Poly(ADP-ribose) polymerase requires DNA for activity, and the catalytic activity of this enzyme is directly coordinated to the number of DNA strand breaks in DNA. Use of molecular techniques and the complete amino acid sequence of the enzyme, established during the past granting period, should allow us to learn considerably more about the mechanism and role of this enzyme in cells exposed to stressful environments.

In AIM I, we proposed to extend our preliminary data on the insertion of full-length polymerase cDNA into various inducible and non-inducible expression vectors and retroviral vectors in both sense and antisense orientations. This would allow us to either inhibit (i.e., through antisense mRNA expression) or intensify the translation of polymerase in a variety of eukaryotic cells. A complementary approach was proposed in AIM II where various functional domains of the polymerase as well as site-directed mutants were to be constructed into inducible expression vectors to test whether selective inhibitors can be favorably used in cells.

Once it was verified that both the engineered mRNAs and appropriate peptides were expressed in vivo procedures in AIM III we proposed to test for cytotoxicity and DNA repair potential and mutagenesis repair of the various reconstructed cells obtained in AIMS I AND II.
A. A COMPREHENSIVE LIST OF THE OBJECTIVES OF THE RESEARCH EFFORT AND STATEMENT OF WORK

It has long been recognized that NAD is a major metabolite of the eukaryotic cell nucleus. The enzymes involved in both the synthesis and breakdown of NAD are associated with chromatin within the nucleus. The rate of NAD synthesis in the eukaryotic nuclei is extremely high ($10^5$ molecules/sec/cell). Approximately 95% of this replaces the NAD that is catabolized in the nucleus (for the poly ADP-ribosylation modification of nuclear proteins) and only 5% maintains the cytoplasmic NAD for growth.

The enzyme poly(ADP-ribose) polymerase requires DNA for activity, and it is significant that the catalytic activity of this enzyme is directly coordinated to the number of DNA strand breaks in DNA, both in vitro as well as in vivo. Accordingly, significant reductions in cellular NAD levels reflect increased poly ADP-ribosylation, due to DNA breaks, and hence it is of significance to this project that 30 organophosphorus and methyl carbonate insecticides in doses as low as 0.6 parts per million cause lowering of NAD. The levels of NAD were directly correlated with teratogenesis. It is our opinion that the poly ADP-ribosylation modification of chromatin-associated proteins plays an important function during the repair of DNA strand breaks in cells due to a variety of environmental toxic agents.

Our laboratory was the first to isolate and clone a full-length cDNA for this enzyme. We also showed that this cDNA, in an appropriate vector, can be expressed in eukaryotic cells. This has permitted direct experiments, using recombinant DNA techniques to test for the role of this enzyme in DNA repair and recovery from toxic agents. For example, we expect to over-or under-produce the polymerase, inhibitory domains or site-directed mutators of enzymes in cells exposed to environmental toxic agents to assess the effects on repair and cell survival. Accordingly, the use of the molecular techniques as well as the complete amino acid sequence of the enzyme, established during the past granting period, should allow us to learn considerably more about the mechanism and role of this enzyme in cells exposed to stressful environments.

An initial approach was developed to establish techniques to express, regions, orientation, site-directed and mutations of the polymerase cDNA in animal cells and confirm that these products are targeted to the nucleus.

In AIM I of the last three year project, we initially proposed to extend our preliminary data on the insertion of full-length polymerase cDNA into various inducible and non-inducible expression vectors and retroviral vectors in both sense and antisense orientations. This would allow us to either inhibit (i.e. through antisense mRNA expression) or intensify the translation of polymerase in a variety of eukaryotic cells. A complementary approach was proposed in AIM II where various functional domains of the polymerase as well as site-directed mutants (based upon sequence data obtained during the earlier granting period) were proposed to be constructed into inducible expression vectors to test whether selective inhibitors can be favorably used in cells.

