3

[a] Two- to three-day-old Wistar-Furth rats were injected subcutaneously with 7×10^7 PFU of virus. Animals were monitored by palpation for tumor development over an 8-month period.

[b] Due to replication deficiencies of virus Ad9ΔE1B in 293 cells, the dose used to infect rats with this virus (8×10^6 PFU) was approximately ninefold lower than that used for the other viruses.

[c] Ad26, a nononcogenic subgroup D human adenovirus closely related to Ad9 (31), served as a negative control in this experiment.

TABLE 4. Histologies of wild-type and mutant Ad9-induced tumors[a]

Virus	Tumor sample	Histology
Wild-type Ad9	1	Fibroadenoma
	2	Fibroadenoma
	3	Fibroadenoma
Ad9ΔE1A	1	Fibroadenoma, one area of increased cellularity
	2	Focally cellular fibroadenoma
	3	Fibroadenoma
	4	Cellular fibroadenoma, focally increased mitoses, and focal phyllodes-like tumor[b]
	5	Fibroadenoma
	6	Fibroadenoma
	7	Fibroadenoma
	8	Fibroadenoma
Ad9ΔE1B	1	Fibroadenoma
	2	Fibroadenoma
	3	Fibroadenoma

[a] For histological examination, tumor samples were fixed in 10% formalin, and sections were stained with hematoxylin and eosin.

[b] A section of this tumor contained an area resembling a phyllodes-like tumor, a type of mammary tumor occasionally observed in rats infected by wild-type Ad9 (29).

Mammary tumors contain and express the Ad9 E4 ORF1 gene. As E4 ORF1 is an essential viral determinant for tumorigenesis by Ad9 (32), we next sought to confirm that Ad9 mutant virus-induced mammary tumors retain this gene and express the protein. By PCR amplification or immunoblot analysis, we detected the Ad9 E4 ORF1 gene (Fig. 6A) or its protein expression (Fig. 6B), respectively, in all mammary tumors, including those elicited by viruses Ad9ΔE1A and Ad9ΔE1B. As smaller tumors had arisen in Ad9ΔE1A virus-infected animals, it was noteworthy that the levels of Ad9 E4 ORF1 protein in Ad9ΔE1A-induced tumors were lower than those in both wild-type Ad9-induced and Ad9ΔE1B-induced tumors (Fig. 6B).

DISCUSSION

In this study, we determined the nucleotide sequence of the subgroup D Ad9 E1 region and showed that its gene organization and predicted protein products are highly related to those of E1 regions from other human adenoviruses. Additionally, to investigate the role of the Ad9 E1 region in Ad9-induced mammary oncogenesis, we engineered the same E1A and E1B deletion mutations into both Ad9 E1 region plasmids and Ad9 viruses. We found that while E1A and E1B mutant Ad9 E1 region plasmids displayed significantly impaired focus-forming activity in vitro, the corresponding E1A and E1B mutant Ad9 viruses retained the ability to generate mammary tumors in rats. These results indicate that although the Ad9 E1 region alone or in cooperation with activated *ras* exhibits transforming activity in vitro, this activity is not required for mammary tumorigenesis by Ad9 in vivo. Similar examples in which transformation in vitro fails to predict tumorigenicity in vivo are also known for other viral and cellular transforming proteins (6, 8, 35, 45, 48, 56).

In addition to showing that Ad9 E1 region transforming functions are dispensable for mammary tumorigenesis by Ad9,

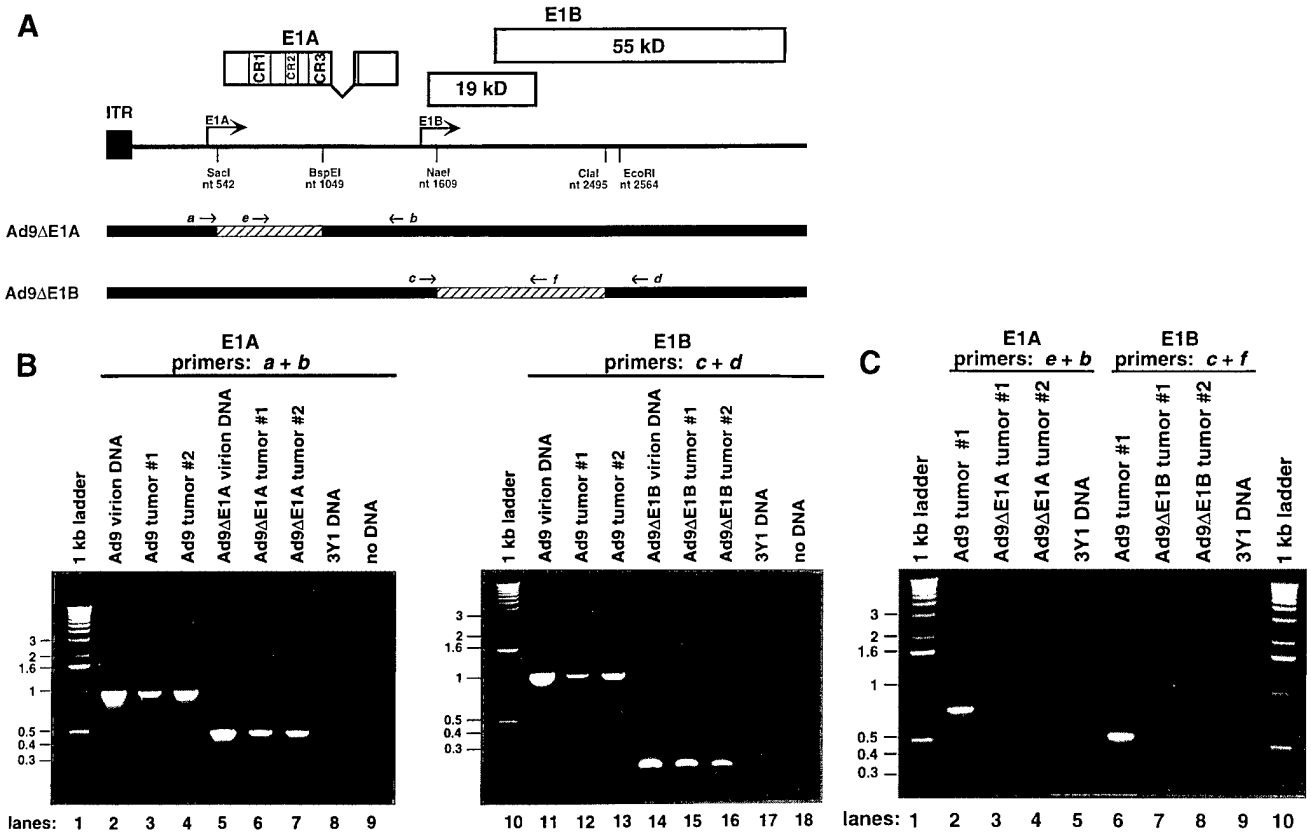

FIG. 5. Tumors induced by viruses Ad9ΔE1A and Ad9ΔE1B do not contain wild-type Ad9 E1 region sequences. (A) Locations of Ad9 E1 region primers used in PCRs. ITR, inverted terminal repeat. (B) PCR 1 utilized either E1A primers *a* and *b* or E1B primers *c* and *d*. Genomic DNA from rat 3Y1 cells or water (no DNA) represented negative controls in these reactions; 1.5 μg of genomic DNA or 10 ng of virion DNA was used as a template. (C) Nested PCR 2 utilized E1A primers *e* and *b* or E1B primers *c* and *f*. In these reactions, DNA from a wild-type Ad9-induced tumor and 3Y1 genomic DNA represented positive and negative controls, respectively; 1/20 of the DNA products from PCR (B) was used as a template for PCR 2. PCR conditions: 30 cycles of denaturation at 94°C for 1 min, annealing at 65°C (E1A reaction 1), 58°C (E1A reaction 2), or 60°C (E1B reactions 1 and 2) for 1 min, and extension at 72°C for 1 min, followed by a final 72°C extension for 15 min. DNA products were separated by agarose gel electrophoresis and visualized with ethidium bromide.

our results further argue that the Ad9 E4 region-encoded ORF1 transforming gene represents the major oncogenic determinant of this virus. In this respect, Ad9 represents the first example of an oncogenic adenovirus for which the E1 region is not the major oncogenic determinant. The fact that the oncogenic avian adenovirus CELO lacks genes related to the human adenovirus E1A and E1B oncogenes (11) further suggests that additional examples non-E1 region oncogenic determinants for adenoviruses will be found.

Although the mechanism by which Ad9 reaches the mammary glands of rats after subcutaneous inoculation has not been established, we hypothesize that the inoculated Ad9 virions are able to directly infect mammary cells to cause tumors in the animals. This idea is based on the fact that rodent cells are generally nonpermissive for replication of human adenoviruses (48), a property that would limit spread of the virus by successive rounds of viral replication in tissues of rats. Moreover, in this study, we found that Ad9 E1A and E1B mutant viruses (Ad9ΔE1A and Ad9ΔE1B, respectively) retained the capacity to generate mammary tumors in these animals. Because E1A and E1B genes encode critical functions needed for efficient replication of adenoviruses (48), these new results with E1A and E1B mutant viruses provide additional support for the idea that viral replication in rats is not required for Ad9 to produce mammary tumors.

