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In Vitro Poly(ADP-Ribosyl)ated Histones H1a and H1t Modulate Rat Testis Chromatin Condensation Differently

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Abstract Rat testis H1 proteins were poly(ADP-ribosyl)ated in vitro. The modifying product, poly(ADP-ribose), was found covalently bound to each histone variant at various extents and exhibited distinct structural features (linear and short, rather than branched and long chains). Interest was focused on the somatic H1a, particularly abundant in the testis, as compared with other tissues, and the testis-specific H1t, which appears only at the pachytene spermatocyte stage of germ cell development. These H1s were modified with poly(ADP-ribose) by means of two in vitro experimental approaches. In the first system, each variant was incubated with purified rat testis poly(ADP-ribose)polymerase in the presence of [^{32}P] NAD. In parallel, poly(ADP-ribosyl)ated H1s were also prepared following incubation of intact rat testis nuclei with [^{32}P] NAD. In both experiments, the poly(ADP-ribosyl)ated proteins were purified from the native forms by means of phenyl boronic agarose chromatography. The results from both analyses were in agreement and showed qualitative differences with regard to the poly(ADP-ribose) covalently associated with H1a and H1t. Comparison of the bound polymers clearly indicated that the oligomers associated with H1a were within 10–12 units long, whereas longer chains (≤20 ADP-R units) were linked to H1t. Individual poly(ADP-ribosyl)ated H1s were complexed with homologous H1-depleted oligonucleosomes (0.5–2.5 kbp) in order to measure their ability to condensate chromatin, in comparison with the native ones. Circular dichroism showed that the negative charges of the oligomeric polyanion, although present in limited numbers, highly influenced the DNA-binding properties of the analyzed H1s. In particular, the poly(ADP-ribosyl)ated H1a and H1t had opposite effects on the condensation of H1-depleted oligonucleosomes. J. Cell. Biochem. 76:20–29, 1999. © 1999 Wiley-Liss, Inc.

Key words: poly(ADP-ribosyl)ation; H1 variants; chromatin condensation; testis

Histone H1 is widely recognized as having a key role in the modulation of chromatin structure [van Holde, 1989]. The potentialities of the H1 nuclear functions are greatly enhanced by the occurrence, in all cell types, of a family of linker histones, structurally related, but with different DNA-binding ability [Cole, 1987; Wolffe, 1995]. The presence of various H1 proteins in different species, cell types, and developmental stages has been related to the regulation of the genomic function, producing chromatin regions of different stability [Weintraub, 1984; Laybourn and Kadonaga, 1991; Hill et al., 1990; Zlatanova and Yaneva, 1991].

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During spermatogenesis in the rat, drastic changes occur in the H1 population of the germ cells that accompany the structural and functional dynamic alterations of chromatin leading to sperm maturation [Meistrich, 1985; Seyedin and Kistler, 1980; Cole et al., 1986]. The somatic H1a is unusually prominent and the testis-specific variant, H1t, appears only at the end (mid to late pachytene) of the meiotic prophase [Seyedin and Kistler, 1980; Cole et al., 1986]. Both variants have been proposed to play main roles in the metabolic nuclear processes driving the chromatin assembly toward the fiber shape in the spermatozoon [Cole et al., 1986; De Lucia et al., 1994; van Wert et al., 1996].

Circular dichroism analyses of rat testis H1-depleted polynucleosomal fractions, reconstituted with homologous H1 variants (a,d-e,c,t), confirmed the general knowledge that these proteins interact differently with DNA and sug-

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gest that in this tissue as well, they are able to induce various levels of chromatin condensation, with the minimal effect measured in the presence of the testis-specific H1t [De Lucia et al., 1994; Markose and Rao, 1989].

Studies aimed at understanding the structural bases underlying such a different behavior have shown that the DNA-condensing property of the H1 histones resides in the correct structuring of the N- and C-terminal tails [Khadake and Rao, 1997; Moran et al., 1985; Wellman, 1996]. As opposed to the rat testis somatic variants, at the C-terminus, H1t lacks most of the tandem repeated DNA-binding motifs, specifically with regard to a (16-mer TPKK) peptide [Khadake and Rao, 1995]. This amino acid sequence appears to be important in bringing about close packing and condensation of DNA [Khadake and Rao, 1995].