Once it was verified that both the engineered mRNAs and appropriate peptides are expressed in vivo, procedures in AIM III we proposed to test for cytotoxicity and DNA repair potential and mutagenesis repair of the various reconstructed cells obtained in AIMS I AND II. It is anticipated that these studies will ultimately contribute new information on the mechanisms of the poly ADP-ribosylation modification and how cells recover from damage caused by specific environmental toxic agents such as polycyclic hydrocarbons, pesticides etc.
AIM I: Construction of poly(ADP-Rib) polymerase cDNA in sense and antisense orientations into expression vectors with inducible promoters.

A. Choice of vectors and cloning strategies.
B. Stable Transfection into various eukaryotic cells: Quantitation of mRNA and expressed proteins after induction.
C. Effects of induced constructions on endogenous polymerase synthesis.

AIM II: Expression in cells of poly(ADP-Rib) polymerase functional domain peptides and site-directed mutants.

A. Cloning strategies and biochemical verifications of expressed peptides driven by various inducible promoters.
B. Effects on cellular poly ADP-ribosylation and cell viability.

AIM III: Cytotoxicity and DNA repair studies with environmental toxic agents.

General Overview of the Interrelationships of the Three Aims:

As discussed above the program has had three interrelated aims. As indicated in the cartoon the first stage of the work involved the recombinant construction of polymerase cDNA into a family of selected expression vectors which in most cases will possess an inducible promoter. As indicated in I (above) the cDNA will be inserted in both sense and antisense orientations and also site-directed mutants. This was accomplished during the first and second years. In AIM II we will be concerned with construction (I-D) and expression of site-directed mutants and potential inhibitory peptides in vitro in order to eventually modulate the activity of poly(ADP-Rib) polymerase in cells, upon induction during DNA repair. As indicated in II, the various expression vectors were stably transfected into a variety of eukaryotic cells generally by co-transfection with a selectable gene. As indicated (II-A-C) we might expect various levels of overexpression and underexpression of poly(ADP-Rib) polymerase. In the case of the site-directed mutants and the inhibitory peptides (II D) we anticipate cells with reduced capacity for ADP-ribosylation. Biochemical and molecular biology characterizations (III, above) of the gene products of the various transfected cells are currently being used in cytotoxicity and DNA repair analysis. These have included: (III-A) Southern analysis to confirm integrated copies of the cDNA; (III B) both Northern and primer extension analysis of cellular mRNA to confirm that upon induction actual expression of the foreign gene occurs; (III-C) immunoprecipitation of poly(ADP-Rib) polymerase in vivo after induction. Finally, using the well characterized cells obtained above a variety of cytotoxicity, mutagenicity DNA repair protocols (IV) were initiated to indicate the effects on recovery of cells from various DNA damaging as occasioned by environmental toxic agents when a requirement for ADP-ribosylation is encountered.
I VECTOR CONSTRUCTS

I) Endogenous levels B) 10-20 fold overexpression ("sense") and 3' truncated stable mRNA

II EXPRESSION IN CELLS

A) Endogenous levels B) 10-20 fold overexpression ("sense") and 3' truncated stable mRNA
C) Reduced levels ("antisense") D) Reduced activity due to expression of Domain Peptide or site-directed mutant

III BIOCHEMICAL CHARACTERIZATION OF TRANSFECTED CELLS

A) Southern analysis for presence of integrated cDNA copies B) Northern and primer extension for induction of polymerase activity C) 35S Met-MMPT of protein D) "Activity" of protein ± inducer

IV CYTOXICITY AND DNA REPAIR

Transfected cell + induction → DNA damage → Alkaline Unwinding for DNA damage
Transfected cell + induction → DNA damage
B. STATUS OF THE RESEARCH EFFORT

Progress in Year 1

PADPRP GENE EXPRESSION STUDIES-RELATIONSHIP TO FUNCTION

A. PADPRP TRANSCRIPTION IS NOT EFFECTED BY DNA BREAKS


The catalytic activity of PADPRP is totally dependent upon the presence of DNA strand breaks. Having isolated a full-length cDNA for the polymerase, we subsequently evaluated the effect of endogenously and exogenously induced DNA strand breaks on the transcriptional control of this enzyme.