Although tumors elicited by wild-type and E1 region mutant Ad9 viruses in this study were found to be histologically identical, the tumors induced by the E1A mutant Ad9 virus were generally smaller than those generated by both the wild-type and E1B mutant Ad9 viruses. This finding suggests that E1A transforming functions may, in fact, enhance the growth of Ad9-induced mammary tumors. Nevertheless, it must also be considered that, separate from its transforming functions, E1A also serves an important role in the viral life cycle by transcriptionally activating other viral gene regions, including the E4 region (7, 34, 42). In the E1A mutant virus Ad9ΔE1A, we introduced a large deletion extending from the E1A initiation codon through half of CR3, a mutation which in addition to abolishing the transforming potential of E1A would also be expected to block transcriptional activation mediated by this gene. With regard to such a lack of E1A transcriptional activity in virus Ad9ΔE1A, it may be relevant that mammary tumors generated by this virus expressed reduced levels of the E4 ORF1 protein (Fig. 6B). This finding may indicate that E1A plays an accessory role in Ad9 mammary tumorigenesis by transcriptionally activating the viral E4 region and, thereby, elevating expression of the Ad9 E4 ORF1 oncogenic determinant. Similar indirect roles in viral oncogenesis have been ascribed to the bovine papillomavirus type 1 E2 and the Epstein-Barr herpesvirus EBNA2 transactivators, which participate in tumor formation by increasing expression of

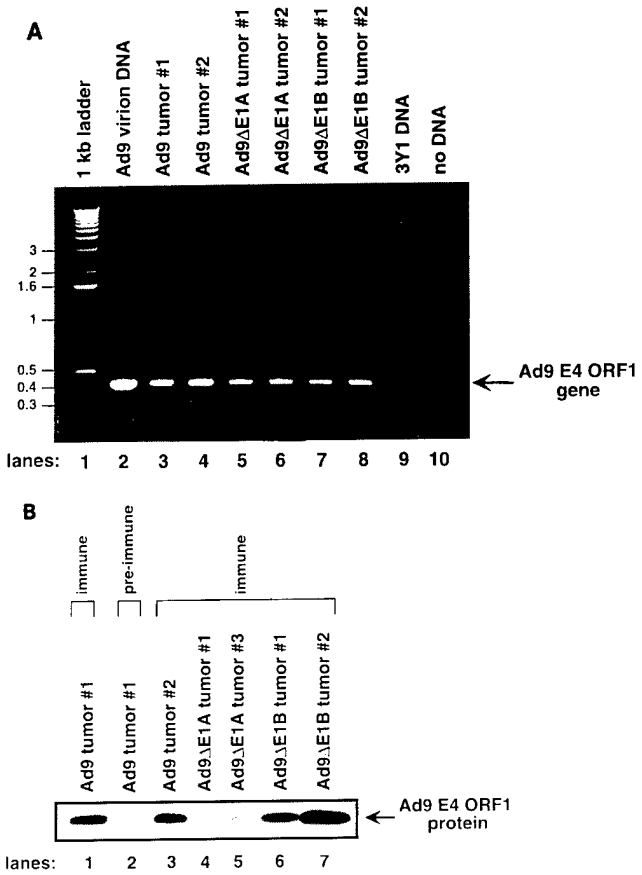

FIG. 6. (A) PCR amplification of the Ad9 E4 ORF1 gene from tumor DNAs. PCRs were performed with Ad9 E4 ORF1 primers as described for Fig. 5 except that a 55°C annealing temperature was used. Genomic DNA from rat 3Y1 cells represented a negative control in these reactions. (B) Detection of Ad9 E4 ORF1 protein in tumors. Tumor lysates containing 3 mg of protein were subjected to immunoprecipitation followed by immunoblot analysis using Ad9 E4 ORF1 polyclonal antiserum. Preimmune serum served as a negative control for immunoprecipitations (lane 2).

the transforming genes of their respective viruses (14, 16, 24, 36, 43).

In addition to promoting tumorigenesis, the oncoproteins of DNA tumor viruses may also contribute to determining which particular tissues are targeted for neoplasia. Comparisons of two related families of viruses, the papillomaviruses (PVs) and fibropapillomaviruses (FPVs), can be used to illustrate this idea. Although members of both families of viruses encode three different, structurally conserved transforming proteins, E5, E6, and E7 (5, 10, 15, 27, 39, 46), PVs and FPVs target distinct tissues in vivo, with PVs causing papillomas in epithelial keratinocytes and FPVs causing fibropapillomas in dermal fibroblasts (27). It has been established that E6 and E7 represent the major transforming proteins of PVs, whereas the E5 gene product is the major transforming protein of FPVs (27). Such observations have led to the hypothesis that the use of functionally different oncogenic determinants contributes to the unique tumorigenic tissue tropisms of PVs and FPVs (27). Likewise, Ad9 causes estrogen-dependent mammary tumors, whereas other oncogenic adenoviruses induce sarcomas in rodents. Therefore, one intriguing possibility is that novel molecular mechanisms which underly the transforming activity of Ad9 E4 ORF1 (41) permit Ad9 to selectively target mammary cells for tumorigenesis.

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Papillomavirus

Oncogenic ~~HPV~~ E6 proteins target the discs large tumour suppressor for proteasome mediated degradation.

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ABSTRACT.

Previous studies have shown that the oncogenic HPV E6 proteins form a complex with the human homologue of the *Drosophila* tumour suppressor protein, discs large (Dlg). This is mediated by residues in the extreme carboxy terminus of the E6 proteins and involves recognition of at least one PDZ domain of Dlg. This region of E6 is not conserved amongst E6 proteins from the low risk papillomavirus types and, hence, binding of HPV E6 proteins to Dlg correlates with the oncogenic potential of these viruses. We have performed studies to investigate the consequences of the interaction between E6 and Dlg. Mutational analysis of both the HPV18 E6 and Dlg proteins has further defined the regions of E6 and Dlg necessary for complex formation. Strikingly, co-expression of wild-type HPV18 E6 with Dlg *in vitro* or *in vivo* results in a dramatic decrease in the amount of Dlg protein, whereas mutants of E6 which fail to complex with Dlg have minimal effect on Dlg protein levels. The oncogenic HPV16 E6 similarly decreased Dlg levels, but this was not observed with the low risk HPV11 E6 protein. Moreover, a region within the first 544 amino acids of Dlg containing the three PDZ domains confers susceptibility to E6 mediated degradation. Finally, treatment of cells with a proteasome inhibitor partially overrides the capacity of E6 to degrade Dlg. These results demonstrate that Dlg is targeted by high risk HPV E6 proteins for proteasome mediated degradation.

INTRODUCTION.

Papillomaviruses (PV) are a group of small DNA viruses that have a specific tropism for squamous epithelial cells and they induce cutaneous and squamous mucosal proliferative lesions in their natural hosts. Some PV types (high risk PVs) are associated with naturally occurring cancers in a number of animal species including humans, and this association has now been well established (zur Hausen and Schneider, 1987; zur Hausen, 1991). Human high risk PVs, such as HPV16 and HPV18, encode two oncoproteins, E6 and E7, whose functions are principally responsible for the transforming capacity of these viruses (Vousden, 1994). These proteins contribute to the oncogenic process by inactivating key cellular proteins involved in the control of cell proliferation. E7 was demonstrated to bind and inactivate the retinoblastoma tumour suppressor protein (pRB) (Dyson *et al.*, 1989; Münger *et al.*, 1989), as well as the related p107 and p130 proteins (Davies *et al.*, 1993). It has also been shown that E7 can induce the degradation of pRB through the ubiquitin pathway (Boyer *et al.*, 1996), resulting in a loss of G1 checkpoint control. The principal activity of the oncogenic associated E6 proteins is the ability to bind to the p53 tumour suppressor protein and stimulate its rapid degradation via the ubiquitin proteolytic pathway (Scheffner *et al.*, 1990). The 100 kDa cellular protein, E6-AP, is also involved in this interaction and, in conjunction with E6, acts as a ubiquitin-protein ligase (Huibregtse *et al.*, 1991, 1993; Rolfe *et al.*, 1995). E6 can also inhibit p53 transcriptional activity independently of its ability to

target p53 for degradation (Pim *et al.*, 1994; Thomas *et al.*, 1995). Nevertheless, there is a growing list of evidence indicating that, in addition to inactivating p53, E6 has other activities which may contribute to its oncogenic potential. It has been shown that HPV16 E6 and bovine papillomavirus type 1 (BPV1) E6 interact with the focal adhesion protein paxillin, resulting in a disruption of the actin cytoskeleton (Tong and Howley, 1997). The E6 oncoproteins also bind to the calcium binding protein ERC55 (E6BP) (Chen *et al.*, 1995), and it has been recently reported that HPV16 E6 stimulates the ubiquitin mediated degradation of c-myc (Gross-Mesilaty, *et al.*, 1998).