In addition to a different distribution of H1 variants along the chromatin [Hill et al., 1990; Zlatanova and Yaneva, 1991] and the structural variability at the N- and C-terminal tails [Khadake and Rao, 1997; Wellman, 1996], the dynamic of chromatin condensation-decondensation seems to include post-translational reversible modifications of H1s [Roth and Allis, 1992; Aubin et al., 1983; Lautier et al., 1993; Boulikas, 1992; de Murcia and Menissier-de Murcia, 1994]. Among these reactions, poly-(ADP-ribosyl)ation is catalyzed by the poly-(ADP-R)polymerase (EC.2.4.2.30, PARP), an enzyme that splits the ADP-ribose moiety from NAD and transfers sequential ADP-ribose units to specific acceptor proteins [Lautier et al., 1993]. Poly(ADP-ribosyl)ation has been included among the factors involved in nuclear events, such as DNA transcription [Lautier et al., 1993], repair [Shieh et al., 1998], and apoptosis [Simbulan-Rosenthal, 1998], often through local modulation of chromatin structure [Poirer et al., 1982]. PARP itself undergoes poly(ADP-ribosyl)ation in the automodification reaction, whereas H1 histone is widely recognized as the preferential acceptor protein in the hetero-poly(ADP-ribosyl)ation [Lautier et al., 1993]. As a result of H1 hyper-ADP-ribosylation, the chromatin is highly decondensed [Lautier et al., 1993; Poirer et al., 1982].

Our previous findings that (1) H1t is the less DNA-condensing [De Lucia et al., 1994], but the most poly(ADP-ribosyl)ated [Althaus and Richter, 1987], variant in the rat testis, and (2) it is preferentially associated with the chromatin fraction enriched in transcriptionally active regions [Quesada et al., 1989], pointed to a functional meaning of the peculiar properties of H1t during spermatogenesis and stimulated research to investigate whether each variant might exhibit different features of the poly(ADP-ribosyl)ation reaction as regulatory signals of their hypothesized roles.

This article presents results that further characterize the poly(ADP-ribosyl)ation reaction of rat testis H1s; in particular, we focused on the modification reaction of H1a and H1t, the two proteins that, for abundance (H1a) and tissue specificity (H1t), might play special roles during cell differentiation in the rat testis. It was specifically analyzed the reaction product covalently bound to the H1 variants, resistant to the most common treatments as perchloric acid extraction of modified proteins, but very sensitive to alkali incubation. Our results converge toward an in vitro poly(ADP-ribosyl)ation of the analyzed histone variants with linear and short chains of ADP-ribose.

In order to investigate the possible role of these histones, we used circular dichroism to examine the effect induced by poly(ADP-ribosylation) of H1a and H1t on their ability to modulate the conformational state of DNA.

MATERIALS AND METHODS

Materials

U^{14}C-NAD^{+}, nicotinamide (U^{14}C)adenine dinucleotide ammonium salt, 248 mCi/mmol, [^{32}P]NAD^{+}, nicotinamide adenine dinucleotide di(triethylammonium) salt adenylate-^{32}P (1,000 Ci/mmol) and MP-Hyperfilm sheets were supplied by Amersham International plc; DNase I (EC 3.1.21.1), RNase A (EC 3.1.27.5, type XII-A), bovine serum albumin (BSA), DNA λ, phenylmethylsulfonylfluoride (PMSF), leupeptin, chymostatin, antipain, pepstatin, spermine, and spermidine were obtained from Sigma Chemical Company. Electrophoretic molecular-weight markers were from Bio-Rad; filters purchased from Millipore (0.45-μm pore size, type HW) were used for enzymatic assay.

