STUDIES ON POLY(ADP-RIBOSE) METABOLISM AND CHROMATIN STRUCTURE

DISSERTATION

Presented to the Graduate Council of the
University of North Texas in Partial
Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Maria E. Cardenas-Corona, B.S., M.S.
Denton, Texas

August, 1988

Cardenas-Corona, Maria Elena, Studies on Poly(ADP-ribose) Metabolism and Chromatin Structure. Doctor of Philosophy (Biochemistry), August, 1988, 163 pp, 11 tables, 24 figures, bibliography, 175 titles. MKJ

Poly(ADP-ribose) metabolism is involved in several chromatin-associated processes. However, the nuclear distribution of endogenous ADP-ribose polymers in the nucleus is unknown.

In these studies, a procedure which allowed the *in vivo* labeling and detection of poly(ADP-ribose) was combined with nuclear fractionation techniques to analyze the nuclear distribution of ADP-ribose polymers. These analyses were conducted in untreated cells and in cells subjected to hyperthermia, N-methyl-N'-nitro-N-nitrosoguanidine, or both treatments. When isolated nuclei from cells subjected to any of these conditions were digested with micrococcal nuclease such that 80% of the DNA was released, 90% of the total poly(ADP-ribose) remained with the nuclease-resistant chromatin.

Nuclear matrix fractions were prepared from isolated nuclei by DNase I digestion and three different salt extraction procedures (2 M NaCl, 0.3 M $(NH_4)_2SO_4$ or 25 mM lithium diiodosalicylate). These matrix fractions

contained about 1% of the total DNA but 50 to 70% of the total poly(ADP-ribose). The nuclear matrix-associated poly(ADP-ribose) represent short polymers, while the polymers extracted with salt contain short and complex chains. Independent lines of evidence suggest that the association of ADP-ribose polymers with the nuclear matrix is covalent.

Probing of nuclear matrices with poly(ADP-ribose) glycohydrolase resulted in the release of a specific subset of polypeptides.

The results from these studies suggest the occurrence of poly(ADP-ribose) metabolism in two compartments of chromatin; one that is nuclear matrix-associated and one that is not. The biological significance of this compartmentalization is conceptualized in a model. This model postulates that, under some physiological conditions, poly(ADP-ribose) metabolism accomplishes the reversible targeting of specific regions of chromatin to the nuclear matrix domain by modulating DNA-protein and or protein-protein interactions.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

NAD^+	- nicotinamide adenine dinucleotide
NMN	- nicotinamide mononucleotide
AMP	- adenosine monophosphate
ADP-ribose	- adenosine diphosphate ribose
PR-AMP	- 2'(5''-phosphoribosyl)5'AMP
DNase	- deoxyribonuclease
TFIIC	- transcription factor IIC
MMTV	- mouse mammary tumor virus
MAR	- matrix associated regions
SAR	- scaffolding associated regions
RNase	- ribonuclease
IgG	- immunoglobulin G
SVPD	- snake venom phosphodiesterase
BAP	- bacterial alkaline phosphatase
MNase	- micrococcal nuclease
Tris	- Tris (hydroxymethyl)aminomethane
HEPES	- N-2-hydroxyethylpiperazine-N'-2-ethanosulfonic acid
MOPS	- 3(N-morpholino)propanosulfonic acid
PIPES	- piperazine-N'-bis(2-ethane-sulfonic acid)
SDS	- sodium dodecyl sulfate

LIS	- lithium diiodosalicylate
spermine	- N,N'-bis(3-aminopropyl)-1,4 butanediamine
spermidine	- N-(3-aminopropyl)-1,4-butanediamine
MNNG	- N-methyl-N'-nitro-N-nitrosoguanidine
BSA	- bovine serum albumin
PMSF	- phenylmethylsulfonyl fluoride
ethacridine	- ethoxyacridine lactate monohydrate
DAPI	- diaminophenylindole
polyA	- homopolymer of adenylic acid
$[(Ap)_{11}A]$	- dodecamer of adenylic acid
$[(Ap)_{7}A]$	- octamer of adenylic acid
$[(Ap)_{4}A]$	- pentamer of adenylic acid
TEMED	- N,N,N',N'-tetramethylendiamine
BCIP	- 5-bromo-chloro-3-indolyl phosphate
NBT	- nitro blue tetrazolium
PBS	- phosphate-buffered saline
NIB	- nuclei isolation buffer
SDS-PAGE gel	- sodium dodecyl sulfate-polyacrylamide electrophoresis
NP-40	- Nonidet P-40
NDB	- nuclei digestion buffer
EDTA	- ethylendiamino-tetracetic acid
TBST	- Tris-buffered saline Tween-20
TCA	- trichloroacetic acid
snRNP	- small nuclear ribonucleoprotein particle
snRNA	- small nuclear RNA

LIST OF TABLES

LIST OF ILLUSTRATIONS

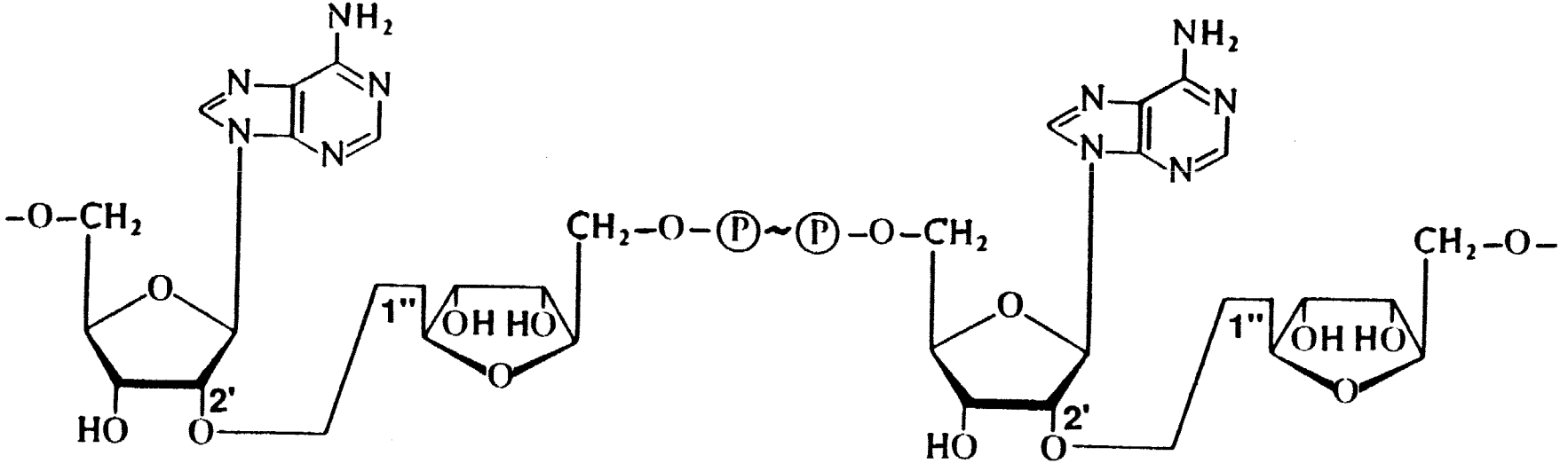
Figure Page

CHAPTER I

INTRODUCTION

The biosynthesis of nicotinamide adenine dinucleotide (NAD^+) from nicotinamide in eukaryotes involves a simple two step pathway. In the first step, nicotinamide condenses with phosphoribosyl pyrophosphate to yield nicotinamide mononucleotide (NMN). This reaction is catalyzed by a soluble cytoplasmic enzyme, nicotinamide phosphoribosyl transferase (1). The nicotinamide mononucleotide must diffuse into the nucleus for the next step, the condensation of the nicotinamide mononucleotide with ATP, to give NAD^+ (2). This reaction is catalyzed by the enzyme NMN adenylyl transferase (NAD^+ pyrophosphorylase), which is exclusively located in the nucleus. Such a localization is difficult to understand in terms of the function of NAD^+ as a coenzyme in many biological oxidation-reduction reactions that occur in the cytoplasm. However, this dinucleotide also participates in other important biological processes for which it is structurally well suited. The NAD^+ molecule contains two energy-rich bonds, a pyrophosphate bond and a glycosylic linkage at the nitrogen of the pyridine ring (3) (Fig.1). In the presence of the appropriate catalysts, the energy-rich

Fig.1. **The chemical structure of NAD+ and group transfer reactions.**


NH2
N
N
N
N
-O-CH2
O
HO
2'
O
1"
OH HO
O
CH2-O-Ⓟ~Ⓟ-O-CH2
NH2
N
N
N
N
O
HO
2'
O
1"
OH HO
O
CH2-O-

bonds allow transfer reactions at both positions (Fig.1). An example of a transfer reaction of type I is the reaction of NAD^+ with *Escherichia coli* polynucleotide ligase, which involves the transfer of AMP to the enzyme with the concomitant release of NMN. This reaction provides energy required during the synthesis of phosphodiester bonds between the 5'-phosphoryl and 3'-hydroxyl termini of "nicked" double-stranded DNA chains (4, 5). The degradation of NAD^+ also takes place at the N-glycosylic linkage (reactions of type II). These reactions involve the transfer of adenosine diphosphate-ribose (ADP-ribose) to suitable acceptors and the release of nicotinamide and H^+. There are two different general classes of enzymes that catalyze the transfer reactions of type II. One of these classes is the NAD^+ glycohydrolases which transfer the ADP-ribose moiety to water. These enzymes also catalyze an exchange reaction between nicotinamide analogs like 3-acetylpyridine and the nicotinamide moiety of NAD^+, leading to the formation of the corresponding NAD^+ analogs (3). In the second class, ADP-ribosyl transferases catalyze the post-translational modification of proteins with ADP-ribose. Among the ADP-ribosyl transferases, two subclasses have been defined: the mono(ADP-ribosyl) transferases and poly(ADP-ribose) polymerase. The mono(ADP-ribose) transferase reactions have been shown to occur in both prokaryotes and eukaryotes, while

poly(ADP-ribosyl)ation seems to be restricted to the nucleus of eukaryotic organisms. Since the present study deals with poly(ADP-ribosyl)ation, the metabolism of polymers of ADP-ribose and the effects of this metabolism on some cellular functions will be reviewed.

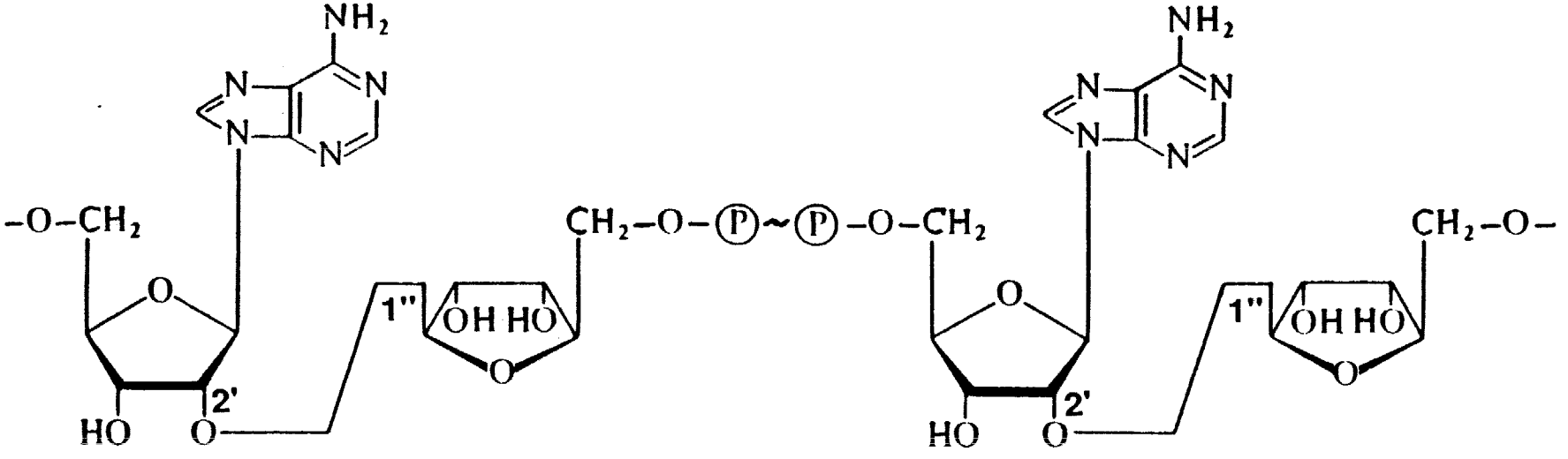
Poly(ADP-ribose) Metabolism

In 1963, Chambon *et al.* (6), made the observation that incorporation of radioactivity from [^{14}C]adenine ATP into acid-insoluble material in a nuclear preparation from hen liver was enhanced 1000-fold by the presence of NMN. In 1965, the same observation was made using a nuclear preparation from rat liver (7). Since the acid insoluble product was resistant to hydrolysis in 0.5 N NaOH at 37°C for 18 h, and no production of 5'AMP was observed upon treatment with snake venom phosphodiesterase, it was concluded that the acid insoluble material was not polyadenylic acid (7, 8). It was shown by several laboratories that NAD^+ was the true substrate for the nuclear enzyme and that the product could be cleaved by snake venom phosphodiesterase to a nucleotide containing two ribose and two phosphate residues per adenine. This nucleotide was later found to be 2'-(5"-phosphoribosyl)-5'AMP (PR-AMP) (9-11).

The elucidation of the structure of PR-AMP and other evidence indicated that the acid-insoluble product was a homopolymer of ADP-ribose units linked together by ribose (1"-2') ribose-glycosidic linkages (Fig.2) (9, 11-14). In addition, branching was observed in large ADP-ribose polymers synthesized in calf thymus nuclei (15) and in cultured cells (16). The structure of the branching portion was identified to be ribose (1'''- 2'') ribose (1''-2') (15-17). Up to 6 branching points have been identified in purified polymers of an average size of 244 residues (18).

Since its discovery, the natural occurrence of poly(ADP-ribosyl)ation activity has been shown in many different species, tissues, and cell types ranging from mammals to plants, fungi, slime molds and algae (for review see 19). It has been consistently shown that the cellular distribution of this activity is confined to the nucleus. Furthermore, a tight association with chromatin was established by Ueda *et al.* (for review see 20), and this was later confirmed by other investigators. This observation led to the definition of poly(ADP-ribose) polymerase as a chromatin-bound enzyme. This enzyme has been purified and characterized from many different sources (for review see 19) and has been shown to contain three functional domains. Following limited proteolysis of the pure enzyme, a 54 kDa fragment was characterized as the NAD^+-binding domain, a 46 kDa

Fig.2. **Structure of poly(ADP-ribose) indicating the (1"- 2')ribose-glycosidic linkages.**


NH2
N
N
N
N
-O-CH2
O
1"
OH HO
O
HO
2'
O
CH2-O-Ⓟ~Ⓟ-O-CH2
NH2
N
N
N
N
O
1"
OH HO
O
HO
2'
O
CH2-O-

polypeptide was identified as the DNA-binding domain and a 22 kDa fragment was shown to be the site of automodification (21-23). The activity of the enzyme is absolutely dependent on DNA strand breaks (24) and it is stimulated by Mg^{2+} and, to a lesser extent, by polycations such as histones and polyamines (25). Recently, the cloning and expression of the cDNA for human poly(ADP-ribose) polymerase has been achieved by two groups (26, 27). Independently, Kurosaki and coworkers have reported the primary structure for this enzyme as deduced from the cDNA sequence (28). These investigators have made the interesting observation that the DNA-binding domain of the enzyme exhibits a sequence homology with localized regions of transforming proteins such as c-fos and v-fos. Furthermore, this domain contains a unique sequence element which resembles the essential peptide for nuclear localization of SV40 and polyoma virus large T antigens. In view of these facts, the authors have speculated about the possibility that the physiological function of poly(ADP-ribose) polymerase depends on its ability to bind DNA and thus control cell transformation, as exemplified by these oncogene products (28).

There are four main reactions attributable to poly(ADP-ribose) polymerase: 1) The initiation reaction, which is the covalent attachment of the ADP-ribosyl moiety from NAD^+ to an acceptor protein; 2) the elongation reaction, whereby further

ADP-ribose moieties are attached to the protein-bound ADP-ribosyl residue; 3) the branching reaction, i.e., the introduction of an ADP-ribose residue which branches off from a linear portion of the polymer; and 4) the NAD^+ase activity, which catalyzes the cleavage of NAD^+ into nicotinamide and ADP-ribose.

Inhibitors of poly (ADP-ribose) polymerase have become extremely important tools in the attempt to elucidate the biological functions of poly(ADP-ribosyl)ation reactions. These inhibitors include nicotinamide, benzamide, and its substituted analogs. An important question in studies involving these inhibitors is whether any changes observed in cellular physiology can be ascribed to suppression of poly(ADP-ribose) synthesis or to side effects of the inhibitor. A series of reports have been focused on the potential metabolic side effects of these inhibitors. Inhibitory effects of high levels of 3-aminobenzamide on *de novo* purine and pyrimidine biosynthesis have been reported by Cleaver *et al.* (29, 30), but were not confirmed by others (31-33). Furthermore, 3-aminobenzamide inhibits deoxyglucose uptake and nicotinamide methylation in cultured cells at concentrations much higher than those normally used to inhibit poly(ADP-ribosyl)ation (34, 35). Thus, in view of these observations and the lack of poly(ADP-ribosyl)ation-deficient mutants, these inhibitors remain at present the

best tools for the study of the biological functions of poly(ADP-ribosyl)ation.

In eukaryotic cells, three enzymes are known to catalyze catabolic reactions of poly(ADP-ribose). The first enzyme, poly(ADP-ribose) glycohydrolase, catalyzes the cleaveage of ribose-ribose linkage in an exoglycosidic mode in both linear and branched polymer chains. The second enzyme, a phosphodiesterase with pyrophosphatase activity, cleaves the pyrophosphate bond of poly(ADP-ribose) in an exonucleolytic fashion. The protein proximal ADP-ribose residue is cleaved by the enzyme (ADP-ribosyl)protein lyase.

Poly(ADP-ribose) glycohydrolase was discovered by Miwa and Sugimura in 1971 (36), and it has been extensively purified (37, 38). The Km of the rat liver phosphodiesterase is 28 μM whereas the Km of poly(ADP-ribose) glycohydrolase is below 2 μM, which is similar to the estimated concentration of constitutive poly(ADP-ribose) in the cell. Hence, poly(ADP-ribose) glycohydrolase seems to be the most important activity in the catabolism of this polymer *in vivo*. The (ADP-ribosyl)protein lyase was discovered and partially purified by Hayaishi *et al.* (39). The physiological role of this enzyme is also being studied in human cells which appear to lack of this activity (40).

The physiological regulation of poly(ADP-ribose) catabolism has not been characterized. However, the

observation that the half-life of polymer residues synthesized as a result of carcinogen treatment of cells is approximately 2 min (41) and that the half-lives of constitutive polymer residues, which are degraded in a biphasic way, are 5 min and 7.75 h (42), suggests that the chain length, polymer concentration and branching complexity are likely to affect this regulation. Recently Jonsson *et al.* (43) made the observation that ADP-ribose polymers accumulated when cultured cells are treated with hyperthermia were degraded with a half-life of 10 min. From their studies, these authors concluded that this alteration in the catabolism of poly(ADP-ribose) involves regulation of poly (ADP-ribose) glycohydrolase activity (43).

Protein Acceptors for Poly(ADP-ribose).

Poly(ADP-ribosyl)ation of nuclear proteins has been observed in reconstituted systems *in vitro*, in intact cells, in nucleotide-permeable cells, in isolated nuclei and in polynucleosomal preparations. In many cases, the same acceptor proteins have been observed regardless of the system used. Table I shows the protein acceptors identified in intact cells or tissues under various physiological conditions. Consistently, poly(ADP-ribose) polymerase and the histones H1, H2A and H2B have been identified as major acceptors (For review see reference 44). Among other

TABLE I

Acceptor proteins for poly(ADP-ribose) identified in intact cells or tissues under various physiological conditions

Protein	Tissue or cell line
Poly(ADP-ribose) polymerase	HeLa cells Yoshida hepatoma AH7974 cells Mouse fibroblasts C3H10T1/2
Topoisomerase I	Yoshida hepatoma AH7974 cells
Histone H1	Rat liver HeLa cells Yoshida hepatoma AH7974 cells Mouse fibroblats C3H10T1/2
Histone H2A	Rat liver Ehrlich ascites tumor cells
Histone H2B	Ehrlich ascites tumor cells Rat liver Yoshida hepatoma AH7974 cells Mouse fibroblasts C3H10T1/2
Histone H3	Rat liver Yoshida hepatoma AH7974 cells
Histone H3d	Mouse fibroblasts C3H10T1/2
Histone H4	Ehrlich ascites tumor cells Yoshida hepatoma AH7974 cells
Protein A24	Mouse fibroblasts C3H10T1/2
High mobility group proteins	Mouse testis
High mobility group proteins 1, 14 and 17	Mouse mammary tumor cells HeLa cells
Low mobility group proteins	Mouse testis
Nuclear lamins	HeLa cells

acceptors that have been observed *in vivo* are the histones H3, H3d, H4 and some of the low and high mobility group non-histone proteins (44). At present, the effect of poly(ADP-ribosyl)ation on the physiological activity of these proteins is unknown.

It is interesting to note that a number of DNA metabolic enzymes such as topoisomerase I (45), topoisomerase II (46), DNA polymerase α (47), DNA polymerase β (48), terminal deoxynucleotidyl transferase (47), DNA ligase I (47) and Ca^{2+}, Mg^{2+}-dependent endonucleases (48) are substrates of poly(ADP-ribose) polymerase *in vitro*. In all these cases, poly(ADP-ribosyl)ation of these enzymes results in the inhibition of their activity.

A unique group of proteins, represented by nuclear matrix proteins, has recently been identified as acceptors of poly(ADP-ribose) *in vitro* (49, 50) and *in vivo* (51). These studies have identified the nuclear lamins as the major acceptors among the nuclear matrix proteins. In addition, a protein recovered with the nuclear matrix with the electrophoretic mobility of poly(ADP-ribose) polymerase was shown to be poly(ADP-ribosyl)ated.

Poly(ADP-ribosyl)ation, DNA Synthesis and Cell Cycle Progression.

Earlier experiments have indicated a correlation between the proliferative activity of tissues and cells and the activity of poly(ADP-ribose) polymerase *in vitro*. However, numerous reports have also indicated that this is not always the case (for review see reference 52). In more recent and careful studies by Adolph and Song (51), cell cycle specific changes in the pattern of poly(ADP-ribosyl)ated proteins have been found. By using [^{3}H]adenosine and [^{32}P]NAD as *in vivo* and *in vitro* radioactive labels, respectively, it was observed that the highest level of modification took place during the S phase of the cell cycle (51). A period of minimal modification was detected early in G1 phase (51). The predominant acceptors during interphase (S and G1 phases) were histones H2A, H2B and H1. For metaphase chromosomes a major acceptor was observed, which appears to be poly(ADP-ribose) polymerase (53, 54). For both isotope labels, the lamins, prominent proteins of the nuclear matrix, were identified as major acceptors of poly (ADP-ribose) (51). At present, the structural or functional significance of this cell cycle dependent poly(ADP-ribosyl)ation remains to be determined.

A direct effect of poly(ADP-ribosyl)ation on DNA replication has been suggested by Lonn and Lonn (55). In addition to the 100 and 200 nucleotide intermediates (Okazaki fragments) formed during DNA replication, they have described the occurrence of 10 kilobase (kb) intermediates formed by ligation of Okazaki fragments (56). These authors demonstrated a large accumulation of 10 kb intermediates in cells treated with 3-aminobenzamide, which suggests that poly(ADP-ribosyl)ation is necessary for the ligation of these intermediates (55). Furthermore, upon removal of 3-aminobenzamide, ligation of the 10 kb intermediates was resumed (56). Therefore, it was concluded that poly(ADP-ribosyl)ation of chromatin may be responsible for inducing chromatin conformational changes necessary to facilitate ligation of these intermediates.

Recovery of Cells from DNA Damage.

As early as 1955, numerous reports established that alkylating agents and ionizing radiations lower intracellular NAD^+ levels. In 1975, studies with isolated nuclei and nucleotide-permeable cells demonstrated that NAD^+ depletion caused by treatment of cells with DNA damaging agents was due to an enhanced utilization of NAD^+ as a substrate for poly(ADP-ribose) biosynthesis (57, 58). In 1979 Juarez-

Salinas *et al.* reached the same conclusion using intact cells (59). In addition these authors ruled out the possibility of NAD^+ depletion being due to its degradation by an NAD^+ glycohydrolase (60). It was later shown that the extent of stimulation of poly(ADP-ribose) biosynthesis, following DNA alkylation damage, did not correlate with the number of alkylated sites in the DNA (61). A series of experiments that involved digestion of isolated nuclei with DNase I (62), and in vitro assays with purified poly(ADP-ribose) polymerase and DNA fragments (24) clearly demonstrated that DNA strand breaks were responsible for the activation of poly(ADP-ribose) polymerase.

A possible role for poly(ADP-ribosyl)ation in DNA repair was indicated by studies of Shall and coworkers. This group observed that DNA strand breaks produced by treatment of cells with the DNA alkylating agent dimethyl sulfate were ligated within 80 min (63). In contrast, upon exposure of cells to 3-aminobenzamide following treatment with dimethyl sulfate, DNA strand breaks persisted for up to 5 h. In addition, it was observed that 3-aminobenzamide potentiated the cytotoxicity of dimethyl sulfate (63). From these observations, the authors concluded that poly(ADP-ribosyl)ation plays a role in the ligation of DNA strand breaks. Similar results were obtained by Wielkens *et al.* (64) and Cleaver *et al.* (65). However, they concluded from

their studies that the accumulation of DNA strand breaks in the presence of 3-aminobenzamide was due to an increased rate of incision rather than to a decreased rate of ligation. Additional support of an involvement of poly(ADP-ribosyl)ation on DNA incision also comes from studies of the regulation of a Ca^{2+}, Mg^{2+}-dependent endonuclease by poly(ADP-ribosyl)ation (48).

An alternative line of evidence to explain the cytotoxic effects of poly(ADP-ribosyl)ation inhibitors, aside from direct effects on repair replication, was provided by Jacobson *et al* (66) and Boorstein and Pardee (67). Independently, both groups reported that in dividing cells, following treatment with a DNA alkylating agent, the presence of 3-aminobenzamide resulted in an lengthened S phase and accumulation of cells in G2 with a delayed entry into mitosis. The conclusion that can be drawn from these experiments is that, following DNA damage, poly(ADP-ribosyl)ation reactions are required for normal DNA replication and cell cycle progression.

The reciprocal exchange of DNA between homologous regions of sister chromatids, termed sister chromatid exchange, involves recombinational events. Therefore, as for DNA-excision repair, DNA strand breakage and ligation mechanisms should be required. This raised the question of a possible relationship between sister chromatid exchange

events and poly(ADP-ribosyl)ation. The answer was provided by studies in which an increase in sister chromatid exchange events in the presence of poly ADP-ribosylation inhibitors was observed (68). However, a definitive mechanistic explanation for these results is not available at this time. The most likely explanation has come from Ferro and Olivera, who have shown inhibition of topoisomerase I activity by poly(ADP-ribosyl)ation *in vitro* (45). Since this enzyme is able to catalyze both intra and intermolecular strand exchange reactions (69), they suggested that the increase in sister chromatid exchange frequencies elicited by poly(ADP-ribosyl)ation inhibitors could result from the failure to inhibit DNA topoisomerase I activity in the vicinity of DNA strand breaks.

Poly(ADP-ribosyl)ation, Cellular Differentiation and Gene Expression.

A large body of available information supports the idea that poly(ADP-ribosyl)ation is involved in cell differentiation processes (for review see 70). This information comes from studies which suggest that a specific pattern of changes in poly(ADP-ribosyl)ation accompanies certain differentiation processes. In some processes, such as the differentiation of myoblasts into muscle fibers, an increase in poly(ADP-ribose) polymerase activity was observed

and inhibitors of this enzyme blocked differentiation (71). In other differentiation systems, such as Friend murine erythroleukemia cells (72), a decrease in poly(ADP-ribose) polymerase activity was shown. In addition, the morphological differentiation or terminal differentiation in the latter system was induced by inhibitors of poly(ADP-ribose) polymerase. These apparently contradictory results could be explained in terms of effects of the inhibitors in mono(ADP-ribosyl)ation as well as in poly(ADP-ribosyl)ation. A very important observation was recently made in studies by Rankin *et al.* (73) in where the effects of (ADP-ribosyl)ation inhibitors on both, mono(ADP-ribose) transferases and poly(ADP-ribose) polymerase were evaluated. It was observed that concentrations of inhibitors at the μM range block poly(ADP-ribosyl)ation, whereas mM concentrations are required to inhibit mono(ADP-ribosyl)ation, both, *in vivo* and *in vitro* (73). Thus, since in the aforementioned cellular differentiation studies mM concentrations of inhibitors were used, it is very likely that the effects observed are the result of a block on mono as well as on poly(ADP-ribosyl)ation.