Sequence analysis of the E6 proteins derived from high risk mucosotropic HPVs reveals a high degree of homology in their C-terminal domains. This highly conserved region of E6 is not involved in the ability of E6 to bind and promote the degradation of p53, suggesting that other activities important for cell transformation may be regulated by this region (Crook *et al.*, 1991; Pim *et al.*, 1994). Recent studies have shown that this conserved C-terminal motif of oncogenic E6 proteins mediates an interaction with the PDZ domain-containing protein hDlg, the human homologue of the *Drosophila* tumour suppressor, discs large protein (DlgA) (Lee *et al.*, 1997; Kiyono *et al.*, 1997). In fact, this E6 motif (XS/TXV/L) is remarkably similar to the C-terminal peptide domain XS/TXV which has been shown previously to interact with the hydrophobic groove of the PDZ sites (Doyle *et al.*, 1996; Songyang *et al.*, 1997). PDZ domains (PSD-95/disc large/ZO-1) are 80-90 amino acid motifs present in several proteins of distinct origin and function as specific protein-recognition modules (Ponting and Phillips, 1995; Saras and Heldin, 1996; Kim, 1997).

They can target cytoplasmic proteins to form complexes at the inner surface of the plasma membrane, and are important for clustering membrane proteins, as well as for linking signalling molecules in multiprotein complexes at specialised membrane sites (Kim *et al.*, 1995; Marfatia *et al.*, 1996; Kim, 1997). hDlg-1 (homologous to the rat SAP97 and hereafter termed DLG) belongs to the family of proteins termed MAGUKs (membrane-associated guanylate kinase homologues) (Lue *et al.*, 1994; Muller *et al.*, 1995). These proteins share amino-terminal PDZ domains, an SH3 domain and a carboxy-terminal guanylate kinase (GuK) homology domain. MAGUKs are multi-functional proteins localized at the membrane-cytoskeleton interface at cell-cell junctions, where they have structural as well as signalling roles (Anderson, 1996). hDlg is expressed in a variety of cell types including epithelia, where it is localized at regions of cell-cell contact (Lue *et al.*, 1994). It associates with the membrane cytoskeleton by binding the 4.1 protein, and also interacts with the Shaker-type K^+ channels and the adenomatous polyposis coli (APC) tumour suppressor protein through its PDZ domains (Marfatia *et al.*, 1996; Kim *et al.*, 1995; Matsumine *et al.*, 1996). *Drosophila* DlgA (Woods and Bryant, 1991, 1993a) is involved in cell growth control, maintenance of cell adhesion and cell polarity in both embryonic and adult tissues, and also functions by blocking cell invasion during development (Woods *et al.*, 1996; Goode and Perrimon, 1997). Mutations in *dlg* cause epithelial cells to lose polarity and undergo neoplastic proliferation (Woods *et al.*, 1996).

Taking into account the activity of E6 with respect to p53, we initiated a series of studies to investigate the effects of E6 upon the steady-state levels of DLG. We show both *in vitro* and *in vivo*

that co-expression of HPV18 E6 with DLG results in a dramatic reduction in the steady-state levels of DLG expression. Mutational analysis of both E6 and DLG shows that this effect correlates with the ability of E6 and DLG to form a protein complex. A similar activity is exerted by HPV16 E6 but not by the low risk HPV11 E6 and, in addition, the reduction in DLG levels by E6 can be reversed by proteasome inhibition. These results demonstrate that oncogenic HPV E6 proteins target DLG for proteasome mediated degradation both ~~*in vitro* and *in vivo*.~~ in cells.

RESULTS

The Thr156 residue of the HPV18 E6 protein is important for binding to DLG *in vitro*.

Recent reports have shown the binding of oncogenic E6 proteins to DLG, a PDZ domain-containing protein (Lee *et al.*, 1997; Kiyono *et al.*, 1997). These studies highlighted the importance of the highly conserved C-terminus of the E6 proteins for this interaction, and the similarity of this region to previously defined PDZ domain-binding motifs (Doyle *et al.*, 1996; Songyang et al, 1997). The E6 proteins derived from low-risk HPV types do not have this consensus motif and were unable to bind to DLG, providing a correlation between biological risk of cervical cancer and the ability to bind DLG. It was also shown that the last C-terminal amino acid residue (Leu) of HPV16 E6 was critical for the interaction (Kiyono *et al.*, 1997), and only a conserved substitution to another hydrophobic amino acid allowed E6 to retain binding activity. To further analyse the E6-DLG interaction, we first investigated the importance of E6 Thr156 for binding DLG, since this residue represents a potential PKA phosphorylation site (Kühne *et al.*, manuscript in preparation) and is also part of the PDZ domain-binding motif (Figure 1). We constructed two different HPV18 E6 mutants: Thr156Glu and Thr156Val and tested them for the ability to bind DLG *in vitro*. Wild type and mutant E6 proteins were expressed by *in vitro* translation and subjected to GST pulldown analyses by incubation with glutathione-S-transferase (GST)-DLG fusion protein (Lee *et al.*, 1997). The results shown in Figure 2 demonstrate that both

mutants are greatly reduced in their ability to bind DLG compared with wild type HPV18 E6. As an additional control, another mutant, Arg153Lys, bearing a point mutation in the C-terminus of E6 upstream of the PDZ domain-binding motif, was also included. As can be seen, the Arg153Lys mutant binds DLG as efficiently as the wild type protein. These results demonstrate that the Thr residue at position 156 within the consensus C terminal binding motif of E6 is necessary for interaction with DLG. This is in agreement with the crystal structure of the PDZ domain-peptide complex, which showed that the hydroxyl oxygen of the T/S residue in the motif of the peptide binding-site is directly involved in the interaction with the PDZ domain (Doyle *et al.*, 1996).

HPV18 E6 binds to all three PDZ domains of DLG *in vitro*.

Previous studies have shown that HPV16 E6 binds principally to the second PDZ domain of DLG (Kiyono *et al.*, 1997). Since HPV-18 E6 has been reported to bind DLG more strongly than HPV16 E6 (Kiyono *et al.*, 1997; Robert Weiss and ~~Ron Javier~~ R.J., personal observations), we were interested in identifying which DLG PDZ domains were necessary for complex formation with HPV18 E6. A series of GST-DLG deletion constructs (Figure 3A) (Lee *et al.*, 1997) were used to assess HPV18 E6 binding. Similar amounts of each DLG fusion protein were immobilized on PVDF membrane and then incubated with purified radiolabelled HPV18 E6 protein probe. The binding of the HPV18 E6 protein to the DLG fusion proteins was ~~then~~ determined by autoradiography. The results shown in Figure 3B demonstrate that HPV18 E6 binds equally well

to each of the three PDZ domains of DLG. This binding is specific since the HPV18 E6 probe did not react with the DLG constructs NT and SH3/GuK, which do not contain PDZ domains. This is in contrast to the results previously obtained with HPV16 E6 which was found to bind largely to DLG PDZ2 (Kiyono *et al.*, 1997) These divergent results are likely to explain the apparent stronger affinity of HPV18 E6 for DLG compared with HPV16 E6.

Degradation of DLG protein by HPV18 E6 *in vitro*.

A major activity of the oncogenic associated E6 proteins is the ability to target a number of their cellular target proteins, such as p53, c-myc and Bak, for ubiquitin mediated degradation (Scheffner *et al.*, 1990; Gross-Mesilaty *et al.*, 1998; Thomas and Banks, 1998). Therefore, we were interested in determining whether HPV18 E6 had a similar activity with respect to DLG. To examine this, we first performed an *in vitro* degradation assay. DLG and HPV18 E6 were translated *in vitro* with rabbit reticulocyte lysate, and were ~~then~~ subsequently mixed together and incubated at 30°C for 60min. The proteins were then run on SDS PAGE, and DLG protein was detected by autoradiography (Figure 4A). The results show a reduced level of DLG protein following incubation with wild type E6. In contrast, no change in the levels of DLG ~~are~~ were obtained when it ~~is~~ was incubated either with the E6 Thr156Glu mutant, which cannot bind DLG, or with control reticulocyte lysate primed with water. For comparison, the same assay was performed with p53 and, as can be seen in Figure 4B, both the wild type and the mutant Thr156Glu E6 proteins similarly reduced the level of p53. This is in agreement with previous

results which showed that the C-terminus of E6 is not involved in E6 mediated degradation of p53 (Crook *et al.*, 1991; Pim *et al.*, 1994). Equivalent results were also obtained when the experiment was performed by simultaneously co-translating the E6 and DLG proteins and then monitoring DLG levels by SDS PAGE and autoradiography (Figure 4C). In this case, DLG was also co-translated in the presence of HPV16 E7 as an additional negative control. These data demonstrate that the wild type HPV18 E6 protein specifically stimulates the degradation of DLG *in vitro* and that this activity is dependent on the ability of E6 to interact with DLG.

DLG is targeted by HPV18 E6 for degradation *in vivo*.

