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Active Poly(ADPribose) Metabolism in DNAase- and Salt-Resistant Rat Testis Chromatin With High Transcriptional Activity/Competence

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Abstract A chromatin fraction, named pP fraction, was prepared from rat testis nuclei, which had been digested with nuclease in order to separate soluble and insoluble chromatin. This fraction resembled nuclear matrix as it was highly resistant to DNAase digestion, had a high content of proteins compared to the low DNA percentage, and a noticeable transcriptional activity. Moreover, poly(ADPribosyl)ation system (i.e., poly(ADPR)polymerase, poly(ADPribose), and acceptor proteins) was still present at high levels. In order to study whether it might be identified as the protein support surrounding chromatin loops, this pP fraction was further analyzed after 3 M NaCl extraction. The 3 M NaCl extract and the highly insoluble pellet, named Nuclear Matrix Pellet, were characterized as it regards DNA, newly synthesized RNA and proteins. Furthermore, poly(ADPribose) metabolism was analyzed by measuring both poly(ADPribose) polymerase and poly(ADPribose) glycohydrolase activities, poly(ADPribose) distribution and by identifying protein acceptors. The final pellet had features of nuclear matrix containing less than 10% DNA and high percentage of proteins; 28% of newly synthesized RNA was still associated with this fraction. Long and branched polyADPribose were found in the nuclear matrix-like pellet, although ADPribose acceptors (mainly H1 and core histones) appeared to be modified mostly with short ADPribose oligomers. Longest and branched polymers were retained on the top of protein gel, likely bound to automodified poly(ADPribose) polymerase. J. Cell. Biochem. 89: 688–697, 2003.

Key words: poly(ADPribose) polymerase; transcription; rat testis chromatin; poly(ADPribose)

Poly(ADPribosyl)ation is a reversible post-translational modification of proteins which appears to be somehow involved in the modulation of DNA metabolism [Ziegler and Oei, 2001]. Poly(ADPribose) is the long and branched polyanion synthesized in the reaction catalyzed by any member of the poly(ADPribose) polymerase (PARP) family, which requires NAD^+ as substrate and transfers ADPribose units to specific acceptor proteins [Althaus and Richter, 1987; Lautier et al., 1993; de Murcia and Menissier-de Murcia, 1994; Jacobson and Jacobson, 1999; Scovassi and Poirer, 1999].

Targets of this group of enzymes are proteins (histones, non-histones, enzymes, including PARP itself) involved in a number of cellular functions, from DNA repair and transcription to cellular transport [Das and Berger, 1986; Boulikas, 1993; Malanga and Althaus, 1994; De Flora et al., 1997; D'Amours et al., 1999; Burkle, 2000; Lupi et al., 2000]. Among poly(ADPribose) acceptors the structural proteins binding DNA weaken their interaction with the nucleic acid as a consequence of the alteration in the charge balance introduced by the polymer. On the other hand, poly(ADPribosyl)ation can reversibly activate or inhibit functional proteins like enzymes or transcription factors.

Activation of PARP by nicked DNA leads to stimulation of DNA repair apparatus and to regulation of transcription and replication machineries [Durkacz et al., 1980; Hough and

Abbreviations used: ADPR, adenosine diphosphate ribose; NMP, Nuclear Matrix Pellet; P, insoluble chromatin; PARP, poly(ADPribose) polymerase; PMSF, phenyl methyl sulphonyl fluoride; pP, highly insoluble chromatin; S, soluble chromatin; S3M, 3 M NaCl extract from pP fraction; sS, soluble chromatin from nuclease digested P.

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Smulson, 1984; Boulikas, 1993; Simbulan-Rosenthal et al., 1999, 2000; Shall and de Murcia, 2000].

PARP is also important for telomere maintenance and for suppressing recombination at DNA ends, and binds DNA sequences that anchor chromatin to the nuclear matrix [Wesierska-Gadek and Sauermann, 1985; Cardenas-Corona et al., 1987; Alvarez-Gonzales and Ringer, 1988; Smith and de Lange, 2000]. This sub-nuclear structure can be envisaged as the check-point of condensed/decondensed state of chromatin and governs the main nuclear functions. Many of the above mentioned nuclear functions are under control of nuclear matrix, where the initiation sites of duplication and transcription are localized, and regulation of DNA compaction in chromosomes, needed to reach an efficient mitotic segregation, takes place [Tubo and Berezney, 1987; Zlatanova and van Holde, 1992; Berezney et al., 1995; Tong et al., 2001].

The link between PARP activity and transcriptionally active/competent chromatin is widely recognized, although PARP modes of action are still controversial. A number of evidence demonstrates that PARP is preferentially associated with actively transcribing/competent chromatin [Hough and Smulson, 1984; Wesierska-Gadek and Sauermann, 1985; Cardenas-Corona et al., 1987; Alvarez-Gonzales and Ringer, 1988; Meisterernst et al., 1997; Malanga et al., 1998; Malanga and Farina, 2000; Tong et al., 2001; Ziegler and Oei, 2001].