Cellular differentiation requires chromatin rearrangements which result in the expression of sets of specific genes. Given the potential of poly(ADP-ribose) to induce local alterations in chromatin structure (to be

discussed in detail below), it is reasonable to assume that the role of poly(ADP-ribosyl)ation in cell differentiation may rely on its ability to regulate gene expression. In support of this assumption, it is possible to enumerate various experimental results which suggest a direct involvement of poly(ADP-ribosyl)ation on gene expression.

A protein factor (TFIIC) which abolishes non-specific transcription at nicked sites on DNA, without affecting specific transcription at a promoter site, has been identified as poly(ADP-ribose) polymerase (74, 75). Upon incubation of the TFIIC with NAD^+ and DNA, this factor loses 40% of its transcription inhibition activity (75). The ability of the factor to suppress random transcription initiation at nicks is associated with its DNA-binding activity. Presumably, auto poly(ADP-ribosyl)ation of TFIIC reduces its affinity for DNA resulting in the exposure of nicks in the DNA template (76). It remains to be seen whether poly(ADP-ribose) polymerase exhibits this function under physiological conditions.

An inverse correlation between poly(ADP-ribosyl)ation of the high mobility group proteins 14 and 17 and glucocorticoid-mediated gene expression of mouse mammary tumor virus (MMTV) has been reported (77). Furthermore, it has been shown that poly(ADP-ribosyl)ation inhibitors synergistically enhance glucocorticoid-induced MMTV gene

expression and nuclear binding of the glucocorticoid (78). While the molecular mechanisms await to be elucidated by more rigorous experimentation, the available information supports the concept of poly(ADP-ribosyl)ation as a regulator of gene expression.

Poly(ADP-ribosyl)ation and Chromatin Structure.

A relationship between poly(ADP-ribosyl)ation and major chromatin functions such as DNA replication, DNA repair and gene expression has been well established. A factor common to all these chromatin functions is that they require changes in chromatin organization. Indirect evidence suggesting that poly(ADP-ribosyl)ation may fundamentally alter chromatin structure comes from the fact that most of the poly(ADP-ribose) acceptor proteins identified so far have the capacity to interact with DNA (see Table 1). Therefore, it is reasonable to postulate that poly(ADP-ribosyl)ation may alter the interaction of these proteins with DNA, and this could be a way to generate local changes in chromatin organization. Direct evidence suggesting that this could be the case comes from studies by Poirier *et al.* (79) and De Murcia *et al.* (80). With the use of electron microscopic techniques, it has been demonstrated that incubation of condensed polynucleosomes with NAD^+ and poly(ADP-ribose) polymerase induces relaxation of the condensed structure (79). Under

these conditions the major acceptor protein is histone H1. Earlier studies by Thoma *et al.* (81) have demonstrated that poly(ADP-ribosyl)ated histone H1 exhibits a reduced DNA binding affinity. This possibility was examined in the polynucleosome relaxation experiments. Moreover, it was concluded in these experiments that poly(ADP-ribosyl)ation of histone H1 did not induce its detachment from DNA but rather affected the function of H1 in initiating the condensation of polynucleosomes (79). Furthermore, it has been shown that poly(ADP-ribose) glycohydrolase activity causes recondensation of relaxed, poly(ADP-ribosyl)ated polynucleosomes (80). Recently, it was reported that hyperpoly(ADP-ribosyl)ation of histone H2B in polynucleosome preparations causes the dissociation of DNA from the histone octamer (82). This finding is particularly interesting in view of the identification of histone H2B as a predominant poly(ADP-ribose) acceptor following treatment of hepatoma cells with a DNA alkylating agent (83). It was therefore suggested that the detachment of DNA from the histone octamer could be related to the increased micrococcal nuclease sensitivity of repair patches in DNA (82).

Poly(ADP-ribosyl)ation results in the addition of a large, complex, negatively charged polymer to a protein. Thus, it is likely that not only the covalent modification of proteins but also the electrostatic non-covalent interactions

of the polymer with other macromolecules is responsible for generating changes in chromatin organization.

Since the studies presented in this dissertation will deal with the nuclear distribution of ADP-ribose polymers, some aspects of nuclear structure will be introduced next.

The Nuclear Envelope.

In 1950, the electron microscopists Callan and Tomlin observed that the two major compartments in the eukaryotic cell, the nucleus and the cytoplasm, are separated by a membranous barrier perforated by pores (84). This membranous barrier, named the nuclear envelope, possesses major structural components which include an inner and outer nuclear membrane, pore complexes and the nuclear lamina. The outer membrane, which is continuous with the endoplasmic reticulum, has bound ribosomes and other endoplasmic reticulum characteristics. The inner membrane is closely associated with chromatin. The pore complexes are protein superstructures that occur at regions where the inner and outer membrane are joined to form pores. Both the protein composition and the functional role of the pore complexes is poorly understood (85). However, circumstantial evidence suggests that the pore complexes mediate active transport of macromolecules between the nucleus and cytoplasm (86).

The nuclear lamina is a fibrillar protein meshwork that covers the nucleoplasmic surface of the inner nuclear membrane (for review see reference 87). While the structure of the nuclear lamina is very similar in different cell types and species, the protein composition shows some differences. In a variety of organisms, three lamin polypeptides (A, B and C) with molecular weights ranging from 60 to 70 kDa have been described (88, 89). Several studies suggest that the nuclear lamina provides a framework for organization and regulation of nuclear envelope structure (87). During interphase, the three nuclear lamin polypeptides form a polymer-like assembly which is stable to non-ionic detergents and salt extractions (90). However, during mitosis, when the nuclear envelope breaks down, they disassemble and disperse throughout the cytoplasm (90, 91). During this period, lamins A and C become soluble while lamin B is associated with membrane vesicles (90). During telophase, the lamins reassemble around the two daughter nuclei concomitantly with nuclear envelope reformation. Since it has been observed that, during mitosis, the lamins become transiently hyperphosphorylated, it has been proposed that phosphorylation regulates their assembly and disassembly (92). Modification of the nuclear lamins A, B and C by poly(ADP-ribosyl)ation has also been reported (50, 51). However, changes in the extent of this modification during

the cell cycle have not been well characterized. The nuclear lamina has been postulated to provide an anchoring site at the nuclear periphery for interphase chromosomes (93); However, this function has not been experimentally studied.

The Nuclear Matrix and Its Associated Functions.

The interphase nucleus from a wide variety of eukaryotic cells ranging from yeast to humans contains a nuclear matrix structure. Morphologically, this structure contains residual elements of the nuclear pore complex and lamina, residual nucleolar structures and an internal protein network composed of ribonucleoproteins and non-histone proteins associated with a fibrous insoluble protein mesh (94-97). The nuclear matrix has been operationally defined as the round shaped nuclear structure which remains after digestion of nuclei with nucleases, usually DNase I, and extraction with salt buffers of high ionic strength, commonly 1 to 2 M NaCl (reviewed in 98 and 99). The protein fraction of the nuclear matrix accounts for 5 to 15% of the total nuclear protein, depending on the isolation procedure (94 and reviewed in 98). In addition, the nuclear matrix contains tightly attached to it a small fraction, usually less than 1%, of the total DNA and from 30 to 80% of the nuclear RNA (94, 100 and reviewed in 98).

Currently, there is a growing interest in the nuclear matrix based on its suggested involvement in DNA replication, DNA repair replication, organization of DNA into loop domains, gene expression, RNA processing, steroid hormone action and carcinogenesis. Perhaps the best studied functions in which a role for the nuclear matrix has been suggested are DNA replication and chromatin organization via the attachment of specific DNA regions. It has been observed that newly synthesized DNA can be preferentially recovered with the nuclear matrix (101, 102). Based on this finding, it has been proposed that DNA replication occurs on the nuclear matrix, either with DNA being reeled through fixed sites of replication on a static structure (101) or on components of a flexible dynamic structure being translocated along the DNA (99). In further support of this hypothesis, DNA polymerase α (103), topoisomerase II (104), DNA primase (103) and RNase H (105), all enzymes involved in DNA replication, have been recovered with the nuclear matrix.

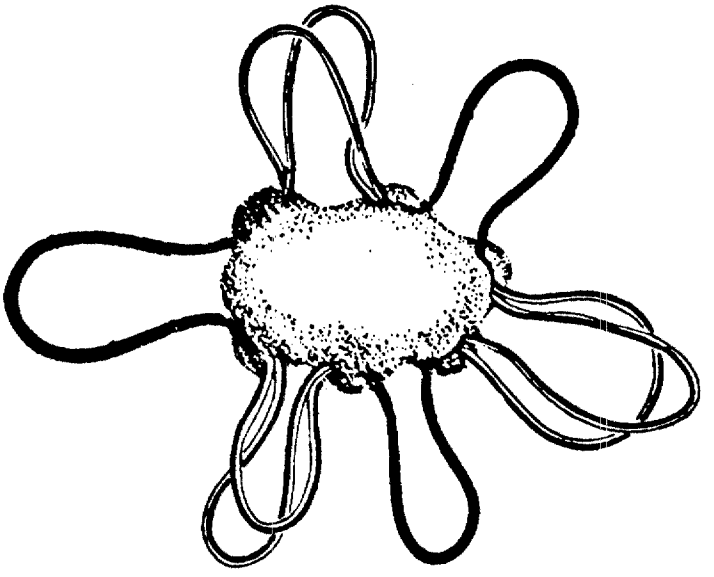
Recently two research groups, working with two different gene systems, have reported the identification of a 200 bp DNA sequence which confers specific nuclear matrix attachment to transcriptionally active genes (106, 107). Characterization of these nuclear matrix associated regions (MAR) (107), or scaffolding associated regions (SAR) (106) revealed that they contain consensus topoisomerase II binding

sites, and that frequently they reside next to transcriptional enhancer sequences (107). These unique characteristics have led to the proposal that MAR sequences may have a major role for transcriptional activation of nearby genes (107). It has been proposed that the genome is organized into loop domains that have both structural and functional roles (108 and see Fig.3 for representation). The observation that there are at least 10,000 MAR sequences per nucleus (107), which could organize the genome into loops ranging in size from 5 to 112 kbp (106, 109), provides strong support for this proposal. Thus, MAR sequences may function as anchoring sites at the base of the DNA loops.

As for DNA replication, evidence suggesting a role of the nuclear matrix in DNA repair replication, RNA processing, steroid hormone action and carcinogenesis rest primarily upon the recovery of biological active molecules in the nuclear matrix fraction (Table II). It remains to be determined whether the recovery of a given molecule in the DNase I and high salt resistant fraction truly indicates that the nuclear matrix is involved in a particular nuclear function. However, it should be emphasized that, in several cases, a particular biologically active molecule has been consistently recovered with the nuclear matrix fraction isolated under different procedures, suggesting that its nuclear matrix association is not artifactual.

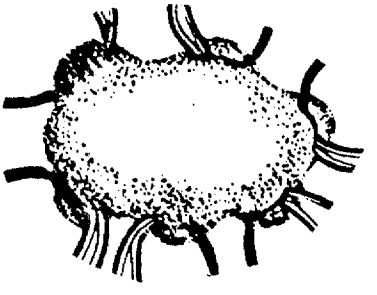
Fig.3. **The nuclear matrix concept.** Schematic representation showing the loops of DNA attached at its base to the nuclear matrix. DNase digestion of isolated nuclei followed by salt extraction results in the isolation of the nuclear matrix. The darker loops represent condensed DNA loops and the double lined loops show relaxed DNA loops engaged in replication.

THE NUCLEAR MATRIX CONCEPT



The Nuclear Matrix organizes
DNA into Loop Domains

DNase / Salt extraction



Nuclear Matrix

TABLE II

Functional molecules recovered with the nuclear matrix fraction

Molecules associated with DNA synthesis	Newly replicated DNA DNA synthesized during repair DNA primase RNAase H DNA polymerase α DNA polymerase β DNA replication forks Topoisomerase II Topoisomerase I
Molecules associated with DNA organization	Supercoiled DNA loops DNA binding proteins
Molecules associated with gene expression	Active genes RNA polymerase II Rapidly labeled RNA or HnRNA[a] RNA splicing intermediates Small nuclear RNA
Molecules associated with hormone action	Estrogen binding sites Androgen binding sites Sites which bind androgen-receptor complex Thyroid hormone binding sites
Molecules associated with viral replication	Viral T antigens Viral DNA binding proteins Viral DNA
Molecules associated with neoplastic transformation	Carcinogen adducts to macromolecules Tumor-associated antigens of viruses Oncogene products

[a]Heterogeneous nuclear RNA

Research Prospectus.

Although it has been clear since its discovery in 1966 (9) that poly(ADP-ribose) metabolism is confined to the nucleus, very limited research concerning the intranuclear localization of this metabolism has been done. Analysis of the distribution of poly(ADP-ribose) polymerase in the nucleosomal array of chromatin has been undertaken by Smulson *et al.* (110). Their results suggested a preferential localization of the enzyme within the linker DNA (110). In contrast to these results, visualization of poly(ADP-ribose) polymerase in chromatin by immunoelectron-microscopy indicated an association of the enzyme with the nucleosomal core (111). While further studies are needed to reconcile these discrepancies, a different aspect of the intranuclear distribution of poly(ADP-ribosyl)ation activity emerged with the finding by two groups that a small fraction of poly(ADP-ribose) polymerase is closely associated with the nuclear matrix (49-51). These results are very interesting in view of the increasing evidence indicating that actively transcribed genes, replicating DNA domains and DNA regions undergoing repair replication are associated with the nuclear matrix.

Nuclear compartmentalization of chromatin appears to play an important role in the regulation of chromatin

functions such as *de novo* DNA replication, DNA repair replication, carcinogenesis and gene expression. Likewise, regulation of these chromatin functions by poly(ADP-ribosyl)ation of nuclear proteins, due to the potential of this polymer to reversibly bring about changes in chromatin conformation *in vitro*, has been proposed. Furthermore, there is the postulation that these changes could be mediated by covalent and non-covalent interactions of poly(ADP-ribose) with chromatin components as well as by the fast turnover of these polymers. Hence, this proposal implies that close proximity of poly(ADP-ribose) with chromatin regions engaged in these functions is essential. Thus, an analysis of the nuclear distribution of ADP-ribose polymers will further help in the support of this proposal or in the formulation of new ones. The goal of the studies presented in this dissertation is to analyze the nuclear distribution of ADP-ribose polymers generated *in vivo*.

CHAPTER II

MATERIALS

Biologicals.

C3H10T1/2 cells (clone 8) were obtained from Dr. C. Heidelberger, Comprenhensive Cancer Center, University of Southern California (Los Angeles, CA). Dulbeccos modified Eagle's medium and gentamicin sulfate were purchased from Life Technologies (Grand Island, NY). Bovine calf serum was obtained from Hyclone Laboratories (Logan, UT).

Topoisomerase II antiserum developed in rabbits was a generous gift of Dr. L. F. Liu of the Department of Biological Chemistry, Johns Hopkins University School of Medicine (Baltimore, MD). The IgG fraction against topoisomerase I was purchased from Immunovision (Springdale, AR). The anti-poly(ADP-ribose) polymerase monoclonal antibody $C^{II}20$ was generously provided by Drs. D. Lamarre and G. Poirier of Centre de Recherche en Cancerologie L' Hotel-Dieu de Quebec (Quebec, Canada). The conditioned medium from a anti-RNP hybridoma, IgG 2a(k) cell line, containing the monoclonal antibody 2.73 against a 70 kDa protein, was kindly donated by Dr. S. Hoch from the Augouron Institute (La Jolla,

CA). The anti-rabbit, anti-human and anti-mouse IgG fractions were purchased from Promega Biotec (Madison, WI).

Radioisotopes.

[2-8,^{3}H]adenine (35 Ci mmol^{-1}), [2-^{14}C]thymidine (52 mCi mmol^{-1}), Tran [^{35}S]-label^{TM} (1100 Ci mmol^{-1}) and [2-^{14}C] uridine (52 mCi mmol^{-1}) were purchased from ICN Radiochemicals (Irvine, CA). [^{35}S]methionine (800 Ci mmol^{-1}) was obtained from Amersham (Arlington Heights, Ill). Ecolite was from Westchem (San Diego, CA). Scintillation vials were obtained from Fisher Scientific (Pittsburgh, PA), and fluorographic enhancer (Enlightning) was purchased from NEN Research Products (Boston, MA).

Enzymes.

Deoxyribonuclease (DNase I), ribonuclease (RNase), from bovine pancreas, snake venom phosphodiesterase (SVPD) from *Crotalus adamanteus* and micrococcal nuclease (MNase) from *Staphylococcus aureus* were purchased from Cooper (Freehold, NJ). DNase I from bovine pancreas, trypsin, type XIV from bovine pancreas, and bacterial alkaline phosphatase (BAP) from *Escherichia coli* were obtained from Sigma (St. Louis,

MO). Poly(ADP-ribose) glycohydrolase was kindly provided by Drs. L. Menard and G. Poirier of Centre de Recherche en Cancerologie L' Hotel-Dieu de Quebec (Qebec, Canada).

General Supplies.

Adenosine, adenosine 5'-monophosphate (AMP), deoxyadenosine, adenosine 5'-diphosphoribose (ADP-ribose), Tris(hydroxymethyl)aminomethane (Tris), piperazine-N'-bis(2-ethane-sulfonic acid) (PIPES), 3(N-morpholino)propanesulfonic acid (MOPS), N-2-hydroxyethylpiperazine-N'-2-ethanosulfonic acid (HEPES),2-mercaptoethanol, sodium dodecyl sulfate (SDS), triton x-100, nonidet P-40 (NP-40), lithium diiodosalicylate (LIS), N,N'-bis(3-aminopropyl)-1,4-butanediamine (spermine), N-(3-aminopropyl)-1,4-butanediamine (spermidine), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), glycine, bovine serum albumin (BSA), prestained molecular weight standards (MW-SDS-blue, 27 to 180 kDa), pepstatin and phenylmethylsufonyl fluoride (PMSF) were purchased from Sigma (St. Louis, MO). Aprotinine (trasylol) was obtained from Boehringer Mannheim (West Germany). L-methionine, and trypan blue were from Grand Island Biochemical (Grand Island, NY). 6,9-Diamino-2-ethoxyacridine lactate monohydrate (ethacridine) was obtained from Aldrich Chemical (Milwaukee, WIS). Diamidinophenylindole (DAPI) was from Serva Feinbiochemical (Heidelberg, FRG). The homopolymers of adenylic acid:

(polyA) of variable chain length (100 to 120 residues); the dodecamer [$(Ap)_{11}A$]; the octamer [$(Ap)_7A$] and the pentamer [$(Ap)_4A$] of adenylic acid were from Pharmacia (Piscataway, NJ). The electrophoresis-grade reagents polyacrylamide, N,N'-methylene-bis-acrylamide, N,N,N',N'-tetramethylendiamine (TEMED), ammonium persulfate and bromophenol blue as well as the chromatography materials polypropylene Econo-Columns (0.8-in I.D) and the cation exchange resin Bio Rex 70 (200-400 mesh) were obtained from Bio Rad (Richmond, CA). The following chemicals and materials used for protein blot analysis: alkaline phosphatase substrates, 5-bromo-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT), were obtained from Promega Biotec (Madison, WI); nitrocellulose membrane filters (pore size 0.2 μm), were purchased from Schleicher and Schuell (Keen, NH) and non-fat dry milk from Carnation (Los Angeles, CA). Guanidine hydrochloride was from Fluka (Lake Ronkonkoma, NY). The supplier for the reverse-phase Altex ultrasphere ODS column (250 x 4.6-mm I.D.) was Beckman (Berkeley, CA). The silica-based columns for gel filtration GF-250 and GF-450 (250 x 9.4-mm I.D.) were from Du Pont (Newton, CO). The supplier for X-ray developer, X-ray fixer and BB-5 X-ray film was Eastman Kodak Co. (Rochester, NY). All other chemicals were reagent grade. The radiolabeled poly(ADP-ribose) standards and dihydroxyboronyl-Bio Rex resin were prepared by different coworkers in this laboratory as previously described (112).

CHAPTER III

METHODS

Cell Culture and Cell Labeling.

C3H10T1/2 mouse fibroblasts (clone 8) were grown in a humidified, 5% CO_2-air incubator at 37°C and maintained in Eagle's basal medium containing 10% fetal calf serum (heat inactivated at 56°C for 30 min) and 2 μg ml^{-1} of gentamicin sulfate. Cells were seeded at 1 x 10^6 cells per 150 cm^2 flask in 25 ml of medium. To label the cells with [^{14}C]thymidine, 10 ml of fresh medium containing 0.5 μCi ml^{-1} of [^{14}C]thymidine was added to the cell cultures when they reached a cell density of approximately 2 x 10^6 cells per 150 cm^2 flask, and incubation was continued for 48 h at which time the cells reached confluence. For [^{3}H]adenine labeling, 50 μCi ml^{-1} of this radioisotope was added to confluent cells in 10 ml of medium and cultures were incubated for 16 h. For metabolic labeling of proteins, when cultures reached a cell density of 4 x 10^6 per 150 cm^2 flask, 10 ml of methionine free Dulbecco's modified Eagle medium, supplemented with 3 μg ml^{-1} methionine and containing 50 μCi ml^{-1} [^{35}S]methionine or Tran [^{35}S] label™ was added to the cultures, and incubation

was continued for 24 h. Tran [^{35}S] label^{TM} is a mixture of [^{35}S]labeled compounds which contains 70% methionine, 20% cysteine, 7% methionine sulfoxide, 2% cysteic acid and 1% other [^{35}S]labeled compounds.

Cell Treatment.

The synthesis and accumulation of poly(ADP-ribose) in intact cells was induced by treating the cells with hyperthermia or MNNG or a combined hyperthermia-MNNG treatment. For hyperthermic treatment, labeling medium was discarded and 45^{o}C prewarmed medium was added. The cultures were incubated at 45^{o}C for 30 min. The medium was then replaced by 37^{o}C prewarmed medium and cultures were transferred to a 37^{o}C incubator. When cells were treated with hyperthemia and MNNG, following transferral of cells at 37^{o}C for 30 min, 10 μg ml^{-1} of MNNG was added to the cultures and incubation was continued for an additional 30 min. For treatment of cells with MNNG, the medium was removed and replaced with 37^{o}C prewarmed medium containing 10 μg ml^{-1} MNNG and cultures were incubated at 37^{o}C for 30 min.

Isolation of Cell Nuclei.

All nuclei isolation steps were performed at 4°C and essentially as previously indicated (83). For isolation of nuclei at pH 3.1, the culture medium was discarded and cells were washed twice with ice cold phosphate buffered saline (PBS) (0.01 M sodium phosphate, pH 6.5, containing 150 mM NaCl). Cells were scraped from the flask and collected by centrifugation at 400 x g for 5 min. The cell pellet was suspended in 1 ml of NIB (nuclei isolation buffer, 25 mM citric acid, pH 3.1, 0.5% NP-40 and 0.5 mM PMSF), incubated on ice for 10 min, and then homogenized with a Dounce homogenizer. It was determined that 40 strokes with a type A pestle were enough to release the nuclei without appreciable nuclei breakage. At this point in some experiments, aliquots were removed for DNA and poly(ADP-ribose) quantification or for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). An equal volume of NIB-1M sucrose was added to the cell homogenate which then was centrifuged through a 3 ml-cushion of NIB-1 M sucrose at 3000 x g for 10 min. The nuclear pellet was washed once with NIB-0.25 M sucrose without NP-40. Routinely at all the nuclear fractionation steps, small aliquots (3 to 5 µl) were removed, mixed with an equal volume of trypan blue (0.4% solution in PBS) and observed by phase-contrast microscopy.

For the isolation of nuclei at pH 6.5, the same procedure was performed except that the NIB contained 10 mM PIPES, pH 6.5, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40 and 0.5 mM PMSF.

Digestion of Nuclei with MNase and Nucleosome Extraction.

Nuclei isolated at pH 6.5 were suspended at 5 x 10^6 nuclei ml^{-1} in NDB (nuclei digestion buffer, 10 mM PIPES, pH 6.5, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM $NaCl_2$, 0.25 M sucrose and 0.5 mM PMSF) and digested at 30°C with MNase at 250 U mg^{-1} of DNA. One unit corresponds to a change in optical density of 1.0 at 260 nm at 37°C and pH 8.0 under the specified conditions. At different times, aliquots of 200 µl were removed from the digestion mixture and centrifuged at 5000 x g for 7 min at 4°C and the pellet was extracted for nucleosomes as previously described (113). Pellets were resuspended in 0.5 ml of lysis buffer containing 10 mM PIPES, pH 6.5, 1 mM ethylendiamino-tetracetic acid (EDTA) and 0.5 mM PMSF, and kept on ice for 30 min with periodic, vigorous resuspension with a glass road. The suspension was then subjected to centrifugation for 7 min at 5000 x g at 4°C. The supernatant containing the nucleosomes was kept on ice, and the pellet was reextracted for 30 min with lysis buffer containing NaCl at 110 mM instead of 10 mM. After centrifugation at 5000 x g for 7 min at 4°C the supernatants

were pooled and the poly(ADP-ribose) and DNA content in the supernatant (MNase sensitive-chromatin fraction) and in the pellet (MNase resistant-chromatin fraction) was determined.

Nuclear Matrix Preparation with NaCl and $(NH_4)_2SO_4$ Extraction.

Approximately 6 x 10^6 nuclei isolated at pH 6.5 were digested with 250 µg ml^{-1} of DNase I (Sigma) in 1 ml of NDB for 40 min at 23°C. In the experiments for protein analysis by SDS-PAGE, DNase I from Cooper was used and the digestion was conducted for 1 h at 4°C. After centrifugation at 750 x g at 4°C, the supernatant was saved for further analysis and the pellet was resuspended by gentle vortexing in NIB-0.25 M sucrose without NP-40 and extracted with an equal volume of a cold solution containing either 0.6 M $(NH_4)_2SO_4$, 3 mM $MgCl_2$, 20 mM PIPES, pH 6.5, or 4 M NaCl, 10 mM EDTA, 20 mM PIPES, pH 6.5. After 10 min at 4°C, the nuclear matrices were pelleted by centrifugation at 1500 x g for 10 min at 4°C. In some experiments, the extraction of nuclei with 2 M NaCl was repeated three times as indicated above.

To prepare the nuclear matrix fraction by DNase I and RNase digestion, the nuclear pellet was resuspended in 1 ml of NDB, digested with 250 µg ml^{-1} of DNase I for 15 min at 23°C. At this point 110 µg ml^{-1} of RNase were added into the

digestion mixture and the incubation was continued for 30 min. Following centrifugation at 750 x g for 10 min at 4°C the supernatant was saved for DNA, RNA and poly(ADP-ribose) determination and nuclear matrices were prepared by extracting the pellet with 2 M NaCl as indicated above.

Preparation of Nuclear Matrices with Lithium Diiodosalicylate (LIS) Extraction.

The nuclear matrix fraction prepared using LIS-extraction was obtained as described (106) with some modifications. Except where otherwise indicated all the steps were performed at 4°C. The cell pellet containing 6 x 10^6 cells was resuspended in 1.5 ml of buffer A containing 3.75 mM PIPES, pH 6.5, 0.05 mM spermine, 0.125 mM spermidine, 20 mM KCl, 0.1% digitonin and 1% thiodiglycol. Cells were homogenized as indicated above (see isolation of nuclei). At this point, aliquots were removed and kept on ice for further analysis. After centrifugation at 400 x g for 5 min the pellet was resuspended and washed twice in 7.5 ml of buffer A. The nuclear pellet was resuspended in 200 µl of buffer B (5 mM PIPES, pH 6.5, 0.25 mM spermidine, 2 mM EDTA, 2 mM KCl and 0.1% digitonin) and incubated at 37°C for 30 min. After incubation, 1.5 ml buffer B at room temperature containing 25 mM LIS and 0.5 mM PMSF was added dropwise to the nuclear suspension. After each drop, the suspension was gently

shaken and finally the nuclei were kept at room temperature for 5 min. Following centrifugation at 800 x g for 10 min at 20°C, the supernatant was removed and stored at 4°C for further analysis. The LIS-extracted nuclear pellet was washed at 25°C with 1 ml of buffer C (20 mM PIPES, pH 6.5, 0.125 mM spermidine, 0.05 mM spermine, 20 mM KCl, 70 mM NaCl, 10 mM $MgCl_2$, 0.1% digitonin and 0.5 mM PMSF). After centrifugation at 800 x g and 20°C the supernatant was saved at 4°C. The pellet was washed 4 times at 25°C by gentle resuspension without vortexing in 1 ml, 0.75 ml, 0.5 ml and 0.25 ml, respectively, of buffer C without digitonin but containing 0.1 mg ml^{-1} BSA. After centrifugation at 800 x g and 20°C the supernatant was pooled with the other supernatants to measure poly(ADP-ribose) and DNA. The pellet was resuspended in 0.5 ml of buffer C containing 0.1 mg ml^{-1} BSA, and digested with 250 µg ml^{-1} of DNase I for 20 min at 30°C. The digestion mixtures were centrifuged at 1500 x g for 10 min at 4°C and the supernatant and pellet were saved for poly(ADP-ribose) and DNA determination.