Having shown that HPV18 E6 could induce the degradation of DLG *in vitro*, we were next interested in analysing whether this would take place *in vivo*. To test this possibility, we performed a series of *in vivo* degradation assays. Human 293 cells were co-transfected with influenza hemaglutinin (HA) epitope-tagged DLG (Kim and Sheng, 1996) and wild type or the Thr156Glu mutant of HPV18 E6. After 24 hrs, the cells were harvested and the levels of DLG were ascertained by western blot analysis using an anti-HA monoclonal antibody. The results shown in Figure 5A (upper panel) indicate that the levels of DLG were greatly reduced in the presence of wild type HPV18 E6 but ~~not at all~~ were unaffected in the presence of the E6 Thr156Glu mutant. Therefore, HPV18 E6 also targets DLG for degradation *in vivo*, and this effect correlates with the ability of E6 to bind DLG *in vitro*. ~~We also~~ To investigated whether E6

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proteins derived from other genital HPV types could also promote the degradation of DLG, we examined the ~~Therefore, the *in vivo* degradation assay was also performed with~~ HPV16 E6 and HPV11 E6 proteins in similar assays. ~~As can be seen in~~ Figure 5 (upper panel), HPV16 E6 ~~can~~ also promote degradation of DLG, albeit to a lesser extent than HPV18 E6, whereas no reduction in the levels of DLG was observed in the presence of HPV11 E6. HPV16 E6 has previously been shown to bind DLG both *in vivo* and *in vitro*; in contrast, HPV11 E6 lacks the consensus PDZ domain-binding motif and cannot bind DLG (Lee *et al.*, 1997; Kiyono *et al.*, 1997). Thus, high risk HPV E6 proteins share the ability to target DLG for degradation *in vivo*, but low risk E6 proteins which fail to bind DLG do not.

To exclude the possibility that the reduced levels of DLG expression obtained *in vivo* in the presence of E6 were due to sequestration within insoluble complexes rather than degradation, we analysed the levels of the DLG protein in the insoluble fractions of cells in both the presence and absence of HPV18 E6. The results obtained are shown in Figure 5A (lower panel) and demonstrate that in the insoluble fractions, the levels of DLG are reduced in the presence of HPV18 E6 and to a somewhat lesser extent with HPV16 E6. In contrast there is no decrease in the levels of DLG in the presence of either the Thr156Glu mutant or HPV11 E6 This result confirms that E6 reduces the total amount of DLG within the cell, and that the reduced levels of DLG observed are not due to the generation of protein complexes which fail to be extracted under the conditions of these assays.

In order to exclude that differences in the ability of the E6 proteins to target DLG for degradation ~~was~~ are not due to differences in levels of E6 expression, a parallel p53 *in vivo* degradation assay

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was also performed. Saos-2 cells were transfected with a p53 expression plasmid together with either HPV18, HPV16, or HPV11 E6 or the Thr156Glu HPV18 E6 mutant. The cells were harvested after 24hrs and the results obtained are shown in Figure 5B. As can be seen, both HPV18 and HPV16 E6 induce complete degradation of the p53 protein. This indicates that the differences in the ability of these two E6 proteins to degrade DLG most likely reflects intrinsic differences in their respective abilities to target DLG for degradation rather than differences in their levels of expression. Likewise the Thr156Glu mutant displays almost wild type levels of p53 degradation. This confirms that this mutant protein is expressed and, in addition, that this region of E6 is not involved in the p53 interaction. Finally, a weak reduction in p53 levels was obtained following transfection with HPV11 E6. This is in agreement with our previous observations (Strorey et al., 1998) and also demonstrates functional expression of this protein.

To determine which regions of DLG render it susceptible to E6 mediated degradation, we performed *in vivo* degradation assays using two truncated derivatives of DLG: DLG-NT3PDZ (amino acids 1-544) contain~~s~~ing the N terminus and the three PDZ domains of the protein, and DLG-SH3GuK (aminoacids 539-end) contain~~s~~ing the GuK and SH3 motifs but lack~~s~~ing the PDZ domains. Both mutants were HA-tagged and the degradation assay was performed as described above. The results shown in Figure 6 demonstrate that the levels of the DLG-NT ~~are~~ were reduced by wild-type E6 to a level similar to that of the full length DLG (DLG FL) protein. In contrast, the levels of the DLG-CT protein, which lacks the three PDZ domains, were completely unaffected by the presence of the HPV18 E6 protein. These results demonstrate that the binding of E6 to DLG is

required for the degradation of DLG *in vivo* and, in addition, that the sequences necessary for efficient degradation of DLG by HPV18 E6 lie within the first 544 amino acid residues of the protein, encompassing the three PDZ domains.

HPV18 E6 stimulates the degradation of DLG via the proteasome pathway.

To further characterise the mechanism by which HPV18 E6 promotes degradation of DLG, we examined the role of the proteasome proteolytic pathway. ~~The~~ An *in vivo* degradation assay was performed as described above, but the transfected cells were treated with the proteasome inhibitor (N-CBZ-LEU-LEU-LEU-AL) for 2 hrs before protein extraction. The levels of DLG were again ascertained by western blotting and the results obtained are shown in Figure 7. The presence of HPV18 E6 ~~again~~ results in a dramatic decrease in ~~the~~ levels ~~of the~~ DLG protein and this ~~is~~ was not ~~obtained~~ observed with the Thr156Glu mutant. Interestingly, addition of proteasome inhibitor results in a partial restoration of DLG protein levels in the presence of HPV18 E6, indicating proteasome involvement in the E6 mediated degradation of DLG. Complete restoration of DLG levels has so far not been attainable and the reasons for this are under further investigation. It is also interesting to note that DLG levels in the absence of E6 are also increased in the presence of the proteasome inhibitor, suggesting that DLG may normally be regulated via the proteasome.

To further investigate whether DLG is regulated by ubiquitination, we performed a series of *in vivo* ubiquitination assays. Cells were transfected with plasmids expressing full length DLG, and the two

truncated derivatives NT3PDZ and SH3GuK. After 24hrs cells were extracted under denaturing conditions in the presence of guanidium hydrochloride and DLG proteins were detected by western blot analysis. The results obtained are shown in Figure 8 panel A and, as can be seen, ladders characteristic of ubiquitination were detected with the full length DLG and the NT3PDZ derivative. In contrast, the SH3GuK derivative, which was not susceptible to E6 mediated degradation did not exhibit laddering characteristic of ubiquitination. To confirm that the full length DLG protein was indeed ubiquitinated *in vivo*, cells were transfected with a "six Histidine" tagged DLG expression plasmid and a plasmid expressing HA tagged ubiquitin. After 24hrs, cells were extracted under denaturing conditions and extracts affinity purified on Ni^{++} agarose columns as described previously (Kühne and Banks, 1998). Bound ubiquitin was then assessed by western blot analysis with the anti HA monoclonal antibody, and the results obtained are shown in Figure 8 panel B. As can be seen, in cells expressing either DLG alone, or in the presence of HPV18 E6, specific retention of high molecular weight ubiquitin ladders were obtained. These results confirm that DLG is ubiquitinated both in the absence and presence of HPV18 E6 and demonstrates that DLG levels are normally most likely regulated in the cell by ubiquitination.

While these results are interesting, no data are given to address why E6 increases the rate of DLG degradation in cells.

DISCUSSION

The high risk HPV E6 proteins share several stretches of homology in different regions of the protein that define specific functional domains. The C-terminus is very highly conserved, and it has been shown to be involved in the binding to cellular proteins bearing PDZ domains. The conserved C-terminal motif of E6 also contains an RXXT consensus sequence for the cyclic AMP-dependent protein kinase A (PKA). We have shown that the Thr156 of HPV18 E6 is critical for the *in vitro* binding to DLG, with both neutral and acidic charge mutations abolishing binding. Interestingly, in the case of the binding of the K^+ channel protein Kir2.3 to the PDZ domain-containing protein PSD-95, it has been reported that the Ser residue at the C-terminus of Kir2.3 is vital for the interaction (Cohen *et al.*, 1996). Moreover, this Ser residue on Kir2.3 is also a substrate for PKA and phosphorylation of the Ser residue was found to negatively regulate the association with the PSD-95 protein. In addition, the crystal structure of the PDZ domain-peptide binding complex showed that the hydroxyl oxygen of the T/S residue in the consensus motif of the peptide binding-site is directly involved in the interaction (Doyle *et al.*, 1996). Taken together, these data suggest that potential PKA phosphorylation of E6 may also negatively regulate E6 binding to DLG.

HPV E6 proteins can stimulate the ubiquitin-mediated degradation of a number of the cellular proteins to which they bind. A number of these cellular targets are tumour suppressors, and it has been proposed that the degradation of such cellular control proteins is responsible for the oncogenic activity of E6 (Scheffner *et al.*, 1990; Gross-Mesilaty *et al.*, 1998; Thomas and Banks, 1998). We show,

both *in vitro* and *in vivo*, that the recently reported E6 target protein, DLG, is also susceptible to enhanced degradation in the presence of HPV18 E6. Using two different experimental approaches, we demonstrate that DLG protein is less stable in the presence of E6 *in vitro*. In addition, an *in vivo* degradation assay consistently demonstrated reduced levels of DLG protein in 293 cells expressing wild-type HPV18 E6. The Thr156Glu E6 mutant, which fails to bind to DLG, had no effect on the stability of DLG *in vitro*, indicating that E6's binding to DLG is important for the stimulation of degradation. In the *in vivo* assays, the Thr156Glu mutant was also greatly reduced in its ability to stimulate DLG degradation.