These results have been confirmed in our laboratory by studying the correlation between poly(ADPribosyl)ation and structural and functional changes of rat testis chromatin [D'Erme et al., 1991; De Lucia et al., 1996; Faraone-Mennella et al., 1999; Quesada et al., 2000].

We had previously demonstrated that in rat testis chromatin loops and nuclear matrix a tightly bound form of PARP is present, and this enzyme form is likely associated with matrix-residual DNA, where chromatin loops are anchored [D'Erme et al., 1991]. In parallel, we had followed a different experimental approach that consisted to separate soluble and insoluble chromatin fractions by digesting rat testis nuclei with DNAase I [De Lucia et al., 1996] and avoiding their high salt extraction, that was used elsewhere [D'Erme et al., 1991; Quesada et al., 2000]. The presence of sites hypersensitive to DNAase I has been correlated generally with decondensation of chromatin regions undergoing transcriptional activity [Wu, 1980]. The results that we obtained confirmed the preferential association of most poly(ADPribose) with a highly insoluble, DNAase resistant chromatin fraction and evidenced also a non-random distribution of poly(ADPribose) among the different fractions [De Lucia et al., 1996]. We hypothesized the existence of two classes of polymers with likely different functional roles, one associated with the chromatin of transcriptional active domains, and the other one in the most insoluble fraction [De Lucia et al., 1996].

The mild and progressive nuclease digestions of rat testis nuclei, incubated with NAD^+, had fractionated the nuclear pellet into various soluble (DNAase sensitive, fractions S and sP) and an extremely insoluble (DNAse resistant, fraction pP) chromatin [De Lucia et al., 1996]. The fraction pP had been considered as comparable to nuclear matrix due its extreme resistance to nuclease digestion and its low DNA percentage [De Lucia et al., 1996]. Moreover, it contained still a high percentage of newly synthesized RNA.

Here we present evidence that pP chromatin can be further fractionated by high salt extraction that gives rise to a new insoluble fraction named NMP (nuclear matrix pellet), containing DNA $<$20 kbp, in the size range described for nuclear matrix, and a 3 M extract with DNA size much higher than 20 kbp.

The characterization of NMP fraction as it regards polyADPribosylation system, proteins and RNA is reported and show patterns resembling those described for the nuclear matrix. Furthermore we provide a more extensive and complete analysis of all rat testis chromatin fractions, as it regards particularly the distribution of specifically sized DNA, RNA and proteins, poly(ADPribose) turnover (PARP and PARG activities), and identification of ADPribose acceptor proteins, prepared under non-denaturing conditions (i.e., avoiding the mild acidic extraction previously used).

MATERIALS AND METHODS

Materials

[^{32}P]NAD^+, nicotinamide adenine dinucleotide di(triethylammonium)salt (adenylate ^{32}P), 1,000 Ci/mmol, and [^{32}P]UTP, 1,000 Ci/mmol, were supplied by Amersham International; DNAase I (EC 3.1.21.1), phenylmethyl sulpho-

nyl fluoride (PMSF), leupeptin, chimostatin, antipain, pepstatin, spermine, and spermidine were obtained from Sigma Chemical Company, Milano, Italy.

Electrophoretic markers were purchased from Sigma (code P1677) and MBI-Fermentas (*Eco*RI + *Hind*III λ DNA, code no. SM03193; pUC, code no. SM03033); PVDF filters (0.45 μm pore size, type HA) were from Bio-Rad, Milano, Italy.

Preparation of Chromatin Fractions

Nuclei from adult rat testes (6 g) were isolated according to De Lucia et al. [1996]. Isolated nuclei were resuspended in 15 mM Tris-HCl, pH 7.5, 0.25 M sucrose, and digested with DNAase I (41 U/mg DNA), in the presence of 0.66 mM $MnCl_2$ for 2 min at 30°C. Nuclease activity was terminated by addition of 7.5 mM Na–EDTA. Digested nuclei were suspended in 10 mM Tris-HCl buffer, pH 7.5, 1 mM Na–EDTA, 1 mM phenylmethylsulfonil fluoride, PMSF (lysis buffer) and lysed by incubation for 1 h at 0°C with intermittent gentle agitation by passage through a Pasteur pipette. Lysed nuclei were centrifuged for 20 min at 12,000*g*. The supernatant was collected and the pellet re-extracted twice as above in lysis buffer.

The pooled supernatant are referred to as "soluble chromatin" (fraction S) and the material remaining after extraction is referred to as fraction P or insoluble fraction. Fraction P was further digested with DNAase I (41 U/mg; 26 μg) for 5 min at 0°C under the same conditions as above. After centrifugation, the new soluble (sP) and insoluble (pP) fractions were obtained.

The latter pellet, pP, was extracted overnight in 10 mM Tris-HCl buffer, pH 7.4/20 mM Na–EDTA/3 M NaCl. After centrifugation at 12,000*g* for 20 min, the supernatant was collected and pellet (NMP) was re-extracted twice for 1 h as above. The three supernatants were pooled (S3M supernatant) and NMP fraction was suspended in 24 mM Tris-HCl buffer, pH 6.8, containing 0.6 M urea, 10% glycerol, 2.3% SDS. Assaying enzymatic activities urea and SDS were avoided.

