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Poly(ADP-ribosyl)ation of proteins associated with nuclear matrix in rat testis*

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We have previously demonstrated that a significant percentage of poly(ADPR) polymerase is present, as a tightly-bound form, at the third level of chromatin organisation defined by chromosomal loops and nuclear matrix. The present work is focused on the study of poly(ADP-ribosyl)ation of proteins present in these nuclear subfractions.

It has been shown that, due to the action of poly(ADPR) polymerase, the ADP-ribose moiety of [¹⁴C]NAD is transferred to both loosely-bound and tightly-bound chromosomal proteins, which in consequence are modified by chain polymers of ADP-ribose of different lengths. Moreover, histone-like proteins seem to be ADP-ribosylated in chromosomal loops and nuclear matrix associated regions of DNA loops (MARS).

A hypothesis can be put forward that the ADP-ribosylation system is functionally related to the nuclear processes, actively coordinated by the nuclear matrix.

Our interest in studying the poly(ADP-ribosyl)ation system associated to the nuclear matrix came from the supposition that the nuclear matrix and ADP-ribosylation of nuclear proteins appear to be implicated in the same processes.

It is now possible to have a better insight into the internal structure of the interphase nucleus. The nuclear framework now characterised as a nonhistone protein scaffold supporting the attachment points of DNA loops [1], seems to influence gene replication and activity. It is widely accepted that the fundamental biological processes of the cell nucleus are associated

with the nuclear matrix. There is experimental evidence that DNA replication [2], transcription [3], mRNA processing [4] and DNA repair [5] are actively coordinated by the nuclear matrix framework.

Poly(ADP-ribosyl)ation, a post translational protein modification, seems also to play a crucial, yet unclear, biological role in these processes [6].

DNA dependent poly(ADPR)¹ polymerase (PARP), present in all eukaryotes, is a very versatile enzyme that transfers the ADP-ribose moiety of NAD to acceptor proteins. It catalyses the formation of linear or branched poly(ADP-

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¹Abbreviations: ADPR, ADP-ribose; Gu-HCl, guanidine hydrochloride; MARS, matrix associated region(s) of chromatin; PARP, poly(ADPR) polymerase; PMSF, phenylmethylsulphonyl fluoride.

ribose)-protein conjugates through a multistep process consisting of the initiation, elongation and branching reactions. The product of these reactions is a homopolymer which varies in complexity, containing up to 200 ADP-ribose residues and multiple branching points up to a proportion of 3%. Poly(ADPR) catabolism is achieved in cells by a poly(ADPR) glycohydrolase [7] and an ADP-ribosyl protein lyase [8] which cleaves the last ADP-ribose residue left on the acceptor by glycohydrolase. The half life of the poly(ADPR) varies with the length of the polymer and the nature of the acceptor.

The proteins susceptible to ADP-ribosylation identified so far, are both histone and non-histone proteins [6, 9]. PARP itself is the major polymer acceptor protein and the automodified enzyme is less active, probably because its affinity for DNA decreases [10]. It has been stated that elongation of the polymer is most probably of a distal mode [11] and is accomplished in a distributive fashion [12]: PARP acts as a dimer with an intermolecular mechanism of action [13].

It is thought that PARP plays a role in the maintenance of genetic integrity. As an example, PARP is defective in the cells from patients suffering from *Xeroderma pigmentosum*, which are unable to excise pyrimidine dimers, induced by ultraviolet radiations [14]. Mathis & Althaus [15] showed that changes in chromatin structure, which accompany repair by DNA excision, were inhibited in cells devoid of poly(ADPR). Furthermore, Satoh *et al.* [16] have shown that poly(ADPR) metabolism is involved in base excision repair but not in nucleotide excision repair.

Moreover, poly(ADPR) is involved in many cellular functions during which genetic information must be decoded and transmitted from cell to cell. PARP has been found associated with the active regions of transcription [17]. The expression level of the PARP gene varies during differentiation of cells, e.g. in neuronal cells [18], HL-60 cells [19], *etc.* In general, PARP seems to be more active in the S and G2 phases of the cell cycle [20].

In all cellular events involving poly(ADP-ribosylation), the state of chromatin represents a signal. It has been shown that poly(ADP-ribosylation) could affect chromatin structure by direct covalent modification of chromosomal proteins and by specific histone electrostatic

interactions [6, 9]. de Murcia *et al.* [21] visualised by electron microscopy modulation of the chromatin superstructure induced by synthesis and degradation of poly(ADPR).