We then extended the study to E6 proteins derived from other genital HPV types. The *in vivo* degradation assays showed that while HPV16 E6 could reduce DLG levels, albeit somewhat less efficiently than HPV18 E6, the HPV11 E6 protein had minimal effect. The C-terminal motif of HPV16 E6, ETQL, is not identical to the consensus XTXV PDZ-binding domains, although a previous report has shown that HPV16 E6 ~~binds~~ is capable of binding DLG ~~efficiently~~ *in vitro* and *in vivo* (Kiyono *et al.*, 1997). In contrast, the C-terminus of HPV11 E6 lacks any homology with the consensus PDZ binding site, and ~~it has been shown that it cannot~~ does not bind to DLG *in vitro* (Lee *et al.*, 1997). The differences in the ability of different E6 proteins to stimulate the degradation of DLG correlates with their oncogenic potential, suggesting that this activity of E6 may contribute to the process of transformation and carcinogenesis mediated by the high-risk HPVs.

Previous studies have shown that among the three PDZ domains of DLG, the second PDZ domain is essential for HPV16 E6 binding

(Kiyono *et al.*, 1997), as is the case for APC binding to DLG (Matsumine *et al.*, 1996). In contrast, we have shown that HPV18 E6 binds efficiently to each of the three isolated PDZ domains *in vitro*, although the assays used are not identical and this may account for some of the differences. However, the data suggest that the HPV16 ~~and~~ HPV18 E6 ~~proteins bind~~ DLG slightly differently, and this may be a reflection of differences in the consensus PDZ binding domains of the two proteins. This may also account for the previous report which demonstrated that HPV18 E6 bound DLG with a higher affinity than HPV16 E6 (Kiyono *et al.*, 1997; Robert Weiss and ~~Ron Javier~~ R.J., personal observations). The *in vivo* degradation assays would also tend to support this conclusion since HPV16 E6 is consistently weaker than HPV18 E6 in its ability to degrade DLG. It will be interesting to determine whether this is reflected in differences in the biology of HPV16 and HPV18, ~~although~~ as there are a number of reports which indicate a poorer prognosis for individuals with cervical tumours harbouring HPV18 DNA rather than HPV 16 DNA (Burnett et al., 1992; Franco, 1992; Zhang et al., 1995).

The *in vivo* degradation assays performed with the deleted derivatives of DLG showed that the derivative bearing the amino terminus and the three PDZ domains ~~was still~~ retained susceptible to degradation by E6. In contrast, deletion of this region renders ~~the~~ DLG resistant to E6 mediated degradation, and demonstrates that the region of DLG which confers susceptibility to E6-mediated degradation lies within the first 544 residues of the protein. A complete mutational analysis will be required to determine the minimal region of DLG which confers susceptibility to E6.

Several other proteins derived from different oncogenic viruses have been shown to bind to PDZ domain-containing proteins through conserved C-terminal motifs. These include the Tax oncoprotein derived from human T cell leukaemia virus (Rousset *et al.* 1998) and the product of the E4 region ORF1 transforming gene product (9ORF1) of human adenovirus type 9 (Ad9). The subgroup D Ad9 requires the viral ~~9orf1~~ 9ORF1 gene for oncogenicity (Javier *et al.*, 1992; Javier, 1994). The 9ORF1 protein contains a C-terminal consensus PDZ binding domain and was shown to bind to DLG and other PDZ-containing proteins, such as mouse 9BP-1. Binding of 9ORF1 to 9BP-1 sequesters this cellular factor within punctate protein aggregates in the cytoplasm of cells (Lee and Javier, manuscript in preparation). In contrast, binding of E6 to DLG led to reduced levels of DLG in both the soluble and insoluble fractions of the cells. This suggests that the reduction in DLG levels in the presence of E6 is a consequence of increased degradation rather than sequestration within insoluble protein aggregates.

We next investigated whether the reduction in DLG protein levels in the presence of E6 was the result of proteasome mediated degradation. The results demonstrated that the treatment of cells with a proteasome inhibitor partially stabilised DLG protein in the presence of E6, although complete protection was never attained. ~~and the reasons for this are still under investigation~~. We also noted that DLG levels also increased following proteasome inhibition in the absence of E6 protein, indicating that DLG may be normally regulated via the proteasome. This suggested that the degradation of DLG is proteasome mediated, with ubiquitination being the most likely mechanism. This was investigated further with a series of assays designed to determine whether DLG was indeed

ubiquitinated. In the absence of E6, full length DLG and the NT3PDZ derivative were found to form high molecular weight complexes indicative of ubiquitinylation. Strikingly, the SH3GuK derivative of DLG which was not suscetible to E6 mediated degradation did not form these high molecular weight complexes. Using His tagged DLG and affinity chromatography together with HA tagged ubiquitin it was further possible to demonstrate that DLG specifically bound ubiquitin both in the absence and presence of HPV18 E6 protein. These results enable us to conclude that DLG is indeed normally regulated through ubiquitination and subsequent degradation via the proteasome.

You may need to speculate about how E6 increases degradation as its increases in ubiquitination are evident in your assays.

The biological significance of the degradation of DLG by E6 in natural infections can be addressed by considering the functions of DLG. DLG is expressed in epithelial cells, the natural host cells of HPV (Lue *et al.*, 1994). Epithelia form sheets of polarised cells, with the apical and basolateral sides separated by tight junctions (Kim, 1997), and DLG is required for the organization of these junctional complexes. Tight junctions form a continuous intercellular contact and their absence is associated with defective cell-cell adhesion, a loss of cell polarity and unregulated proliferation (Anderson, 1996; Woods *et al.*, 1996). Maintenance of cell polarity is crucial for normal differentiation, and the localization of many signalling receptors to the basolateral membrane is apparently mediated by PDZ proteins (Woods and Bryant, 1993b). Therefore, reducing the levels of DLG may contribute to a disorganisation of these tightly controlled signal transduction pathways. This could lead to a loss of cell control proliferation or an alteration of the proper pattern of keratinocyte differentiation. This may contribute to the p53-independent

[How do these results compare with p53]

Is E6AP involved here?

transforming activity of E6, as well as to its ability to inhibit Ca^{2+} induced differentiation of keratinocytes (Sherman and Schlegel, 1996). Significantly, the well-characterized Dlg-A of *Drosophila* does not simply inhibit individual cell behaviour but, rather, is a component in a development pathway essential for blocking cell proliferation, invasion and migration in a defined pattern. In addition, it was shown that invasive cells bearing certain Dlg-A mutations resemble malignant cells in several ways, including migratory ability (Goode and Perrimon, 1997). The fact that high risk HPV E6 proteins are able to degrade DLG, may also contribute to the invasive characteristic of cancerous cells infected by these viruses.

In summary, degradation of DLG would be expected to induce alterations in signal transduction pathways, cytoskeletal organization and cellular migration. These alterations could contribute to the transformed undifferentiated phenotype of cells expressing high risk HPV E6 proteins. The elucidation of which of the functions of DLG need to be abrogated by the virus during infection will contribute not only to the understanding of the viral biology, but also to the understanding of the mechanisms leading to cell transformation and carcinogenesis. Further studies are in progress to address this question as well as to determine whether other PDZ domain-containing proteins can be targeted by the E6 oncoproteins.

METHODS

α GST-DLG antibodies?

Plasmids.

The E6, E7 and p53 proteins were cloned under the control of the CMV promoter into the pCDNA-3 expression plasmid. The E6 mutants were constructed using PCR directed mutagenesis. HA-tagged SAP97 expression plasmid was kindly provided by Kyung-Ok Cho. Deleted derivatives of SAP97 were expressed from the CMV promoter in the GW1 plasmid (Kim and Sheng, 1996). pGEX-2T-SAP97 was described previously (Lee *et al.*, 1997) as was the HA-tagged ubiquitin expression plasmid (Treier *et al.*, 1994).

Cells and tissue culture.

Human 293 and Saos-2 cells were grown in DMEM with 10% foetal calf serum. All the transient transfections were carried out using the calcium phosphate precipitation procedure as described previously (Matlashewski *et al.*, 1987).

GST fusion protein expression and binding assays.

GST-SAP97 fusion protein bound to glutathione-linked agarose was prepared as described previously (Thomas *et al.*,1995). The binding was performed by mixing the *in vitro* translated E6 proteins with aliquots of the resins and incubating at 4°C for 1 h in binding buffer (50 mM Tris-HCl pH7.5; 150mM NaCl; 1mM EDTA). After removal of the supernants, the resin was washed four times with binding buffer containing 0.4% Nonidet-P 40 for 40 mins. Bound protein was eluted and analysed by SDS-PAGE and autoradiography. Protein blotting assays using truncated

derivatives of GST-DLG fusion proteins with radiolabelled HPV18 E6 were done as described previously (Lee *et al.*, 1997).

In vitro degradation assay.

The experiments were performed as described previously (Pim *et al.*, 1994). Briefly, the DLG, p53, E6 and E7 proteins cloned in pCDNA-3 were translated *in vitro* using the TNT coupled rabbit reticulocyte system (Promega), according to the manufacturers instructions, in the presence of [^{35}S]-cysteine. The translated products were quantitated by gel electrophoresis and Phospho Imager. Assays for the E6-mediated degradation of DLG or p53 were performed by mixing the *in vitro* translated proteins and incubating at 30°C for 60 min. The extent of degradation was determined by gel electrophoresis and autoradiography. For the cotranslation assays, pCDNA-3 plasmids encoding the different proteins were used as templates for simultaneous translation in the TNT system at 30°C for 90 min. The samples were then analysed by gel electrophoresis and autoradiography.