All procedures were performed at 4°C, except where indicated. Proteinases were irreversibly inhibited by the presence of 1 mM PMSF in all steps except for nuclei digestion and lysis in which leupeptin 10 μg/ml, chimostatin 10 μg/ml, antipain 5 μg/ml, and pepstatin 5 μg/ml were added.

Gel Electrophoresis and Western Blotting

Proteins of soluble and insoluble chromatin fractions were analyzed on 12% polyacrylamide slab gels in the presence of 0.1% SDS as described previously [De Lucia et al., 1994] and electrotransferred onto PVDF membrane (Bio-Rad) at 200 V for 2 h at 4°C in the same buffer used for the electrophoretic run.

Images of stained gels and filters and autoradiographic patterns were acquired by a phosphor imager (mod. FX, Bio-Rad).

For immuno-blot experiments procedures and buffers were according to Harlow and Lane [1988]. PVDF sheets were treated for 3 h with the blocking solution (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.5% (v/v) Tween-20, and 3% (w/v) gelatin. Incubation with commercial anti-PARP antibodies (Santa Cruz, Santa Cruz, CA; rabbit anti-human PARP, H-250, 1:2,000, v/v) was performed for 15 h at room temperature in the same solution supplemented with 0.3% gelatin).

The blots were washed several times with TBS–Tween and antibody binding was detected by using phosphatase conjugated goat anti-rabbit IgG from Bio-Rad [Harlow and Lane, 1988]. Phosphatase reaction was revealed by using a kit for chemiluminescence (Super Signal West Dura Extended Substrate, 34075, Pierce, Pierce, MA) and reading by the phosphor imager (Bio-Rad).

Poly(ADPribose)Polymerase Assay

Poly(ADPribose) polymerase (PARP; E.C. 2.4.2.30) activity was assayed under standard conditions [De Lucia et al., 1996] for 10 min at 25°C in the presence of 0.4 mM [^{32}P]NAD (20,000 cpm/nmol).

The reaction mixture (final volume 50 μl) contained 100 mM Tris-HCl, pH 8.0, 1.0 mM DTT, 10 mM $MgCl_2$, 4 mM NaF, 40 U DNAase I (Sigma), 10 μg calf thymus DNA, and, as enzyme source, an amount of nuclei or chromatin fractions corresponding to 1.0 μg of DNA.

The reaction was stopped with ice-cold 20% trichloroacetic acid and the radioactivity present in the acid-insoluble material, collected on a HAWP filter (0.45 μm, Millipore), determined on a Beckman LS 1701 liquid scintillation spectrometer.

One enzymatic unit was defined as the enzyme activity catalysing the incorporation, per minute at 25°C, of one μmol of ADP-ribose into

acid-insoluble material. Activity was expressed as milliunits (mU).

Poly(ADPribose) Glycohydrolase Assay

The assay was performed according to Bernardi et al. [1997]. Duplicate aliquots (1μg DNA) of each chromatin fraction were incubated in the presence of [^{32}P]NAD$^+$ under the conditions of PARP assay. After incubation the reaction mixture of one aliquot (mixture A) was stopped by adding 20% trichloroacetic acid (final) and, after filtration on Millipore membrane, measuring the incorporated radioactivity. This labeling was taken as the amount of [^{32}P]poly(ADPribose) available as substrate of PARG.

The second mixture (mixture B) was adjusted to 50 mM K-phosphate buffer, pH 7.5/50 mM KCl, 10 mM 2-mercaptoethanol, and BSA (100 μg/ml) in a final volume of 60 μl, and incubated at 37°C for 10 min. Proteins were precipitated with 20% trichloroacetic acid and radioactivity measured as described in the previous section. Each assay was repeated three times. PARG activity was calculated as difference of labeling between mixture A and B, and taking into account the specific radioactivity of the starting [^{32}P]NAD$^+$ (20,000 cpm/nmol). The reported values were mean of three determinations. Under these conditions poly(ADPribose) (1.9 μM) produced by endogenous PARP and available for PARG activity was less than the substrate concentration (12 μM) described for standard assay with purified poly(ADPribose) [Bernardi et al., 1997]. One PARG unit is defined as the amount of enzyme required to produce 1nmol of ADPribose per minute at 37°C under assay conditions.

Production of free ADPR was also checked by analysing PARG products by TLC according to Bernardi et al. [1997] (data not shown).

Poly(ADP-ribosyl)ation Reaction

Intact nuclei were resuspended (1/1; w/v) in 0.25 M sucrose containing 10 mM Tris-HCl, pH 8.0, 5 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 50 mM NaCl, 5 mM NaF, 1 mM PMSF, 10 μg/ml leupeptin, and incubated with 0.2 mM [^{32}P]NAD (22,000 cpm/nmol) for 20 min at 20°C. The reaction was terminated by diluting with the same buffer and chilling on ice.

