

Report for AFOSR-89-0053

The Key Involvement of Poly (ADP-Ribosylation) in Defense Against Toxic Agents in
Molecular Biology Studies

Mark E. Smulson, Ph.D.
Georgetown University School of Medicine
Department of Biochemistry and Molecular Biology
Washington D.C. 20007

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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-Rib) 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 application that 38 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. This data is presented in the next page. 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 will permit 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 propose to over-or under-produce the polymerase, inhibitory domains or site-directed mutations 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 which have been 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 has been during the last year to carefully 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**, 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 will 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) will be constructed into inducible expression vectors to test whether selective inhibitors can be favorably used in cells.

Once it is verified that both the engineered mRNA's and appropriate peptides are expressed *in vivo*, procedures in **AIM III** will 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 a polycyclic aromatic hydrocarbon, 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.

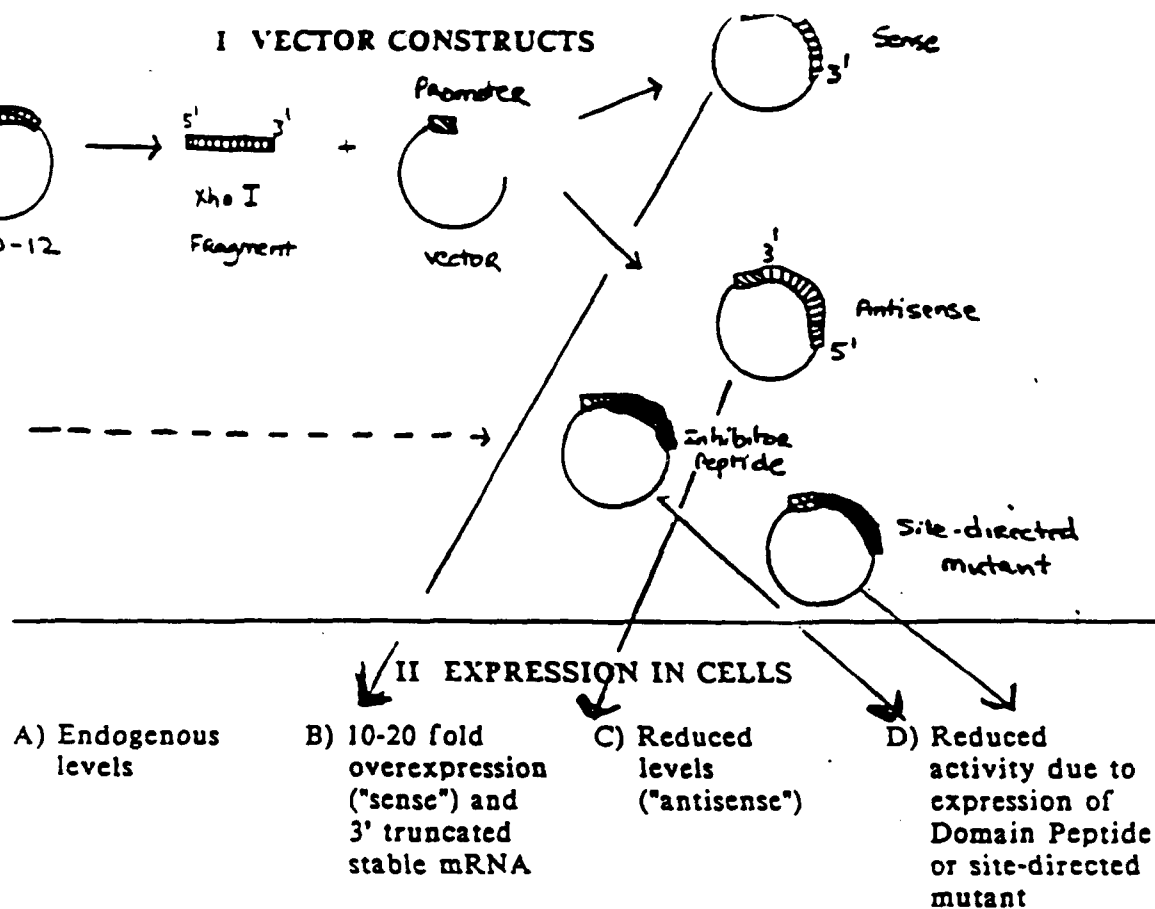
AIM III: Cytotoxicity and DNA repair studies with environmental toxic agents.

GENERAL OVERVIEW OF THE INTERRELATIONSHIPS OF THE THREE AIMS:

As discussed above the program has three interrelated aims. As indicated in the cartoon the first stage of the work involves 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 has been 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 vivo* 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 will be 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 proposed prior to cytotoxicity or DNA repair analysis. These will include: (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) will be 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.



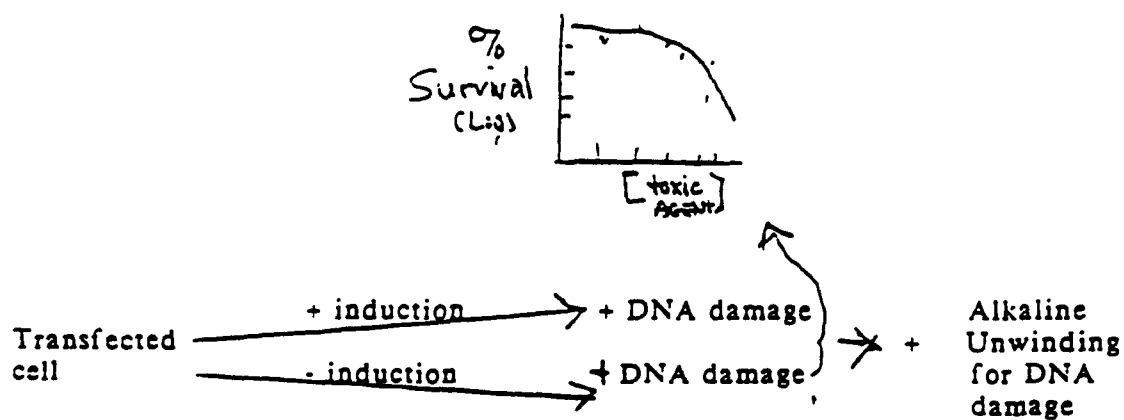
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III BIOCHEMICAL CHARACTERIZATION OF TRANSFECTED CELLS