In vivo degradation assay.

Cells were harvested in extraction buffer (250 mM NaCl, 0.1% NP40, 50 mM HEPES pH 7.0, 1% aprotinin) 24 hrs after transfection. Equal amounts of proteins were separated by SDS-PAGE and transferred to nitrocellulose. The levels of remaining HA-tagged Dlg were determined by immunoblot analysis using the anti-HA monoclonal antibody (Boeringher-Mannheim). The levels of remaining p53 were determined by immunoblot analysis using a pool of anti p53 monoclonal antibodies, pAb1801, 1802 and 1803 (Banks *et al.*, 1986). The blots were developed using the

Amersham ECL technique according to the manufacturer's instructions. Cells were treated with the proteasome inhibitor (N-CBZ-LEU-LEU-LEU-AL, 40μM), as indicated in the text, two hours prior to protein extraction.

In vivo ubiquitination assays.

Cells were transfected with His tagged DLG together with a plasmid expression HA-tagged ubiquitin. After 24hrs cells were harvested as described previously (Kühne and Banks, 1998) and extracts affinity purified on Ni^{++} agarose columns and bound ubiquitin measured by western blot analysis with anti HA monoclonal antibodies.

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FIGURE LEGENDS.

Figure 1. Alignment of the C-terminal ends of E6 proteins derived from HPV types associated with genital infections. HPV-18, 16, 45, 31, 33 and 51 are high risk HPVs associated with cervical cancer. HPV-11 is a low risk type associated with benign genital lesions. The alignment was performed relative to the last cysteine of the second zinc-finger of the E6 sequence. The conserved PDZ consensus binding site is shown.

Figure 2. Binding of HPV-18 E6 to GST-DLG in *vitro.*

Panel A. Radiolabelled *in vitro* translated E6 proteins were incubated with GST-DLG or GST alone as a control. The bound E6 proteins were assessed by autoradiography. Wild type (WT) and the mutant E6 proteins tested are indicated, and amount of input protein is shown on the left of the gel.

Panel B. Coomassie Blue stained gel showing equal levels of fusion protein loading.

Panel C. Sequence of the C terminal domain of the wild-type and mutant E6 proteins used in the assay. The amino acid substitutions are shown with respect to the wild type HPV18 E6 sequence and are underlined.

Figure 3. HPV 18 E6 binds to all three individual PDZ domains of DLG *in vitro.*

Panel A. Schematic representation of the full-length (FL) and truncated DLG proteins used in the binding assays.

Panel B. GST-DLG fusion proteins were transferred to a PVDF membrane, which was then incubated with purified radiolabelled HPV18 E6 protein ($5x10^5$ cpm/ml), and washed extensively. Bound E6 was detected by autoradiography. Arrows indicate the positions of truncated GST-DLG fusion proteins which did not bind the E6 protein probe.

Panel C. Coomassie stain of the PVDF membrane after performing the binding assay showing equal levels of fusion protein loading.

Figure 4. E6-mediated degradation of DLG *in vitro.*

Panel A. *In vitro* translated DLG was incubated in the presence of *in vitro* translated wild type E6, Thr156Glu E6 mutant (E6Glu) or water-primed lysate for 0 or 60min as indicated. The amount of DLG remaining after the incubations was assessed by SDS PAGE and autoradiography. The positions of E6 and DLG is indicated.

Panel B. The same assay was carried out with p53 protein as a positive control. The amount of p53 protein remaining after the incubations was assessed by SDS PAGE and autoradiography. The positions of E6 and p53 is indicated.

Panel C. DLG protein was cotranslated *in vitro* with E6, the Thr156Glu (E6Glu) mutant or E7 for 90 min at 30°C as indicated. The samples were then analysed by SDS-PAGE and autoradiography. p53 served as a positive control in these assays and the left hand three lanes show E6, E6Glu and E7 translated alone. Arrows indicate the positions of DLG, p53, E7 and E6.

Figure 5. E6 mediated degradation of DLG *in vivo.*

Panel A. 293 cells were transfected with 4 μg of HA-tagged DLG expression plasmid, in the absence (DLG) or presence of 4μg of

HPV18 E6 (18), the Thr156Glu (glu) mutant, HPV16 E6 (16) and HPV11 E6 (11). After 24 hrs, the proteins were extracted (soluble and insoluble fractions as indicated) and equal amounts were separated by SDS-PAGE. The levels of DLG were ascertained by Western blot analysis with anti-HA monoclonal antibody (Boeringher-Mannheim).

Panel B. A parallel transfection was performed in Saos-2 cells with 4μg of p53 expression plasmid in the absence or presence of 4μg HPV18 E6 (18), the Thr156Glu (glu) mutant, HPV16E6 (16) and HPV11 E6 (11). After 24hrs, the proteins were extracted and equal amounts were separated by SDS-PAGE. The levels of p53 were ascertained by Western blot analysis with a pool of anti-p53 monoclonal antibodies (Banks *et al.*, 1986).

Figure 6. *In vivo* degradation comparing full-length DLG with truncated mutants.

The *in vivo* degradation assay was performed as in Figure 5. All transfections contained 4μg of the HA-tagged DLG expression plasmid and 4μg of the indicated E6 expression plasmids. The HA-tagged proteins used are also indicated (FL indicating full length), and arrows show the position of the wild type and mutant DLG proteins. The truncated DLG mutants are described in Figure 3.

Figure 7. HPV-18 E6 enhances DLG degradation via the proteasome.

An *in vivo* degradation assay was performed as described for Figure 5. Cells were transfected with DLG alone (DLG) or together with either HPV18E6 (18E6) or the Thr156Glu mutant (18E6glu) and proteasome inhibitor was added to the cells 2 hrs before

harvesting as indicated (+). Residual DLG was ascertained by Western blotting with the anti-HA antibody.

Figure 8. DLG is ubiquitinated *in vivo*.

Panel A. 293 cells were transfected with HA-tagged full length DLG (lane 4) the DLGNT3PDZ derivative (lane 3) and the DLGSH3GuK derivative (lane 2) or control expression plasmid (lane 1). After 24hrs cells were harvested in the presence of 2M Guanidium Hydrochloride and proteins analysed by western blot with anti HA-specific monoclonal antibodies (upper panel). A plasmid expressing β-galactosidase (pCDNA3-LacZ) served as an internal transfection control and the parallel western blot was developed with anti β-galactosidase antibodies (lower panel).

Panel B. A plasmid expressing "six Histidine" tagged DLG (pCDNAHis-DLG) was expressed in the presence of a plasmid expressing HA tagged ubiquitin (pHA-Ubi) in 293 cells. The DLG protein was affinity purified on Ni++ agarose under denaturing conditions. Western blots were performed with anti GST-DLG antibody (left panel) or anti HA-ubiquitin antibody (right hand panel). The lanes represent cells transfected with pHA-Ubi (1), pCDNAHis-DLG and pHA-Ubi (2) and pCDNAHis-DLG, pHA-Ubi and pCDNA-18E6. The postions of the His-DLG and HA-ubi ladders are indicated.

+ ? His.

Fig. 1

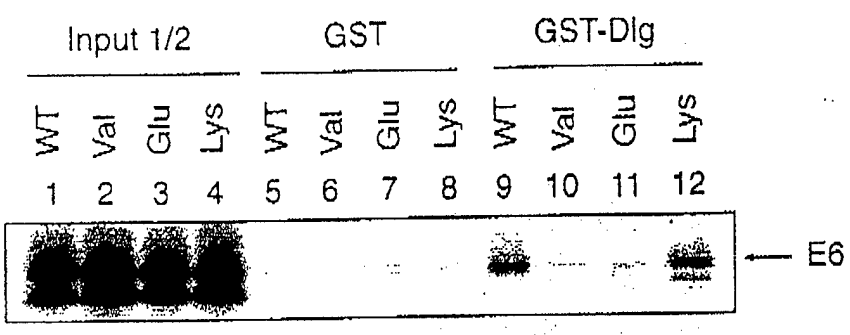
```
HPV18    AGHYRGQCHS CCNRARQERL QRRRETQV
HPV45    AGQYRGQCNT CCDQARQERL RRRRETQV
HPV16    RGRWTGRCMS.CC.....RSS RTRRETQL
HPV31    GGRWTGRCIA CW......R. RPRTETQV
HPV33    SGRWAGRCAA CW......R. SRRRETAL
HPV51    AGRWTGQCAN CW.....QRT RQRNETQV

HPV11                    CLHCWTTCMEDLLP

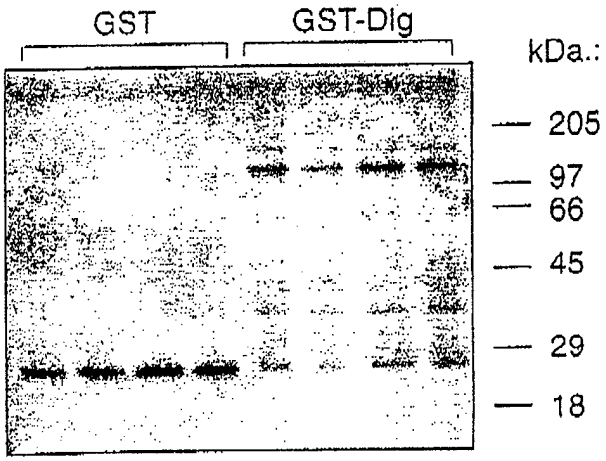

PDZ consensus                    XTXV
                                  S
```