The nuclei, collected by centrifugation at 15,000*g* for 15 min at 4°C, were washed with incubation buffer to remove unbound radioactivity.

The different chromatin fractions were then prepared from the incubated nuclei by the procedure described in a previous section.

Fraction P was further on digested twice with DNAase I (800–1,000 U/mg DNA) in 60 mM Tris-HCl, pH 7.5 buffer, containing 60 mM NaCl, 20 mM $MgCl_2$, 10 μg/ml leupeptin and 10 μg/ml chimostatin at 37°C for 1 h to obtain the fraction Pp. The latter was further extracted with 3 M NaCl as reported above.

Poly(ADPribose) was analyzed as previously described [De Lucia et al., 1996]. Briefly, intact [^{32}P]poly(ADPribose) moieties incorporated into the proteins of fraction S, P and pP were detached by incubating the samples at 60°C for 3 h with 10 mM Tris, 1 mM EDTA, pH 11. Samples were extracted with CHCl3/isoamyl alcohol (49:1, v/v), dried in Speed-vac and dissolved in 50% urea, 25 mM NaCl, and 4 mM EDTA, pH 7.5, to be analyzed on 20% polyacrylamide slabgel (5.0 × 7.0 × 0.1 cm) as described by Panzeter and Althaus [1990].

De Novo Synthesis of RNA

RNA synthesis was followed by incubation of nuclei in the presence of [^{32}P]UTP [Greenberg and Ziff, 1984]. Reaction mixture (0.55 ml) contained nuclei from 1.5 g tissue, 0.2 mM unlabeled nucleotides (ATP, GTP, CTP) and [^{32}P]UTP (20 μl; 1,000 Ci/mmol, 10 μCi/μl, Amersham) in 10 mM Tris-HCl buffer, pH 8.0/5 mM $MgCl_2$/300 mM KCl, and was incubated 15 min at 27°C. The reaction was stopped by chilling on ice. [^{32}P]Nuclei were washed three times with the same buffer and pellet was treated as described above to prepare chromatin fractions. RNA was measured as acid-insoluble radioactivity. Aliquots of each fraction were precipitated by 25% trichloroacetic acid (final concentration) in the presence of 5 mM Na-pyrophosphate, filtered through Millipore filters (0.45 μ), washed with 10% trichloroacetic acid and counted for radioactivity.

Miscellanea

Protein concentration was determined by a commercial method (Pierce) using bovine serum albumin (BSA) as a standard. DNA content was determined on the basis of the absorbance at 260 nm (1.0 $A_{260\ nm} = 50$ μg/ml DNA) or by diphenylamine method [Burton, 1968]. Electrophoresis on agarose (1%) gels of DNA associated

with chromatin fractions was performed according to Sambrook et al. [1989].

RESULTS

Soluble and Insoluble Chromatin Fractions

Rat testis chromatin fractions, named S and P, were obtained by DNAase digestion of nuclei (15×10^6/g tissue), whereas sP and pP chromatins were produced by a further incubation of P fraction with nuclease following the protocol previously described [De Lucia et al., 1996]. Over 50% proteins were still associated to the DNAase resistant pP chromatin with a protein/DNA ratio >1.

Thus, the whole nuclease insoluble pellet pP was salt extracted under conditions used to prepare nuclear matrix, in the presence of 3 M NaCl; the 3 M extract (S3M) and a residual pellet (NMP) were obtained.

Figure 1A shows percent distribution of DNA (7 mg in nuclei) and proteins (11.4 mg in nuclei) among soluble (S, sP, S3M) and insoluble (P, pP, NMP) fractions, with values in good agreement with previous results for S, sP, P, and pP [De Lucia et al., 1994].