During retinoic acid or dimethyl sulfoxide-induced differentiation of HL-60 human leukemia cells, which may involve DNA breaks as well as other changes in chromatin, mRNA levels for the polymerase increased very early and remained high for up to 48 after which it decreased to pre-induced levels.

Polymerase transcript levels did not change, however, during the induction of DNA strand breaks by dimethyl sulfate, a variety of other alkylating agents, X irradiation, or UV irradiation in several mammalian cell lines. It appears that in sharp contrast to the catalytic requirement of the polymerase, the induction of transcription of the polymerase gene may not be a strand break-dependent process.

A Control

<table>
<thead>
<tr>
<th>A</th>
<th>V-79 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2 3 4</td>
</tr>
<tr>
<td>2</td>
<td>1 2 3 4</td>
</tr>
<tr>
<td>3</td>
<td>1 2 4 8</td>
</tr>
<tr>
<td>4</td>
<td>1 2 4 8</td>
</tr>
</tbody>
</table>

G DMS

C Normal Human Fibroblasts

DNA STRAND BREAKS DO NOT INDUCE POLYMERASE GENE EXPRESSION

HYPER-EXPRESSION OF PADPRP DURING TRANSIENT TRANSFECTION: EFFECT ON DNA REPAIR RATES

Since PADPRP expression did not respond to DNA damage, we tested the effect of plasmid-induced increases in the transcripts.

The feasibility of genetically modulated PADPRP activity (ie. AIM I below) is illustrated by expression studies performed during the first year. The data below illustrates a DNA transient transfection of Cos cells utilizing pCD-12 and pCD-19, a partial cDNA for PADPRP, which is deleted by approximately 400 bases at the 5' region of the cDNA. After 48 hr the cells were assayed for PADPRP. The data indicates nearly a three-fold increase of specific activity.
for PADPRP over endogenous levels was present by the transient transfection. The same extracts analyzed by "activity gel" analysis or by immunoprecipitation with anti-PADPRP (B&C) indicates that there is a 10-15 fold increase in PADPRP enzyme mass in these cells.

We then examined DNA repair in these transfected cells. Cells were irradiated with 200rad (cGy) on ice and immediately allowed to repair at 37°C. In the data illustrated, we employed the alkaline elution method in collaboration with the laboratory of Kurt Kohn (NIH). The differences in the amount of SSB-released during a 15 min repair period in cells hyperexpressing polymerase were compared to control treatments. The data suggests that the DNA repair initial rate in the transfected cells with the sense mRNA was nearly twice that of control cells (i.e. 766 break freq. versus 387). To confirm that the increased rate of induced DNA strand breaks was due to increased poly (ADP-ribosylatation) of Cos cells, the effects of the polymerase inhibitor 3-AB was also studied. The inhibitor reduced the increase in repair due to expressed polymerase.

<table>
<thead>
<tr>
<th>Cell Treatment</th>
<th>Time (hours)</th>
<th>DNA Break Frequency (Rad-Equivalents)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>1000</td>
</tr>
<tr>
<td>Mock-Transfected</td>
<td>15</td>
<td>766</td>
</tr>
<tr>
<td>pcD-12</td>
<td>0</td>
<td>2020</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>3000</td>
</tr>
</tbody>
</table>

**Table 2. Hyper-expression of poly(ADP-ribose) polymerase increases the rate of X-ray induced DNA break occurring in Cos cells.**

**EXPRESSION OF HUMAN PADPRP IN E. COLI AND IN YEAST-RELATIONSHIP TO FUNCTION**


Using the bacterial expression vector described in more detail below in AIM I, we have placed the native human PADPRP gene, fused to ubiquitin, in front of the lpl-promoter. By heat induction, we have demonstrated the expression of a catalytically active 113 Kda PADPRP enzyme fused to ubiquitin, as well as several polymerase deletion mutants which are catalytically inactive and will be used for the experiments described in AIM I. A typical induction is shown below:

As shown above by Coomassie stain, detectable amounts of either full-length Ub-polymerase or a polymerase deletion mutant, containing the NAD binding domain, are made within 1
minute of induction. By 40 mins. after induction, approximately 5-20% of the total protein is represented by PADPRP. Enzymatically the PADPRP is nearly indistinguishable from the native enzyme. This conclusion is based on the ADP-ribose acceptor pattern, the apparent Km for NAD and its activation by DNA strand breaks.