Fig. 2

A



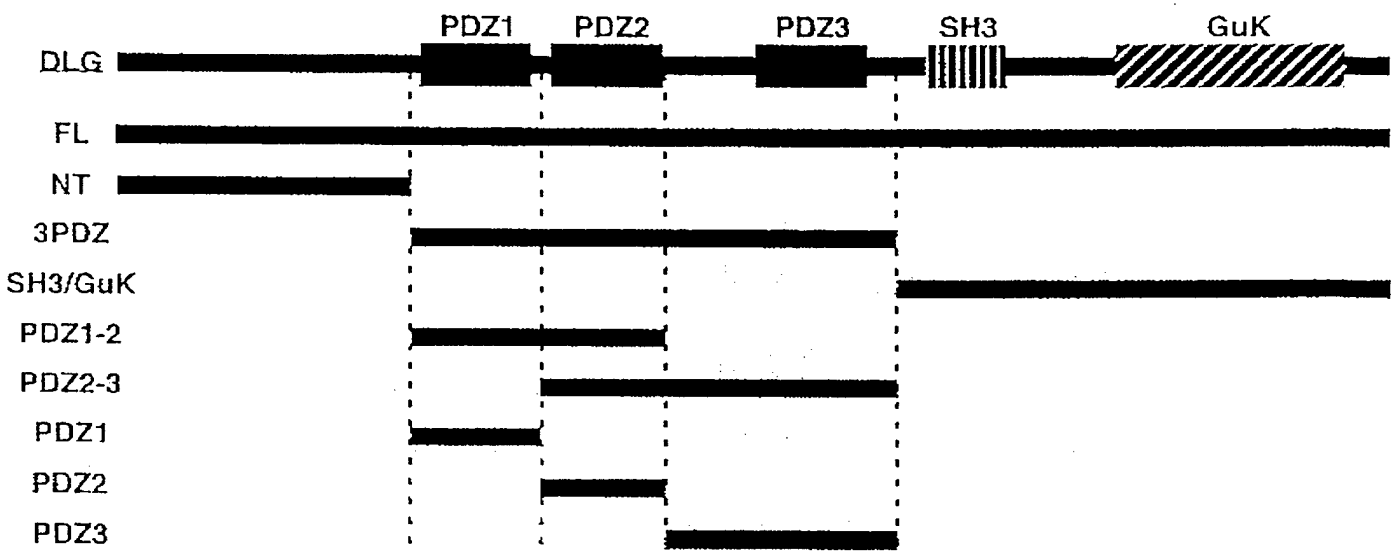
B

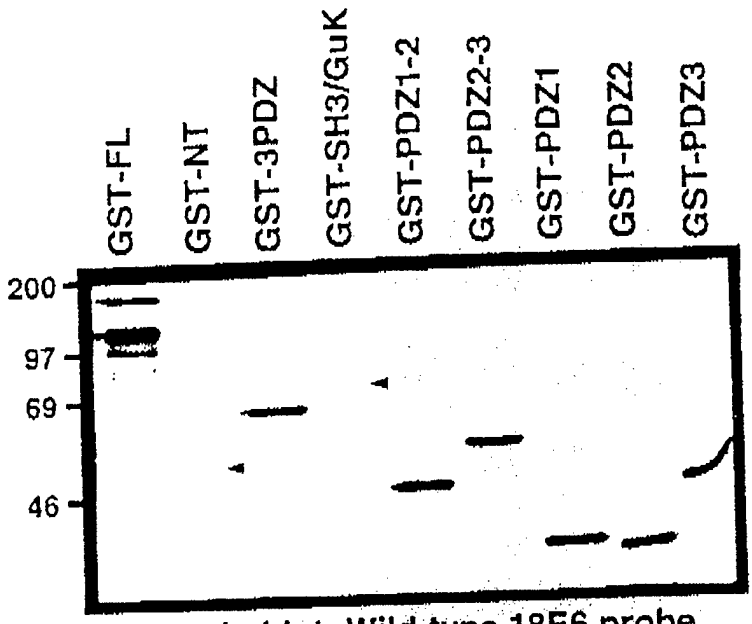


C

WT	QRRRETQV
Val	QRRRE<u>v</u>QV
Glu	QRRRE<u>e</u>QV
Lys	QR<u>k</u>RETQV

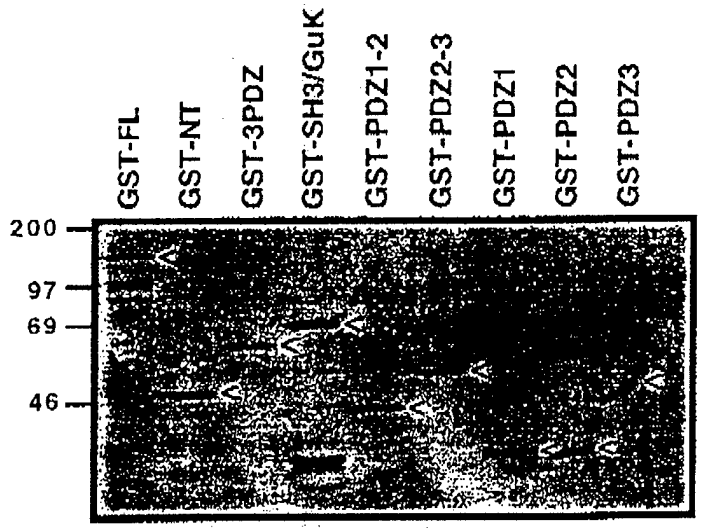
3A.


PDZ1
PDZ2
PDZ3
SH3
GuK
DLG
FL
NT
3PDZ
SH3/GuK
PDZ1-2
PDZ2-3
PDZ1
PDZ2
PDZ3



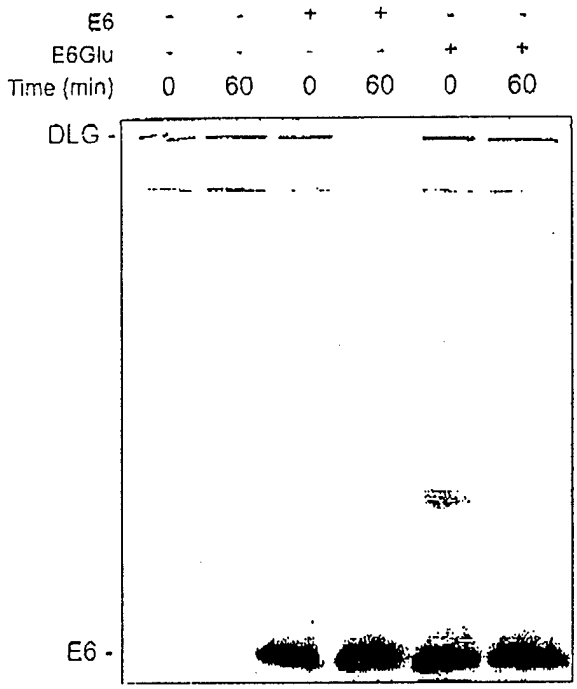
3B

Protein blot: Wild-type 18E6 probe

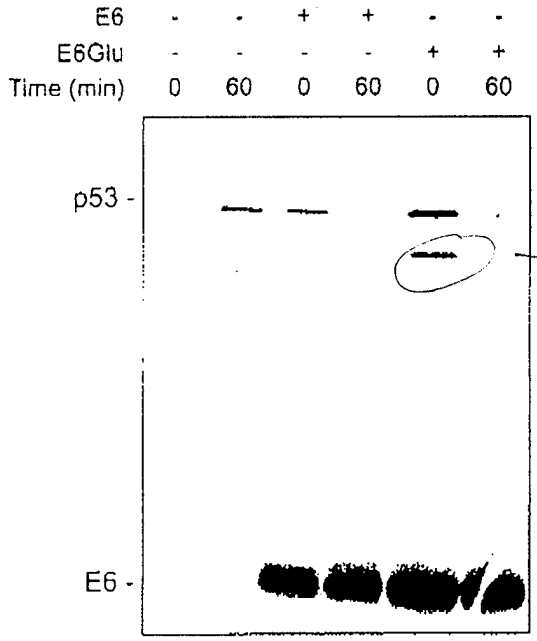


3C

Coomassie stained membrane


E6 - - + + - -
E6Glu - - - - + +
Time (min) 0 60 0 60 0 60
DLG -
E6 -

4A


E6 - - + + - -
E6Glu - - - - + +
Time (min) 0 60 0 60 0 60
p53 -
E6 -

4B

?