Digestion of Nuclear Matrix with Poly(ADP-ribose) Glycohydrolase.

Isolated nuclear matrices from 6 x 10^6 cells were resuspended in 250 µl of NIB-0.25 M sucrose without NP-40 containing 1 mM AMP and 5 mM EDTA and digested with 5 U ml^{-1} of poly(ADP-ribose) glycohydrolase. Following incubation at 30°C for 1 h, the reaction mixture was centrifuged at 3000 x g for 10 min at 4°C and samples from the pellet and supernatant were saved for further analysis. In some experiments, in order to inhibit poly(ADP-ribose) glycohydrolase, the digestion of the nuclear matrix with this enzyme was performed in the presence of 100 µM ethacridine.

Quantification of Poly(ADP-ribose).

The procedure for determination of poly(ADP-ribose) has been described elsewhere (18, 114) except for minor modifications. The samples from the homogenate, nuclei and nuclear fractions were precipitated for 1 h on ice with 20% TCA with 1 mg ml^{-1} BSA added as carrier. After centrifugation at 1700 x g for 15 min at 4°C, the supernatants were removed and the pellets were dissolved in 200 µl of 88% formic acid and diluted to 3 ml with water. Samples were reprecipitated with 0.8 ml of 100% TCA for

30 min on ice. Following centrifugation at 1700 x g for 10 min at 4°C, the pellets were dissolved in 1 M KOH, 100 mM EDTA and alkaline hydrolysis was performed at 37°C for 2 h. The hydrolyzed material was adjusted to 10 ml with application buffer (250 mM ammonium acetate, pH 9.0, containing 1 M guanidine- hydrochloride and 10 mM EDTA) and adjusted to pH 9.0 with concentrated acetic acid. Affinity chromatography was carried out by applying the samples onto a 12.5 x 4.5-mm dihydroxylboryl-Bio-Rex 70 Econo-Column (0.5 ml of packed resin) freshly preequilibrated with 10 ml of application buffer. The unbound material was removed by washing the column with 25 ml of application buffer followed by 10 ml of
1 M ammonium bicarbonate, pH 9.0, containing 10 mM EDTA. Poly(ADP-ribose) was eluted from the column with 5 ml of water. The amount of radioactivity contained in the eluate was determined by liquid scintillation counting. Routinely a radiolabeled poly(ADP-ribose) standard was used in each experiment to correct for recovery. The results shown are corrected for the recovery obtained which usually was 80 to 90%.

Quantification of DNA.

The procedure used to quantify DNA by fluorescence has been previously described (115). In general, samples from the different cell fractions were solubilized with 2 ml of 0.2% triton X-100 containing 10 µl ml^{-1} 1N NaOH. After 5 min aliquots of 200 µl were mixed with 2.5 ml of DAPI reagent. The DAPI reagent was prepared as follows: 0.4 volumes of DAPI working solution (2.5 µg ml^{-1} in HEPES, pH 7.0 and 18 mM Na_2SO_4); 3.6 volumes assay buffer and 6 volumes of water. After 90 min at room temperature, the relative fluorescence was measured at 10X sensitivity with a 360 nm excitation and 450 nm emission filter in a Perkin-Elmer fluorescence spectrophotometer series 650-40. The content of DNA in the samples was estimated by using standards containing 0.25 to 2.5 µg ml^{-1} of calf thymus DNA.

For cells labeled with [^{14}C]thymidine, the relative amounts of DNA were estimated by liquid scintillation counting of TCA-precipitable material.

Reversed-Phase Chromatography.

In order to confirm that the radioactivity eluted from the boronate affinity column consisted only of poly(ADP-ribose), the eluted material was lyophilized, dissolved in 5

ml of water and lyophilized again. The lyophilized sample was dissolved in 1 ml of 50 mM MOPS, pH 7.4 and 20 mM $MgCl_2$, digested with 1 U each of SVPD and BAP for 3 h at 37°C. Prior to application onto reversed-phase high pressure liquid chromatography, 100 nmoles of adenosine and deoxyadenosine were added to the digested material which was then filtered through a 0.22 µM type GS Millipore membrane. Samples were applied, in a total volume of 1 ml, to a Beckman-Altex ultrasphere-ODS reversed-phase column (250 x 4.6-mm I.D.) using a Beckman model 330 liquid chromatograph. The injector was equipped with a sample loop of 1 ml. An Instrumentation Specialties Company UA-5 ultraviolet absorbance monitor was used to measure the absorbance at 254 nm. The absorbance monitor was equipped with a type 6 optical unit, 19 µl flow cell, and 254 band pass filter. The column was eluted isocratically at a flow rate of 1 ml min^{-1} with a mixture of 7 mM ammonium formate buffer, pH 5.8, containing a proportion of methanol which varied from 10 to 13%, depending on the age of the column, so that the retention-time for deoxyadenosine and ribosyladenosine did not exceed 12 to 14 min. Fractions of 0.5 ml were collected using a Frac-100 fraction collector from Pharmacia. The radioactivity in the fractions was determined by liquid scintillation counting.

Molecular Sieve Chromatography of Poly(ADP-ribose).

Poly(ADP-ribose) polymers purified by boronate chromatography were lyophilized, dissolved in water and lyophilized again. The lyophilized material was dissolved in 50 µl of a solution containing 0.5 U polyA, 0.125 U of each $[(Ap)_4A]$, $[(Ap)_7A]$ and $[(Ap)_4A]$ and 0.2 mM of ADP-ribose and AMP. The samples were fractionated by size exclusion chromatography in a Du Pont spherical silica GS-450 and GS-250 columns (250 x 9.4-mm I.D) connected in series. The running buffer used was 0.1 M sodium phosphate, pH 6.8, at a flow rate of 1 ml min^{-1}. Absorbance at 254 nm was monitored and fractions of 0.5 ml were collected. The radioactivity in the fractions was determined as described above.

Analysis of Proteins by SDS-PAGE.

For this analysis, $[^{35}S]$-labeled cells were used and all the buffers used during the cell fractionation contained in addition to 0.5 mM PMSF, 100 mg ml^{-1} pepstatin and 100 KIU (kallikrein-inhibition units) aprotinin as protease inhibitors. The supernatants from the DNase I digestion, $(NH_4)_2SO_4$ extraction and poly(ADP-ribose) glycohydrolase digestion were precipitated in 30% TCA for 2 h at 4°C. After centrifugation at 10,000 x g for 15 min at 4°C, the pellets

were resuspended and washed 6 times with 1 ml of ether and the residual solvent was evaporated by spining the samples in a speed vacuum concentrator for 1 h at room temperature. The ether-dried pellets as well as the pellets from different cell fractions were dissolved in 50 to 75 µl of a buffer containing 62 mM Tris-HCl, pH 6.8, 1.25% SDS, 12.5% glycerol, 178 mM 2-mercaptoethanol and 0.01% bromophenol blue, and boiled for 3 min. The samples were subjected to electrophoresis in an SDS 9 to 18% polyacrylamide slab gel (16 x 20-cm, 0.75-mm thick) according to the method of Laemmli (116). Electrophoresis was preformed in a Protean-II cell apparatus (Bio Rad) at 4°C at a constant current of 9 mA for approximately 12 h.

Protein Blot (Western Blot) Analysis.

Following SDS-PAGE analysis of the nuclear fractions, the gel was briefly soaked in transfer buffer containing, 20 mM Tris-base, 150 mM glycine, 0.05% SDS and 20% methanol at a final pH of 8.3. Proteins were transferred from the gel to nitrocellulose membrane filters (0.2 µm pore) soaked in transfer buffer at 0.5 mA cm^{-2} during 90 min using the semi-dry polyblot apparatus from American Bionetics. Filters were soaked in 50 ml of 5% non-fat milk solution prepared in TBST (Tris buffered saline tween-20, containing 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% tween-20 and 0.02% NaN_3). For

probing with antibodies the filters were incubated overnight in a 30 ml solution of the respective antibody in TBST. After washing the filters with 50 ml of TBST for 10 min five times, these were transferred to a 30 ml solution containing the appropriate anti-IgG-alkaline phosphatase conjugate in TBST and incubated for 1 h. Filters were washed as indicated above, and developed by incubation for various periods of time (usually 5 to 15 min, depending on the antibody) with the alkaline phosphatase substrates (BCIP an NBT) in a 100 mM Tris-HCl, pH 9.5 buffer containing, 100 mM NaCl and 5 mM $MgCl_2$. It is important to note that: all the filter incubations were done at room temperature and under constant gentle shaking; the milk, the antibody as well as the anti-IgG-alkaline phosphatase solutions could be used for up to three times without any appreciable loss in sensitivity. These solutions were stored at -20°C. The efficiency of protein transfer and binding to nitrocellulose filters was monitored by including prestained molecular weight standards for protein (ranging from 27 to 180 kDa) in all the gels to be transferred.

Fluorography of Gels.

Gels were fixed and equilibrated for 1 h in a solution of 5% glycerol, 40% methanol and 10% acetic acid. Following removal of this solution, gels were soaked for 30 min in 100 ml of fluorographic enhancer (Enlightning^{TM}). Finally, gels were dried at 60^{o}C for 2 h in a slab gel dryer model 483 from Bio Rad, and exposed to Kodak X-Omat BB-5 film at -70^{o}C.

CHAPTER IV

RESULTS

Recovery of DNA and Poly(ADP-ribose) During the Isolation of Nuclei.

In the present study, the nuclear distribution of ADP-ribose polymers was examined in untreated cells and in cells in which polymer levels were increased by treatment with either the alkylating agent MNNG or a brief hyperthermic treatment or both treatments. Previous studies have shown that MNNG results in the production of DNA strand breaks, which leads to a rapid activation of poly(ADP-ribose) polymerase (59, 60), while hyperthermic treatment results in increased polymer levels due to a decreased activity of poly(ADP-ribose) glycohydrolase (43). Except where otherwise indicated, the data presented in this study was obtained from cells that were treated with MNNG following a brief period of hyperthermia.

To obtain a preparation of nuclei from C3H10T1/2 mouse fibroblasts free of cytoplasmic debris, a procedure which involved gentle mechanical homogenization of cells, treatment with the non-ionic detergent NP-40 and centrifugation through

a 1 M sucrose cushion was used (83). At each step of the isolation procedure, samples were examined by phase-contrast microscopy to monitor structural integrity and cytoplasmic contamination. The final nuclear preparation consisted of a population of intact, round-shaped nuclei which were apparently free of cytoplasmic remnants. The recovery of nuclei through all the isolation steps was estimated on the basis of the DNA recovered with the nuclear fraction. DNA recovery was determined by measuring the radioactivity in the whole cell homogenate and in the nuclear fractions from cells prelabeled with [^{14}C]thymidine, or by quantification of fluorescence following DNA extraction and formation of the DNA-diamidinophenylindole complex (115). The results, depicted in Table III, show that the nuclear isolation performed by this procedure resulted in an efficient recovery of nuclei as judged by the DNA content, which was 81 to 83% when measured by fluorescence or radiolabel, respectively.

A highly sensitive yet simple radiolabeling method to determine the poly(ADP-ribose) content of intact cultured cells has recently been developed in this laboratory (18, 114). This method involves: 1) the labelling of confluent cell cultures with [^{3}H]adenine, which is efficiently transported into the cells and incorporated into the cellular NAD^+ pool; 2) the separation of ADP-ribose polymers from the acid-soluble nucleotide pool by TCA-precipitation; 3) alkaline digestion of the TCA-precipitated material to

TABLE III

Stability of poly(ADP-ribose) and yield of DNA during the isolation of nuclei at pH 3.1 or 6.5

Cells prelabeled with [^{14}C]thymidine and [^{3}H]adenine were treated with hyperthermia and MNNG. Following this treatment the cell nuclei were isolated at pH 3.1 or 6.5, and the poly(ADP-ribose) and DNA content were determined as described in Chapter III.

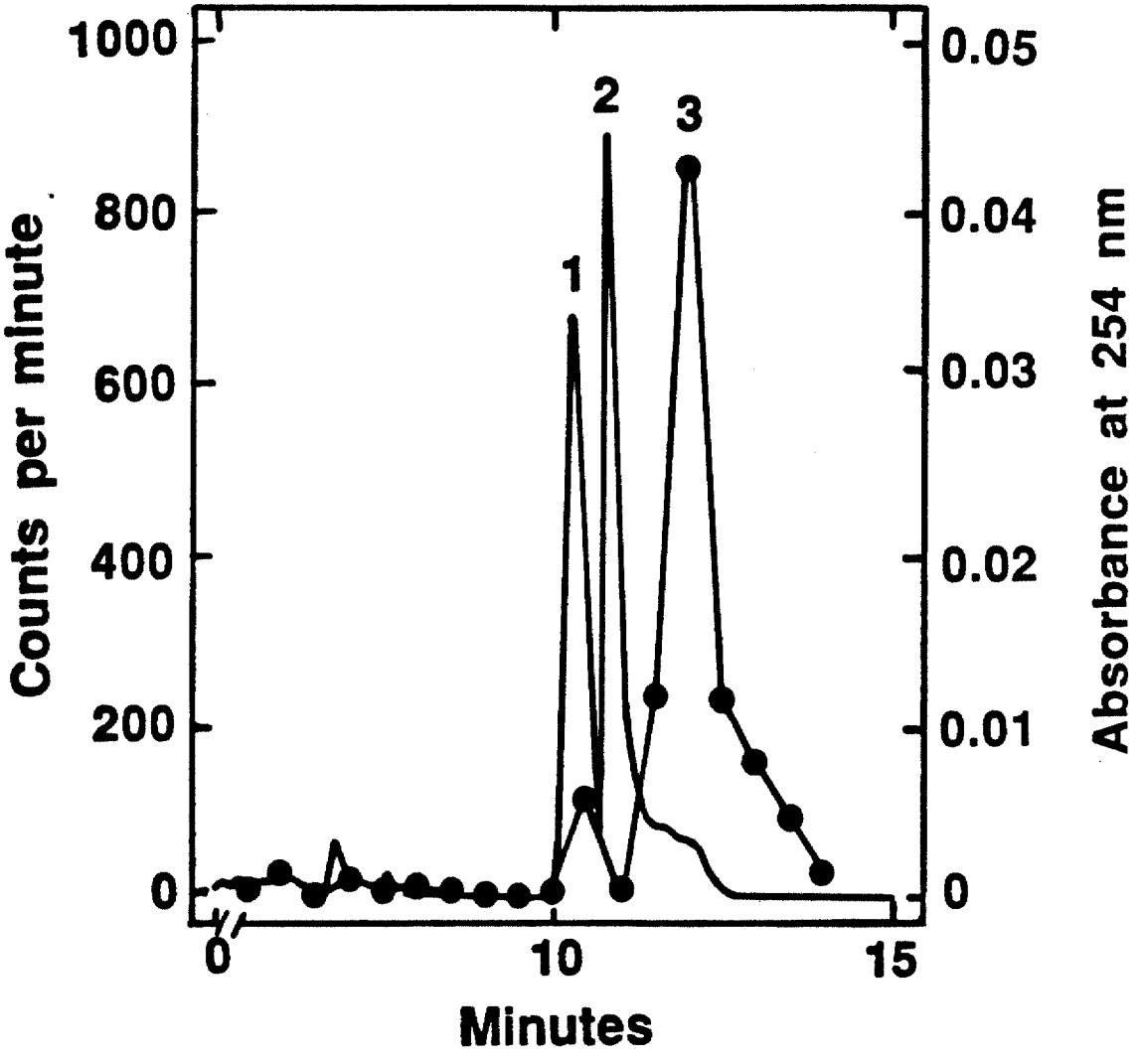
Fraction	DNA				poly(ADP-ribose)	
	Fluorescence		Radiolabel (^{14}C)		Radiolabel (^{3}H)	
	μg	%	*10^3 cpm*	%	*10^3 cpm*	%
At pH 3.1						
Homogenate	77	100	638	100	304	100
Nuclear	62	81	526	83	250	82
At pH 6.5						
Homogenate	NDa	ND	791	100	291	100
Nuclear	ND	ND	732	92	295	100

[a]ND, not determined.

release polymers from protein and to hydrolyze RNA and monomeric ADPR residues and 4) isolation of the ADP-ribose polymers by boronate column chromatography followed by quantification of radioactivity in the eluted fraction. It is important to note that in this procedure the TCA-precipitation should be performed twice. Following the first precipitation with 20% TCA, the pellet is redissolved in 88% formic acid, diluted with water and precipitated again with TCA. Failure to do this results in an unacceptable level of contamination of trapped adenine nucleotides in the eluted material and, consequently, in an artifactual overestimation of poly(ADP-ribose). To show that the radiolabel eluted from the boronate column consisted only of poly(ADP-ribose), the eluted material was digested with snake venom phosphodiesterase and bacterial alkaline phosphatase. This digestion results in the generation of ribosyladenosine and diribosyladenosine, from the linear internal and branched residues, respectively, as well as adenosine from the terminal residues (18). Following digestion, the samples were subjected to reversed-phase high performance liquid chromatography. A typical result is shown in Fig. 4. From the relative amount of ribosyladenosine to adenosine, which was 9 to 1, it was estimated that a minimum of 90% of the radiolabel was contained in poly(ADP-ribose).

Most of the protein-poly(ADP-ribose) conjugates so far

Fig.4. **HPLC reversed-phase chromatography of poly(ADP-ribose) digested with SVPD and BAP**. Peaks 1,2 and 3 indicate adenosine, deoxyadenosine and ribosyladenosine, respectively. (_____), Absorbance at 254 nm. (●), radioactivity. For experimental details see Chapter III.


1000
800
600
400
200
0
Counts per minute
0.05
0.04
0.03
0.02
0.01
0
Absorbance at 254 nm
1
2
3
0
10
15
Minutes

described are linked via a carboxylate ester linkage to either glutamic acid residues or carboxyl terminal lysine residues (117). These linkages are unstable in an even midly basic milieu (118). Adamietz and Rudolph (83) have shown that isolation of nuclei at pH 3.1 in a buffer containing citric acid results in a good yield of monomeric and polymeric ADP-ribose residues attached to protein. Therefore, the isolation of nuclei from C3H10T1/2 cells was performed initially at pH 3.1 and the poly(ADP-ribose) content of the cell homogenate and nuclear fraction was determined as indicated above. Table III shows that 82% of the total cellular poly(ADP-ribose) was recovered in the nuclear fraction. This is in good agreement with the recovery of nuclei which, as mentioned above, was 81 to 82% (Table III). Although no adverse effects of pH 3.1 on the nuclear distribution of ADP-ribose polymers has been reported, the isolation of nuclei was also performed in buffers at pH 6.5. The results, depicted in Table III, show that isolation of nuclei at this pH also allowed the efficient recovery of poly(ADP-ribose) with the nuclear fraction, as well as an efficient yield of nuclei. In view of this result, all further experiments were conducted at pH 6.5.

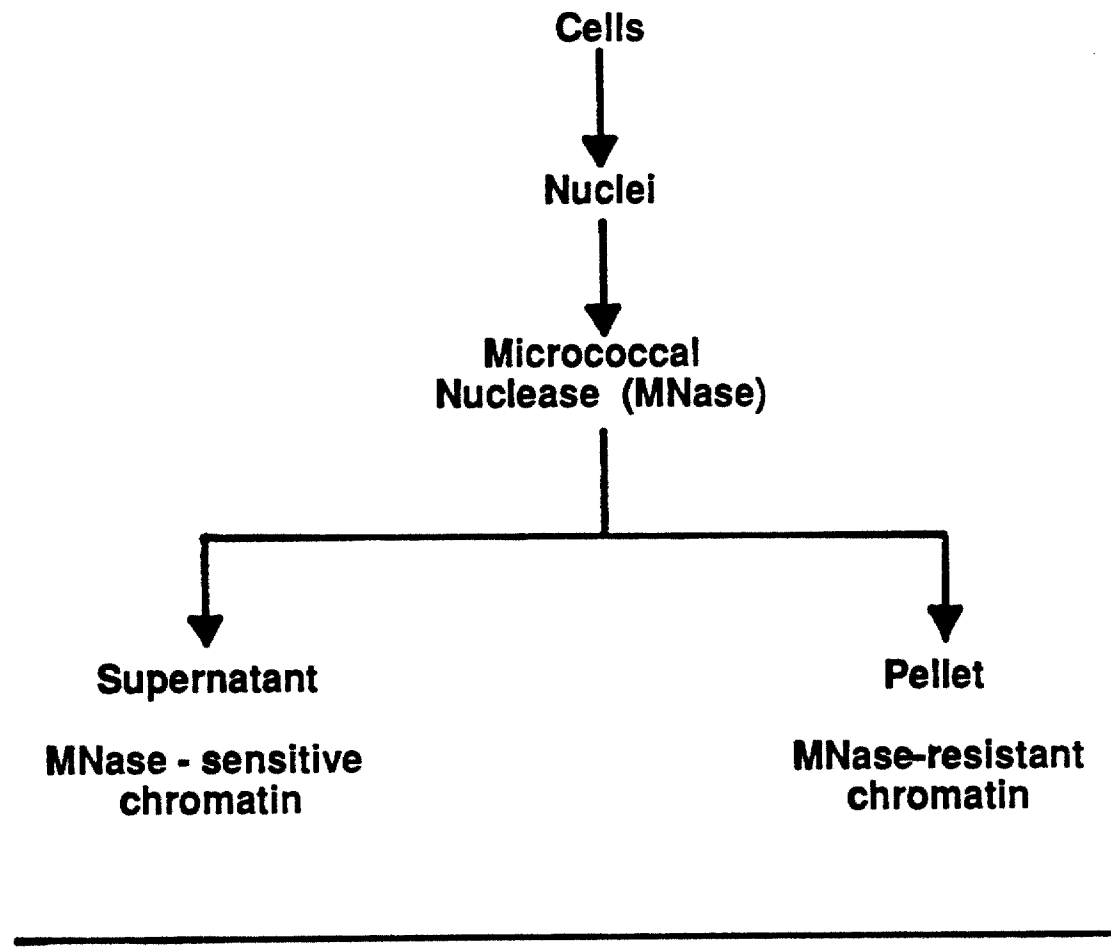
Are ADP-ribose Polymers Randomly or not Randomly Distributed in Chromatin?

Digestion of chromatin with nucleases has been frequently used as a tool to fractionate chromatin. One of the nucleases most commonly used in chromatin fractionation studies is MNase. This enzyme specifically cleaves the linker DNA between nucleosome core particles. In addition, it has been generally established that chromatin regions undergoing active transcription, *de novo* replication and repair replication are hypersensitive to MNase digestion. This fact has led to the conclusion that regions of chromatin active in RNA and DNA synthesis have a more relaxed conformation than inactive regions (for review see 119). Thus, digestion of chromatin with MNase results in the generation of two chromatin fractions: MNase-sensitive and MNase-resistant chromatin, which differ in structural and functional organization.

The question of whether or not poly(ADP-ribose) is randomly distributed within chromatin was first approached by examining for polymer association with chromatin regions differing in their sensitivity to MNase. The flow-chart for the generation of MNase-sensitive and MNase-resistant chromatin fractions is shown in Fig. 5.

Initial experiments were designed to determine optimal

Fig.5. **Flow-chart of the steps involved in the preparation of MNase-resistant and MNase-sensitive chromatin fractions.**


Cells
Nuclei
Micrococcal
Nuclease (MNase)
Supernatant
MNase - sensitive
chromatin
Pellet
MNase-resistant
chromatin

conditions for the MNase digestion of isolated nuclei. Fig. 6 shows the release of DNA into the MNase digestion supernatant as a function of increasing nuclease units at 30 min of incubation. The plateau of release was reached at an MNase concentration of 250 U mg^{-1} of DNA. A further increase in the concentration of MNase did not significantly affect the DNA fraction rendered soluble by the nuclease. Thus, all further chromatin fractionations were carried out at a MNase concentration of 250 U mg^{-1} of DNA in the isolated nuclei.

The fraction of ADP-ribose polymers released with the MNase-sensitive chromatin was determined in experiments where dividing C3H10T1/2 cells were labelled with [^{14}C] thymidine at 0.5 μCi ml^{-1} of culture medium during one cell doubling time, followed by a 16 h period of labeling with [^{3}H]adenine at 50 μCi ml^{-1} of culture medium. Fig. 7 shows the release of DNA and ADP-ribose polymers as a function of time during MNase digestion of isolated nuclei. After 30 min of digestion, when over 80% of the DNA had been released, 90% of the total poly(ADP-ribose) still remained in the MNase-resistant chromatin fraction. These data show that ADP-ribose polymers are not randomly distributed in chromatin but rather they exhibit a predominant association with the chromatin fraction resistant to MNase digestion. However, the possibility that these polymers confer resistance to the nuclease digestion cannot be ruled out at this point.

Fig.6. **Digestion of isolated nuclei as a function of increasing concentrations of MNase.** Isolated nuclei from cells radiolabeled with [^{14}C]thymidine were digested for 30 min at 30^{o}C with the indicated units of MNase per mg of DNA. The TCA-soluble radioactivity was determined as described in Chapter III.

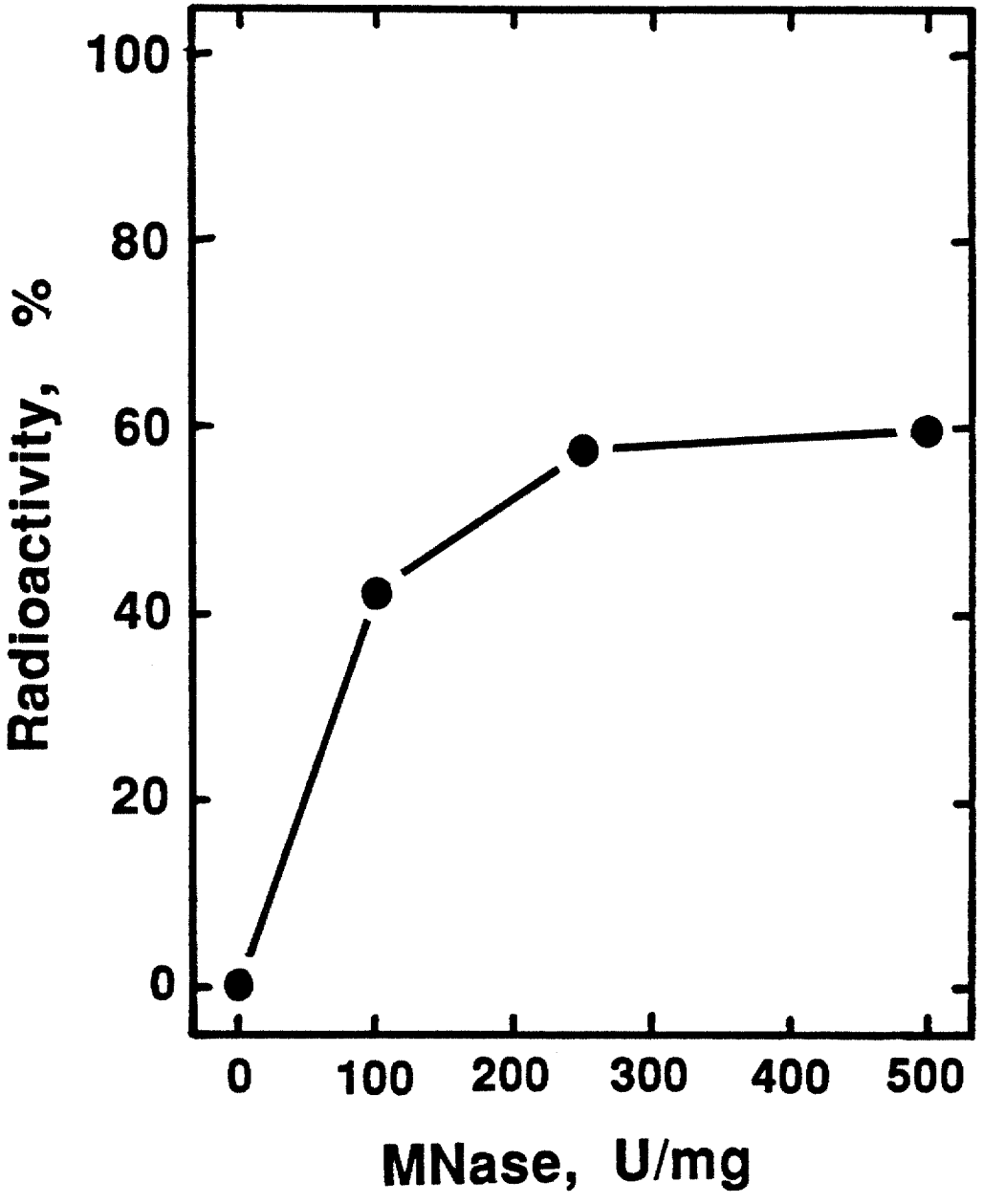
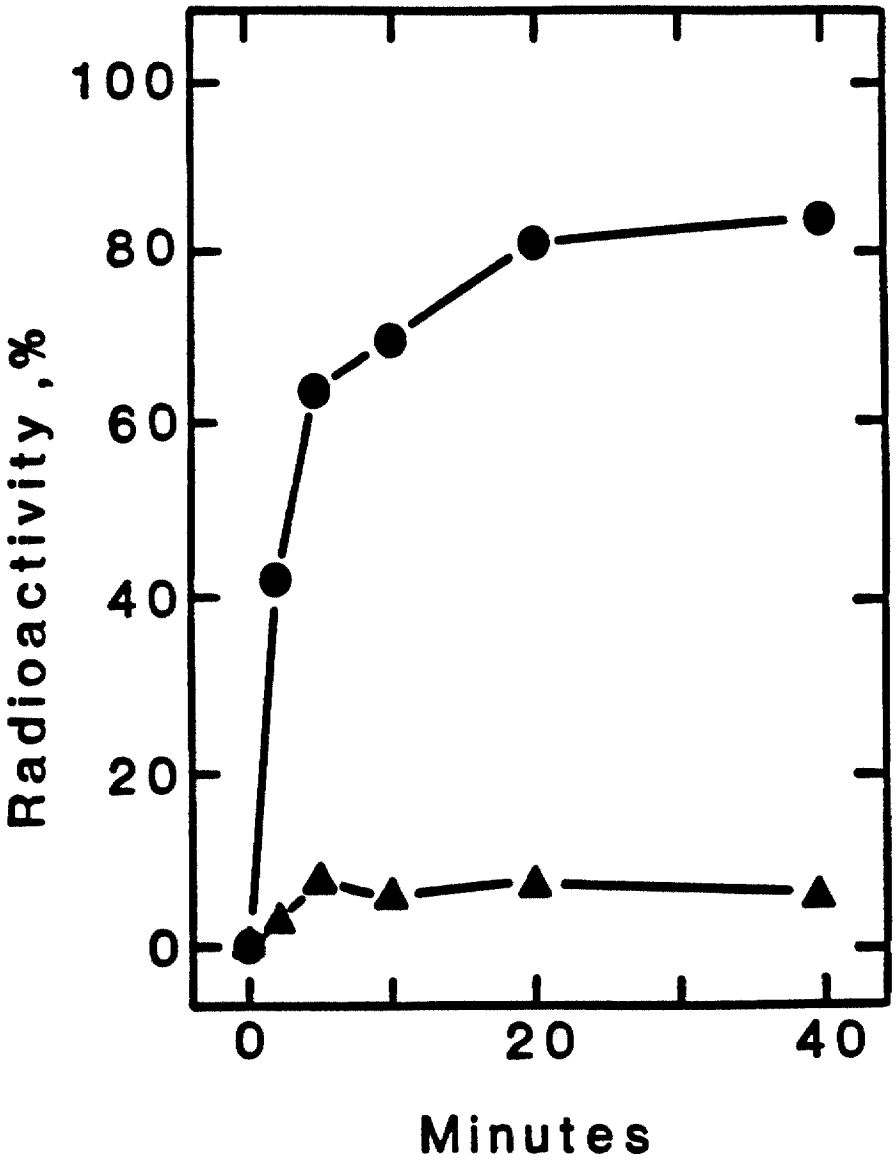

100
80
60
40
20
0
Radioactivity, %
0
100
200
300
400
500
MNase, U/mg

Fig.7. **DNA and poly(ADP-ribose) released during the digestion of isolated nuclei with MNase.** Nuclei from hyperthermia-MNNG- treated cells isolated at pH 6.5 were digested with MNase. At the indicated times aliquots were removed from the digestion mixtures and extracted twice for nucleosomes. The content of DNA (●) and poly(ADP-ribose) (▲) in the supernatant was determined. All experimental details were as described in Chapter III.