A possible mechanism of poly(ADP-ribosylation) reaction, proposed by Althaus [22] takes into account all the described features of the poly(ADP-ribosylation) reaction and tries to give the functional meaning of the reaction. According to this mechanism, histone proteins are reversibly detached from the chromatin by the concerted action of poly(ADPR) polymerase and poly(ADPR) glycohydrolase; the long ADP-ribose chains linked to the PARP enzyme would be responsible for the dissociation of the chromatin structure, and would then be degraded by the de-ADP-ribosylating enzyme to allow reassociation of histones to DNA.

The use of rat testis as an experimental model came from the knowledge of a high level of poly(ADP-ribosylation) in that tissue, correlated to germinal cell differentiation [23]. In a previous paper we reported that, in rat testis, a tightly bound form of PARP could be identified which did not seem to be an intrinsic component of the nuclear matrix, but only indirectly associated to this structure and localised rather in the matrix associated regions of the chromatin [24]. Our experiments were then carried out in order to determine both the entity of the ADP-ribosylation reaction in the nuclear matrix and the identity of the nuclear ADP-ribose-protein conjugates.

MATERIALS AND METHODS

Materials. [U-¹⁴C]NAD⁺, nicotinamide [U-¹⁴C]adenine dinucleotide ammonium salt (248 mCi/mmol), was supplied by Amersham International PLC; [³²P]NAD⁺, nicotinamide adenine dinucleotide di(triethylammonium) salt ([³²P]adenylate), 30 Ci/mmol, was purchased from DuPont New England Nuclear; DNase I (EC 3.1.21.1), snake venom phosphodiesterase (EC 3.1.4.1), alkaline phosphatase (EC 3.1.3.1), phenylmethylsulphonyl fluoride (PMSF), leupeptin, spermine and spermidine were obtained from Sigma Chemical Co. Electrophoretic molecular mass markers were purchased from Pharmacia, X-Omat RP films from Kodak and nitrocellulose filters (0.45 µm pore size, type HA) from Millipore.

Isolation of nuclei and chromatin fractions.

Rat testis nuclei were isolated by homogenisation and differential centrifugation, as previously described [23, 24], except that Ca^{2+} and Mg^{2+} were replaced by 0.15 mM spermine, 0.75 mM spermidine and 1 mM EDTA/EGTA. Proteases were irreversibly inhibited by 1 mM PMSF and 0.1 mM leupeptin.

Chromatin, dissociated for 1 h at 4°C by high-salt treatment [24], was subsequently fractionated into stripped chromatin loops and nuclear matrix by use of differential sucrose density centrifugation, as described elsewhere [24].

An alternative procedure was as described by Tubo & Berezney [25] and consisted essentially of endogenous digestion of isolated nuclei, followed by a three times repeated extraction with a high salt buffer (2 M NaCl, 0.2 mM MgCl_2 , 1 mM PMSF in 10 mM Tris/HCl, pH 7.4). The nuclear matrix was then re-suspended in one third volume of low-salt buffer (0.2 mM MgCl_2 , 1 mM PMSF in 10 mM Tris/HCl, pH 7.4).

With both procedures of nuclear matrix preparations a high amount of residual DNA (25%–10%) was often obtained. The so-called DNA-rich nuclear matrix had then to be submitted to extensive digestion with DNase I (600 U/mg of DNA) and RNase A (100 U/mg of DNA) for 12–20 h at 4°C. The digested nuclear matrix fraction contained 2%–3% of the total nuclear DNA that represented the matrix associated regions of DNA loops.

In other experiments, an aliquot of the DNA-rich nuclear matrix was sonicated by two 20-s pulses at the lowest setting possible (Branson Sonifier, Model 145W). The supernatant (matrix extract) and the pellet (sonicated matrix) were finally re-suspended in low-salt buffer.

Nuclear proteins extraction. Acid-soluble proteins contained in nuclei, in nuclear matrix or in stripped chromatin loops were extracted three times by 1 h stirring at 4°C in 0.2 M H_2SO_4 , and centrifuged at $10000 \times g$ for 15 min. The extracts were pooled and proteins precipitated with 6 vols. of ice-cold acetone at –20°C overnight. Precipitates were collected by centrifugation at $18000 \times g$ for 20 min at –10°C.