- A) Southern analysis for presence of integrated cDNA copies
- B) Northern and primer extension $\pm$ induction
- C) ^{35}S Met-IMMPT of protein
- D) "Activity" of Polymerase $\pm$ inducer

IV CYTOTOXICITY AND DNA REPAIR



B. STATUS OF THE RESEARCH EFFORT (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 last 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 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 such as described in the previous technical report.* 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 as described in the previous year's technical report. *The information obtained during the current year will aid in the ability to genetically modulate by gene engineering the amount of this protein in cells.*

Progress on the expression of a partial functional domain for PADPRP and its effect on catalytic activity in cells. In the previous technical report we discussed studies involving the expression of the DNA binding domain of PADPRP (i.e. the *Bam*HI fragment) under control of the mouse metallothionein promoter. The human DNA binding region with this promoter was transfected into mouse 3T3 cells and initial results reported at that time suggested that: 1). the mouse metallothionein promoter was quite leaky in these transfected cells and hence the transcription of the analog PADPRP occurred in an uncontrolled fashion and caused cells to grow very slowly and 2). expression of this analog DNA binding domain appeared to enhance the exogenous activity for poly ADP-ribosylation, as assessed in a permeabilized cell assay. However, during the recent year several complications have arisen with these same transfected cell lines. For example, the catalytic activity for PADPRP now appears to possibly be inhibited rather than stimulated by the constitutive expression of the analog fragment of PADPRP. To alleviate these problems the DNA binding domain *Bam*HI fragment of PADPRP has been cloned into an alternative expression vector which contains the sheep metallothionein promoter. This promoter has been reported to be much more stringently regulated in cells. We have transfected this cloned vector stably into Hela cells rather than mouse 3T3 cells because of the better growth properties of these cells in tissue culture; several G418 resistant clones have been isolated and they are currently being characterized for integration of the expected DNA binding domain. *During the next year we intend to utilize these cells to clarify whether such an approach can be used to inhibit or stimulate ADP-ribosylation in cells.*

Establishment of Hela cells with stably integrated antisense PADPRP cDNA. To complement the approaches described above we have also attempted to use 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 last year we

have 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.

Northern Analysis. These experiments were performed during the last year 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 ^{32}P 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 last 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 the past year 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 terminus) 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 the last year a preferential gene repair assay has been utilized 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 last 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.

C. CUMULATIVE CHRONOLOGICAL LIST OF WRITTEN PUBLICATIONS AND TECHNICAL JOURNALS

PUBLICATIONS

Thraves, P.J., Kasid, U., and **Smulson, M.E.**; Selective Isolation of Domains of Chromatin Proximal to both Carcinogen-Induced DNA Damage and Poly(Adenosine-Diphosphate-Ribosylation), Cancer Research, 45, 386-391 (1985).

Kasid, U.N., Hough, C., Thraves, P., Dritschilo, A., and **Smulson, M.E.**; The Association of Human c-Ha-ras Sequences with Chromatin and Nuclear Proteins, Biochem., Biophys. Res., Commun., 128, 226-232 (1985).

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S. Prasad, P. Thraves, Y. Kanaï, **M. Smulson** and A. Dritschilo; A Dot-Blot Method for Screening Polyclonal and Monoclonal Antisera to Poly(ADP-ribosyl), J. Immunol. Methods, 116, 79-85 (1989).

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Bhatia, K.G., Pommier, Y., Giri, C., Fornace, A.J., Imaizumi, M., Breitman, T., Cherney, B.W., **Smulson, M.E.**; Expression of the poly(ADP-ribose) polymerase gene following natural and induced DNA strand breakage and effect of hyperexpression on DNA repair, Carcinogenesis, 11, 123-128 (1990).

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Bhatia, K., Kang, V., Stein, G.S., Bustin, M., Cherney, B.W., Notario, V., Haque, S., Huppi, K., and **Smulson, M.E.**; Cell cycle regulation of an exogenous human poly(ADP-ribose) polymerase cDNA introduced into murine cells. J. Cellular Physiology, 144, 345-353 (1990).

D. LIST OF PROFESSIONAL PERSONNEL

Mark E. Smulson, Ph.D.
Veronica Kang, M.S. (50%)

Ruchuang Ding, M.D. (50%)
Noelle Lewis (20%)

Barry Cherney, Ph.D. (20%)

E. INTERACTIONS (COUPLING ACTIVITIES)

RECENT INVITED SYMPOSIUM CHAPTERS AND LECTURES

Smulson, M.E.: "The Poly (ADP-ribose) polymerase gene: Direct or indirect Involvement in DNA Repair and Malignancy?" Fifth International Conference on Environmental Mutagens, Cleveland, OH, July 10-14 (1989).

Smulson, M.E.: "Alterations in the Poly(ADP-ribose) polymerase gene(s) with respect to DNA Repair and Carcinogenesis" Gordon Research Conference on Mammalian DNA Repair, January 23-28 (1989) Oxnard, CA.

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