In Vitro Poly(ADP-Ribosyl)ation of H1s

Method I

Purification of rat testis H1 variants. Perchloric acid-soluble proteins were extracted with 5% (w/v) perchloric acid (1: 2.4, w/v) from whole testis according to Nicholas and Goodwin [1982]. The H1 fraction was precipitated with 3.5 vol

acetone and collected by centrifugation. The precipitate, dissolved in 0.1% trifluoroacetic acid (TFA), was analyzed on a C_4 column (Vydac ODS, cm 0.5 × 25; 0.5-μm particles) by high-pressure liquid chromatography (HPLC) according to De Lucia et al. [1994]. A linear gradient (20–60%) of solvent B (95% CH_3CN) in solvent A (0.1% TFA) was applied at the first minute for 80 min. This elution system ensured the resolution of all the variants, except H1d-e, which coeluted in the same fraction. The fractions corresponding to the H1s were concentrated by a Speed-vac centrifuge and used for the following analyses. Protein concentration was measured by the Bradford procedure [Bradford, 1976], using BSA as the standard.

Purification of rat testis poly(ADP-ribose) polymerase. The pure enzyme was prepared following the procedure described by Burtscher et al. [1986]. In the final step, the enzyme fraction was collected as the precipitate after dialysis against an ammonium sulfate-saturated solution. The specific activity of the purified enzyme was 73 mU/mg protein.

Enzyme activity was assayed and the enzymatic unit defined as previously reported [De Lucia et al., 1996]. Briefly, aliquots of the analyzed fractions were incubated at 25°C for 10 min in the presence of 0.64 mM ^{14}C- or [^{32}P]NAD (10,000 cpm/nmol) in 80 mM Tris-HCl buffer, pH 8.0, containing 14 mM β-mercaptoethanol, 5 mM NaF, 10 mM $MgCl_2$, in a final volume of 0.125 ml. The total potential activity was determined under the same conditions, adding to the reaction mixture rat testis H1 (5 μg), calf thymus DNA (5 μg), and bovine pancreatic DNase I (40 U), and measured as 20% TCA-insoluble radioactivity [De Lucia et al., 1996].

Labeling of H1s with [^{32}P]. The whole H1 histone fraction or the single variant were incubated in the presence of purified PARP (3.6 μU/assay). The reaction mixtures, prepared as described above, did not contain $MgCl_2$, and the [^{32}P]NAD concentration was reduced to 0.2 mM (12,000 cpm/nmole). Under these conditions, the H1s are preferentially poly(ADP-ribosyl)ated, although they behave as enzyme activators too. The control assay was performed in the absence of H1. The incorporated [^{32}P]poly-(ADP-ribose) was measured as 20% TCA-insoluble radioactivity [De Lucia et al., 1996].

In large-scale experiments, the reaction mixtures (from 1.25 to 3.75 ml, final volume) were prepared under the same conditions. The incubation was prolonged to 15 min, and the reaction was stopped by cooling on ice.

The reaction mixture was loaded on a Sephadex G-50 column (10 times the volume of the loading sample) to remove free nucleotides. The fraction at V_0 was collected and equilibrated in 50 mM Mops, pH 8.2. The native H1 was separated from the poly(ADP-ribosyl)ated forms by a chromatography on phenyl boronic agarose (PBA-30, 400 μl/1.25-ml reaction mixture), at 4°C [Scovassi et al., 1993]. After an extensive wash of the column with 6 M guanidine hydrochloride in 50 mM Mops, pH 8.2, the poly(ADP-ribosyl)ated proteins were eluted with 6 M guanidine hydrochloride in 0.2 M NaH_2PO_4/Na_2HPO_4 buffer (pH 5.5). The protein fraction was dialyzed against H_2O. In some experiments, the radioactive fraction from PBA column was extracted with 5% (w/v) PCA in order to separate the automodified enzyme from the ^{32}P-labeled variants.