4C

E6
E6Glu
E7
p53
p53+E6
DLG
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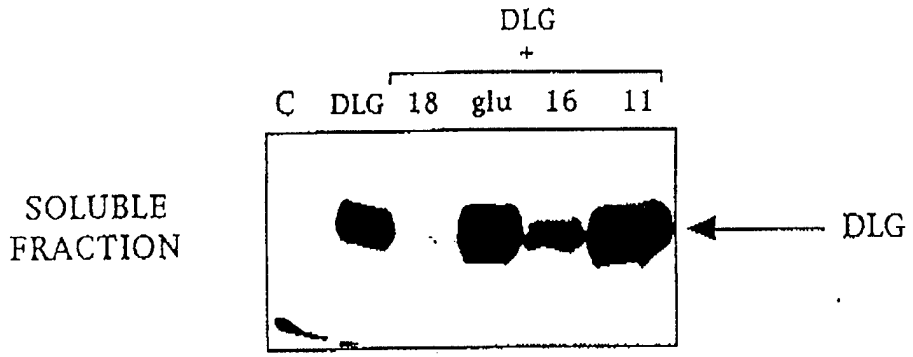
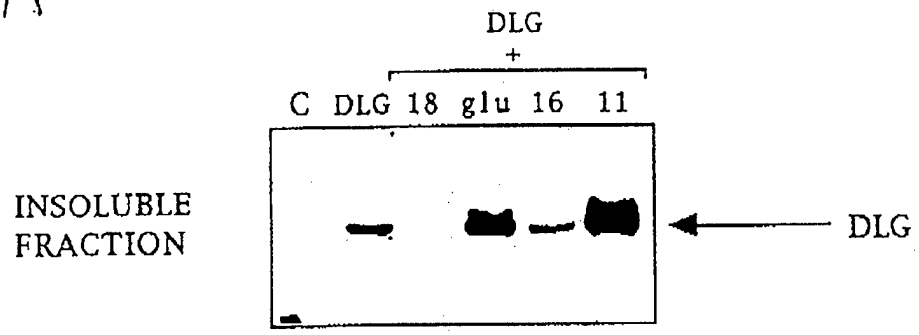
DLG + E7
DLG
p53
E7
E6
E7 ?


DLG
+
C DLG 18 glu 16 11
SOLUBLE
FRACTION
DLG

5A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+
C DLG 18 glu 16 11
INSOLUBLE
FRACTION
DLG

5B

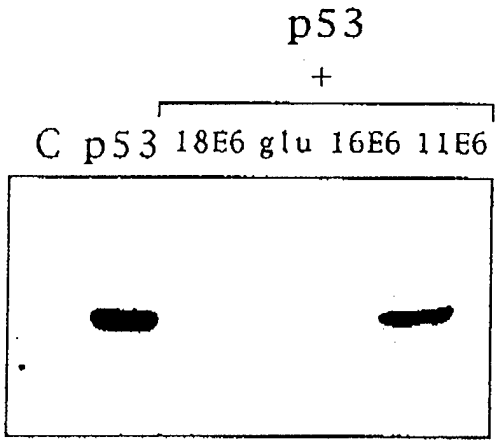

p53
+
C p53 18E6 glu 16E6 11E6

Fig. 6

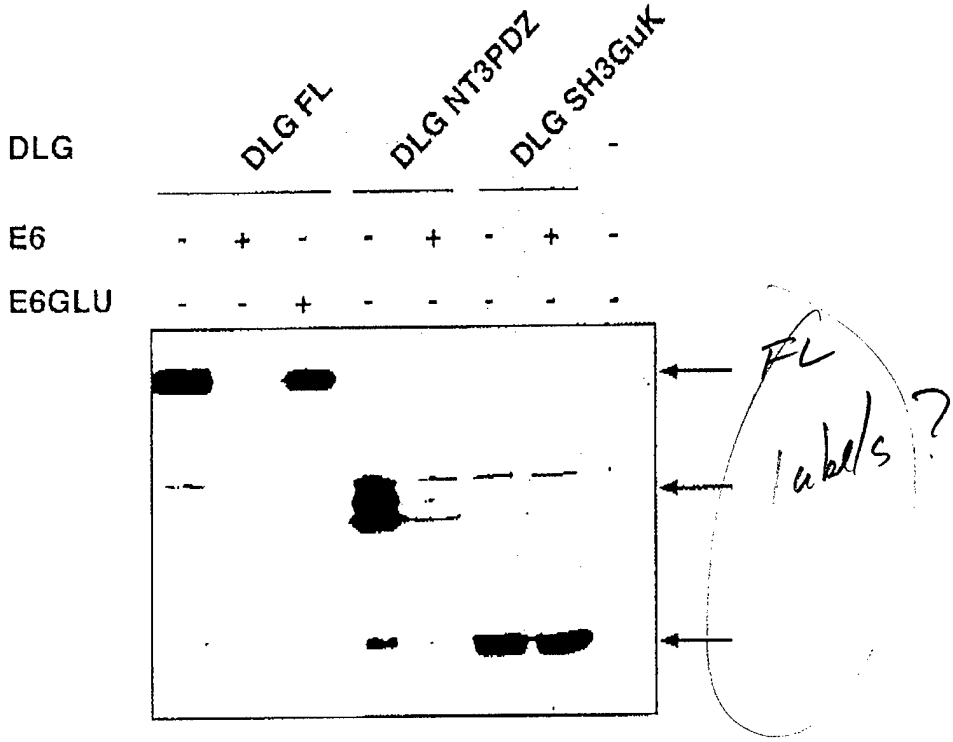
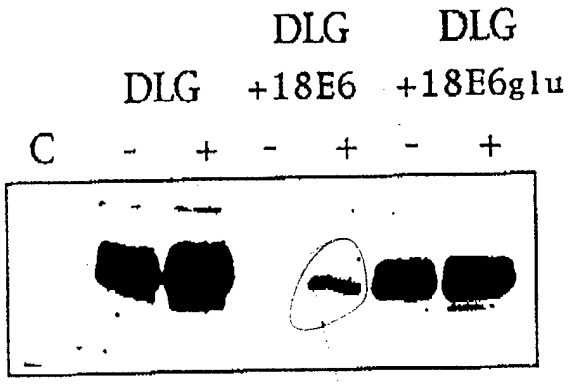


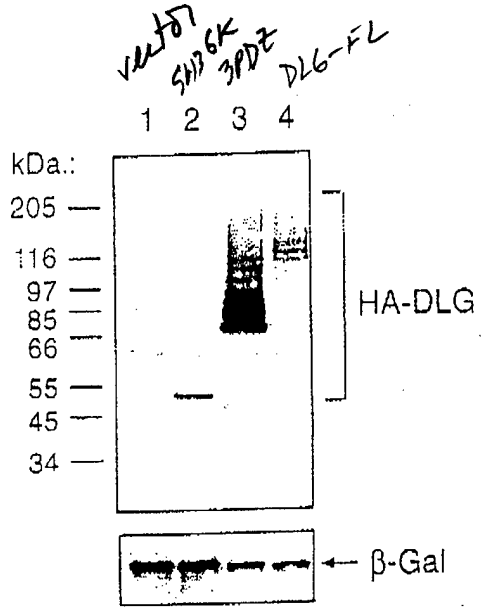
Fig 7



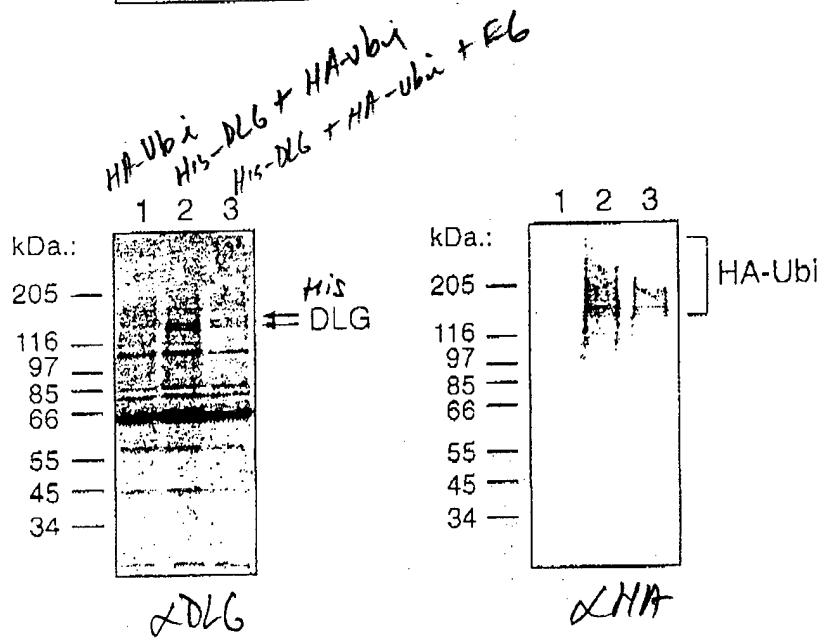
why not
wt levels?
proteosome-independent
degradation?

Fig. 8

A


vector
SH3 GK
3PDZ
DLG-FL
1 2 3 4
kDa.:
205
116
97
85
66
55
45
34
HA-DLG
β-Gal

B


HA-Ubi
His-DLG + HA-Ubi
His-DLG + HA-Ubi + E6
1 2 3
kDa.:
205
116
97
85
66
55
45
34
His
DLG
αDLG
1 2 3
kDa.:
205
116
97
85
66
55
45
34
HA-Ubi
αHA