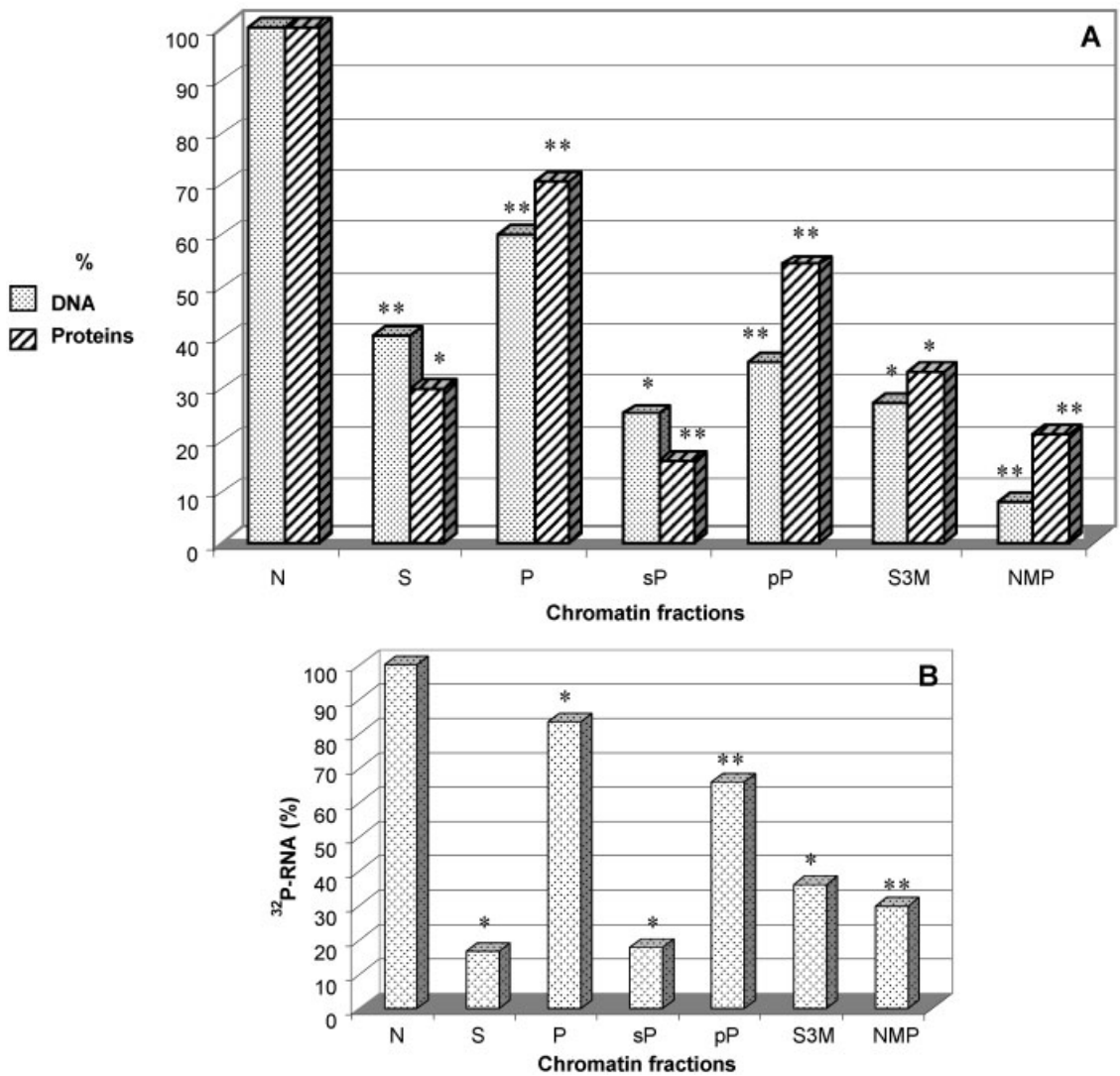

Fig. 1. Percent distribution of proteins, DNA (**A**) and RNA (**B**) in different rat testis chromatin fractions. Total proteins (11.4 mg), DNA (7 mg) and ^{32}P-RNA (10^6 cpm) in nuclei were taken as 100%. DNA, measured by Burton assay, was 1.05 ± 0.15 mg/g tissue (wet weight; mean of four independent determinations). The abbreviations on abscissa refer to the chromatin fractions as indicated in the text. Error %: <2 (*); ≤6 (**).

As it regards DNA, the extraction with 3 M NaCl of pP pellet produced the S3M fraction containing most of the DNA present in pP chromatin, and a residual pellet with 8% of nuclear DNA and more than 20% proteins. The electrophoretic patterns of the nucleic acid showed that soluble fractions S and sP were enriched of short fragments (0.1–2 kbp) whereas longer fragments (<21 kbp) were evident in the NMP fraction. The size of DNA associated with 3M supernatant (S3M) was too large to penetrate gel pores (Fig. 2).

Proteins found in pP chromatin were 54% of those in nuclei (11.4 mg; 100%), further split up into S3M (33%) and NMP (21%) with a protein/DNA ratio in the latter (2.62) doubling that in S3M (1.2). It must be underlined that proteins were not extracted by mild acids (sulfuric acid, perchloric acid) and that the whole chromatin fractions were analyzed under SDS–PAGE conditions. Protein patterns showed the presence of core histones in all fractions with variable amounts of H1, LMG, and HMG among soluble and insoluble chromatin (Fig. 3A). Particularly enriched in non-histones was the sP soluble chromatin, whereas in S3M supernatant histones were prevalent. In NMP H1 occurred in traces as compared to core histones. NMP protein pattern was highly heterogenous as it regards non-histones, including LMGs and HMGs.

Transcriptional activity was followed by analyzing the distribution of newly synthesized [^{32}P]RNA after incubation of nuclei with [^{32}P]UTP. In nuclei incorporated radioactivity (10^6 cpm) corresponded to 0.48 nmol RNA (100%). Over 60% labeling remained in pP chromatin and was further dissociated into S3M (35%) and NMP (28%) fractions (Fig. 1B).