Whole nuclear matrix proteins were solubilised in 0.5 M Tris/HCl, pH 8.8, containing 6 M guanidine hydrochloride (Gu-HCl), 75 mM dithiothreitol, 5 mM EDTA. After 1 h at room temperature, the samples were centrifuged for 2 h at $150000 \times g$ in a Beckman Ti60 rotor.

Poly(ADP-ribosyl)ation reaction. Intact nuclei were re-suspended (50×10^6 nuclei/ml) in 0.25 M sucrose containing 10 mM Tris/HCl, pH 8, 14 mM 2-mercaptoethanol, 10 mM MgCl_2 , 60 mM NaCl, 4 mM NaF, 1 mM PMSF, and incubated with 0.2 mM NAD^+ and 1 μCi of [^{14}C]- NAD^+ or [^{32}P] NAD^+ per 25×10^6 of nuclei for 40 min at 20°C. The reaction was terminated by the addition of 8 mM 3-aminobenzamide and the nuclei, collected by centrifugation at 3000 r.p.m. for 15 min at 4°C, were washed twice with the incubation buffer to remove unbound radioactivity. The different chromatin fractions were then prepared from the incubated nuclei by the previously described procedures.

Poly(ADP-ribose) polymerase assay. In a typical assay, the reaction mixture (final volume 250 μl) contained: 100 mM Tris/HCl, pH 8, 14 mM 2-mercaptoethanol, 10 mM MgCl_2 , 4 mM NaF, 1 mM dithiothreitol, 400 μM [^{14}C] NAD^+ (10000 c.p.m./nmol), 12 μg DNase I, 10 μg DNA, 10 μg histone H1 and, as enzyme source, an amount of nuclei or chromatin fraction corresponding to 30 μg of protein. After 10 min incubation at 20°C, the reaction was stopped with ice-cold trichloroacetic acid and the radioactivity present in the acid-insoluble material, collected on a HAWP (0.45 μm) filter, determined on a Beckman LS8100 liquid scintillation spectrometer. One enzymatic unit was defined as the enzyme activity catalysing the incorporation, per minute at 20°C, of one μmole of ADP-ribose into acid-insoluble material.

Electrophoretic analysis. Acid-soluble nuclear proteins were analysed by electrophoresis on urea/acetic acid/20% polyacrylamide slab-gels (pH 2.9) and SDS/7%–15% polyacrylamide slab-gels, as described by Nicholas & Goodwin [26]. Each labelled sample was run in duplicate and either stained with Coomassie Brilliant Blue R-250 or autoradiographed. Fluorographic analysis of ^{14}C -labelled samples was performed after conditioning the gel with ENTENSIFY universal autoradiography enhancer (DuPont NEN).

Analysis of reaction products. Intact [^{32}P]poly(ADP-ribose) moieties incorporated into the proteins were detached by incubation at 60°C for 3 h with 10 mM Tris/NaOH, pH 12, 1 mM EDTA. Samples were extracted with phenol/ CHCl_3 /isoamyl alcohol (49:49:2, by vol.), dried in a Speed-Vac and dissolved in 50% urea, 25 mM NaCl and 4 mM EDTA, pH 7.5, to be

analysed on 20% polyacrylamide slab-gel [27]. Alternatively, [^{14}C]ADP-ribosylated proteins were subjected to enzymatic digestion overnight at 18°C with snake venom phosphodiesterase (0.25 U) and alkaline phosphatase (2.5 U) in 250 mM NH_4 -acetate, pH 9, in the presence of 10 mM MgCl_2 . Samples deproteinised and neutralised with 0.1 M NaOH were analysed by reverse-phase HPLC as described by Kielbauch *et al.* [28].

Proteins and DNA assay. Protein concentration was determined using the protein assay reagent (Pierce) and bovine serum albumin as a standard. DNA content was determined on the basis of the absorbance at 260 nm ($1.0 A_{260}$ = about 50 μg of DNA/ml).