Method II

Purification of poly(ADP-ribosyl)ated H1s from rat testis nuclei. *Isolation of nuclei*: Testes were collected from adult male rats (Wistar CF-1, 45–50 days old). Nuclei were isolated by the procedure of Utakoji et al. [1968], except that calcium and magnesium ions were replaced with 0.15 mM spermine, 0.75 mM spermidine, and 1 mM EDTA/EGTA; 0.1 mM PMSF was used as protease inhibitor. The purity and integrity of nuclei were checked by a phase-contrast microscope. *Incubation of intact nuclei with [^{32}P]NAD*: The intact nuclei (from 10-g testes) were suspended in 10 mM Tris-HCl buffer (pH 8.0)/0.25 M sucrose/10 mM $MgCl_2$/5 mM NaF/50 mM NaCl/5 mM β-mercaptoethanol/1 mM PMSF/leupeptin,10 mg/ml, (incubation buffer), at a concentration of 80–100 × 10^6 nuclei/ml, and incubated for 20 min at 25°C in the presence of 0.2 mM [^{32}P]NAD (22,000 cpm/nmol). The reaction was stopped by a twofold dilution of the mixture with cold incubation buffer and centrifuging at 2,500g for 10 min to remove the supernatant with free radioactivity; the pellet was washed twice with the same buffer. Labeled nuclei were extracted twice with 0.5 M NaCl in 10 mM Tris-HCl buffer (pH 8.0)/1 mM PMSF/leupeptin, 10 mg/ml (1:1, w/v) by homogenization with Polytron at maximum speed (2 min). The ^{32}P-extracts were pooled, dialyzed to reduce salt concentration, equilibrated in 0.1% TCA, and analyzed by RP-HPLC as described by De Lucia et al. [1994]. The fractions correspond-

ing to the single ^{32}P-labeled H1 variants were concentrated by a Speed-vac centrifuge. The radioactive H1s were separated from the unlabeled proteins by PBA chromatography (see above).

Gel Electrophoresis and Autoradiography of ^{32}P Proteins

The protein samples were analyzed using 12% polyacrylamide slab gels in the presence of SDS as described by Nicholas and Goodwin [1982]. The ^{32}P-electrophoresed proteins were exposed to MP-Hyperfilm (RPN36) films for a time ranging from 24 and 98 h.

Analysis of ^{32}P-poly(ADP-ribose)

^{32}P-poly(ADP-ribose) was detached from modified proteins by incubation of the labeled fraction at 60°C for 3 h with 10 mM Tris-HCl/1 mM EDTA/8 mM NaOH, pH 11.0. After extraction with $CHCl_3$/isoamyl alcohol (48:2, v:v), the products were dried in Speed-vac, dissolved in 50% (w/v) urea/25 mM NaCl/4 mM EDTA, pH 7.5, and analyzed on 20% polyacrylamide slab gel (20 × 25 × 0.1 cm) as described elsewhere [Panzeter and Althaus, 1990]. To perform separate evaluation of the types of products associated specifically with the enzyme and with the H1 variants, particularly H1a and H1t, the eluates from PB agarose were extracted by 5% (w/v) perchloric acid. After centrifugation in Microfuge (Eppendorf) at maximum speed for 10 min, both the acidic supernatant (H1) and the pellet (enzyme) were analyzed to identify the bound products.

Preparation and H1 Depletion of Chromatin Fractions

Isolated nuclei were digested with DNase I (41 U/ml DNA), in the presence of 0.66 mM $MnCl_2$ for 2 min at 37°C according to De Lucia et al. [1996]. Digested nuclei were suspended in 15 mM Tris-HCl buffer pH 7.5/1 mM EDTA/1 mM PMSF (lysis buffer) and lysed by incubation for 1–2 h at 0°C with intermittent gentle agitation by passage through a Pasteur pipette [Huang and Cole, 1984]. Lysed nuclei were centrifuged for 20 min at 9,000 rpm and the supernatant (soluble chromatin) digested for 30 min at 25°C with pre-boiled RNase A (type XII, Sigma;10 mg/ml). Native oligonucleosomes of different lengths were obtained from the soluble chromatin by centrifugation on a 5–35% (w/v) continous sucrose gradient for 14 h at 25,000 rpm (Beckman SW28 rotor) (11). The 0.5- to 2.5-kbp fractions were selected for the following experiments. The size of oligonucleosomal DNA was measured by gel electrophoresis in 1% or 2% (w/v) agarose containing 0.1% (w/v) SDS and ethidium bromide (0.5 μg/ml) [De Lucia et al., 1994]. DNA concentration was estimated by A260 (1 U A_{260} = 50 μg DNA).