Poly(ADPribosyl)ation in Chromatin Fractions and Protein Acceptors of Poly(ADPribose)

PolyADPribosylation system in rat testis chromatin fractions was studied by analyzing PARP and PARG activity, polyADPribose levels, and protein acceptors of ADPribose.

PARP was mostly solubilized by DNAase digestion and only 17% activity remained in Pp fraction (Fig. 4A). NaCl extraction (3 M) of this sample left 5% PARP activity in nuclear matrix-like pellet. The wide enzyme distribution was confirmed by immunoblotting analysis that showed the presence of PARP in all fractions (Fig. 3C).

The largest amount of PARG activity was solubilized after the second nuclease digestion with a 25% left in pP fraction (Fig. 4A). High salt extraction did not remove too much of this

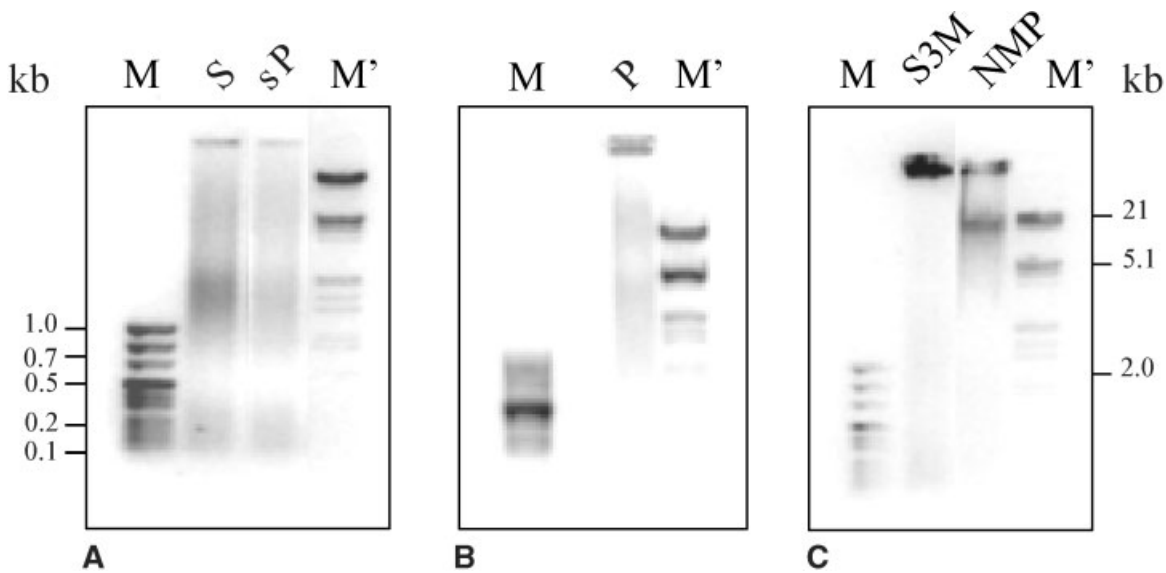

Fig. 2. Agarose (1%) gel electrophoresis of rat testis chromatin fractions. The abbreviations on abscissa refer to the chromatin fractions as indicated in the text. **A**: S and sP fractions; (**B**) pellet P; (**C**) 3 M NaCl supernatant (S3M) and residual pellet (NMP). (M) λ DNA; (M′) pUC marker as indicated in "Materials and Methods." DNA (1 μg) was loaded/lane. DNA was evidenced by including in the gel 1% ethidium bromide (0.5 μl) and fluorescence detected by P-imager (Bio-Rad).

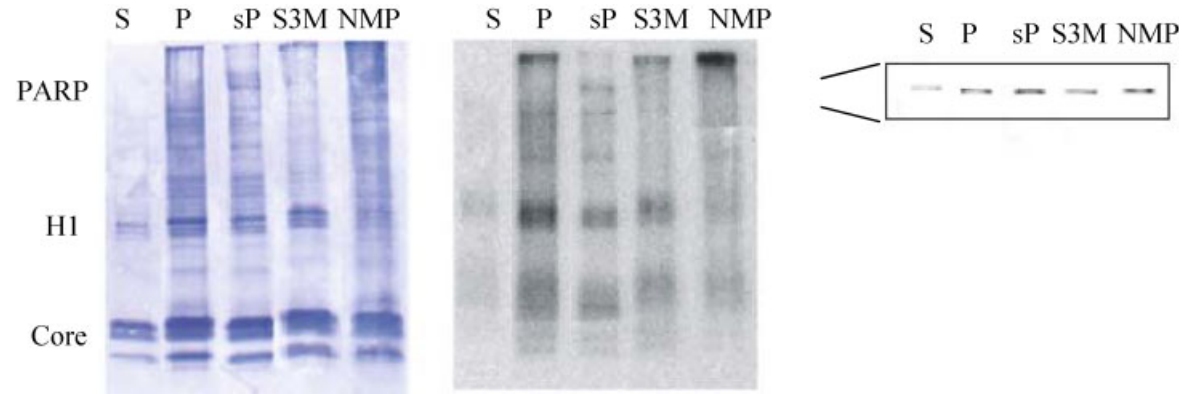

Fig. 3. SDS–PAGE and Western-blotting of rat testis chromatin fractions. Loading of proteins was normalized per DNA (1 μg DNA in duplicate of each fraction, except for NMP, 0.35 μg DNA). After electrophoresis proteins were blotted on PVDF membrane. One lane of the blotted proteins was stained with 0.5% Coomassie blue (**A**); the duplicate lanes were exposed to P-imager to acquire the autoradiographic patterns (**B**) and thereafter analyzed with anti-PARP polyclonal antibodies (**C**). Separately, polyacrylamide gels, after blotting, were stained with Coomassie in order to check the complete transfer of proteins onto filter. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

activity as about 19% was still found in NMP chromatin.