It should be noted that in the absence of the ubiquitin portion we were unable to synthesize significant amounts of PADPRP. However, the fusion protein with ubiquitin is catalytically active, made in copious amount and will therefore be quite useful for site-directed mutation studies which are outlined in greater detail in AIM I of this renewal application. A protease is available to specifically cleave the Ub-PADPRP junction.

**HUMAN POLY(ADP-RIbose) POLYMERASE IS FUNCTIONAL IN SCHIZOSACCHAROMYCES POMBE (MS IN PREP.)**

The full length cDNA for human PADPRP has been introduced into the yeast Schizosaccharomyces pombe under the transcriptional control of the SV40 early promoter. A number of haploid stably transformed strains which express the human RNAs constitutively have been generated. Phenotypically, these strains showed an absolute requirement, for normal growth and survival, on the addition of NAD+ precursors to supplement those in the culture media.

**Recent Progress of a Partial Functional Domain for PADPRP and its Effect on Catalytic Activity in Cells**

These experiments were performed during the first and second year and constitute a pilot research example of the types of experiments that will be done to measure modulated PADPR activity and toxic agents.

**Construction of pBS-1 plasmid.** A 1.88 kb fragment encompassing the whole DNA binding domain and part of automodification domain of human PADPRP cDNA was purified from pCD12, the plasmid containing the original full length clone for poly ADP-Rib polymerase. Such a fragment, extending from the Bam H1 site within the SV40 early region promoter to the Bam H1 site at the nucleotide position 1599 into the PADPRP coding region was subcloned in both sense and antisense orientations into the unique BgIII site of the expression vector p341-3. This vector allows the inducible expression under the the control of mouse metallothionein I promoter, and provides adequate signals for transcription and translation termination within the SV40 early polyadenylation region located immediately downstream from the BglII cloning site. Subclones were isolated by transformation into E.coli C600. Plasmid DNA was purified by double Cscl density gradient centrifugation.

**Isolation of mouse cell lines expressing the DNA binding domain of the human Poly(ADP-ribos)e polymerase.** NIH/3T3 fibroblast cells (5x10^5) were transfected with a DNA mixture of the recombinant expression vector having the inserted Bam H1 fragment in sense orientation and pSV2neo which carries the G418 selectable marker in molar ratio of 40:1. Control cells were transfected with p343-3 and the pSV2neo in the same molar ratio. Transfection was done by modified calcium phosphate precipitation method. Transfected cells were selected for G418 resistance 48 hours post-transfection. After two weeks of G418 selection, 20 individual clones were picked up and expanded into individual cell lines. The remaining colonies were trypsinized and expanded as pool cell line.

**Northern blot analysis of the human mRNA in NIH/3T3 cells.** In Northern analysis, total RNA from three representative clones (E3, E8 and E9) after electrophoresis was blotted onto Zeta probe membrane. The cells were grown in DMEM containing 10% dialyzed FCS supplemented with ITS. Induction of cells growing in log phase was performed for 16-18 h with zinc and
cadmium at 50 and 0.5 mM final concentrations respectively. A human PADPRP cDNA insert hybridized with approximately 2.6-kb mRNA (Figure 3) both in the presence (+) (lanes 2, 4, and 6) and absence (-) (lanes 1, 3, and 5) of induction. It appeared from these results that the mouse metallothionein I promoter of the expression vector is leaky under this growth condition of NIH/3T3 fibroblasts.

**Northern analysis showing the expression of the DNA binding domain**

- C - E3 - C - E3
- 28S' - 3.7 kb
- 18S'

**Poly(ADP-ribose)polymerase activity.** E3 and C cells were grown in conditioned medium and induced for 16-18 h and were made permeable to nucleotide by standard method. ADP-ribose incorporation was measured in acid-insoluble material by filter binding assay. We noted a significantly higher ADP-riboseylation in E3 cells compared with C cells (Table 1).