100
80
60
40
20
0
Radioactivity, %
0
20
40
Minutes

The MNase-resistant chromatin fraction, prepared by digestion of isolated nuclei with MNase, contains, in addition to approximately 20% of the total DNA, a nuclear structure that has been termed the nuclear matrix. The nuclear matrix concept has been previously introduced in Chapter I. To further pursue the nuclear localization of ADP-ribose polymers, the possibility of their association with the nuclear matrix was examined.

Isolation and Characterization of the Nuclear Matrix Fraction from C3H10T1/2 Cells.

The nuclear matrix has been operationally defined as the residual structure remaining after extensive nuclease digestion of isolated nuclei followed by extraction with buffers of high ionic strength. For the purpose of extensive DNA digestion, most nuclear matrix isolation procedures utilize DNase I, which because of its relatively low DNA sequence specificity, allows digestion at a high number of sites on the DNA (120). Likewise, the salt most commonly used is NaCl, but in some procedures KCl, $(NH_4)_2SO_4$, and LIS have been used (106, 121, and 122). The salt concentration used in various methods varies from 1 to 3 M in the case of NaCl and KCl (122 and 123), while 0.3 M $(NH_4)_2SO_4$ and 25 mM LIS have been generally used (106 and 122).

Fig. 8 shows the flow-chart of the steps involved in the preparation of nuclear matrices. It is important to note that, throughout the procedure, excessive centrifugal force and vortexing were avoided to prevent formation of tightly packed pellets and concomitant disruption of nuclear structures. Observation under the phase-contrast microscope revealed that after digestion of isolated nuclei with 250 U ml^{-1} DNase I, a structure that still retains the rounded shape characteristic of intact nuclei was obtained. However, high salt extraction resulted in apparently shrunken structures (not shown). After washing to remove salt, matrix preparations were examined by electron microscopy. A typical result is shown in Fig. 9. The nuclear matrix assumed a more or less rounded shape and was surrounded by elements of the nuclear envelope and pore complex. An extensive internal structure consisting of residual nucleoli and a fibrillar network could also be observed.

To further characterize the nuclear matrix preparations, dividing C3H10T1/2 cells were radiolabeled with [^{35}S]methionine at 50 μCi ml^{-1} in methionine-free medium containing 3 μg ml^{-1} methionine. This methionine concentration, which represents 10% of that contained in the complete medium, was required to allow normal cell growth (Fig. 10). The cells were incubated for 24 h to allow one more cell division. Under these radiolabeling conditions, no effect on cell division was observed (data not shown). The

Fig.8. **Flow-chart of the steps involved in the preparation of nuclear matrices.**

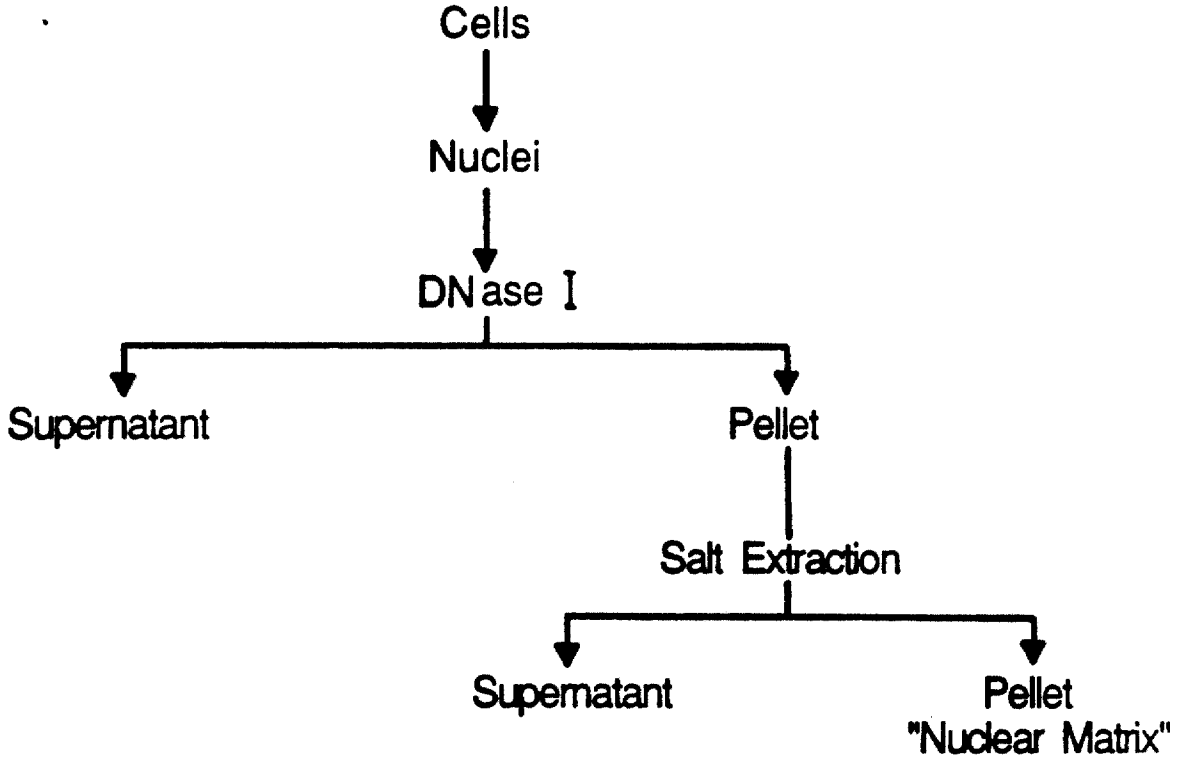

Cells
Nuclei
DNase I
Supernatant
Pellet
Salt Extraction
Supernatant
Pellet
"Nuclear Matrix"

Fig.9. **Electron micrograph of the nuclear matrix preparation from C3H10T1/2 mouse fibroblasts**. Nuclei and nuclear matrix were isolated as described in Chapter III. Sample preparation for thin-sectioning and subsequent electron microscopy was performed by Dr. Lawrence X. Oakford (Texas College of Osteophatic Medicine, Fort Worth TX.) as described in reference 123. Magnification X 21,400. PC, pore complex; Ne, nuclear envelope; RN, residual nucleoli.

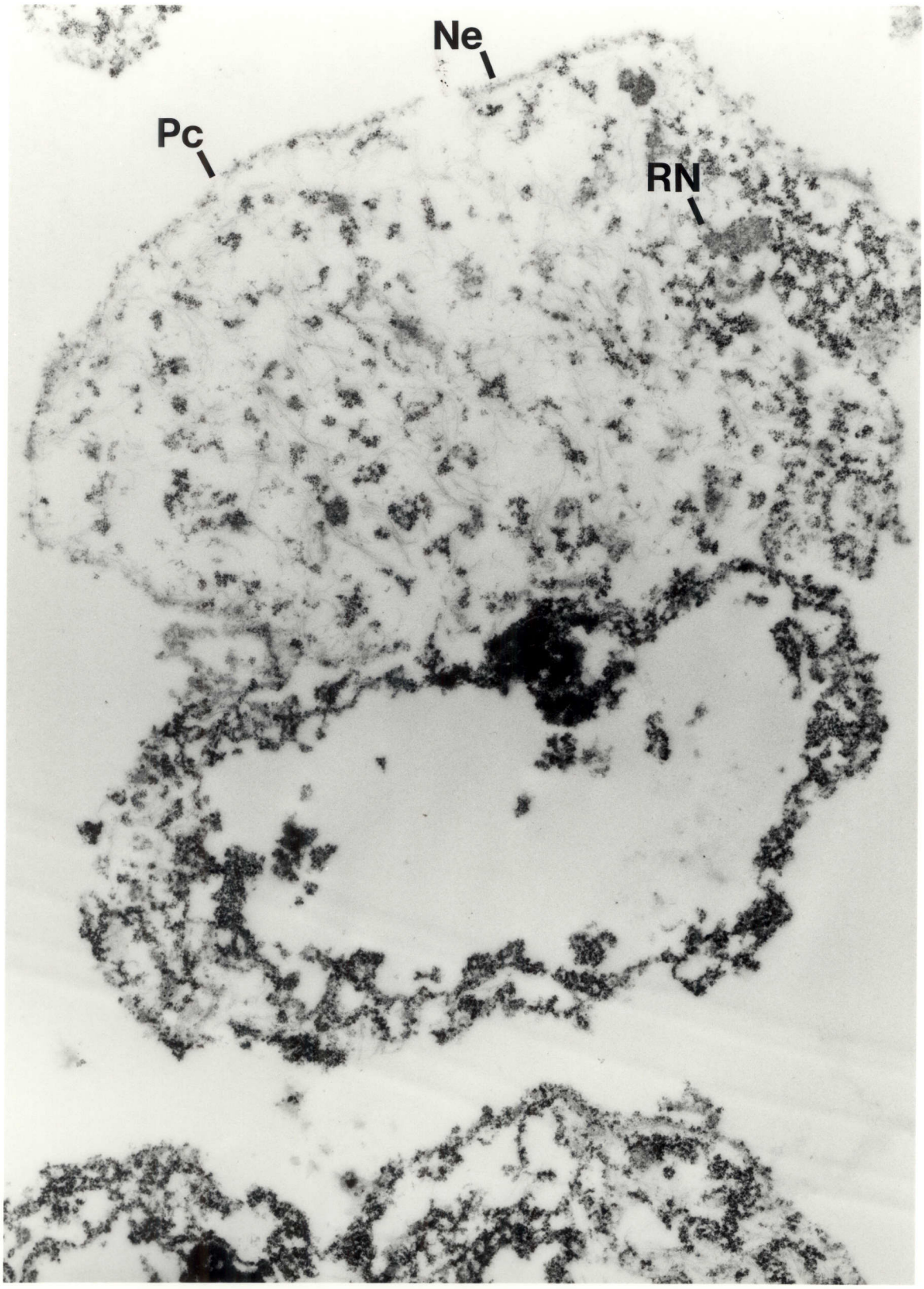
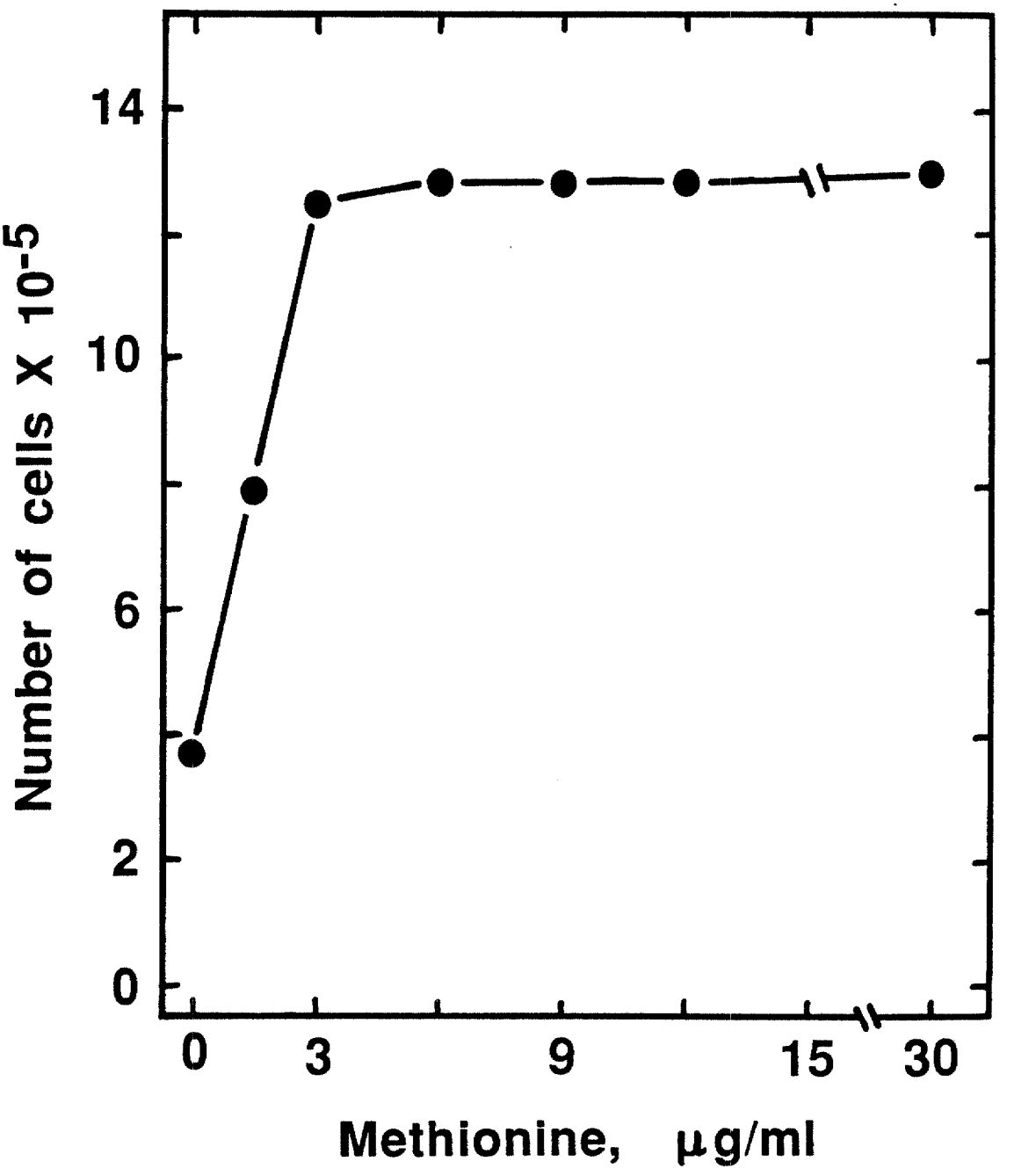

Ne
Pc
RN

Fig.10. **Cell growth of C3H10T1/2 cells at different concentrations of methionine in the culture-medium.** Cells seeded at a density of 2.8 x 10^4 per 35 mm dish were grown in complete medium. At 48 h, when the the cells reached a density of 3 x 10^5 cells per dish, the medium was discarded and fresh medium containing the indicated methionine concentration was added. Incubation was continued for 24 h and the cells were harvested and counted using a Coulter Counter.

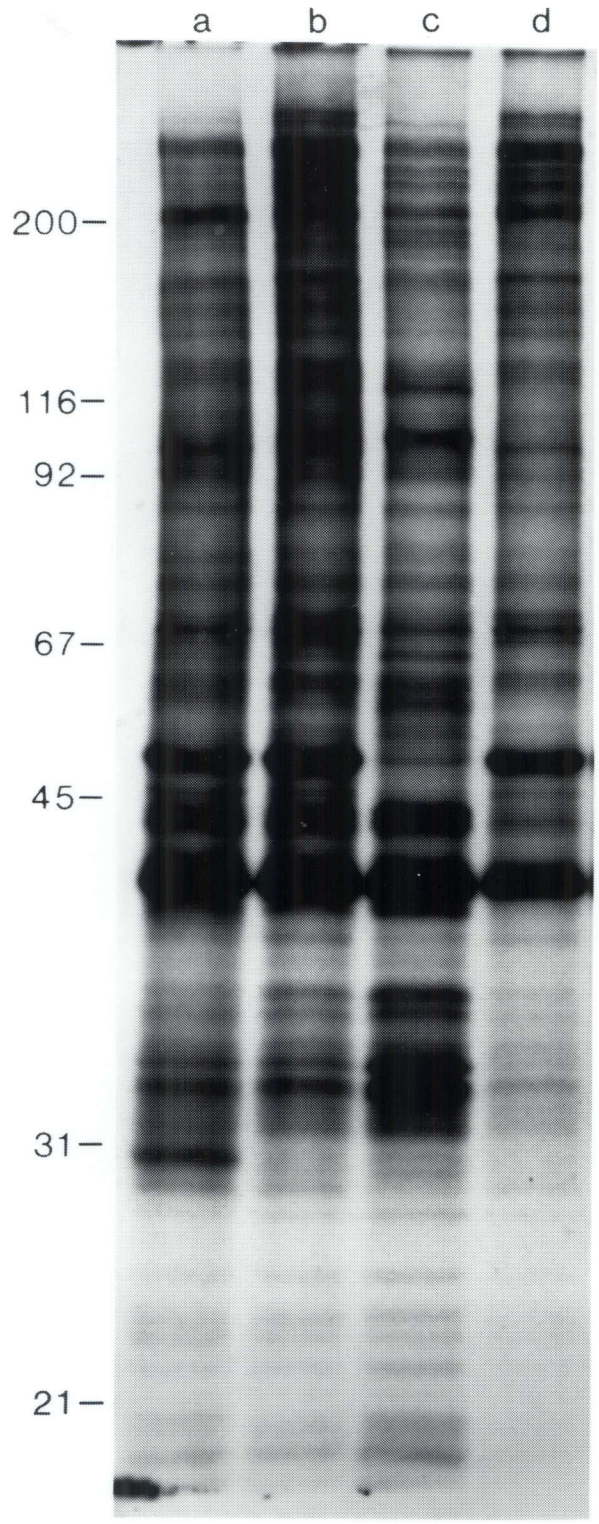

14
10
6
2
0
Number of cells X 10-5
0
3
9
15
30
Methionine, µg/ml

cells were fractionated and samples from the isolated nuclei, DNase I digestion supernatant, high salt extraction and nuclear matrix were analyzed by SDS-PAGE. Fig. 11 shows an autoradiograph from this analysis. The release of many nuclear polypeptides in the molecular weight range of 200 to 10 kDa was observed in both the DNase I digestion supernatant (lane b) and high salt extract (lane c). The salt fraction was particularly enriched in histone proteins as compared to the total nuclear fraction (lane a). The extraction of histone proteins by treatment of nuclei with high salt buffers has been previously described (106, 121-123). Other polypeptides rendered soluble by DNase I digestion and high salt extraction were those in the molecular weight range of 30 to 40 kDa. The nuclear matrix fraction was highly enriched in polypeptides of molecular weight above 45 kDa and practically devoid of low molecular polypeptides and histones (lane d) (Fig. 11).

Poly(ADP-ribose) Content of Nuclear Matrices.

The poly(ADP-ribose) and DNA content were measured in the different fractions generated during the preparation of nuclear matrices (Fig. 8). When intact nuclei were treated with DNase I, only a small fraction of poly(ADP-ribose) was released, although 88% of the DNA was rendered soluble by

Fig.11. **Protein composition of nucleus and nuclear fractions from C3H10T1/2 mouse fibroblasts.** The protein patterns of total nucleus (lane a), DNase digestion supernatant (lane b), 0.3 M $(NH_4)_2SO_4$ extract (lane c) and nuclear matrix (lane d) were obtained on a 9 to 18% linear gradient polyacrylamide gel. The figure shows the autoradiograph of the gel. Numbers on the left, molecular weight for myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (92 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa) and soybean trypsin inhibitor (21 kDa).


a
b
c
d
200
116
92
67
45
31
21

DNase I digestion (Table IV). In contrast, when the resulting pellet was extracted with 2M NaCl, 48% of the poly(ADP-ribose) was released from the soluble fraction along with most of the remaining DNA. It is interesting to note that the release of poly(ADP-ribose) by the high salt treatment was not dependent on prior nuclease digestion, as about the same release was observed when undigested nuclei were extracted with NaCl (Table IV). Following NaCl extraction, one half of the total poly(ADP-ribose) still remained in the nuclear matrix fraction even though this fraction contained less than 1% of the total DNA. The association of poly(ADP-ribose) with the nuclear matrix fraction could be explained in two ways: either the polymers are associated with the nuclear matrix *in vivo* or this association occurred *in vitro* during the preparation of the nuclear matrices. The possibility of an *in vitro* association was addressed in experiments in which radiolabelled polymer, previously isolated from nuclear matrices, was added to unlabelled nuclei during the preparation of the nuclear matrix fraction. The fact that the matrices obtained from these nuclei did not contain significant radiolabel (data not shown) does not completely rule out the second explanation, but strongly favors the first.

During the course of this study, there was some concern with the controversy over possible artifacts in the chemical

TABLE IV

Isolation of nuclear matrix with NaCl extraction

Nuclei from cells treated with hyperthermia and MNNG isolated at pH 6.5 were digested with with DNase I. Control samples not digested with DNase I were also included. Nuclear matrices were prepared by extracting the nuclei with a buffer containing 2 M NaCl as described in Chapter III.

	DNA		Poly(ADP-ribose)	
	Undigested			
Fraction	10^3 cpm	%	10^3 cpm	%
Nuclear	330	100	214	100
Digestion supernatant	10	3	8	4
2 M NaCl extract	31	9	93	43
Nuclear matrix	290	88	113	53
	DNase I-digested			
Nuclear	329	100	187	100
Digestion supernatant	291	88	10	5
2 M NaCl extract	38	12	90	48
Nuclear matrix	<2	<1	87	47

composition of the nuclear matrix. This controversy has been mainly centered on the hypertonic salt extraction commonly used in the last step of the nuclear matrix isolation. Thus, nuclear matrices were also isolated by two additional procedures which utilize extraction at a lower ionic strength. In one of these procedures, digested nuclei were extracted with 0.3M $(NH_4)_2SO_4$. The other procedure, as originally described, involved the extraction of histones with a low salt buffer containing 25 mM LIS followed by digestion of DNA by restriction enzymes (106). This procedure results in a fraction, termed the nuclear scaffold, which contains approximately 30% of the total DNA. It was found that extensive digestion of the LIS-extracted nuclei with DNase I released approximately 99% of the DNA into the digestion supernatant (Table V). This raises the possibility that the nuclear structure recovered after DNase I digestion could be similar to the nuclear matrix isolated by other procedures. Its actual composition, however, remains to be analyzed. Tables V and VI show the distribution of poly(ADP-ribose) among the subnuclear fractions obtained in the preparation of nuclear matrices using $(NH_4)_2SO_4$ and LIS. When $(NH_4)_2SO_4$ was used, 73% of the poly(ADP-ribose) was detected in the matrix fraction (Table VI), while 57% remained in this fraction when LIS extraction was used (Table V). As indicated for NaCl extraction, the release of

TABLE V

Isolation of nuclear matrix with LIS extraction

Nuclei from cells treated with hyperthermia and MNNG isolated at pH 6.5 were extracted with 25 mM LIS as previously described (109 and Chapter III). LIS-extracted nuclei were digested or not with DNase I.

	DNA		Poly(ADP-ribose)	
	Undigested			
Fraction	10^3 cpm	%	10^3 cpm	%
Nuclear	185	100	190	100
25 mM LIS extract	0	0	78	42
Digestion supernatant	4	2	<1	<1
Nuclear matrix	182	98	109	58
	DNase I-Digested			
Nuclear	182	100	190	100
25 mM LIS extract	<1	<1	81	43
Digestion supernatant	210	99	<1	<1
Nuclear matrix	2	1	108	57

TABLE VI

Isolation of nuclear matrix with $(NH_4)_2SO_4$ extraction

Experimental details were as described in the legend of Table IV except that nuclear matrices were prepared by extracting the nuclei with a buffer containing 0.3 M $(NH_4)_2SO_4$ as described in Chapter III.

	DNA		Poly(ADP-ribose)	
	Undigested			
Fraction	10^3 cpm	%	10^3 cpm	%
Nuclear	379	100	239	100
Digestion supernatant	<1	<1	8	3
0.3 M $(NH_4)_2SO_4$ extract	7	2	39	16
Nuclear matrix	372	98	192	80
	DNase I-Digested			
Nuclear	313	100	192	100
Digestion supernatant	274	88	9	5
0.3 M $(NH_4)_2SO_4$ extract	32	10	43	22
Nuclear matrix	3	1	140	73

poly(ADP-ribose) by $(NH_4)_2SO_4$ or LIS treatment was not dependent on prior nuclease digestion, since about the same release was observed with undigested nuclei (Tables V and VI).

The data concerning the nuclear distribution of poly(ADP-ribose) presented in Tables IV-VI was obtained with isolated nuclei from cells treated with hyperthermia and MNNG. As stated above, although by different mechanisms, MNNG or hyperthermic treatment both lead to accumulation of poly(ADP-ribose). Therefore, it was reasonable to consider the possibility that the difference in mechanism could result in a difference in the nuclear distribution of these polymers. Table VII shows the results obtained when this possibility was experimentally tested. A higher poly(ADP-ribose) content was observed in nuclear matrices from cells treated with hyperthermia or hyperhermia-MNNG as compared with untreated cells or cells treated with MNNG. The implication of this result in the turnover of the ADP-ribose polymers will be discussed below.

TABLE VII

The effect of cell treatment in the nuclear distribution of poly(ADP-ribose)

Isolated nuclei from untreated or cells treated with hyperthermia, MNNG or both treatments were fractionated and the poly(ADP-ribose) content was determined in the different fractions. Experimental details were described in Chapter III.

	Treatment			
	None	Hyperthermia	MNNG	Hyperthermia MNNG
Fraction	%			
Nuclear	100	100	100	100
Digestion supernatant	5	3	1	5
0.3 M $(NH_4)_2SO_4$ extract	41	34	36	22
Nuclear matrix	44	63	48	73

About 50% of the Total ADP-ribose Polymers are Tightly Associated to the Nuclear Matrix.

A set of experiments was designed to further examine the association of ADP-ribose polymers with the nuclear matrix. In one type of experiment, following DNase I digestion, the insoluble nuclear fraction was extracted three times with 2 M NaCl. The results in Table VIII show that about 60% of the polymers resisted the multiple salt extraction and remained with the nuclear matrix fraction.