The analysis of radioactivity distribution in different chromatin fractions, isolated from rat testis nuclei incubated with ^{32}P-NAD^{+} and digested with DNAase, confirmed previous results of a preferential association of radioactivity with soluble chromatin (Fig. 4B). Taking as 100% the radioactivity incorporated into nuclei (2.5×10^6 total cpm), the residual labeling in Pp chromatin (38%) dissociated between S3M supernatant (21%) and NMP fraction (17%). In the latter fraction ^{32}P-poly(ADPR)/DNA ratio was noticeably high (Fig. 4B).

Electrophoretic patterns of reaction products confirmed the results obtained previously, showing exclusively short oligomers (<8 ADPR residues) associated with soluble chromatin, and short, long, branched polymers in the insoluble fractions (data not shown).

Electrophoretic and autoradiographic patterns of proteins from different chromatin fractions, after Western blotting, showed the main labeling in correspondence of core and H1 histone bands (Fig. 3B). In sP lane, the automodified PARP was also evident, and some non-histones in the mass range between those of H1 (30 kDa) and PARP (116 kDa) appeared to be ADPribosylated. In P and NMP fractions, a strong labeling was localized at the top of the lanes and several non-histones were labeled.

Comparison of stained and autoradiographic patterns revealed that proteins were modified by short oligomers of ADPribose, as the electrophoretic mobility of labeling was slightly retarded as compared with the stained bands. The presence of proteins modified with larger and branched polymers was likely indicated by labeling at the top of filter in P and NMP fractions.

DISCUSSION

In a previous paper, we reported the results obtained with a protocol applied to prepare DNAase sensitive and resistant chromatins from rat testis nuclei and, in parallel, following the distribution of the newly synthesized ^{32}P-RNA in nuclei. In particular, after extensive DNAase digestions we obtained an extremely DNAase insoluble fraction, named pP chromatin, where high levels of new RNA were still found.

The strong DNAase resistance and the high transcriptional activity of pP pellet suggested that this chromatin could be nuclear matrix-like, although it was obtained applying a protocol not conventional to prepare nuclear matrix [De Lucia et al., 1994].

In this paper we present evidence that, when treated with high salt (3 M NaCl), pP fraction can be dissociated into a S3M supernatant and a pellet characterized by features similar to nuclear matrix. The DNA (8%) associated to the latter fraction had a size (<21 kbp) corresponding to that described for Matrix Associated Regions (MARs) which represent the sites anchoring DNA loops onto the protein support [Georgiev et al., 1991; Berezney et al., 1995].