RESULTS AND DISCUSSION

Figure 1 shows a comparison of different rat testis nuclear matrix preparations. With the use of both procedures reported in Materials and Methods, nuclear matrix preparations were often obtained which differ, with regard to DNA, proteins, PARP and poly(ADPR) content. In fact, rat testis nuclei preparations did not seem to share the same sensibility to the action of endogenous DNases, probably because of the peculiar composition of the tissue (germinal cells at different stages of differentiation). The content of DNA underwent always

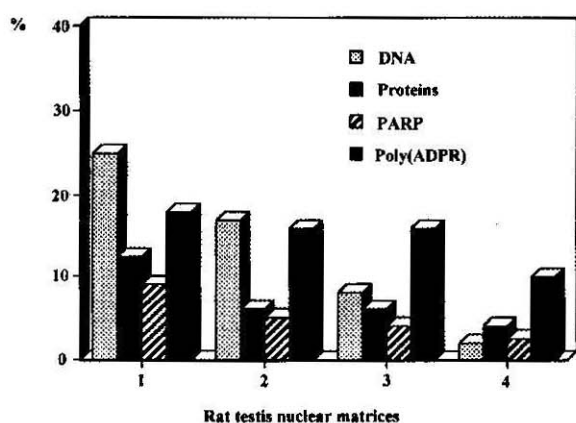


Fig. 1. Residual DNA, nuclear proteins, PARP and poly(ADPR) content in different rat testis nuclear matrices.

The histogram shows these values as percentage of the amounts determined in intact isolated nuclei. The different nuclear matrix preparations reported (1–4) are indicative of the different types of nuclear matrix that can be obtained from rat testis nuclei, independently of the experimental procedure used.

larger changes than that of proteins or of PARP activity. Moreover, the tightly-bound enzymatic activity recovered in a DNA-rich nuclear matrix (40%–20% DNA) represents 20%–10% of the total PARP nuclear activity and decreases to a minimal value of 2.5% in a nuclear matrix containing 2% DNA as matrix associated regions of chromatin (MARS).

On the other hand, the decrease of DNA and tightly-bound proteins was paralleled by a decrease in PARP but not in the content of poly(ADPR). The percentage of poly(ADPR) which was associated to nuclear matrices prepared from nuclei incubated with 200 μM [^{14}C]NAD varied from 20% to 10%. It is noticeable that the poly(ADPR) content was not directly related to the amount of both DNA or protein, since the poly(ADPR)/protein ratio was about three times higher in a nuclear matrix containing only 2% of residual DNA, than in a DNA-rich matrix (Fig. 1). These results seem to indicate that the nuclear matrix is enriched in ADP-ribosylated proteins. Moreover, drastic treatments (DNase/RNase digestion or sonication) of nuclear matrices are not effective in solubilising most (60%–40%) of the ADP-ribose associated to nuclear matrix. Instead, ADP-ribose was completely dissociated by the use of 6 M Gu-HCl, thus confirming the high level of ADP-ribosylation of the intrinsic matrix components (Table 1).

We have examined the level of ADP-ribosylation in the nuclear matrix, considering both the extent of the modification and the size of the ADP-ribose oligomers.

Poly(ADPR) associated to acid-soluble proteins extracted from nuclei and nuclear matrix was digested with phosphodiesterase and alkaline phosphatase and analysed by reverse-phase HPLC (not shown). These analyses led to the determination of the average length of the poly(ADPR) chain using a formula based on the ratio between adenosine derived from the last residue of each chain and ribosyl-adenosine derived from the hydrolysis of each residue of each chain. The results indicated that polymers as large as 8–13 residues on average were incorporated into proteins extracted from nuclei, whereas polymers not larger than 5 residues modified tightly-bound chromosomal proteins, still bound to nuclear matrix associated regions of chromatin (MARS). Moreover, the longest polymers were present in the Gu-

Table 1
Stability to different treatments of
poly(ADP-ribose)- associated to rat testis nuclear
matrix

The data represent the distribution of DNA, proteins and poly(ADP-ribose) in the matrix extract (supernatant) and nuclear matrix (pellet) after the treatments indicated, giving as 100% their content in a DNA rich nuclear matrix. ND, not detected.

Treatment	DNA (%)	Proteins (%)	Poly(ADP-ribose) (c.p.m. %)
DNase + RNase			
Supernatant	100	44	36
Pellet	—	56	64
Sonication			
Supernatant	33	40	34
Pellet	66	60	66
6 M Guanidine-HCl			
Supernatant	ND	100	100
Pellet	—	—	—

HCl extract together with intrinsic nuclear matrix proteins.