<table>
<thead>
<tr>
<th>Cells</th>
<th>Induction</th>
<th>Permeabilized Cells</th>
<th>Synchronized cell extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>181.52</td>
<td>20278.400</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>175.18</td>
<td>2064.617</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>254.67</td>
<td>2781.456</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>319.29</td>
<td>3373.540</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>95.54</td>
<td>3742.42</td>
</tr>
</tbody>
</table>

Similar activation was also noted with other clones compared with C cells (data not shown). However, when the permeabilized cells were preincubated with DNase I (200 mg/mL) in ice for 10 min the difference in the activities between E3 and C cells was almost abolished. In contrast, in an in vitro assay of ADP-riboseylation using sonicated cell extract we found no significant difference in activity between E3 and C cells (Table 1).

These experiments were unexpected in the sense that we anticipated that expression of this analogue truncated part of the enzyme would inhibit activity. However, the stimulation of activity is quite significant and was exploited in various strategies later in this project.

**Growth kinetics.** During the first year, we noted that the clones expressing the tail-truncated human PADPRP had quite reduced proliferative capacity compared with control or parental NIH/3T3 cells. Accordingly, we examined the growth rate of one representative clone (E3) as well as C cells. When density-arrested cells were diluted into fresh medium to allow reentry into the cell cycle E3 cells exhibited slower initial growth rate compared with the control cells.

**DNA synthesis in synchronized cells.** E3 and C cells were synchronized by double thymidine block according to the standard method and the cells were released to enter the S phase. The
rate of DNA synthesis was monitored by incubating the cells in triplicate on a 35-mm plate with 2 nCi of $^3$H-thymidine in 2 ml of medium for 1 h at 37°C and by measuring the incorporation of radioactivity in trichloroacetic acid-insoluble materials at 1 h intervals up to 9 h. It appears from the data that there is no significant difference in the length of the S phase between E3 and C cells. However, the total radioactive thymidine incorporation in E3 cells is remarkably lower than that in C cells. The cells were counted after release of thymidine block, and the data were normalized in terms of number of cells. We noted that the initial rate of $^3$H-thymidine incorporation is very similar if not same. Therefore, the difference in the total amount of thymidine uptake is due to the difference in the number of cells in the plate, although the experiment was started with the same number of cells in each plate. These data clearly indicated that E3 cells exhibit differential growth patterns in different stages of proliferation compared with C cells.

**Fluorescence activated cell sorter analysis.** During the first year we initiated the use of fluorescence activated cell sorter analyses to confirm the proliferative stage-specific differential growth patterns of the cells expressing the tail-truncated PADPRP. DNA per cell distribution. Histograms of cells, harvested from early and mid-log phases, stained with propidium iodide after fixation in ethanol and RNase digestion are shown below. Both sets of samples were analyzed under identical settings of laser power, electronic amplification and optics. The histograms of DNA from cells, harvested in early log phase reveal that the distributions of E3 cells in G2 phase is significantly higher than that of C cells, while the E3 cells harvested from mid exponential phase exhibited no difference in cell cycle distributions compared to the corresponding control cells.
We have developed a model system for molecular perturbation of this protein modification system by expressing a genetically engineered N-terminal partial human PADPRP cDNA in murine fibroblast cells during the first year. Such a tail-truncated cDNA under the control of mouse metallothionein I promoter encodes the whole DNA binding and a part of automodification domains. We rationalized that if this partial polypeptide, catalytically inactive, but capable of binding to DNA strand breaks, will interfere with the function of the endogenous enzyme in vivo, we might be able to perturb at the molecular levels the various functions of PADPRP.