Some procedures for preparation of nuclear matrices include, in addition to DNase I treatment, digestion with RNase (124, 125). Thus, it was decided to analyze the poly(ADP-ribose) content of nuclear matrices prepared with both DNase I and RNase digestion followed by extraction with 2 M NaCl. Although the treatment with RNase solubilized 85% of the nuclear RNA, as measured by TCA-precipitation of [^{14}C]uridine counts incorporated into RNA (Fig. 12), nearly 50% of ADP-ribose polymers resisted the digestion with the nucleases and the multiple salt extractions (Table IX).

In another type of experiment, following digestion with DNase I, nuclei were extracted with buffers of increasing salt concentration. The gradual increase up to 0.4 M $(NH_4)_2SO_4$ in the extraction buffer resulted in the linear release of up to approximately 50% of the polymers (Fig. 13).

TABLE VIII

The fraction of poly(ADP-ribose) associated with the nuclear matrix is resistant to multiple high salt extractions

Nuclei from cells treated with hyperthermia and MNNG isolated at pH 6.5 were digested with DNase I. Digested nuclei were extracted three times with a buffer containing 2M NaCl (See Chapter III for experimental details).

Fraction	DNA		Poly(ADP-ribose)	
	10^3 cpm	%	10^3 cpm	%
Nuclear	358	100	93	100
Digestion supernatant	326	91	5	5
2 M NaCl extract	25	7	27	29
2 M NaCl extract	2	>1	2	2
2 M NaCl extract	2	>1	1	1
Nuclear matrix	1	>1	58	62

Fig.12. **RNA digestion of isolated nuclei.** Nuclei isolated from [^{14}C]uridine labeled cells were treated with DNase I followed by digestion with 110 μg ml^{-1} RNase at 23°C. At the indicated times, aliquots from the digestion mixture were removed and centrifuged. The radioactivity released into the supernatant was determined (See Chapter III for experimental details).

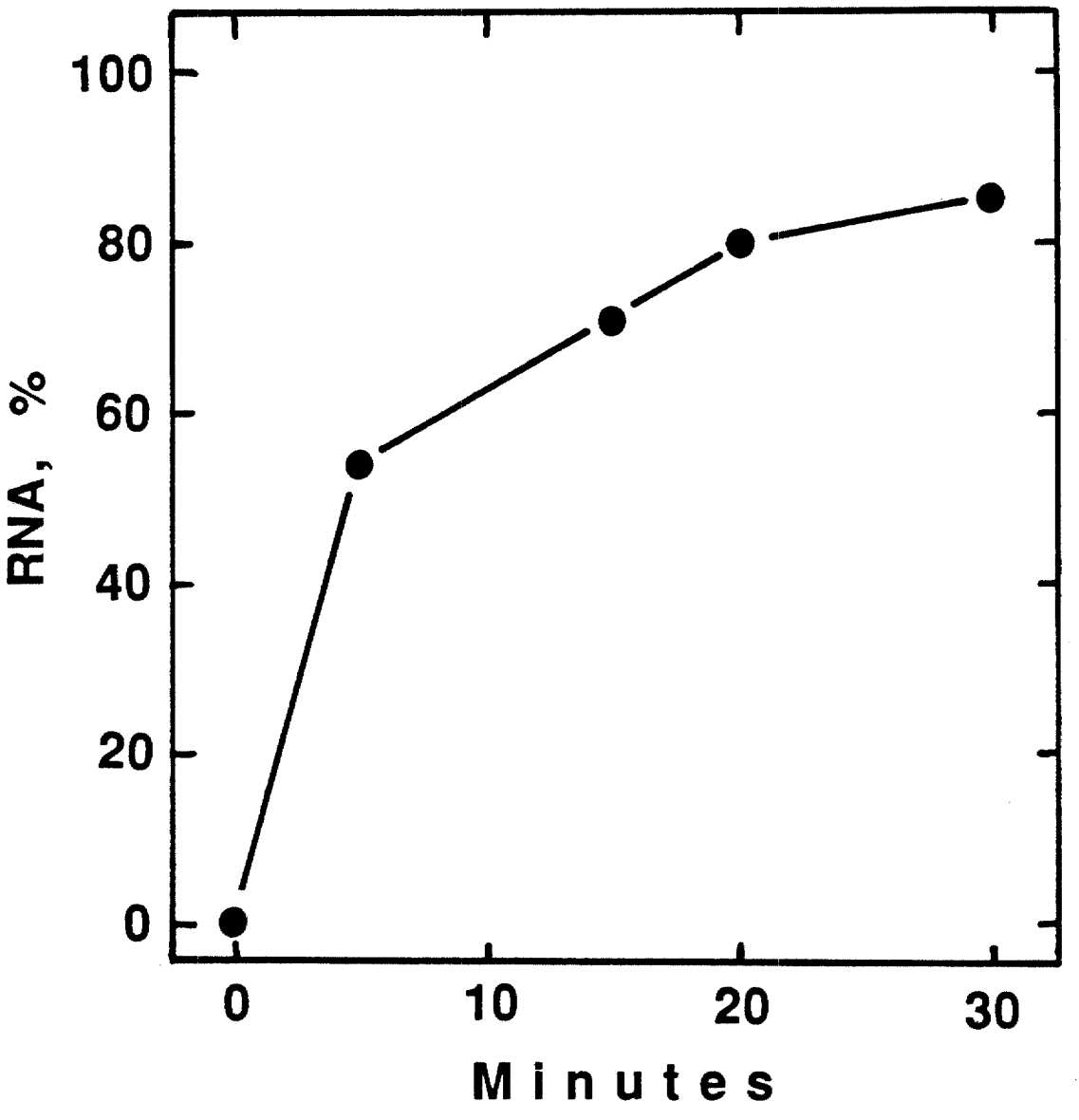

100
80
60
40
20
0
RNA, %
0
10
20
30
Minutes

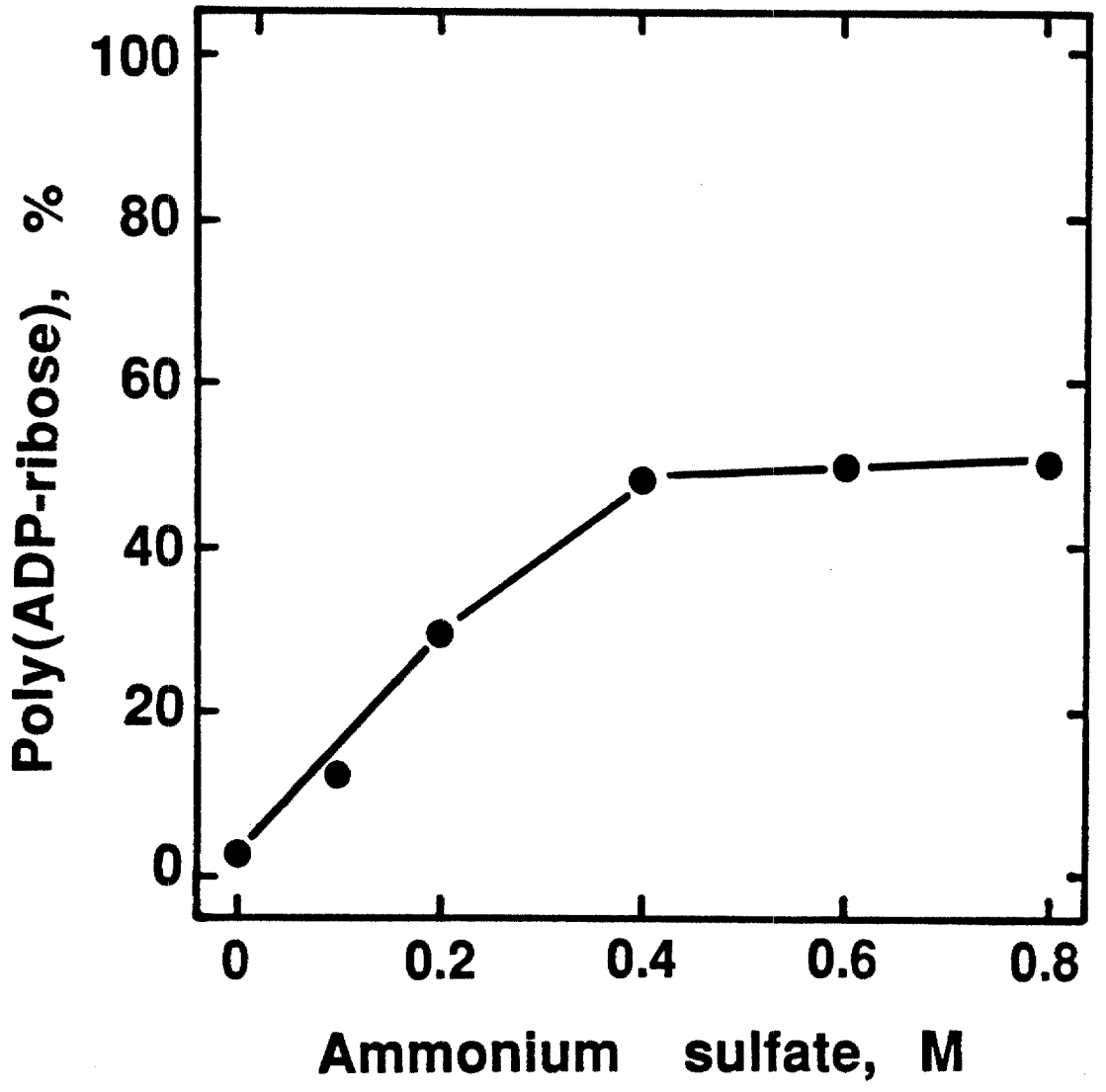
TABLE IX

The fraction of poly(ADP-ribose) associated with the nuclear matrix is resistant to DNase I-RNase digestion and multiple high salt extractions

Experimental details were as indicated in the legend to Table VIII with the exception that following DNase I digestion the isolated nuclei were digested with RNase as described in Chapter III.

Fraction	DNA		Poly(ADP-ribose)	
	10^3 cpm	%	10^3 cpm	%
Nuclear	411	100	69	100
Digestion supernatant	366	89	6	9
2 M NaCl extract	35	9	25	36
2 M NaCl extract	>1	>1	3	4
2 M NaCl extract	>1	>1	2	2
Nuclear matrix	>1	>1	33	48

Fig.13. **Extraction of poly(ADP-ribose) from DNase digested nuclei as a function of salt concentration in the extraction buffer.** DNase I digested nuclei were extracted for 10 min at 4°C with the indicated concentrations of $(NH_4)_2SO_4$ in the extraction buffer. The content of poly(ADP-ribose) in the salt extract was determined as indicated in Chapter III.


Poly(ADP-ribose), %
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
Ammonium sulfate, M

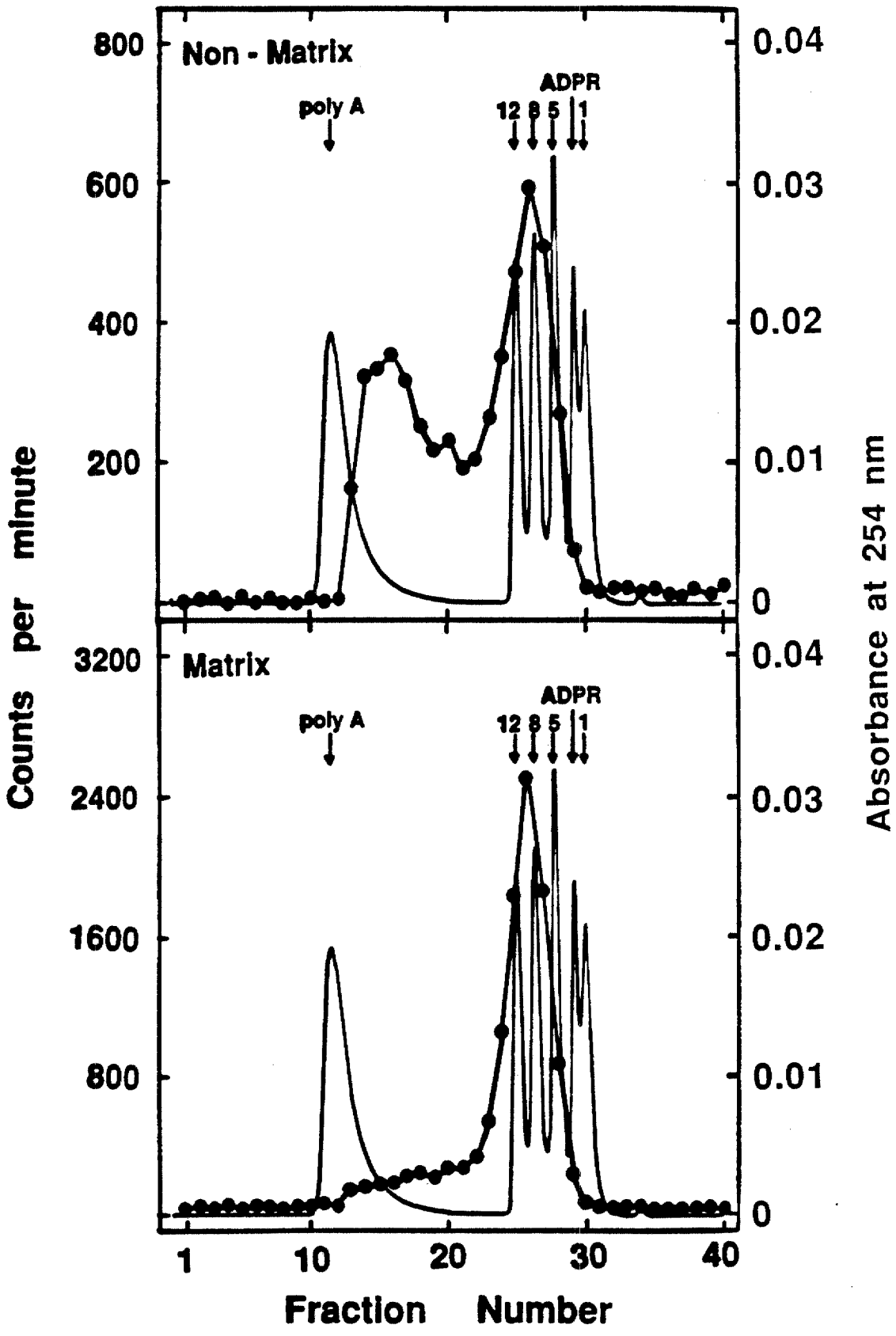
Moreover, a further increase up to 0.8 M $(NH_4)_2SO_4$, did not result in the extraction of additional polymers (Fig. 13). These results illustrate that the association of ADP-ribose polymers to the nuclear matrix is tight.

Relative Size of the Nuclear Matrix-Associated and non-Nuclear Matrix-Associated ADP-ribose Polymers.

Thus far two classes of ADP-ribose polymers have been defined by this study: the polymers extracted with high salt buffers which are termed here "non-matrix-associated", and the polymers resistant to salt extraction termed "nuclear matrix-associated".

Previous studies from this laboratory have indicated a heterogeneity in the size and complexity of ADP-ribose polymers generated *in vivo* (18). Furthermore, these studies have demonstrated the effectiveness of molecular sieve chromatography in the sizing of these polymers (18). Therefore, this methodology was applied in the present study to determine whether there were any differences in size between the nuclear matrix and non-nuclear matrix-associated polymers. Thus, [^{3}H]adenine-labelled ADP-ribose polymers from the salt extract and nuclear matrix fractions were isolated and subjected to molecular sieve chromatography. The results from this analysis are shown in Fig. 14. The

Fig.14. **Molecular sieve chromatography of non-matrix and matrix-associated ADP-ribose polymers.** Non-matrix (upper panel) and matrix-associated (lower panel) polymers. The peaks for the positions of polyA, $(Ap)_{11}A$ (12), $(Ap)_7A$ (8), $(Ap)_4A$ (5), ADPR and AMP (1) are indicated. (_____) absorbance at 254 nm and (●) radioactivity.


Non - Matrix
poly A
ADPR
12 8 5 1
800
600
400
200
0
0.04
0.03
0.02
0.01
0
Matrix
poly A
ADPR
12 8 5 1
3200
2400
1600
800
0.04
0.03
0.02
0.01
0
1
10
20
30
40
Fraction Number
Counts per minute
Absorbance at 254 nm

nuclear matrix fraction showed a peak at a low molecular weight, whereas, in the salt extracted fraction, two peaks, corresponding to high and low molecular weight polymers were observed. Gel filtration analysis of ADP-ribose polymers generated *in vivo* have revealed that the high molecular weight peak represents large complex, multi branched polymers, while the low molecular weight peak contains short polymers (18). Thus, it was concluded that the non-matrix associated polymers contained both complex and short polymers, while the matrix associated poly(ADP-ribose) represents primarily short chain length polymers.

This analysis allowed a further classification of ADP-ribose polymers, based on their nuclear distribution and size. Thus, three classes could be defined: non-matrix-associated short polymers and complex polymers, and matrix-associated short polymers. It was of interest to determine which polymers, complex or short, were more labile to extraction with salt. To this end, DNase I digested nuclei were extracted at different $(NH_4)_2SO_4$ concentrations and subjected to molecular sieve chromatography. The results are shown in Figs. 15 and 16. At salt concentrations lower than 0.4 M only short chain-length polymers were extracted, whereas at 0.4 M or higher concentration, the complex chain length polymers were sequentially solubilized in the salt extract (Figs. 15 and 16, upper panels). As a confirmation,

Fig.15. **Molecular sieve chromatography of ADP-ribose polymers extracted from DNase digested nuclei with 0.2 M and 0.4 M $(NH_4)_2SO_4$.** Purified polymers from the supernatants (upper panels) and pellets (lower panels) of the 0.2 M (panels A and C) and 0.4 M (panels B and D) $(NH_4)_2SO_4$ extracted nuclei. All symbols are as in Fig. 14.

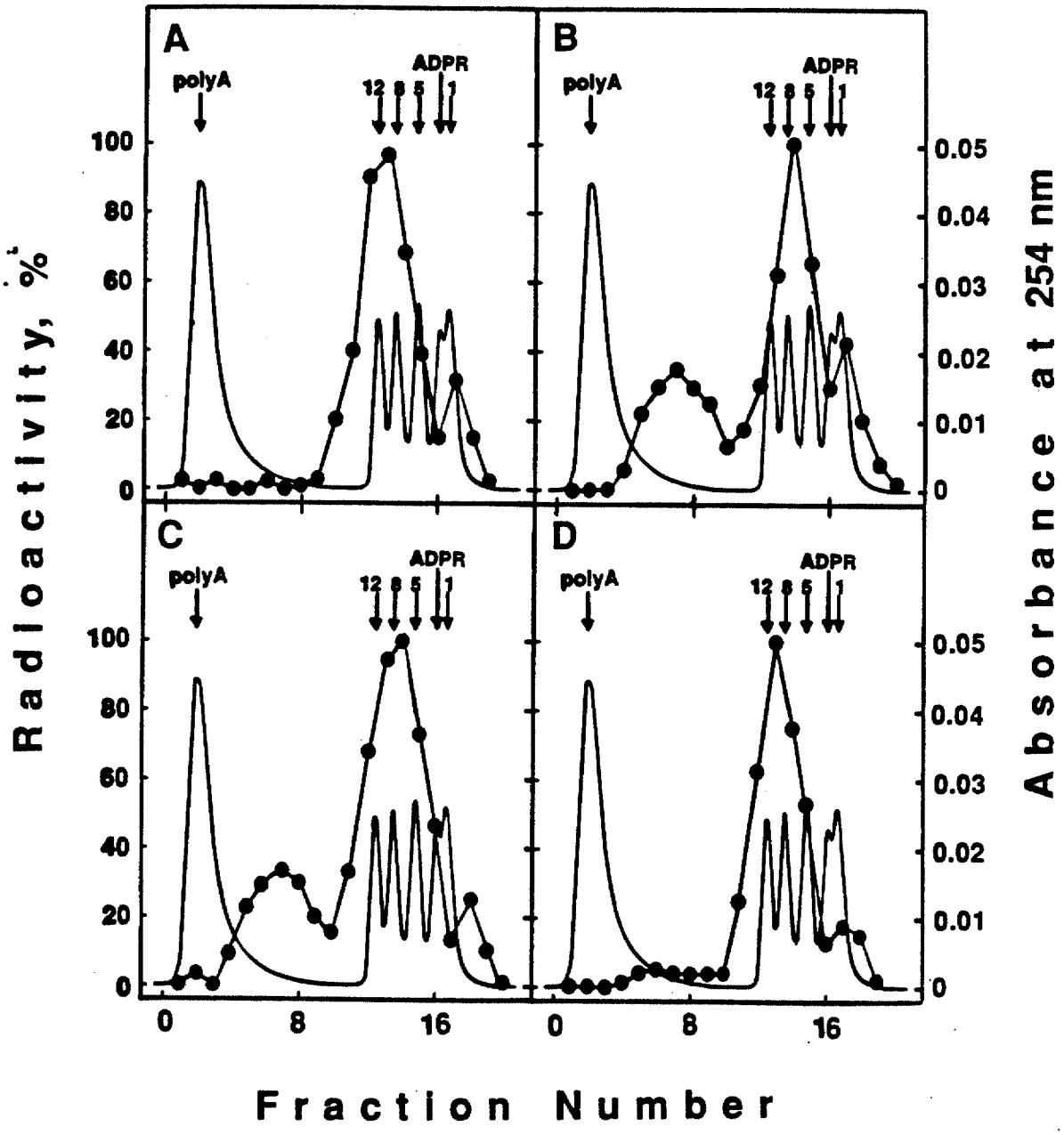
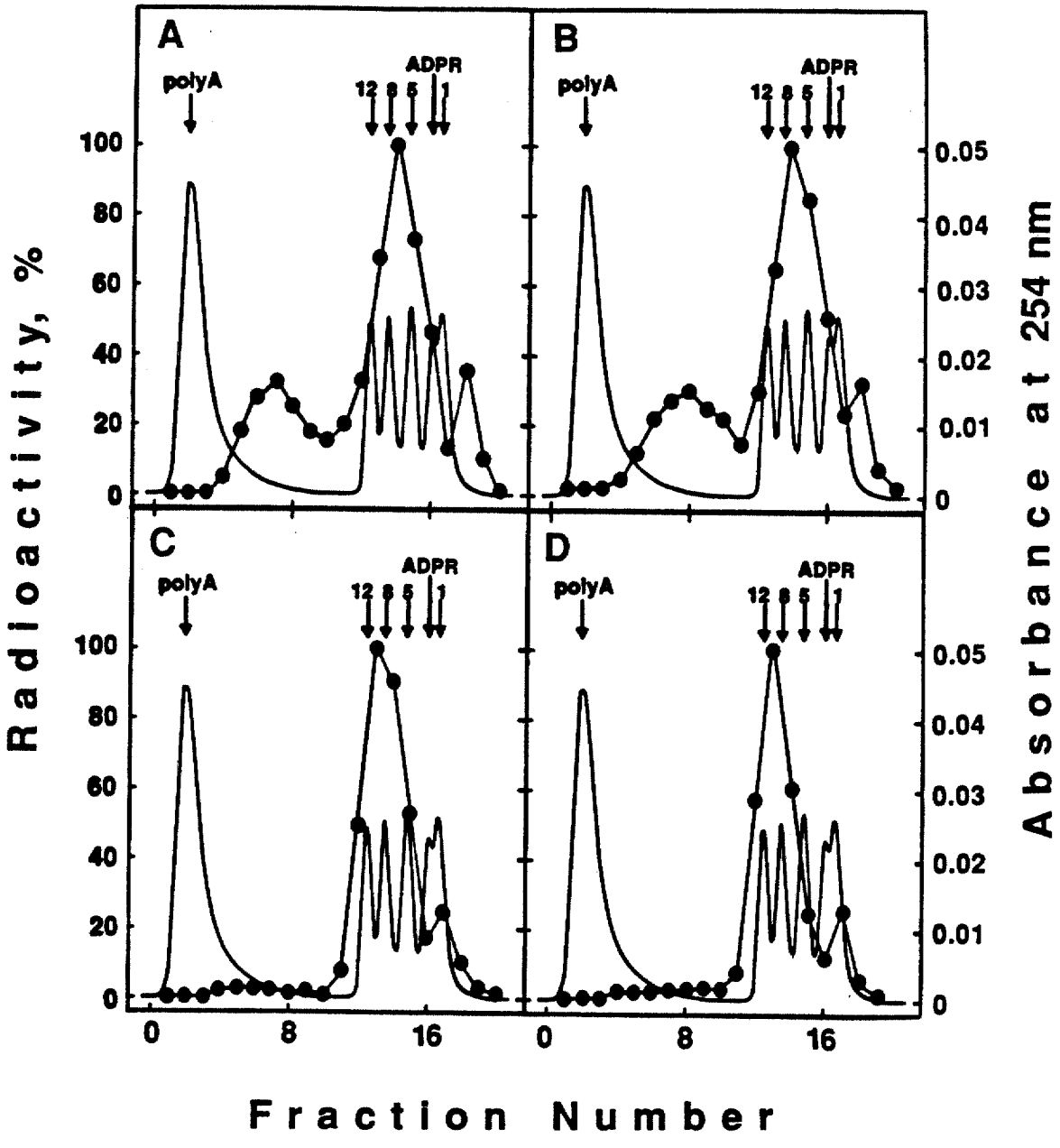

A
B
C
D
polyA
ADPR
12
8
5
1
100
80
60
40
20
0
0.05
0.04
0.03
0.02
0.01
0
8
16
Radioactivity, %
Absorbance at 254 nm
Fraction Number

Fig.16. **Molecular sieve chromatography of ADP-ribose polymers extracted from DNase digested nuclei with 0.6 M and 0.8 M $(NH_4)_2SO_4$.** Purified polymers from the supernatants (upper panels) and pellets (lower panels) of the 0.6 M (panels A and C) and 0.8 M (panels B and D) $(NH_4)_2SO_4$ extracted nuclei. All symbols are as in Fig. 14.


A
B
C
D
polyA
ADPR
12
8
5
1
Radioactivity, %
Absorbance at 254 nm
Fraction Number
0
8
16
20
40
60
80
100
0.01
0.02
0.03
0.04
0.05

the complementary result was seen in the salt extracted pellets. At $(NH_4)_2SO_4$ concentrations up to 0.4 M these pellets contained long and short polymers; while further extraction with salt concentrations above 0.4 M resulted in the depletion of long polymers (Figs. 15 and 16, lower panels).

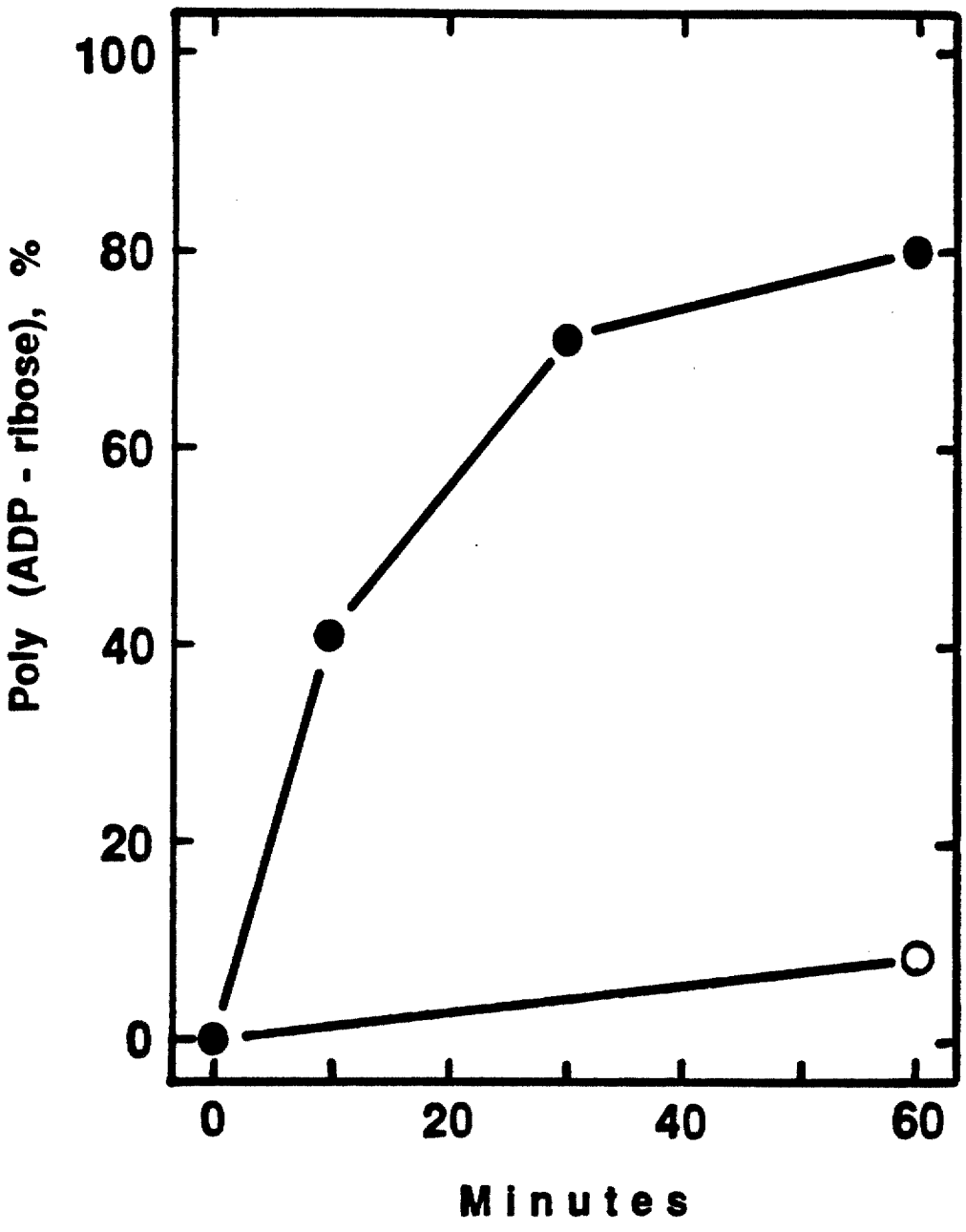
Probing of the Nuclear Matrix with Poly(ADP-ribose) Glycohydrolase.