The H1-depleted oligonucleosomes were prepared by exposure of the native chromatin to 0.6 M NaCl and subsequent centrifugation for 14 h through a 5% (w/v) sucrose cushion in the presence of 1 mM EDTA and 0.6 M NaCl [De Lucia et al., 1994]. The depletion of H1 was checked by analyzing the chromatin associated proteins by SDS-PAGE.

Circular Dichroism Analyses

Circular dichroism analyses were performed with a Jasco spectropolarimeter (J-750) equipped with a Hewlett Packard computer, at 21°C; 1-mm light-path cells and a 0.5-mdeg/FS sensitivity were used.

The spectra of chromatin fractions were recorded at 220–320 nm. The molar ellipticity, θ, expressed as degrees × cm^2 × $dmol^{-1}$, was normalized to the number of DNA base pairs.

Reconstitution experiments were performed in 5 mM Tris-HCl buffer, pH 7.5/60 mM NaCl. The whole H1s or the individual H1 variants, in either the native or poly(ADP-ribosyl)ated form, were mixed with H1-depleted oligonucleosomes (A_{260} = 0.3–0.6) and left 5 min before collecting data. The amount of H1s was increased from 0.2 to 2 molecules/nucleosome. Control experiments were performed by reconstituting H1-depleted chromatin with poly(ADP-R) in the same amount found associated with each H1 variant in the related experiments. An analogous control was performed with PARP alone.

The oligonucleosome concentration was determined as A_{260} (1 U A_{260} = 50 μg DNA) and all chromatin samples originated from one stock solution. Measurements on native, depleted, and reconstituted fractions were repeated in duplicate on two different chromatin preparations at an interval of several weeks.

RESULTS

In Vitro Poly(ADP-Ribosyl)ation of Rat Testis H1 Variants

The first step of this work was to poly(ADP-ribosyl)ate the linker histone proteins by in

vitro incubation with PARP. The interest was focused onto the poly(ADP-ribose) covalently bound to the histones (i.e., the number of polymers specifically targeting acceptor proteins and not removed under the experimental conditions used). The total radioactivity, incorporated by in vitro incubation of each H1 somatic (a,c,d-e) and testis-specific (t) variant in the presence of purified PARP and [^{32}P] NAD, is shown in Figure 1A. In the absence of H1 and Mg^{2+}, a scarce radioactivity was present in the control containing only PARP, whereas the highest value of radioactivity was associated with the H1t mixture as compared with the other H1 variants.

After electrophoretic analysis of the incubated mixtures shown in Figure 1A (Fig. 1B), the autoradiography showed that all the radioactive bands were shifted toward the origin of the gel, compared with the Coomassie-stained histone variants, as a result of the bound poly-(ADP-ribose). The mobility of the modified H1t (Fig. 1B, b, lane t) was the most retarded as compared with the stained protein (Fig. 1B, a, lane t). Showing the amount of all proteins normalized by weight, Figure 1B (b) clearly shows that H1t was preferentially poly(ADP-ribosyl)ated.

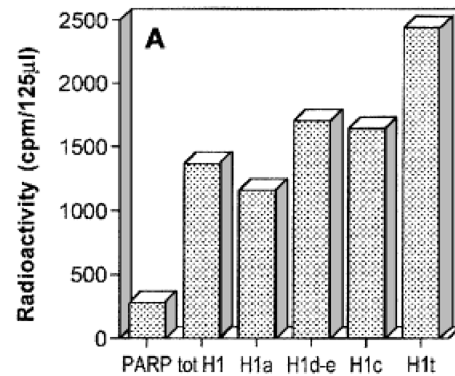
In order to evaluate the contribution of the automodified PARP and to assign the modification levels to the involved proteins correctly, the poly(ADP-ribosyl)ated H1 variants were prepared alternatively, by incubating rat testis nuclei with [^{32}P] NAD (Method II). The RP-HPLC (Fig. 2A) selectively separated the enzyme from the histones, and the radioactivity of each H1 peak was measured on TCA-precipitated aliquots of the fractions. The results were (pmoles of ADP-R per nmol of H1)—H1a, 11.0; H1d-e, 16.0; H1c, 9.5; H1t, 29.0—and indicated that the ^{32}P-product associated with H1t was three times that bound to H1a.