Previously it has been shown by this laboratory that PADPRP mRNA accumulates during the late S phase in synchronized HeLa cells, suggesting a possible requirement of de novo PADPRP in late S and/or early G2 phases. However, it is unclear whether this role of PADPRP is catalytic or structural. Our observation indicates that NIH/3T3 cells expressing the DNA binding domain of human PADPRP grow at slower rate in early exponential phase compared with C cells or parental NIH/3T3. Detailed cell cycle analyses of these cells during this first year demonstrate that this is due to an arrest in G2 phase. This G2 arrest results from the possible interference with the normal function of endogenous PADPRP by the tail-truncated polypeptide. However, the transfectants are able to overcome this growth arrest in the latter phases of growth. One reason may be that the cells are able to reverse the G2 arrest by an activation of the endogenous enzyme, as we observed an increase in polymer synthesis in permeabilized cells, harvested in mid log phase. Or alternatively, the cells develop a compensating mechanism to restore the normal growth status. The expression of tail-truncated polypeptide may have important implications for future studies of the project on DNA repair of toxic agents.

**Progress in Year 2**

**Human PADPRP expression in murine cells.** In order to accomplish the aims of the grant, which essentially involves expression of regions of PADPRP or modulated sequences in eukaryotic cells, and in order to ascertain how this enzyme is involved in recovery of cells from toxic agents during the second year we began studies on the regulation of exogenous forms of PADPRP mRNA in cells. Accordingly, we first evaluated the regulation of expression of the PADPRP gene during growth and replication. We found that in a synchronized population of HeLa cells that were in serum-stimulated WI-38 cells, steady state levels of the polymerase mRNA were highest at late S and S-G2 phases and negligible in early S phase. We noted however that transcription did not solely account for the significant increase in the mRNA levels of PADPRP observed in late S phase by Northern analysis. We found that the stability of the PADPRP mRNA was dependent on the percent proliferating cells in the culture. Accordingly, we found that the polymerase mRNA from cells in early exponential phase was significantly more stable than from cells in stationary phase of asynchronous growth.

To clarify these observation, we utilized a novel heterologous expression system that involved murine NIH/3T3 cells transfected with human full-length PADPRP cDNA under the control of the non-cell cycle-specific promoter. **These types of studies will be important as prototypes for the expression of inhibitory domains of PADPRP.** The cells were synchronized, and a comparison was made of the endogenous (murine) and exogenous (human) polymerase mRNA levels. Both the endogenous and exogenous mRNA were specifically stabilized by the same mechanisms and only during late S phase; therefore, we concluded that mRNA pools for PADPRP are regulated at the post-transcriptional level. More importantly, the presence of extra copies (human) of the PADPRP gene transfected into cells did not provide an increased amount of the total PADPRP mRNA or protein and in fact the sum of the endogenous and exogenous mRNA in the transfected cells was noted to be approximately the same as the level of endogenous transcript in the control cells. This indicated that there might be a limit to the amount of PADPRP...
protein accumulating in the cellular pool and thus levels of PADPRP may be autoregulated. This data will be extremely important and future approaches directed at expressing for example the DNA binding region of the protein.

Establishment of Hela cells with stably integrated antisense PADPRP cDNA. To complement the approaches described above we established the use of antisense mRNA expression as a way to modulate poly ADP-ribosylation in cells, for this grant as well as several other funded projects concurrently underway in the laboratory. During the second year we successfully stably transfected the following recombinant plasmids into Hela S3 cells: pMX18 and pMX49 containing full length PADPRP cDNA inserted into the expression vector pMAMneo in either the sense or anti-sense orientations. This vector contains a glucocorticoid hormone inducible promoter (MMTV) and a selective marker neo gene. Ten clones, transfected with the anti-sense recombinant plasmid, and 3 clones transfected with sense recombinant plasmid were also isolated and characterized.

The clones which were identified as being able to express effectively either sense or antisense PADPRP cDNA after dexamethasone (DX) induction were further identified by a PADPRP enzyme activity assay. In the group of sense cDNA transfected transfectants only one clone was able to increase the cellular PADPRP activity (i.e. 43% after 48 hour induction). However, 5 clones transfected with the anti-sense cDNA demonstrated a 17% to 50% decrease of enzyme activity. The clone #7 (MX18-7), which showed the lowest enzyme activity (50% reduction) following DX induction was selected for further study.