These studies have shown that about 50% of the total polymer residues are associated with the nuclear matrix. There is the possibility that these polymers mediate the association, via covalent or non-covalent interactions, of some proteins to the nuclear matrix. One experimental approach that was used to examine this possibility involved the digestion of the nuclear matrix-associated polymers and analysis of the material released. For this purpose, the enzyme poly(ADP-ribose) glycohydrolase was selected because of its specificity and high affinity for cleavage of the glycosidic ribose-ribose linkages in both linear and branched ADP-ribose polymers (36, 126). The poly(ADP-ribose) glycohydrolase preparation used in these experiments was purified 152-fold (127). This preparation had an Specific Activity of 122 U mg^{-1} protein (in some experiments an 8000-

fold purified enzyme was used). One unit of poly(ADP-ribose) glycohydrolase produces 1 nmol of ADP-ribose min^{-1} under standard conditions (127).

Isolated nuclear matrices from cells treated with hyperthermia and MNNG were digested with 5 U ml^{-1} of poly(ADP-ribose) glycohydrolase for 1 h at 30°C. Fig. 17 shows the time-course of the digestion. After 60 min of treatment, the bulk of ADP-ribose polymer residues were rendered soluble by the action of glycohydrolase. No appreciable polymer degradation was observed during incubations of nuclear matrices in the absence of this enzyme. In order to determine if protein was released by digestion of nuclear matrices with glycohydrolase,dividing cells were labelled with Tran [^{35}S]-label^{TM} under the conditions indicated above for cellular labeling with [^{35}S]methionine. Tran [^{35}S]-label^{TM} is a hydrolysate of *Escherichia coli* grown in the presence of [^{35}S]H_2SO_4. This preparation is used for protein labeling instead of [^{35}S]methionine because it contains a mixture of [^{35}S]-labeled amino acids (see Methods), which provides a higher specific activity when used for metabolic labeling. Nuclear matrices were prepared from the radiolabeled cells and digested with poly(ADP-ribose) glycohydrolase. In these cell fractionation experiments, the addition of three different protease inhibitors to the buffers was important, since otherwise significant protease activity was detected during

Fig.17. **Digestion of nuclear matrix-associated poly(ADP-ribose) with poly(ADP-ribose) glycohydrolase.** Isolated nuclear matrix fraction was digested with 5 U ml^{-1} of poly(ADP-ribose) glycohydrolase at 30°C. At the indicated times aliquots were removed and the content of poly(ADP-ribose) in the digested (●) and undigested (o) incubations was determined as described in chapter III.


100
80
60
40
20
0
Poly (ADP - ribose), %
0
20
40
60
Minutes

the incubation with poly(ADP-ribose) glycohydrolase. Evidence supporting this observation will be shown below. Thus, 1 mM PMSF, 100 KIU ml^{-1} aprotinin and 100 $\mu g\ ml^{-1}$ pepstatin from fresh concentrated solutions were added to all the buffers immediately before use. This combination of protease inhibitors shows a wide inhibition spectrum since it inhibits serine and acid proteases as well as trypsin-like proteases (128). The results, presented in Table X, show that in 60 min of digestion, along with 80% of digested polymers, a net release of 2% of the nuclear matrix protein was detected. In the control incubation without glycohydrolase, only 0.1% of the protein was released together with a small fraction of polymer (Table X). In order to analyze which proteins were released by the probing of nuclear matrices with poly(ADP-ribose) glycohydrolase, samples from the digested pellets and supernatants were analyzed by SDS-PAGE. An autoradiograph of one such gel is shown in Fig. 18. In the supernatant from the control incubation in the absence of glycohydrolase, no significant amount of protein was released (lane b), whereas in the glycohydrolase digestion supernatant proteins were detected (lane d). The enrichment for some proteins in this fraction, as compared with the digested nuclear matrix (lane c) was evident. In particular, polypeptides with molecular weights of 170 kDa, 116 kDa, 100 kDa, 70 kDa, 67 kDa and, 50 kDa were

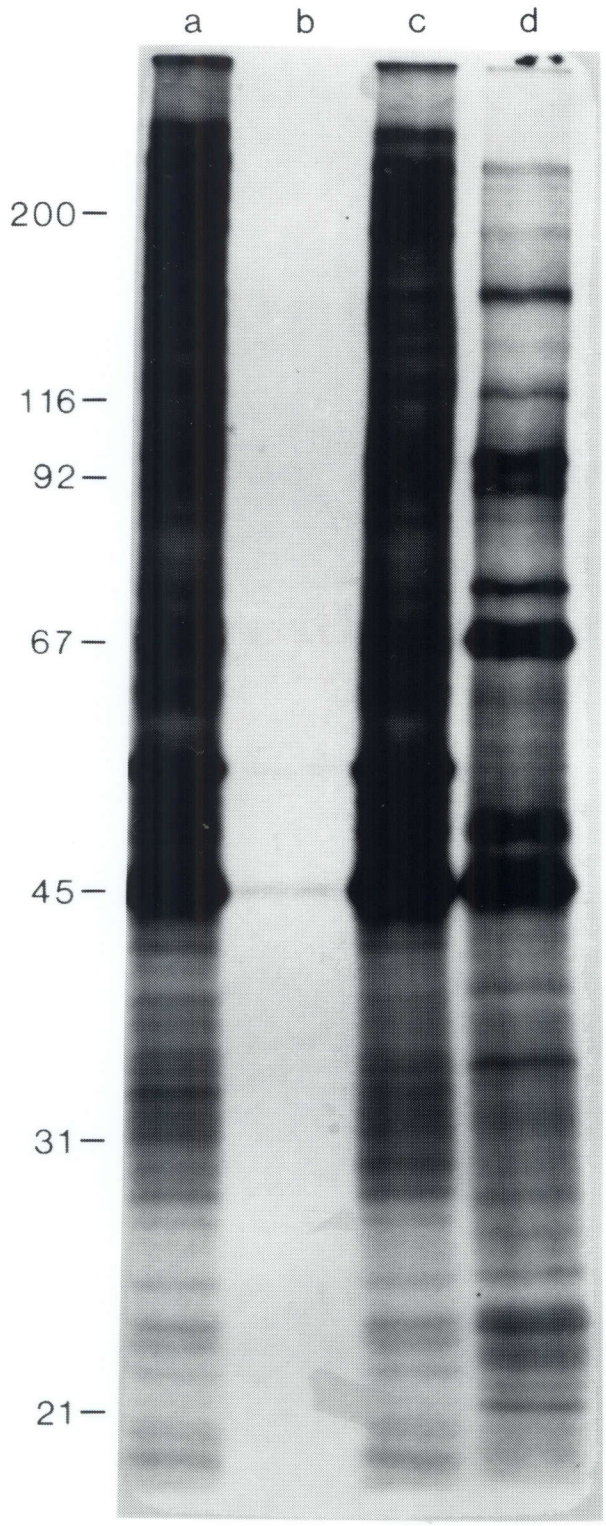
TABLE X

Probing nuclear matrices with poly(ADP-ribose) glycohydrolase

Isolated nuclear matrices from [^{35}S]methionine labeled cells were digested with 5 U ml^{-1} of poly(ADP-ribose) glycohydrolase for 1 h at 30°C. Following centrifugation of digestion mixtures at 3000 x g for 10 min at 4°C the poly(ADP-ribose) content in the pellets was determined. The protein fraction released into the digestion supernatant was estimated by liquid scintillation counting.

	Poly(ADP-ribose)	Protein
Condition	% Released	
Control	8	0.1
Poly(ADP-ribose) Glycohydrolase	80	2.0

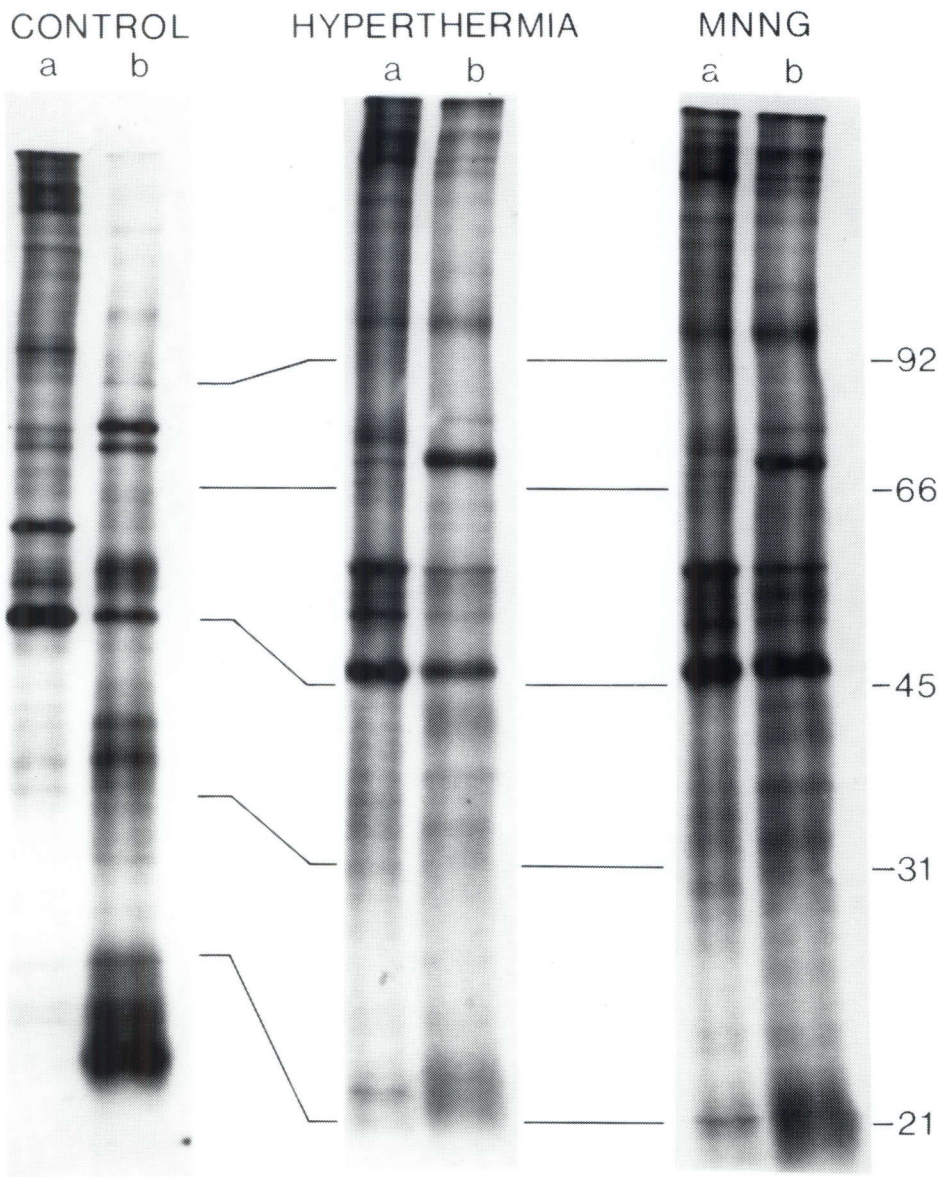
Fig.18. **Protein released from the nuclear matrix by poly(ADP-ribose) glycohydrolase digestion.** Isolated nuclear matrices from [^{35}S]-labeled cells were digested for 1 h at 30°C with 5 U ml^{-1} of poly(ADP-ribose) glycohydrolase. After centrifugation of the digestion mixtures, the pellets (lanes a and c) and supernatants (lanes b and d) from the undigested (lanes a and b) and digested (lanes c and d) nuclear matrices were analyzed by SDS-PAGE. The figure shows the autoradiograph of the gel. Numbers on the left indicate molecular weight in kDa.


a
b
c
d
200
116
92
67
45
31
21

released. In addition, an enrichment of polypeptides in the molecular weight of 15 to 30 kDa, characteristic of the histone proteins, was observed. The prominent release of a polypeptide of 45 kDa was also seen. However this polypeptide, as will be shown below, was also occasionally released during incubation of nuclear matrices without glycohydrolase. Attempts to identify these polypeptides by the use of antibodies raised against nuclear matrix proteins will be described below.

The data shown in Fig. 18 were obtained with nuclear matrices from cells treated with hyperthermia and MNNG. Thus, the possibility of a different electrophoretic pattern in the polypeptides released by poly(ADP-ribose) glycohydrolase treatment of nuclear matrices from untreated or cells treated singly with hyperthermia or MNNG was investigated. The major difference observed in such an experiment was that the release of the 170 kDa polypeptide was not as prominent in untreated and MNNG or hyperthermia treated cells as in the hyperthermia and MNNG treated cells (compare lanes b, Fig. 19 and lane d, Fig. 18). In the untreated cells as compared to the other conditions, only a minor amount of the 100 kDa polypeptide was released, whereas the release of the 70 kDa polypeptide was enhanced while the release of the 67 kDa protein was decreased.

Fig.19. **Proteins released by poly(ADP-ribose) glycohydrolase digestion of nuclear matrices from untreated, hyperthermia and MNNG-treated cells.** The radiolabeling of the cells with [^{35}S]methionine and cell treatments were as described in Chapter III. Digestions of the nuclear matrices were carried out as indicated in the legend to Fig.18. The pellets (lanes a) and supernatants (lanes b) from the digestion mixture were analyzed by SDS-PAGE. The figure shows the autoradiograph of the gel.


CONTROL
a
b
HYPERTHERMIA
a
b
MNNG
a
b
-92
-66
-45
-31
-21

Ethacridine, an Inhibitor of Poly(ADP-ribose) Glycohydrolase Prevents the Release of Proteins During Glycohydrolase Treatment.

The poly(ADP-ribose) glycohydrolase used in these studies was a partially purified preparation from bull testes (127). Although this preparation was shown to be virtually free of nuclease or phosphodiesterase activity (127), there is still a minor possibility that the protein released by digestion of nuclear matrices with this enzyme preparation could be due to the action of contaminating activities rather than to poly(ADP-ribose) glycohydrolase activity. This possibility was further examined by experiments where the digestion with poly(ADP-ribose) glycohydrolase was carried out in the presence of an inhibitor of this enzyme. In 1985, Tavassoli *et al.* (129) reported that a group of DNA intercalating agents inhibite poly(ADP-ribose) glycohydrolase activity. Among these inhibitors, ethacridine was shown to have a potent inhibitory effect at concentrations as low as 50 μM. Thus, experiments were done in which the digestion of isolated nuclear matrix with poly(ADP-ribose) glycohydrolase was performed in the presence of 100 μM ethacridine. Under the digestion conditions used in these experiments (see methods), 100 μM ethacridine caused complete inhibition of the poly(ADP-ribose) glycohydrolase activity (Table XI).

TABLE XI

Inhibition of poly(ADP-ribose) glycohydrolase and protein release by ethacridine

Isolated nuclear matrices from [35S]methionine labeled cells were digested with poly(ADP-ribose) glycohydrolase as indicated in legend to Fig. 17 except that one of the digestions was carried out in the presence of 100 μM ethacridine.

	Poly(ADP-ribose)	Protein
Condition	% Released	
Control	8	0.3
Poly(ADP-ribose) Glycohydrolase	80	2.4
Poly(ADP-ribose) glycohydrolase + Ethacridine	3	0.3

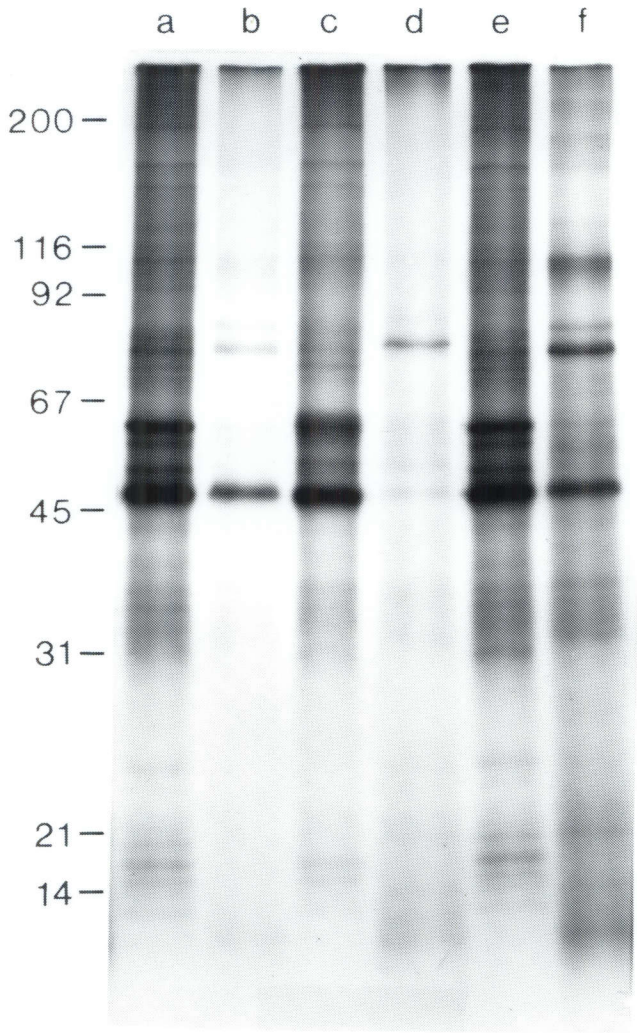
Ethacridine not only blocked the degradation of poly(ADP-ribose) by glycohydrolase but also resulted in the inhibition of protein release (Table XI, also compare lanes d and f on Fig.20).

Attempts to Identify Some of the Proteins Released by Probing the Nuclear Matrix with Poly(ADP-ribose) glycohydrolase.

The association of DNA topoisomerases with the nuclear matrix in *Drosophila*, chicken and mammalian cells, in the case of topoisomerase II (104, 130, 131), and in rat liver and HeLa cells for topoisomerase I (132, 133), has been well documented. From 60 to 80% of the topoisomerase II molecules have been recovered with the nuclear matrix or nuclear scaffold in HeLa cells (131). The molecular weight of topoisomerase II was shown to be 170 kDa, while a molecular weight of 100 kDa has been reported for topoisomerase I (134-136). Moreover, since topoisomerase I is very sensitive to proteolysis, a major proteolytic fragment of approximately 70 kDa which still retains enzymatic activity has consistently been detected (135, 136). Based on these observations, the possibility that the polypeptides of 170 kDa, 100 kDa and 70 kDa represented topoisomerase II and I polypeptides, respectively, was examined.

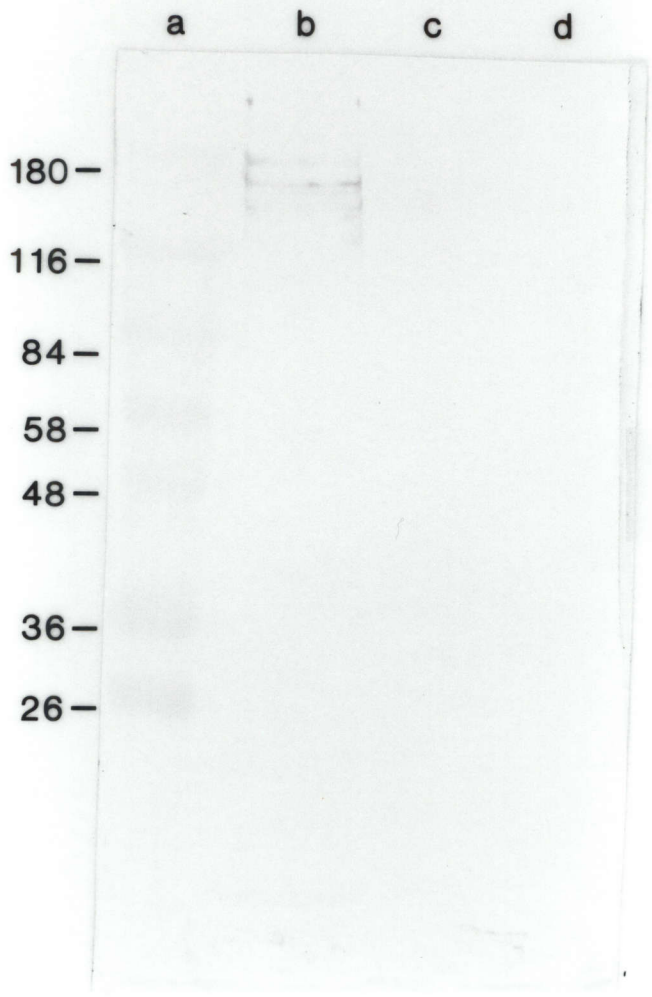
The enrichment of topoisomerase II activity or protein

Fig.20. **Ethacridine, an inhibitor of poly(ADP-ribose) glycohydrolase blocks the release of proteins.** Nuclear matrices were digested with poly(ADP-ribose) glycohydrolase (lanes e and f), as indicated in the legend to Fig. 18, except that one of the digestions was carried out in the presence of 100 μM ethacridine (lanes c and d). Lanes a and b correspond to the control incubation in the absence of glycohydrolase. The figure shows the autoradiograph of the SDS-PAGE analysis of the pellets (lanes a,c and e) and supernatants (lanes b, d and f) of the digestion mixtures.


a
b
c
d
e
f
200
116
92
67
45
31
21
14

in rapidly dividing cells and its loss in non-dividing cells has been reported (137, 138). Thereby, nuclear matrices from growing C3H10T1/2 cells were prepared, digested with poly(ADP-ribose) glycohydrolase and the resulting nuclear fractions were analyzed by SDS-PAGE. The proteins were transferred to nitrocellulose filters and probed with a topoisomerase II antiserum. This antiserum was prepared in rabbit and it was raised against the carboxy terminal half of a recombinant polypeptide of human topoisomerase II (Liu, L. unpublished). Fig. 21 shows the results when topoisomerase II antiserum bound to the filter was detected with an anti-rabbit IgG-alkaline phosphatase conjugate followed by the color reaction of this enzyme with BCIP and NBT as substrates. In the $(NH_4)_2SO_4$ extract, topoisomerase II antigen was detected as a triplet of bands in the molecular weight of around 170 kDa (lane b, Fig. 21). This pattern of bands for topoisomerase II has been observed by other workers (130). However, no antigen was detected in either the pellet or supernatant of nuclear matrices digested with poly(ADP-ribose) glycohydrolase (lanes c and d, Fig. 21). In support of these results, topoisomerase II activity could not be detected when the glycohydrolase-digestion supernatant of isolated nuclei was tested in an specific assay for this enzyme using catenated [^{3}H]kinetoplast DNA as substrate (not shown).

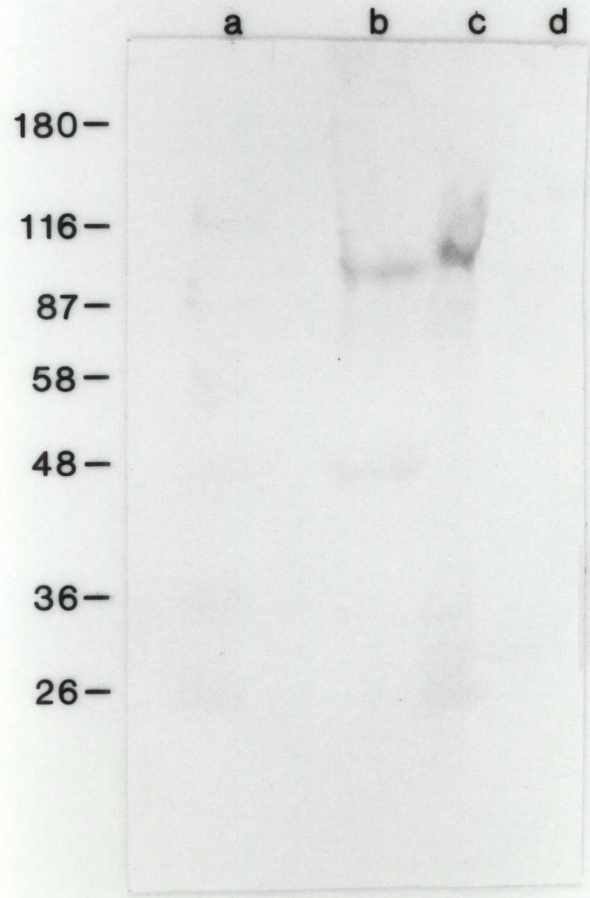
Fig.21. **Probing the nuclear fractions with an anti-topoisomerase II antibody**. The nuclear fractions were analyzed in a 9 to 18% polyacrylamide gradient gel, and the proteins were transferred to a nitrocellulose filter. The filter was screened with a rabbit anti-topoisomerase II antibody. The figure shows the development of the filter with an anti-rabbit-alkaline phosphatase conjugate. (lane a) molecular weight markers, (lane b) $(NH_4)_2SO_4$ extract, (lanes c and d) pellet and supernatant, respectively, from the digestion mixture of nuclear matrices with poly(ADP-ribose) glycohydrolase. Numbers on the left indicate molecular weight in kDa. All experimental details were as described in chapter III.


a
b
c
d
180
116
84
58
48
36
26

For probing of the nuclear fractions with an antibody against topoisomerase I, an IgG fraction from plasma of patients with scleroderma (Scl-70) containing antibodies that react with the 70 kDa fragment of topoisomerase I was used (136). While cross-reacting material at a molecular weight of approximately 100 kDa was detected in the $(NH_4)_2SO_4$ extract and in the pellet from nuclear matrices digested with poly(ADP-ribose) glycohydrolase (lanes b and c, Fig. 22), a signal was not observed in the supernatant from this digestion (lane d, Fig. 22). It should be noted that in an effort to increase the sensitivity of the western blot, the gels were overloaded with protein which resulted in distortion of the bands (Fig. 22).

As stated above, the recovery of a small fraction of automodified poly(ADP-ribose) polymerase with the nuclear matrix has been reported (49-51). Hence, it was reasonable to investigate whether the 116 kDa polypeptide, solubilized by the treatment of nuclear matrices with poly(ADP-ribose) glycohydrolase, was identical with poly(ADP-ribose) polymerase. Thus, purified poly(ADP-ribose) polymerase, as well as proteins from the different nuclear fractions immobilized in nitrocellulose filters, were probed with a anti-poly(ADP-ribose) polymerase monoclonal antibody (139). This monoclonal antibody, termed $C^{II}20$, was raised against the 46 kDa proteolytic fragment of poly(ADP-ribose)

Fig.22. **Probing the nuclear fractions with an anti-topoisomerase I antibody**. Experimental details were as indicated in legend to Fig. 21, except that the nitrocellulose filter was screened with a human IgG fraction containing anti-topoisomerase I antibodies. Numbers and letters are as indicated in the legend to Fig. 21.


a
b
c
d
180
116
87
58
48
36
26

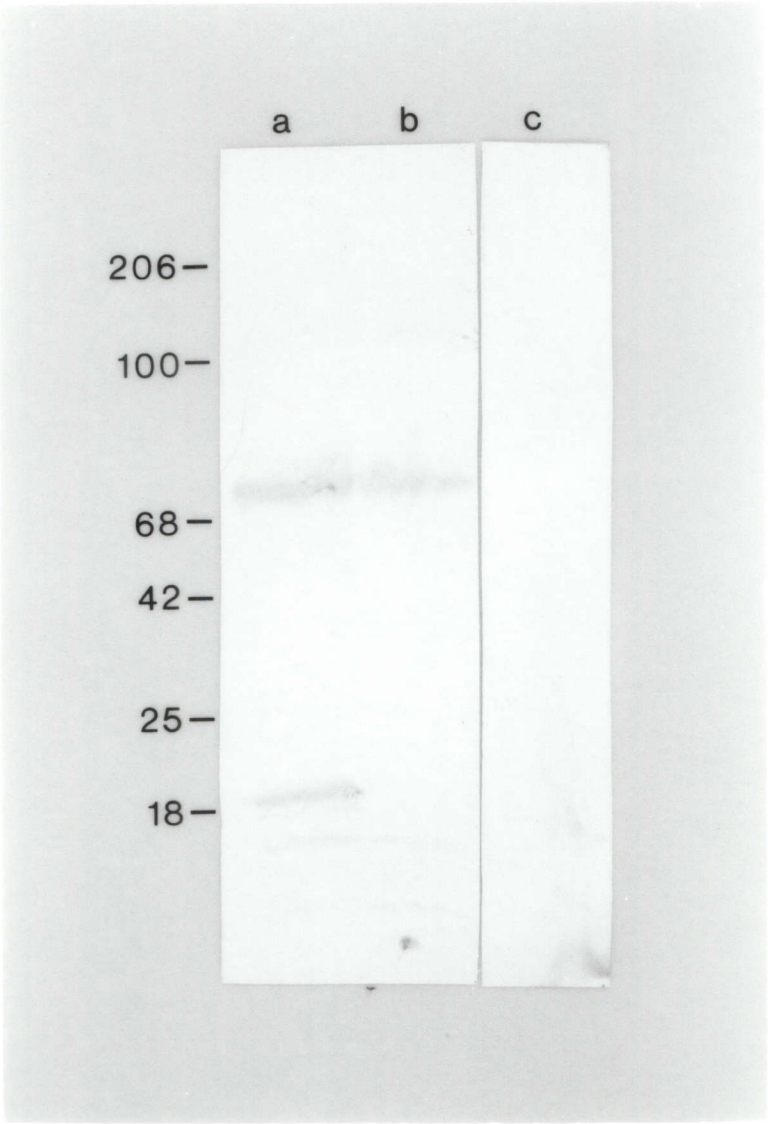
polymerase which contains the DNA binding domain (139). As anticipated, the purified enzyme was detected as a band of 116 kDa, However, antigen for this enzyme could not be detected in the subnuclear fractions, including the poly(ADP-ribose) glycohydrolase digestion supernatant of nuclear matrices (not shown).