To characterise the fraction of poly(ADPR) solubilised by treatment with 6 M Gu-HCl, aliquots of nuclei incubated with 200 μ M [32 P]NAD and of the nuclear matrix isolated from these nuclei, were subjected to alkaline hydrolysis (pH 12), thus detaching the intact polymer, which was then processed for sequencing by 20% polyacrylamide gel electrophoresis and autoradiography. The typical ladder of poly(ADPR) indicates, in the nuclear matrix sample, a polymer population, different from that observed in the nuclei sample, enriched in < 20 moiety chains, and in a top fraction which is known to be due to presence of branched polymers (Fig. 2).

These data are shown in Table 2, together with data on the extent of ADP-ribosylation of loosely-bound (2 M NaCl extractable), tightly-bound (2 M NaCl resistant) chromosomal proteins and total nuclear matrix proteins (6 M Gu-HCl extractable), determined on the basis of the [14 C]ADP-ribose incorporated as acid-insoluble radioactivity. While loosely-bound histone and non-histone proteins contained approximately 100 pmoles of ADP-ribose/mg

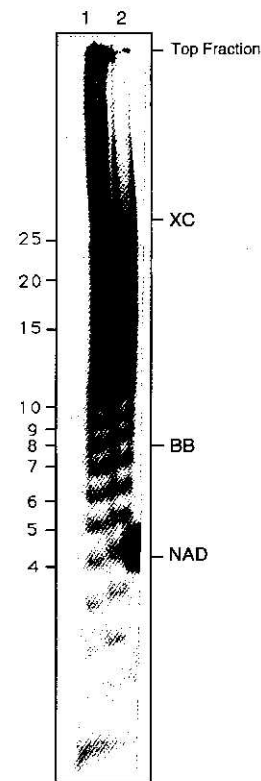


Fig. 2. Autoradiography of [32 P]poly(ADPR). The preparations were produced by incubation of nuclei with [32 P]NAD $^{+}$, detached by alkaline hydrolysis from nuclear matrix (1) and nuclei (2) and analysed on a 20% polyacrylamide sequencing slab-gel. The number of ADP-ribose residues in the chain (4–25) is indicated, referred to the migration of NAD corresponding to (ADPR) $_4$. XC is for xylene-cyanol and BB for bromophenol-blue.

of protein, tightly-bound proteins seemed to be ADP-ribosylated almost 5-fold as intensely, whereas intrinsic nuclear matrix components

Table 2
Protein bound poly(ADP-ribose) in different
nuclear fractions

Fraction	Poly(ADP-ribose)	
	pmol ADPR/mg protein	Number of residues
Loosely-bound chromosomal proteins 2 M NaCl extractable (histone and nonhistone proteins)	110	8–13
Tightly-bound chromosomal proteins 2 M NaCl resistant (histone and nonhistone proteins)	570	2–5
Nuclear matrix proteins 6 M Gu-HCl extractable (nonhistone proteins)	341	< 20

contained an intermediate amount of incorporated poly(ADPR) (341 pmoles/mg of protein).

To identify ADP-ribose acceptors, protein components present in samples obtained from [^{14}C]NAD $^{+}$ incubated nuclei were separated by means of 18% polyacrylamide gel electrophoresis on acetic acid/urea and processed for fluorographic analysis.

Fluorography of [^{14}C]ADP-ribosylated acid-soluble proteins from nuclei and other nuclear fractions (Fig. 3) revealed that radioactivity was associated to the histone-like proteins in stripped chromatin loops and nuclear matrix (10% residual DNA) as well as in nuclei and in the 2 M NaCl extract. Moreover, the component carrying most of the radioactivity appeared to be histone H1 which, in the acidic electrophoretic system, splits into two bands containing separately the testis specific variants H1 a,t and the somatic variants H1 b-e.

Total nuclear matrix ADP-ribosylated proteins solubilised by treatment with 6 M Gu-HCl were separated by 7%–15% gradient polyacrylamide gel electrophoresis in 1% SDS. The corresponding fluorography (Fig. 4) indicated that, among the intrinsic components of the matrix of a molecular mass ranging from 20 kDa to 120 kDa, several proteins were modified; a portion of released poly(ADPR) was also detected in the low molecular mass region of the gel, as a

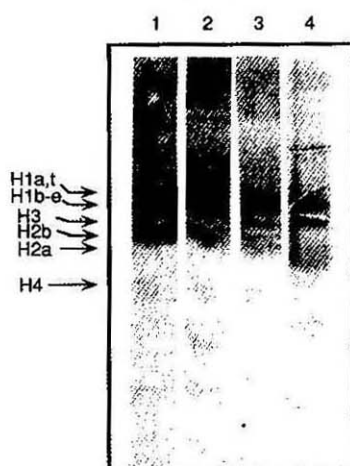


Fig. 3. Fluorography of 0.2 M H $_2$ SO $_4$ extractable [^{14}C]ADP-ribosylated proteins.