Progress in Year 3

Northern analysis. These experiments were performed during year three to verify that induction of antisense mRNA to PADPRP reduced the endogenous levels of Hela cell mRNA for this DNA repair enzyme. Total RNA was isolated from MX18-7 cells using a variety of DX induction periods from 6 to 72 hours. Following electrophoresis and Northern transfer, the RNA was hybridized to a 32P labeled riboprobe representing a 1.7 kb fragment of the 3' end of the PADPRP cDNA. Since this RNA probe was synthesized in the sense orientation, it specifically hybridized only to the anti-sense orientation of PADPRP mRNA. Therefore it was used to study the expression of anti-sense cDNA in MX18-7 cells. Analysis of the data indicated: a) A 3.7 kb band existed in MX18-7 cells, but not in the control cells (Hela S3 transfected with vector alone) indicating the expression of intact anti-sense PADPRP mRNA. b) The expression anti-sense mRNA responded to the induction of DX. The highest expression was detected at 12 to 24 hours after DX induction. c) Significant reduction of the endogenous Hela cell PADPRP mRNA 3.7 kb band at 48 hours of induction and almost no band noted at 72 hours of DX induction strongly suggested the degradation of recombinant mRNA hybridized by sense and anti-sense mRNA, which may in turn block the translation of the sense mRNA and result in the lower enzyme activity in MX18-7 cells.

Western analysis. To test whether the reduction of PADPRP activity noted above was caused by the loss of PADPRP enzyme protein in MX18-7 cells after DX induction, immunological determination of PADPRP was performed during the third year. Whole cell protein extract was separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose paper and reacted with polyclonal anti-human PADPRP antibody. Western blot analysis demonstrated almost total reduction of the 116 kD PADPRP band in MX18-7 cells following 48 to 72 hour DX induction indicating significant loss of PADPRP protein content in these cells. Immunohistological staining data demonstrated the same result.

Expression of an active site mutant analog PADPRP in Hela cells. Other funded projects in the laboratory have been directed at developing site-directed mutations in the PADPRP gene in vectors which can be expressed in E. coli for large scale preparation of such proteins for study.
Some of these mutations will be also suitable for the studies in the current project and during year three one such mutation has been designed and the cells have been stably transfected with this plasmid. There is a convenient Clal site at the 3' (carboxyl termini) of the PADPRP human cDNA which when cut eliminates nucleotides coding for about 30 amino acids which encompasses the NAD-active site of the enzyme. When these base are removed the E. coli expressed enzyme is totally devoid of in vitro catalytic activity. We have cut with Clal and religated the original pCD12 plasmid which encodes the human PADPRP with the SV40 late promoter to simulate an analog of the E. coli situation noted above. This plasmid has been stably transfected into Hela cells along with the neomycin gene and during the next year we intend to characterize the effects of expression of this mutated PADPRP in cells exposed to environmental toxic agents.

Development of preferential gene repair assay. A more precise method to study DNA repair in the various cells engineered for modulated PADPRP activity as described above is required. During year three a preferential gene repair assay was initiated to explore the role of poly ADP-ribosylation in DNA repair caused by toxic compounds on specific transcriptionally active genes. This research has been performed in collaboration with Dr. Vilhelm A. Bohr of the nearby National Cancer Institute. Dr. Bohr, in collaboration with Dr. Philip Hanawalt has developed a technique to measure DNA repair after UV damage in defined genomic sequences. The efficiency of DNA repair is determined by measuring the frequency of pyrimidine dimers at different times after irradiation in genomic restriction fragments containing a DNA sequence of interest. In principle, this assay can be employed with DNA damage caused by any agent and any genomic sequence for which a DNA probe and detailed information concerning its restriction map are available. Dr. Bohr has recently redesigned this assay to study preferential gene repair in cells treated with various types of alkylating agents. For exploring the DNA repair process caused by toxic agents and the role of PADPRP in DNA repair, especially these active genes, after mammalian cells are exposed to lethal and sublethal doses of toxic agents, Hela cells with low PADPRP activity or high activity will be exposed during the next year to different doses of test agents and DNA will be isolated and digested with differing restriction enzymes. Digested DNA will be initially hybridized to probes to study the repair of the dihydrofolate reductase (DHFR) gene. During the third year we have initiated a dose response curve with an alkylating agent in Hela cells to test the feasibility of this method and we anticipate that this assay along with other more classical assays for DNA repair will be studied intensely during the future periods.