Evidence has been provided that the ribonucleoprotein (RNP) particle containing the small nuclear RNA U1 (snRNA) and several other RNP particles are attached to the nuclear matrix (for review see 140). To date, U1 snRNP particles are known to contain at least nine different protein constituents, termed the 70 kDa protein and A, B', B, C, D, E, F and G proteins, with molecular weights ranging from 10 to 30 kDa (141-143). It has been suggested that the 70 kDa protein might be involved in the binding of U1 snRNP to the nuclear matrix, since a substantial fraction of this protein is not released from this nuclear structure by incubation with RNase or DNase (140). Other U1 snRNP-associated proteins are almost completely extracted under these circumstances (140, 144, 145). Interestingly, extraction of U1 RNP particles by means of sonic disruption of nuclei contained the 70 kDa protein, whereas particles isolated at slightly alkaline pH lacked this protein (146). Furthermore, a report has suggested the poly(ADP-ribosyl)ation of ribonucleoproteins of molecular weights of approximately 70

kDa and 30 to 40 kDa in isolated nuclei (147). These observations and the fact that poly(ADP-ribose)-protein adducts are sensitive to mild alkaline pH, suggested the possibility that the 70 kDa protein in the U1 snRNP and the 70 kDa polypeptide which is an acceptor for poly(ADP-ribose) might be identical. In addition, it was also reasonable to hypothesize that poly(ADP-ribose) could be mediating the association of this protein to U1 snRNP particles and perhaps to the nuclear matrix. One of the predictions that can be drawn from this hypothesis is that digestion of poly(ADP-ribose) should remove the 70 kDa polypeptide from the nuclear matrix. Thus, the possibility that the 70 kDa polypeptide released by digestion of nuclear matrices with poly(ADP-ribose) glycohydrolase could be identical with the 70 kDa protein of the U1 snRNP was investigated.

In these studies, the conditioned medium from the 2.73 hybridoma anti-RNP, IgG 2a(k) cell line, containing the monoclonal antibody 2.73 anti-(U1 RNP) was used for the specific detection of the 70 kDa polypeptide from the U1 snRNP (148). Protein blots of the different cell fractions were screened with this antibody. Fig. 23 shows that the 70 kDa polypeptide gave an strong signal in the DNase and $(NH_4)_2SO_4$ combined fractions (lane a) and in the pellet from the digestion of nuclear matrices with poly(ADP-ribose) glycohydrolase (lane b). A signal was not seen in the glycohydrolase digestion supernatant (lane c).

Fig.23. **Probing the nuclear fractions with a monoclonal antibody against the 70 kDa polypeptide from the U1 snRNP** . Experimental details were as indicated in legend to Fig. 21, except that the nitrocellulose filter was screened with a monoclonal antibody against the 70 kDa polypeptide in the U1 snRNP. (lane a) DNase and $(NH_4)_2SO_4$ combined fractions. (lanes b and c) pellet and supernatant, respectively, from the digestion mixture of nuclear matrices with poly(ADP-ribose) glycohydrolase. Numbers on the left indicate molecular weight in kDa.


a
b
c
206
100
68
42
25
18

CHAPTER V

DISCUSSION

Numerous studies have pointed out that the metabolism of poly(ADP-ribose) is associated with chromatin, and poly(ADP-ribose) polymerase has been shown to be a chromatin-bound enzyme. Likewise, inhibitors of poly(ADP-ribose) polymerase have been shown to affect several chromatin-related functions such as DNA repair replication, cellular recovery from DNA damage, sister chromatid exchanges, cellular transformation, gene expression and cellular differentiation. It is clear that the execution of these functions requires dynamic rearrangements in the organization of chromatin superstructure. At present, the most likely explanation for the involvement of the poly(ADP-ribosyl)ation system in such diverse biological functions is based in the extraordinary potential of this system to effect reversible changes in chromatin organization. Several lines of evidence support the idea that poly(ADP-ribosyl)ation of nuclear proteins may modulate, via covalent or non-covalent interactions, DNA-protein interactions at the nucleosomal level of chromatin organization. It is striking that a common feature of all the protein acceptors of ADP-ribose polymers identified thus

far protein acceptors of ADP-ribose polymers identified thus far is their capacity to bind DNA (Table I). Interestingly, it has been shown that in *in vitro* systems poly(ADP-ribosyl)ation of histones and poly(ADP-ribose) polymerase reduces their DNA binding affinity (149-152). In combination these findings provide an explanation of why poly(ADP-ribosyl)ation of polynucleosomes leads to their relaxation *in vitro* (79). Furthermore, poly(ADP-ribosyl)ation of DNA metabolic enzymes *in vitro,* such as DNA polymerases α and β, DNA ligase, topoisomerases I and II, Ca^{+2}, Mg^{+2}-dependent endonucleases, wheat germ RNA polymerase II and terminal deoxynucleotidyl transferase, results in the inhibition of enzymatic activity (45-48). Although not conclusively proven at present, this inhibition may result from a reduction of the DNA-binding affinity of these enzymes.

While most of the reported work has focused on the altered functions of the poly(ADP-ribosyl)ated proteins, only recently two reports were directed to evaluate the potential of the ADP-ribose polymer chains *per se* for influencing changes in chromatin structure. In the first report, Alvarez-Gonzalez and Jacobson (18) were able to show the occurrence of long and multibranched ADP-ribose polymers in intact cells, even under physiological conditions where polymer turnover is rapid. As stressed by these authors, since each polymer residue has two negative charges at

physiological pH, this complex polyanion has the potential to interact with different components of chromatin. In the second report, by Sauermann and Wesierska-Gadek (150), the capacity of purified ADP-ribose polymers to effectively compete with DNA for histone H4 binding was demonstrated. From the aforementioned studies, it seems reasonable to postulate that the influence of poly(ADP-ribose) in DNA-protein interactions may not only depend on the acceptor molecule to which the homopolymer is covalently bound, but also on the localization of the polymer itself and the acceptor molecule within different regions of chromatin. Further support for this postulate awaits results from studies on the nuclear distribution of poly(ADP-ribose) metabolism.

The intranuclear localization of poly(ADP-ribose) polymerase has previously been attempted. Tsopanakis *et al.* (153) published the first report concerning the presence of activity in isolated nucleoli from *Tetrahymena pyriformis* and this observation was later extended to other types of mammalian cell lines (154, 155). The distribution of poly(ADP-ribose) polymerase activity within chromatin is still controversial since one research group (110) claims the association of this enzyme with the linker DNA, whereas a second group (111) observed a preferential association with the nucleosomal core. Other studies have established the association of automodified poly(ADP-ribose) polymerase (49,

51) and the recovery of a small percentage of this enzymatic activity (50) with the nuclear matrix. Interestingly, preliminary observations by Adamietz (156) suggested that in rat liver the most active fraction of poly(ADP-ribose) polymerase is that recovered with a nuclear matrix-like structure.

While these observations concerning the nuclear distribution of poly(ADP-ribose) polymerase have been made, analysis of the nuclear distribution of ADP-ribose polymers has not been previously studied. This has been due to the lack of suitable methodology to enable the detection and quantification of ADP-ribose polymers in subnuclear fractions.

A technique for the radiolabeling of the cellular NAD^+ pool to a high specific activity by using [^{3}H]adenine as the metabolic radiolabel has been developed (18). The combination of this technique with dihydroboronyl Bio-Rex chromatography has allowed the isolation of highly radiolabeled poly(ADP-ribose) which was used to examine the complexity of these polymers (18). This methodology was recently adapted to the estimation, with high sensitivity, of polymer levels in intact cells (114). In the present study, this approach has provided sufficient sensitivity to allow the quantification of the poly(ADP-ribose) content of isolated nuclei and nuclear fractions of cells treated to induce polymer synthesis and accumulation (Tables III-IX).

Likewise, it was possible to analyze the nuclear distribution of basal levels of ADP-ribose polymers in untreated cells (Table VII). The selectivity of this procedure for the exclusive detection of poly(ADP-ribose) in the nuclear and subnuclear fractions was also proven (Fig. 4). Finally, with this technique it was possible to isolate intact polymers from different nuclear fractions in sufficient quantities as to allow their further characterization (Figs. 14-16).

The procedure for the preparation of nuclei used in this study allowed the isolation of intact nuclei free of cytoplasmic debris (not shown). In addition, from the high recovery of DNA and poly(ADP-ribose) in the nuclear preparations (Table III), it can be concluded that throughout the procedure no serious nuclear breakage was produced. Due to the instability of the covalent protein-poly(ADP-ribosyl) bonds at pH values above 7.0 (118), it was important to conduct these experiments at pH values below 7.0 to avoid disruption of these linkages. The isolation of nuclei at pH 3.1 and at pH 6.5 resulted in a good recovery of polymer (Table III). However, pH 6.5 was selected as a compromise between "physiological conditions" and the preservation of protein-poly(ADP-ribose) linkages.

In order to study the distribution of poly(ADP-ribose) under conditions where polymer metabolism is enhanced, C3H10T1/2 cells were treated with hyperthermia and MNNG. Confluent cultures were treated under conditions where hyperthermia and MNNG are only potentially lethal to non dividing cells as compared to dividing cells (18, 157). As indicated above, hyperthermic treatment results in the accumulation of poly(ADP-ribose) by decreasing the turnover of the polymer (43). Treatment of cells with the DNA alkylating agent MNNG results in the activation of the cellular DNA excision repair system. DNA excision repair is often subdivided into four steps: 1) incision near the lesion in DNA; 2) excision of modified or damaged nucleotides; 3) DNA synthesis to form the repair patch and 4) ligation of the DNA nick. At present it is still unclear if step 3 occurs prior to step 2. In this series of events poly(ADP-ribose) polymerase detects the DNA strand break by binding to the damaged DNA, presumably on or near the break. This results in the activation of the enzyme and concomitant synthesis of poly(ADP-ribose). Thus, it should be expected that poly(ADP-ribosyl)ation occurs in chromatin in close proximity with the DNA strand break, and thus near the site where the DNA repair patch will be synthesized.

The studies described in this dissertation have shown that ADP-ribose polymers generated in response to hyperthermia and MNNG are non-randomly distributed in

chromatin, but rather are associated with chromatin domains which are resistant to MNase (Fig. 7). This result was unexpected, in view of previous studies which pointed out that newly synthesized repair patches following exposure to UV light exhibit a transient increase in MNase sensitivity in mammalian cells (158, 159). However, this result can be reconciled with observations made by Mathis and Althaus (160). These authors showed that 20 min following exposure of cells to UV light, DNA repair patches acquired an unfolded configuration. However, from 30 to 60 min the patches progressively recovered a folded configuration and were resistant to DNA crosslinking agents and MNase. In the present study, the elapsed time between the induction of DNA strand breaks by MNNG and the isolation of the MNase fractions was approximately 2 h. Thus, it is likely that the period of nucleosome unfolding has elapsed and that the chromatin has recovered the folded MNase-resistant configuration. The development of experimental systems which allow the analysis of poly(ADP-ribose) distribution at short times (5 to 10 min) following the onset of repair replication should further clarify this matter. Alternatively, it is possible that the presence of these polymers in some regions of chromatin confers MNase resistance to these regions. In attempts to explore this possibility, the MNase sensitivity of cells treated to induce poly(ADP-ribose) accumulation was compared with untreated control cells. No difference in

MNase sensitivity was detected (not shown). However, it can still be argued that the test was done with bulk chromatin. Therefore, analysis of MNase sensitivity of specific regions of chromatin could provide a better system to test the possibility of MNase resistance induced by poly(ADP-ribose).

As indicated above, the MNase-resistant chromatin isolated in these experiments contains approximately 20% of the total DNA and the nuclear matrix. Thus, the possibility of the association of ADP-ribose polymers with the nuclear matrix was examined.

Morphological examination by electron microscopy (Fig. 9), protein patterns in SDS-PAGE (Fig. 11) and analysis of the DNA content (Tables III-VI) of the nuclear matrix preparation isolated in these studies have established the identity of these preparations with nuclear matrices as reported by others (161-163).

When the poly(ADP-ribose) content of these preparations was examined, it was found that 50% of the total polymer residues were associated with the nuclear matrices. The association of poly(ADP-ribose) to the nuclear matrix could be explained in two ways: either the polymers are associated with the nuclear matrix *in vivo* or this association occurred *in vitro* during the preparation of nuclear matrix. Two independent lines of evidence support an *in vivo* association: i) exogenous polymers added to isolated nuclei prior to the

preparation of nuclear matrices do not bind significatively to these matrices; ii) nuclear matrices isolated by extraction of nuclei with buffers containing 2 M NaCl, 0.3 M $(NH_4)_2SO_4$ and 25 mM LIS contained from 50 to 70% of the total poly(ADP-ribose) (Tables IV-VI). This rules out an artifactual association of poly(ADP-ribose) to the nuclear matrix promoted by hypertonic salt extraction.

In contrast to the high poly(ADP-ribose) content of the nuclear matrix, its DNA content was less than 1% of the total (Tables IV-VI). This result raises the interesting possibility that 50% of the total poly(ADP-ribose) is associated with a chromatin fraction which is clustered and tightly bound to the nuclear matrix. However, this possibility has to be reconciled with the observation that the fraction of poly(ADP-ribose) released from the nuclei by salt extraction was not dependent on prior nuclease digestion, as approximately the same fraction of polymers was released by salt extraction of undigested nuclei (Tables IV-VI).

The nuclear matrix-associated ADP-ribose polymers, which represent 50% of the total residues are short polymers, while the polymers extracted by salt contain both short and complex chains (Fig. 14). These results demonstrate that on a molecule basis there are considerable more molecules of polymers in the nuclear matrix, that in the salt-extracted fraction. Therefore, it is very likely that in the nuclear

matrix there are more molecules of protein modified by these polymers. Although, modification of proteins at multiple sites is also consistent with these data.

These studies have shown a small increase in the fraction of nuclear matrix-associated polymers generated following hyperthermia, where polymer half-life is 10 min (43), as compared to basal levels of polymers in untreated cells or polymers synthesized in response to MNNG treatment, where polymer half-life are 5 min and less than 2 min, respectively, (42-43) (Table VII). These results and the observation that only short ADP-ribose polymers (Fig. 14) were detected in the nuclear matrix suggest that turnover for nuclear matrix-associated polymers is faster than for non-matrix-associated polymers. The fact that practically no endogenous poly(ADP-ribose) glycohydrolase activity could be detected with the nuclear matrix in the experiment shown in Fig. 17 does not rule out this possibility, as previous reports have shown that this enzyme has a low affinity for short polymers (126, 164). These observations agree with previous reports (18, 164) which have suggested that ADP-ribose polymer chain length seems to be tightly regulated by poly(ADP-ribose) glycohydrolase *in vivo*.

The association of poly(ADP-ribose) with the nuclear matrix may be mediated by i) non-covalent interactions between this negatively charged polymer and other nuclear

matrix components or ii) covalent attachment to matrix-associated proteins or both. The observations that the salt-extracted polymers are large and multibranched, whereas the nuclear matrix-associated polymers are short (Fig. 14), does not support the idea of strong non-covalent interactions. However, based on other studies (18), an average size of 10 residues for nuclear matrix-associated polymers could be assumed. This gives these polymers a formal negative charge of -20, which may allow non-covalent interactions to be sufficient. In addition, progressive increase of salt concentration in the extraction buffer, from 0.2 M to 0.8 M $(NH_4)_2SO_4$, did not disrupt the association of the short ADP-ribose polymers to the nuclear matrix (Figs. 13, 15 and 16). This indicates that these polymers are tightly bound, perhaps covalently, to the nuclear matrix. Previous studies performed both *in vitro* and *in vivo*, have shown that several nuclear matrix proteins are covalent acceptors for poly(ADP-ribose) (45-51). Whether the nuclear matrix-associated polymers represent an *in vivo* covalent attachment to nuclear matrix proteins will require further analysis.

It has been shown that most histones are extracted from the nucleus at the salt concentrations used here (106, 121, 122, 145). In this regard, it was remarkable that the curve of ADP-ribose polymer release from the nucleus by increasing ionic strength in the extraction buffer (Fig. 13) matched the curve of protein released in an analogous experiment reported

by other authors (Fig. 1 in reference 145). Studies by Menard *et al.* (164) using a reconstituted *in vitro* poly(ADP-ribose) turnover system, under close physiological conditions, have shown that long polymers are synthesized as a result of automodification, while short polymers are synthesized preferentially but not exclusively on histone H1. Additionally, only 2% of the total poly(ADP-ribose) synthetase activity has been detected with the nuclear matrix (50). Altogether, these observations suggest that the short and complex ADP-ribose polymers released by salt extraction may represent chains that are covalently bound to histones and to poly(ADP-ribose) synthetase, respectively, which originally could be either associated or in close proximity to chromatin domains clustered on the nuclear matrix. Conversely, these results are also consistent with the possibility that there are two classes of polymer metabolism, one that is associated with the nuclear matrix and one that is not.

Digestion of isolated nuclear matrices with poly(ADP-ribose) glycohydrolase resulted in the release of a small fraction of protein, 2% of the total (Tables X and XI), composed of a specific subset of polypeptides (Fig. 18). This result raises the interesting possibility that the association of these proteins to the nuclear matrix is mediated by poly(ADP-ribose). The nature of this association remains to be determined. It should be stressed that the

pattern of proteins released was highly reproducible and that the kind of treatment used to stimulate accumulation of poly(ADP-ribose) with few exceptions affected this pattern (Figs. 18 and 19). Considering the different mechanisms by which each of these cell treatments affect the metabolism of poly(ADP-ribose), it is difficult to explain the meaning of these observations. Perhaps they reflect the fact that under diverse conditions, the role of this metabolism in chromatin is, as discussed above, a common one. Thus, further characterization of these proteins will likely provide clues to the functions of poly(ADP-ribose) metabolism in the nuclear matrix. Among the proteins released from the nuclear matrix by poly(ADP-ribose) glycohydrolase, there were prominent polypeptides of 170 kDa, 116 kDa, 100 kDa, 70 kDa, 67 kDa, 55 kDa and a set at 20 to 36 kDa (Fig. 18). The enrichment of some of these polypeptides in the glycohydrolase-released fraction as compared to the nuclear matrix was evident (Figs. 18 and 19).

Experiments were aimed toward the identification of the 100 kDa, 170 kDa, 116 kDa and 70 kDa polypeptides as topoisomerases I and II, poly(ADP-ribose) polymerase and the 70 kDa protein from the U1 snRNP, respectively. The reasons why these proteins were selected have been described in Chapter IV. In addition, both topoisomerases I and II and poly(ADP-ribose) polymerase have been reported to be

associated to the nuclear matrix (104, 130-133, 49-51) to a certain extent. Thus, the possibility of a concerted action of these three enzymes to bring about organizational rearrangements in nuclear matrix-associated chromatin seemed very likely. Therefore, identification of these three enzymes as a part of the set of proteins released by poly(ADP-ribose) glycohydrolase would further support this hypothesis. Toward this end, the protein fraction released by poly(ADP-ribose) glycohydrolase was probed with antibodies directed against these proteins. However, no antigen for these antibodies could be detected in the glycohydrolase-released protein fraction (Figs. 21-22).

In probing for the topoisomerases I and II and 70 kDa protein from U1 snRNP, antigen was detected in other nuclear fractions from the same experiment (Figs. 21-23). In these experiments, approximately the same amount of protein from each nuclear fraction probed was present on the nitrocellulose filters. These observations make it highly unlikely that the lack of detection was artifactual or due to insufficient material. Although purified poly(ADP-ribose) polymerase was detected with good sensitivity in western blots, the screening for this antigen was negative for all the nuclear fractions tested. The reason for this result remains to be determined. The selection of additional antibodies therefore remains to be accomplished for the successful identification of these proteins.

A fraction of poly(ADP-ribosyl)ated histones has been recovered in the nuclear matrix (165). Thus, it is possible that the 20-36 kDa polypeptides released by glycohydrolase are histone proteins. The confirmation of this possibility would strongly support the idea that poly(ADP-ribose) metabolism could regulate interactions between soluble chromatin-proteins and nuclear matrix proteins.

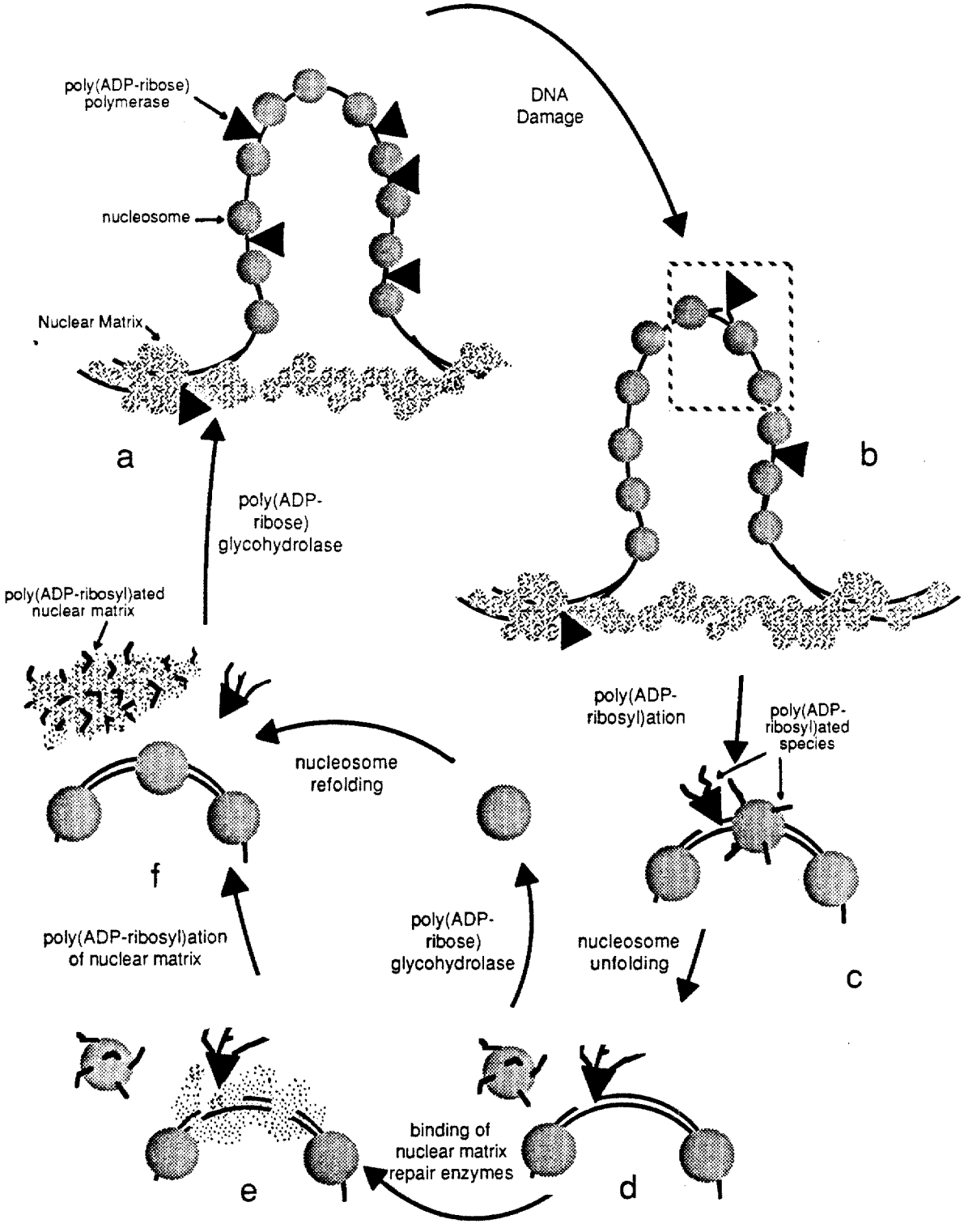
The last part of this discussion will deal with the postulation of a model which attempts to explain the biological significance of the occurrence of polymers of ADP-ribose in two functional compartments of chromatin; one that is nuclear matrix-associated and one that is not. This model is based on observations by other investigators in the area as well as on the observations provided by the present study.

Based on their distribution size and complexity, these studies have defined three classes of ADP-ribose polymers; non-nuclear matrix-associated short and complex polymers and nuclear matrix-associated short polymers. This finding strongly supports, the occurrence of at least three kinds of protein acceptors. In view of the distribution of these hypothetical acceptors in two functional domains of chromatin, it is reasonable to suggest that these polymers may play a role linked to the protein and the domain where they occur. Further, the degree of polymer complexity may reflect this role. Several proteins known to be associated

with the nuclear matrix have been identified as acceptors for poly(ADP-ribose). These include DNA metabolic enzymes which have a role in DNA replication, DNA repair replication and DNA transcription (Tables I and II). Therefore, it is obvious that under some conditions these proteins require access to DNA. The primary role of the poly(ADP-ribosyl)ation system could be to sense this requirement by sensing a DNA strand break, an event that results in the activation of poly(ADP-ribose) metabolism. The model proposed is summarized in Fig. 24.

Under normal conditions, inactive molecules of poly(ADP-ribose) polymerase are distributed throughout chromatin and only a minor fraction in the nuclear matrix (50, 156, 166) (Fig. 24a). Upon DNA damage, the enzyme translocates from these sites of low affinity to a site of higher affinity in or near the DNA strand break (Fig. 24b). This results in the activation of the enzyme which become automodified and also modifies nearby nucleosomal proteins, i.e. histones (Fig 24c). Following DNA damage it has been shown that poly(ADP-ribose) polymerase, histone H1 and H2b are the major poly(ADP-ribose) acceptors *in vivo* (83, 167)Thus, this model predicts that histones and poly(ADP-ribose) polymerase are the acceptors of the non-nuclear matrix-associated short and complex polymers, respectively. The extent and protein

Fig. 24. **Metabolism of poly(ADP-ribose) and the targeting of DNA repair replication to the nuclear matrix.** (a and b) DNA loop fixed at its base by the nuclear matrix protein network. (c-f) show an expanded view of a region of the DNA loop (inside the square in b) in where a DNA strand break has been produced. For further explanation see text.


poly(ADP-ribose) polymerase
nucleosome
Nuclear Matrix
a
DNA Damage
b
poly(ADP-ribose) glycohydrolase
poly(ADP-ribosyl)ated nuclear matrix
poly(ADP-ribosyl)ation
poly(ADP-ribosyl)ated species
nucleosome refolding
f
poly(ADP-ribosyl)ation of nuclear matrix
poly(ADP-ribose) glycohydrolase
nucleosome unfolding
c
binding of nuclear matrix repair enzymes
e
d

spectrum of this modification may be regulated, as suggested by Menard et al., (164) at the turnover level by poly(ADP-ribose) glycohydrolase.

As poly(ADP-ribosyl)ation and turnover progress it reach a point where the degree of modification and spectrum of proteins modified, within the nucleosome, reduces the DNA-protein interactions (161, 164). This results in unfolding of the nucleosomal DNA (Fig. 24d). Nucleosome unfolding following DNA damage has been previously shown (158-160). Likewise, poly(ADP-ribosyl)ation is required for the establishment of unfolded domains of chromatin and normal progression of the excision repair process (168).