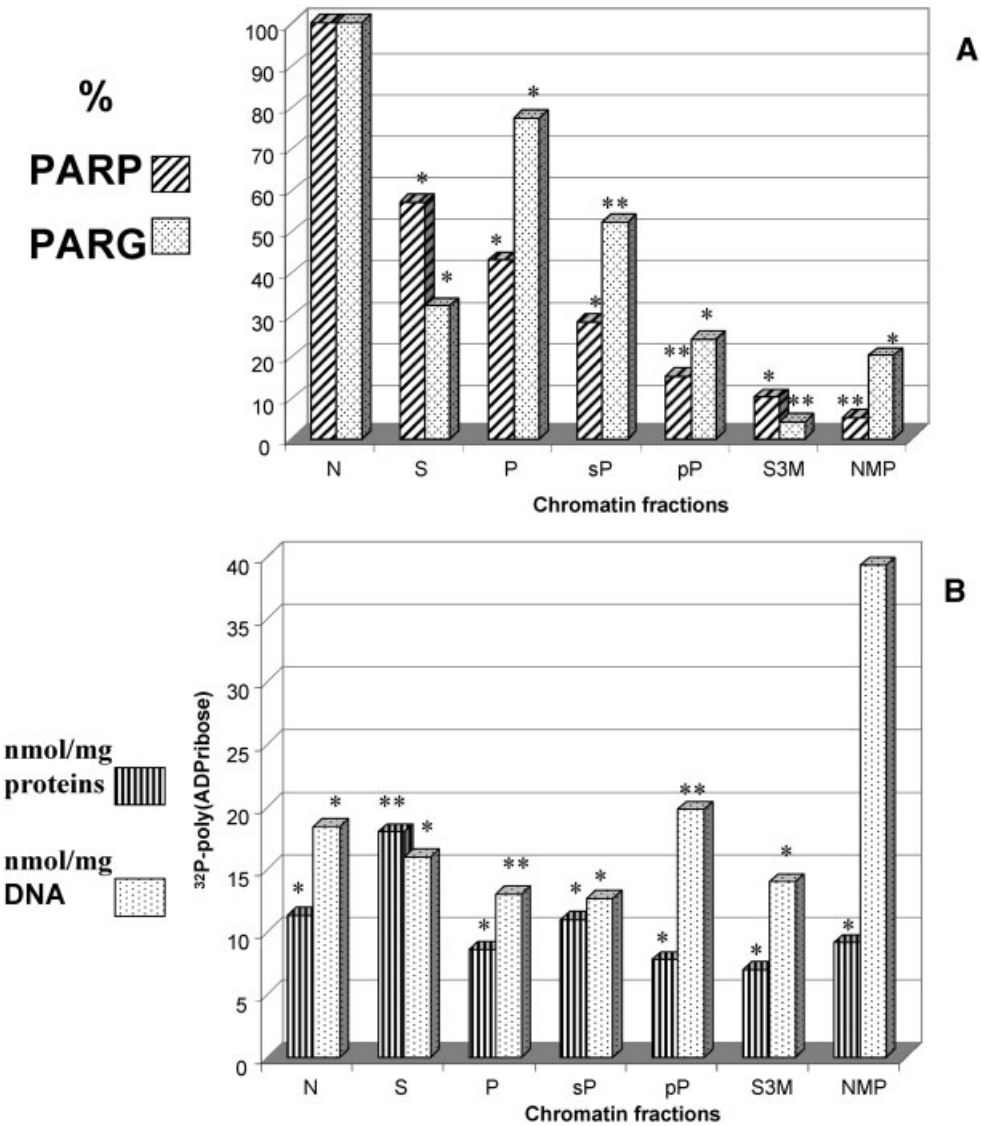

Fig. 4. The poly(ADPribosyl)ation system in rat testis chromatin fractions. **A**: Distribution of poly(ADPribose) polymerase and poly(ADPribose) glycohydrolase activities. In nuclei PARP and PARG corresponded to 1.14 mU/mg protein and 0.05 U/mg, respectively, both taken as 100% activity. **B**: Polymer bound to each chromatin fraction, expressed as ratio of total labelling to both proteins and DNA. Total acid-insoluble radioactivity incorporated into nuclei (2.6×10^6 cpm) corresponded to 42 nmoles ADPribose, taken as 100%. Error %: <1 (*); ≤ 2.5 (**).

The longest fragments of DNA in pP fraction were solubilized in the 3M NaCl supernatant. This DNA likely represents chromatin loops, which can reach up to 200 kbp size. Although we cannot exclude that some loops were still in NMP (see top of gel in Fig. 2), this fraction is enriched in nuclear matrix (high percentage of proteins and new RNA). The fact that after salt extraction only 5% of PARP activity was found in NMP is in line with previous results from our laboratory indicating that this activity is not matrix-intrinsic, but it is associated with DNA anchored on it [D'Erme et al., 1991].

The analysis of ADPribosylated proteins obtained under non-denaturing conditions, led to an autoradiographic pattern more complete than that previously obtained by mild acidic extraction, due the lower acid solubility of proteins bound to ADPribose. In most fractions ADPribose acceptor proteins were identified as PARP itself, H1 and core histones. The fact that the 3M NaCl extraction did not solubilize the modified non-histones observed in NMP fraction renders likely that these proteins, as well as residual histones, are strictly associated to the protein enriched pellet.

Protein acceptors were mainly modified with short oligomers of ADPribose in the soluble fraction (covalent heteromodification), while the most insoluble chromatin, particularly NMP, was characterized by acceptors bound to both long and short polymers, but enriched in branched polymers (top fraction in NMP lane, Fig. 3B). This result likely indicates the presence, in the region including the transcriptional machinery, of auto- and hetero-modification, regulated by the presence of the residual PARG activity. The occurrence of ADPribose polymers of different length and branching is in the direction of a previously proposed hypothesis that structural variability of poly(ADPribose) might have different functional meanings.

It is known that both length and structure of the polymer play an important role in determining auto- and/or hetero-modification [D'Amours et al., 1999]. A contribution to this choice is given by poly(ADPribose) catabolism, a highly ordered process, as demonstrated by the biphasic mechanism of PARG kinetic [Ikejima and Gill, 1988].

The residual PARG in NMP might account for a rapid conversion of long and branched polymers (on PARP) into oligomers charged to heterotargets in order to modulate their functions.

Transcriptional activity requires the presence of different specific proteins and the related rearrangement of rat testis chromatin structure might be regulated directly or not by an active turnover of poly(ADPribose), as the result of a high synchronism between PARP and PARG activities.

The relationship of the observed levels of poly(ADPribose) and its metabolism in rat testis NMP chromatin with transcription is under investigation.

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