Effects of reduced PADPRP levels and overall DNA repair. During the end of the current funding period, alkaline elution studies were initiated to assess the effects of antisense to PADPRP in RNA on methyl methanesulfonate damage to engineered HeLa cells. In brief, the results of these recent experiments were the following. In order to determine whether poly-ADP-ribosylation participates in, or is rate-limiting in, DNA single-strand break (SSB) rejoicing, which might also suggest a role for this nuclear protein modification in replication and recombination, we first established the concentration range in which the alkylating agent, methyl methanesulfonate (MMS) caused sufficient DNA strand breaks to be detectable by the poly-ADP-ribosylation response in PADPRP-as[7] and control cells. It has been consistently observed that NAD is rapidly depleted as a result of the activation of poly-ADP-ribosylation by the introduction of DNA strand breaks in cells either by radiation, alkylating agents, or H2O2. Incubation of control cells with 1 mM MMS for 1 h caused a small depletion of NAD, whereas 5 mM MMS reduced the amount of NAD by greater than 90%. In agreement with the NAD data, a progressive increase in DNA strand breaks with increasing concentrations of MMS was observed as detected by the alkaline elution method. At 2 mM MMS, DNA damage representing 500-600 rad-equivalents was observed in both control and antisense cells. Despite a significant reduction in PADPRP content of the PADPRP-as[7] cells treated with dexamethasone, the alkaline elution curves after 5 h of repair were the same, showing approximately 95% DNA SSB repair for both cell lines and in the absence or presence of hormone. Accordingly, in subsequent experiments, repair capacity was analyzed at
relatively early repair periods (10 - 90 min). After 90 min, 90% of DNA repair was again complete; therefore, no difference in the extent of SSB repair was apparent in PADPRP-as[7] cells, whether induced or uninduced. Thus, minute nuclear amounts of PADPRP seem to be sufficient to allow SSB repair. However, significant differences were noted in the capacity for SSB rejoining at the early time point. On the basis of these results, the initial repair period was examined more closely. In control cells, the SSB rejoining at 10, 20, 30 and 45 min was the same in the absence and presence of dexamethasone; rad-equivalents of SSB decreased from approximately 50 rad to 330, 210, 110 and 90 rads, respectively, representing 34%, 58%, 78% and 82% of SSB rejoining. Similarly, for the PADPRP-as[7] cell line, in the absence of dexamethasone the rejoining rate displayed the approximate same pattern. In contrast, the induced PADPRP-as[7] cells showed a significant reduction in the SSB repair rate. After 10 and 20 min, about 460 rad-equivalents SSB remained, equal to the initial damage, indicating that no SSB rejoining had occurred during this time period. Even after 45 min of post-damage incubation, 330 rad-equivalents of breaks remained in the antisense cells, representing only 25% of SSB rejoining. However, repair resumed very rapidly from 45 to 90 min.

The most interesting of our results is the observation that PADPRP-depleted cells performed very limited DNA repair during early time periods of recovery from MMS-induced DNA strand breaking. Repair of the damage induced by this type of alkylating agent is accompanied by a stimulation of poly(ADP-ribose) synthesis that is induced by strand breakage. PADPRP activity has been shown to increase in cells treated with agents—such as MMS, methylnitrosourea, ionizing radiation, endonucleases, and numerous DNA-alkylating drugs—that induce SSBs.

The establishment of the PADPRP-as[7] cell line has provided a new experimental system for elucidating the role of PADPRP and its mechanism of action in chromatin repair, replication, and recombination reactions, and in transcriptional activation involving DNA strand breaks. This information will be further studied during the subsequent three year renewal period.
C. CUMULATIVE CHRONOLOGICAL LIST OF WRITTEN PUBLICATIONS IN TECHNICAL JOURNALS

Publications


Manuscript Planned for Submission

### D. LIST OF PROFESSIONAL PERSONNEL

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E. INTERACTIONS (COUPLING ACTIVITIES)

Recent Invited Symposium Chapters and Lectures