These proteins were separated by acetic acid/urea/20% polyacrylamide slab gel electrophoresis, from: (1) chromatin; (2) loosely-bound proteins in 2 M NaCl extract; (3) stripped chromatin loops; (4) matrix associated regions of chromatin (MARS). The arrows indicate the mobility of native histone proteins in the acidic electrophoretic system.

consequence of the solubilisation conditions used.

Further analyses will focus on the characterisation of nuclear matrix proteins which represent the poly(ADPR) acceptor in the rat testis nuclear matrix. The need of a drastic treatment, such as 6 M Gu-HCl, to solubilise these proteins, explains the difficulties found so far in the characterisation of these ADP-ribose acceptors. In fact, we are dealing with intrinsic components of the nuclear matrix which, as it is well known, cannot be easily solubilised. The composition of the nuclear matrix was found to be [1, 29] astonishingly complex: despite many efforts, knowledge of the components of the proteinaceous nucleus skeleton is still in its infancy.

In conclusion, our results are in line with recent reports on the existence, within the nucleus, of multiple classes of PARP molecules, representing different functional forms of the enzyme. Several authors have reported the presence of variable amounts of PARP in nuclear matrices isolated from different sources [30–32]. According to Kaufmann *et al.* [33], the recovery of PARP in rat liver nuclear matrix varies with the extraction conditions, since the association of the enzyme to this structure is mediated by formation of intermolecular disulphide bonds.

Thus, the amount of poly(ADPR) (10%) associated with the rat testis nuclear matrix is far

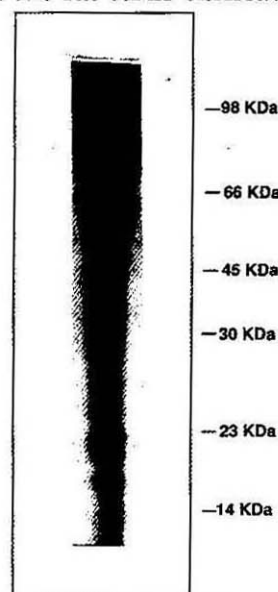


Fig. 4. Fluorography of 6 M Gu-HCl extractable [^{14}C]ADP-ribosylated nuclear matrix proteins.

These proteins were separated by 7%–15% polyacrylamide gradient slab gel electrophoresis with 1% SDS.

from being negligible. Analyses performed to characterise the reaction product have revealed that nuclear proteins are modified by three classes of poly(ADPR) differing in length and complexity. Moreover, the ADP-ribose content, in terms of pmoles/mg of protein, is higher in general in the tightly-bound chromosomal proteins than in the loosely-bound chromosomal components, suggesting the presence of different ADP-ribosylation sites in the two classes of proteins.

It was found that tightly-bound histones were ADP-ribosylated, and that they interacted specifically with the matrix associated regions of the chromatin (A+T rich sequences involved in higher order chromatin organisation and containing the regulatory/enhancer regions of several genes). Tightly-bound proteins are a small class of proteins, characterised by strong bonds with DNA, which are likely to play an important role in gene regulation, being not-randomly distributed on chromatin but associated with DNA stretches enriched in tissue-specific active gene sequences [24]. Interestingly, their modification could imply the involvement of the ADP-ribosylation reaction in the attachment of DNA loops to the nuclear matrix, and/or in some steps of DNA metabolism which occurs in association with the nuclear matrix.

Finally, an interesting hypothesis can be drawn concerning the well known automodification mechanism of PARP. The presence of a portion of poly(ADPR) tightly associated to the nuclear matrix raises the possibility of the occurrence of an auto-ADP-ribosylated form of the enzyme anchored to the nuclear matrix, as already reported by Brauer *et al.* [34] in rat liver. This automodified form of PARP, moreover, has to be considered not an intrinsic component of the matrix but a resting form of this nuclear enzyme.

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