The unfolding of nucleosomes allows DNA-accessibility to DNA repair enzymes, such as DNA polymerase β and topoisomerase I which are nuclear matrix-proteins (Table II), (shown in Fig. 24e). DNA polymerase β is the presumptive enzyme responsible for the synthesis of DNA during repair replication in eukaryotic cells (169). Although, the involvement of topoisomerase I in DNA repair replication has been suggested, it has not been proved (45). Two reports have made the observation that DNA-repair patches produced in response to UV light and γ-radiations were preferentially recovered with the nuclear matrix (170 and 171). These

results raised the possibility that DNA repair replication preferentially occurs in chromatin domains clustered in the nuclear matrix. Also consistent with this possibility is the observation that DNA damage in actively transcribed DNA sequences, known to be closely associated with the nuclear matrix (106 and 107), is repaired before and at a faster rate than the genome overall (172 and 173).

Once the DNA repair enzymes have accomplished their functions, poly(ADP-ribosyl)ation of these proteins reduces their DNA-binding affinity resulting in their removal from DNA, or even, on the inhibition of the enzymatic activity (Fig. 24f). These nuclear matrix proteins could be represented by the acceptors of the nuclear matrix-associated short polymers and/or by the proteins released by probing of the nuclear matrix with poly(ADP-ribose) glycohydrolase. It should be stressed that the poly(ADP-ribosyl)ation of topoisomerases I and II and DNA polymerase β, as well as of other nuclear matrix proteins both *in vivo* and *in vitro* have been described (Table I) (41-47). Finally, the DNA is folded into the original nucleosomal configuration by histones from which the glycohydrolase has removed the poly(ADP-ribose) residues (Fig. 24a)

Previous studies have defined two functional domains in chromatin, one that is closely associated with the nuclear matrix and one that is not (for review see 174, 175). The nuclear matrix-associated domain appears to be most active whereas, the non-matrix associated domain contains chromatin in a highly packed and therefore inactive form. The mechanisms by which chromatin is interconverted into these two domains is at present one of the most challenging questions in the DNA-protein interactions field; poly(ADP-ribose) metabolism may be the answer. Thus, this model postulates that poly(ADP-ribose) metabolism during DNA repair replication accomplishes the reversible targeting of some regions of chromatin to the nuclear matrix domain by modulating DNA-protein and/or protein-protein interactions at the nucleosomal and nuclear matrix level.

BIBLIOGRAPHY

1. Dietrich, L. S., Fuller, L., Yero, I. L., Schneider, W. C., and Martinez, L. (1966) *J. Biol. Chem.* **241**, 188-191

2. Hogeboom, G. H., and Scheneider, W. C. (1952) *J. Biol. Chem.* **197**, 611-620

3. Zatman, L. J., Kaplan, N. O., and Colowick, S. P. (1953) *J. Biol. Chem.* **200**, 197-212

4. Little, J. W., Zimmermann, S. B., Oshinsky, C. K., and Gellert, M. (1967) *Proc. Natl. Acad. Sci. U.S.A.* **58**, 2004-2011

5. Olivera, B. M., and Lehman, I. R. (1967) *Proc. Natl. Acad. Sci. U.S.A.* **57**, 1700-1709

6. Chambon, P., Weill, J. D., and Mandel, P. (1963) *Biochem. Biophys. Res. Commun.* **11**, 39-43

7. Fujimura, S., Sugimura, T., Okabe, K., and Yoshida, T. (1965) *J. Jap. Biochem. Soc.* **37**, 584-590

8. Fujimura, S., and Sugimura, T., (1966) *J. Jap. Biochem. Soc.* **38**, 691-698

9. Chambon, P., Weill, J. D., Dolly, M. T., Strosser, M. T., and Mandel, P. (1966) *Biochem. Biophys. Res. Commun.* **25**, 638-643

10. Sugimura, T., Fujimura, S., Hasegawa, S., and Kawamura, Y. (1967) *Biochem. Biophys. Acta* **138**, 438-441

11. Reeder, R. H., Ueda, K., Honjo, T., Nishuzuka, Y., and Hayaishi, O. (1967) *J. Biol. Chem.* **242**, 3172-3179

12. Nishuzuka, Y., Ueda, K., Nakazawa, K., and Hayaishi, O. (1967) *J. Biol. Chem.* **242**, 3164 3171

13. Hasegawa, S., Fujimura, S., Shimisu, Y., and Sugimura, T. (1967) *Biochem. Biophys. Acta* **149**, 369-376

14. Dolly. J., and Petek, F. (1966) *C.R. Acad. Sci.* (Paris) **263**, 1341-1344

15. Miwa, M., Saikawa, N., Yamaizumi. Z., Nishimura, S., and Sugimura, T. (1979) Proc. *Natl. Acad. Sci. U.S.A.* **76**, 595-599

16. Juarez-Salinas, H., Mendoza-Alvarez, H., Levi, V., Jacobson, M. K., and Jacobson, E. L. (1983) *Anal. Biochem.* **131**, 410-418

17. Kanai, M., Miwa, M., Kuchino, Y., and Sugimura, T. (1982) *J. Biol. Chem.* **257**, 6217-6223

18. Alvarez-Gonzalez, R. and Jacocobson, M. K. (1987) *Biochemistry* **26**, 3218-3224

19. Althaus, F. R., and Richter, Ch. (1987) in *ADP-ribosylation of Proteins. Enzymology and Biological Significance. Molecular Biology Biochemistry and Biophysic.* **37**, 12-15. Springer-Verlag Berlin, Heidelberg, New York, London, Paris and Tokyo.

20. Ueda, K., Kawaichi, M., and Hayaishi, O. (1982) in *ADP-ribosylation Reactions* (Hayaishi, O., and Ueda, K., eds.) pp. 139-141. Acad. Press. London-New York

21. Nishikimi, M., Ogasawa, K., Kameshita, I., Tanaguchi, T., and Shizuta, Y. (1982) *J. Biol. Chem.* **257**, 6102-6105

22. Kameshita, I., Matzuda, Z., Taniguchi, T., and Shizuta, Y. (1984) *J. Biol. Chem.* **259**, 4770-4776

23. Shizuta, Y., Kameshita, I.,Agemori, M., Ushiro, H., Tanaguchi, T., Otsuki, M., Sekimizu, K., and Natori, S. (1985) in *ADP-ribosylation of Proteins* (Althous, F. R., Hilz, H., and Shall, S., eds.) pp. 52-59. Springer-Verlag, Berlin, Heidelberg, New York, Tokyo

24. Benjamin, R. C., and Gill, D. M. (1980) *J. Biol. Chem.* **255**, 10502-10508

25. Kawamura, M., Tonigawa, Y., Kitamura, A., Miyake, Y., and Shimoyama, M. (1981) *Biochim. Biophys. Acta* **652**, 121-128

26. Alkhatib, H. M., Chen, D., Cheerney, B., Bhatia, K., Notario, V., Giri, C., Stein, G., Slattery, E., Roeder, R. G., and Smulson, M. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 1224-1228

27. Suzuki,H., Uchida, K., Shima, H., Sato, T., Okamoto, T., Kimura, T., and Miwa, M. (1987) *Biochem. Biophys. Res. Commun.* **146**, 403-409

28. Kurosaki, T., Ushiro, H., Mitsuuchi, Y., Suzuki, S., Matzuda, M., Matzuda, Y., Katunama, N., Kangawa, K., Matzuo, H., Hirose, T., Inayama, S., and Shizuta, Y. (1987) *J. Biol. Chem.* **262**, 15990-15997

29. Cleaver, J. E., Bodell, W. J., Morgan, W. F., and Zelle, B. (1983) *J. Biol. Chem.* **258**, 9059-9068

30. Milam, K. M., Thomas, G. H., Cleaver, J. E., (1986) *Exp.Cell Res.* **165**, 260-268

31. Hunting, D. J., Gowans, B. J., and Henderson, J. F. (1985) *Mol. Pharmacol.* **28**, 200-206

32. Snyder, R. D. (1984) *Biochem. Biophys. Res. Commun.* **124**, 457-461

33. Ben-Hur, E., Chen, C-C., and Elkind, M. M. (1985) *Cancer Res.* **45**, 2123-2127

34. Grunfeld, C., and Shigenaga, J. K. (1984) *Biochem. Biophys. Res. Commun.* **123**, 785-791

35. Johnson, G. S. (1981) *Biochem. Int.* **2**, 611-617

36. Miwa, M., and Sugimura, T. (1971) *J. Biol. Chem.* **146**, 6362-6364

37. Tavassoli, M., Tavassoli, M. H., and Shall, S. (1983) *Eur. J. Biochem.* **135**, 449-455

38. Tanuma. S., Kawashima, K., and Endo, H. (1986) *J. Biol. Chem.* **261**, 965-969

39. Okayama, H., Honda, M., and Hayaishi, O. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2254-2257

40. Williams, J. C., Chambers, J. P., and Liehr, J. G. (1984) *J. Biol. Chem.* **259**, 1037-1042

41. Wielkens, K., George, E., Press, T., and Hilz, H. (1983) *J. Biol. Chem.* ***258***, 4098-4104

42. Alvarez-Gonzalez, R., Eichenberger, R., Lotscher, P., and Althaus, F. R. (1985) Abstr. in Eur. Worksh. *ADP-Ribosylation of Proteins*, Reading, England Dec 16-18

43. Jonsson, G. G., Jacobson, E. L., and Jacobson, M. K. (1988) *Cancer Res.* in press

44. Althaus, F. R., and Richter, Ch. (1987) in *ADP-ribosylation of Proteins. Enzymology and Biological Significance. Molecular Biology Biochemistry and Biophysic.* **37**, 45-58. Springer-Verlag Berlin, Heidelberg, New York, London, Paris and Tokyo

45. Ferro, A. M., and Olivera, B. M. (1984) *J. Biol. Chem.* **259**, 547-554

46. Darby, M. K., Schmitt, B., Jonstra-Bilen, I., and Vosberg, H. P. (1985) *EMBO J.* **4**, 2129-2134

47. Yoshihara, K., Itaya, A., Tanaka, Y., Ohashi, Y., Ito, K., Teraoka, H., Tsukada, M., Matsukage, A., and Kamiya, T. (1985) *Biochem. Biophys. Res. Commun.* **128**, 61-67

48. Yoshihara, K., Tanigawa, Y., Burzio, L., and Koide, S. S. (1975). *Proc. Natl. Acad. Sci. U.S.A.* **72**, 289-293

49. Adolph, K. W., and Song, M. K. H. (1985) *Biochem. Biophys. Res. Commun.* **126**, 840-847

50. Wesierska-Gadek, J., and Sauermann, G. (1985) *Eur. J. Biochem.* **153**, 421-428

51. Adolph, K. W. (1987) *Biochim. Biophys. Acta* **909**, 222-230

52. Althaus, F. R., and Richter, Ch. (1987) in *ADP-ribosylation of Proteins. Enzymology and Biological Significance. Molecular Biology Biochemistry and Biophysic.* **37**, 93-95. Springer-Verlag Berlin, Heidelberg, New York, London, Paris and Tokyo

53. Song, M. K. H., and Adolph, K. W. (1985) *Biochem. Biophys. Res. Commun.* **115**, 938-945

54. Adolph, K. W., and Song, M. K. H. (1985) *Biochemistry* **24**, 345-352

55. Lonn, U., and Lonn, S. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3996-3999

56. Lonn, U., and Lonn, S. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 104-108

57. Smulson, M., Stark, P., Gazzoli, M., and Roberts, J. H. (1975) *Exp. Cell Res.* **90**, 175-182

58. Whish, W. J. D., Davies, M. I., and Shall, S., (1975) *Biochem. Biophys. Res. Commun.* **65**, 722-730

59. Juarez-Salinas, H., Sims, J. L., and Jacobson, M. K. (1979) *Nature* **282**, 740-741

60. Jacobson, M. K., Levy, V., Juarez-Salinas, H., Barton, R. A., and Jacobson, E. L.(1980) *Cancer Res.* **40**, 1797-1802

61. Skidmore, C. J., Davies, M. I., Goddwin, P. M., Omidiji, O., Zia'ee, A-A., and Shall. S. (1980) in *Novel ADP-ribosylations of Regulatory Enzymes and Proteins* (Smulson, M. E., and Sugimura, T. eds) pp. 197-205, Elsevier, Amsterdam, New York.

62. Miller, E. G. (1975) *Biochem. Biophys. Res. Commun.* **66**, 280-286

63. Durkacz, B. W., Omidiji, O., Gray, D. A., and Shall, S. (1980) *Nature* **283**, 593-596

64. Wielkens, K., Phase, T., and Schaefer, G. (1985) in *ADP-ribosylation of Proteins* (Althous, F. R., Hilz, H., and Shall, S., eds.) pp. 252-263. Springer-Verlag, Berlin, Heidelberg, New York, Tokyo.

65. Cleaver, J. E., and Morgan, W. F. (1985) *Mutat. Res.* **150**, 69-76

66. Jacobson, E. L., Smith, J. Y., Mingmuang, M., Meadows. R., Sims, J. L., and Jacobson, M. K. (1984) *Cancer Res.* **44**, 2485-2492

67. Boorstein, R. J., and Pardee, A. B. (1984) *J. Cell. Physiol.* **120**, 345-353

68. Oikawa, A., Tohda, H., Kanai, M., Miwa, M., and Sugimura, T. (1980) *Biochem. Biophys. Res. Commun.* **97**, 1311-1316

69. Halligan, B. D., Davis, J. L., Edwards, K. A., and Liu, L. F. (1982) *J. Biol. Chem.* **257**, 3995-4000

70. Althaus, F. R., and Richter, Ch. (1987) in *ADP-ribosylation of Proteins. Enzymology and Biological Significance. Molecular Biology Biochemistry and Biophysic.* **37**, 101-106. Springer-Verlag Berlin, Heidelberg, New York, London, Paris and Tokyo

71. Farzaneh, F., Zalin, R., Brill, D., and Shall, S. (1982) *Nature* **300**, 362-366

72. Sugiura, M., Fram, R., Munroe, D., and Kufe, D. (1984) *Dev. Biol.* **104**, 484-488

73. Rankin, P. W., Jacobson, E. L., Benjamin, R. C., Moss, J., and Jacobson, M. K.(1987) in *Niacin, Nutrition, ADP-Ribosylation and Cancer* (Jacobson, E. L. Jacobson, M. K. eds.) in press, Springer-Verlag, Berlin, Heidelberg, New York, Tokyo

74. Matzui, T., Segall, J., Weil. P. A., and Roeder, R. G. (1980) *J. Biol. Chem.* **255**, 11992-11996

75. Slattery, E., Dignam, J. D., Matzui, T., and Roeder, R. G. (1983) *J. Biol. Chem.* **258**, 5955-5959

76. Ohtsuki, M., Sekimizu, K., Agemori, M., Shizuta, Y., and Natori, S. (1984) *FEBS lett.* **168**, 275-277

77. Tanuma, S., Johnson, L. D., and Johnson, G. S. (1983) *J. Biol. Chem.* **258**,

78. Johnson, G. S., and Ralhan, R. (1986) *J. Cellul. Physiol.* **129**, 36-42

79. Poirier, G. G., Murcia, G. de., Jonstra-Bilen, P., Niedergang, C. C., and Mandel, P. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 3423-3427

80. Murcia, G. de., Huletsky, A., Lamarre, D., Gaudreau, A., Pouyety, J., Daune, M., and Poirier, G. G. (1986) *J. Biol. Chem.* **261**, 7011-7018

81. Thoma, F., Koller, and Klug, A. (1979) *J. Cell. Biol.* **83**, 403-427

82. Huletsky, A., Murcia, G. de., Mazen, A., Lewis, P., Chung, D. G., Lamarre, D., Aubin, R. J., and Poirier, G. G. (1985) in *ADP-ribosylation of Proteins* (Althous, F. R., Hilz, H., and Shall, S., eds.) pp. 180-189. Springer, Berlin, Heidelberg, New York, Tokyo.

83. Adamietz, P., and Rudolph, A. (1984) *J. Biol. Chem.* **259**, 6841-6848

84. Callan, H. G., and Tomlin, S. G. (1950) *Proc. R. Soc. Lond. Biol. Sci.* **137**, 367-368

85. Aaronson, R.P., Coruzzi, L. A., and Schmidt, K. (1982) in *The Nuclear Envelope and The Nuclear Matrix.* (Maul, G. G. ed.) pp. 13-29. Alan R. Liss, Inc. New York

86. Franke, W. (1974) *Int. Rev. Cytol. Suppl.* **4**, 71-236

87. Krohne, G., and Benavente, R. (1986) *Exp. Cell Res.* **162**, 1-10

88. Gerace, L., Blum, A., and Blobel, G. (1978) *J. Cell. Biol.* **79**, 546-566

89. Shelton, K., Higgins, L., Cochran, D., Ruffolo, D., and Egle, P. (1980) *J. Biol. Chem.***255**, 10978-10983

90. Gerace, L., and Blobel. G. (1980) *Cell* **19**, 277-287

91. Krohne, G., Franke, W., Ely, S., D'Arcy, A., and Jost, E. (1978) *Cytobiologie* **18**, 22-38

92. Gerace, L., Comeau, C., and Benson, M (1984) *J. Cell Sci. Suppl.* **1**, 137-160

93. Lebkowski, J., and Laemmli, U. (1982) *J. Mol. Biol.* **156**, 325-344

94. Berezney, R., and Coffey, D. S. (1974) *Biochem. Biophys. Res. Commun.* **60**, 1410-1419

95. Agutter, P. S., and Richardson, J. C. W. (1980) *J. Cell. Sci.* **44**, 395-435

96. Bouteille, M., Bourier, D., and Seve, P. (1983) *Int. Rev. Cytol.* **83**, 135-182

97. Hancock, R. (1983) Biol. *Cell.* **46**, 105-121

98. Kaufmann, S., Fields, A. P., and Shaper, J. H. (1986) *Meth. Achiev. Exp. Pathol.* **12**, 141-171

99. Berezney, R. (1979) *in The Cell Nucleus* (Busch, H. ed.) **VII**, 413-456. Acad. Press Inc.

100. Eekelen, C. A. G., and Venrooij, J. V. (1981) *J. Cell. Biol.* **88**, 554-563

101. Pardoll, D. M., Vogelstein, B., and Coffey, D. S. (1980) *Cell* **19**, 527-536

102. Jackson, D. A., and Cook. P. R. (1986) *EMBO. J.* **5**, 1403-1410

103. Tubo, R., and Berezney, R. (1987) *J. Biol. Chem.* **262**, 5857-5865

104. Halligan, B. D., Small, D., Vogelstein, B., Hseieh, T-S., and Liu, L. E. (1984) *J. Cell. Biol.* **99**, 128a-135a

105. Tubo, R., and Berezney. R. (1987) *J. Biol. Chem.* **262**, 1148-1154

106. Mirkovitch, J., Mirault, M. E., and Laemmli, U. K. (1984) *Cell* **39**, 223-232

107. Cockerill, P. N., and Garrard, W. T. (1986) *Cell* **44**, 273-282

108. Paulson, J. R., and Laemmli, U. K. (1977) *Cell* **12**, 817-828

109. Mirkovitch, J., Spierer, P., and Laemmli, U. K. (1986) *J. Mol. Biol.* **190**, 255-258

110. Giri, C. P., West, M. H. P., Ramirez, L. M., and Smulson, M. E. (1978) *Biochemistry* **17**, 3501-3504

111. Leduc, Y., Murcia, G. de., Lamarre, D., and Poirier, G. G. (1986) *Biochim. Biophys. Acta* **875**, 248-255

112. Wielkens, K., Bredehorst, R., Adamietz, P., and Hilz, H. (1981) *Eur. J. Biochem.* **117**, 69-74

113. Aubin, R. J., Dam, V. T., Miclette, J., Brosseau, Y., and Poirier, G. G. (1981) *Can. J. Biochem.* **60**, 295-305

114. Aboul-Ela, N., Jacobson, E. L., and Jacobson, M. K. (1988) *Anal. Biochem.* submitted

115. Sorger, T., and Germinario, R. J.(1983) *Anal. Biochem.* **131**, 254-256

116. Laemmli, U. K. (1970) *Nature* **227**, 680-685

117. Burzio, L. O. (1982) in *ADP-ribosylation Reactions* (Hayaishi, O., and Ueda, K. eds) pp. 103-116, Academic Press, New York

118. Riquelme, P. T., Burzio, L. O., and Koide, S. S. (1979) *J. Biol. Chem.* **254**, 3018-1028

119. Gross, D. S., Garrard, W. T. (1988) *Annu. Rev. Biochem.* **57**, in press

120. Drew, H. R. (1984) *J. Mol. Biol.* **176**, 535-557

121. Benyajati, C., and Worcel, A. (1976) *Cell* **9**, 393-407

122. Capco, D. G., Wau, K. M., and Penuau, S. (1982) *Cell* **29**, 847-858

123. Berezney, R., and Coffey, D. S. (1977) *J. Cell. Biol.* **73**, 616-637

124. Van Eekelen, C. A. G., and Van Venrooij, W. J. (1981) J. *Cell. Biol.* **88**, 554-563

125. Kaufmann, S. H., and Shaper, J. H. (1984) *Exp. Cell Res.* **155**, 477-495

126. Hatekayama, K., Nemoto, Y., Ueda, K., and Hayaishi, O. (1986) *J. Biol. Chem.* **261**, 14902-14911

127. Menard, L., and Poirier, G. G. (1987) *Can. J. Biochem.* **65**, 668-673

128. United States Biochemical Co. *Editorial Comments* (Winter 87-88) **14**, 1-3

129. Tavassoli, M., Tavassoli, M. H., and Shall, S. (1985) *Biochim. Biophys. Acta* **827**, 228-234

130. Berrios, M., Osheroff, N., and Fisher, P. A. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4142-4146

131. Gasser, S. M., Laroche, T., Falquet, J., Tour, E. B. de la., Laemmli, U. K. (1985) J. *Mol. Biol.* **188**, 613-629

132. Nishizawa, M., Tanabe, K., and Takahashi, T. (1984) *Biochem. Biophys. Res. Commun.* **124**, 917-925

133. Van Venrroij, W. J., Stapel, S. O., Houben, H., Habets, W. J., Kallenberg, C. G. M., Penner, E., and Van de Putte, L. B. (1985) *J. Clin. Invest.* **75**, 1053-1060

134. Shelton, E. R., Osheroff, N. and Brutlag, D. L. (1983) *J. Biol. Chem.* **258**, 9530-9535

135. Liu, F. L., and Miller, K. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 3487-3491

136. Maul., G. G., French, B. T., Van Venrooij, W. J., and Jimenez, S. A. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5145-5149

137. Duguet, M., Lavenot, C., Harper, H., Mirambeau, G., and DeRecondo, A. M. (1983) *Nucl. Acids Res.* **11**, 1059-1075

138. Earnshaw, W. C., and Heck M. M. S. (1985) *J. Cell Biol.* **100**, 1716-1725

139. Lamarre, D., Talbot, B., Leduc, Y., Muller, S., and Poirier, G. G. (1986) *Can. J. Biochem.* **64**, 368-376

140. Mariman, E. C., and Van Venrooij, W. J. (1985) in *UCLA Symp. Molec. Cell Biol.* (Smuckler, E. A. and Clawson, G. A. eds.) **26**, 315-329, New York: Alan R. Liss

141. Billings, P. B., and Hoch, S. O. (1984) *J. Biol. Chem.* **259**, 12850-12856

142. Pettersson, I., Hinterberger, M., Mimori, T., Gottlieb, E., and Steitz, J. A. (1984) *J. Biol. Chem.* **259**, 5907-5914

143. Brunel, C., Sri-Widada, J., and Jeanteur, P. (1985) in *Prog. Molec. Subcell. Biol.* (Hahn, S. E., Kopecko, D. J., and Muller, W. E. G. eds) **IX**, 1-52, Berlin: Springer-Verlag

144. Habets, W. J., Berden, J. H. M., Hoch, S. O., and Van Venrooij W. J. (1985) *Eur. J. Immun.* **15**, 992-997

145. Verheijen, R., Kuijpers, H., Vooijs, P., Van Venrooij, W. J. and Ramaekers, F. (1986) *J. Cell Sci.* **80**, 103-122

146. Verheijen, R., Kuijpers, H., Vooijs, P., Van Venrooij, W. J. and Ramaekers, F. (1986) *J. Cell Sci.* **86**, 173-190

147. Kotstka, G., and Schweiger, A. (1982) *Biochim. Biophys. Acta* **969**, 139-144

148. Billings, P. B., Allen, R. W., Jensen, F. C., and Hoch, S. O. (1985) *J. Immun.* **128**, 1176-1180

149. Zahradka, P., Ebisuzaki, K. (1982) *Eur. J. Biochem.* **127**, 579-585

150. Sauermann, G., and Wesierska-Gadek, J. (1986) *Biochem. Biophys. Res. Commun.* **139**, 523-529

151. Mathis, G., and Althaus. F. R. (1987) *Biochem. Biophys. Res. Commun.* **143**, 1049-1054

152. Huletsky, A., Murcia, G. de., Muller, S., Lamarre, D., Hengartner, M., and Poirier, G. G. (1987) in *Niacin, Nutrition, ADP-Ribosylation and Cancer* (Jacobson, E. L. and Jacobson, M. K. eds.) in press, Springer-Verlag, Berlin, Heidelberg, New York, Tokyo

153. Tsopanakis, C., Leer, J. C., Nielsen, O. F., Gocke, E., and Shall, S. (1978) *FEBS Lett.* **93**, 297-300

154. Ikai, K., Ueda, K.,and Hayaishi, O. (1980) *J. Histochem. Cytochem.* **28**, 670-676

155. Kawashima, K., and Izawa, M. (1981) *J. Biochem.* **89**, 1889-1901

156. Brauer, M., Witten, J., Adamietz, P. (1987) in *Niacin, Nutrition, ADP-Ribosylation and Cancer* (Jacobson, E. L. and Jacobson, M. K. eds.) in press Springer-Verlag, Berlin, Heidelberg, New York, Tokyo

157. Jacobson, E. L. Smith, J. Y., Wielkens, K., Hilz, H., and Jacobson, M. K. (1985) *Carcinogenesis* **6**, 715-718

158. Smerdon, M. J. (1986) *J. Biol. Chem.* **261**, 244-252

159. Nissen, K. A., Lan, S. Y., and Smerdon, M. J. (1986) *J. Biol. Chem.* **261**, 8585-8588

160. Mathis, G., and Althaus. F. R. (1986) *J. Biol. Chem.* **261**, 5758-5765

161. Hodge, D. D., Mancini, P., Davis, F. M. and Heywood, P. (1977) *J. Cell. Biol.* **72**, 194-208

162. Lewis, C. D., Lebkowski, J. S., Daly, a. k. and Laemmli, U. K. (1984) *J. Cell. Sci. Suppl.* **1**, 103-122

163. Evans, G., and Hancok, D. C. (1985) *Cell* **43**, 253-261

164. Menard, L., Thibault, L., Gaudreau, A., and Poirier, G. G. (1987) in *Niacin, Nutrition, ADP-Ribosylation and Cancer* (Jacobson, E. L. and Jacobson, M. K. eds.) in press, Springer-Verlag, Berlin, Heidelberg, New York, Tokyo

165. Wesierska-Gadek, J., and Sauermann, G. (1987) Abstr. in *The 8th International Symposium on ADP-Ribosylation*, Fort Worth, TX. U.S.A., May 30-jun 3

166. Lamarre D., Murcia, G. de., Talbot, B., Laplante, C., Leduc, Y., and Poirier, G. G. (1987) in *Niacin, Nutrition, ADP-Ribosylation and Cancer* (Jacobson, E. L. and Jacobson, M. K. eds.) Springer-Verlag, Berlin, Heidelberg, New York, Tokyo

167. Adamietz, P. (1987) *Eur. J. Biochem.* **169**, 365-372

168. Mathis, G., and Althaus, F. R. (1987) in *Niacin, Nutrition, ADP-Ribosylation and Cancer* (Jacobson, E. L. and Jacobson, M. K. eds.) in press, Springer-Verlag, Berlin, Heidelberg, New York, Tokyo

169. Scovassi, A. I., Plevani, P. and Bertazzoni, U. (1980) *Trends Biochem. Sci.* **5**, 335-337

170. Mullenders, L. H. F., Van Kesteren, A. C., Bussmann, C. J. M., Van Zeeland, A. A., and Natarajan, A. T. (1984) *Mutat. Res.* **141**, 75-82

171. Jackson, D. A., McCready, S. J., and Cook, P. R. (1984) *J. cell Suppl.* **1**, 59-79

172. Bohr, V. A., Smith, C. A., Okamoto, D. S., and Hanawalt, P. C. (1985) *Cell* **40**, 359-369

173. Madhani, H. D., Bohr, V. A., and Hanawalt, P. C. (1986) *Cell* **45**, 417-422

174. Gasser, S. M., and Laemmli, U. K. (1987) *Trends genet.* **3**, 16-22

175. Gross, D. S.. and Garrard, W. T. (1987) *Trend Biochem.* **12**, 293-297