The following electrophoretic and autoradiographic analyses (Fig. 2B) confirmed that H1t was modified to a larger extent than the other variants. The lack of labeling at the top of the gel indicated that the enzyme and/or noncovalently bound polymers were removed during histone purification.

Analysis of the [^{32}P] Poly(ADP-ribose)

In the experiments of in vitro incubation of H1s with purified PARP, the polymers of ADP-R detached from the labeled proteins were analyzed by gel electrophoresis, followed by autoradiography (Fig. 3, lanes 1–5). All lanes show the presence of long and branched polymers with comparable size distribution patterns, which included the ADP-ribose chains associated with the automodified PARP (Fig. 3, lane 1).

After 5% (w/v) perchloric acid extraction of the PBA eluates, the longest and branched



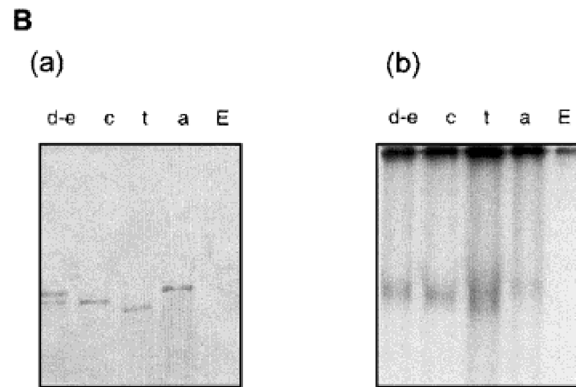
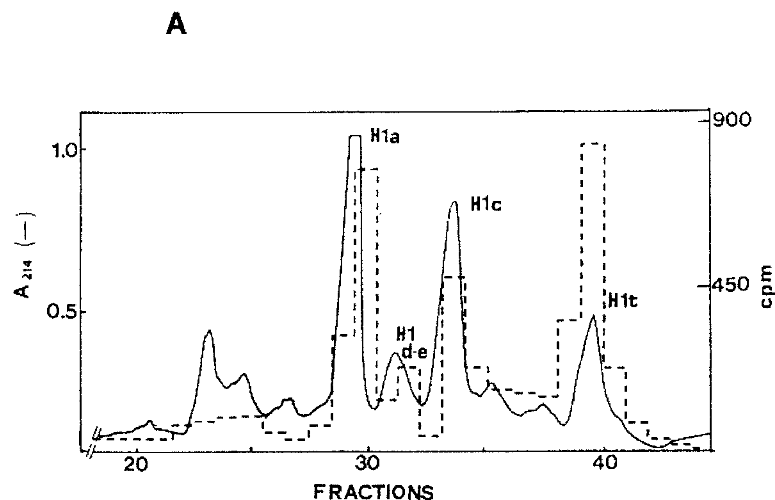


Fig. 1. Poly(ADP-ribosyl)ation of rat testis H1s. **A:** Individual subfractions (5 µg) were incubated in the presence of pure PARP (0.36 µU, 49 ng) and 0.64 mM [^{32}P]NAD (10,000 cpm/nmol) in a final volume of 125 µl. Enzyme activity was measured as 20% trichloroacetic acid-insoluble radioactivity. Poly(ADPR)polymerase (PARP) and tot H1 correspond to the radioactivity incorporated in the absence of H1 and in the presence of unfractionated H1, respectively. **B:** Sodium dodecyl sulfate (SDS)-(15%) polyacrylamide gel electrophoresis (PAGE) of poly(ADP-ribosyl)ated variants. a, electrophoretic pattern of the RP-high-performance liquid chromatography (HPLC)-purified H1s incubated as described in **A**. Letters refer to the analyzed variants (a,c,d-e,t; 5 µg each). **Lane E**, reaction mixture containing PARP in the absence of histone. A negative control with H1t and [^{32}P]NAD in the absence of PARP was also performed, but is omitted here. Staining with 0.5% Coomassie blue in 7% acetic acid/10% methanol. b, Autoradiographs of the samples in a.



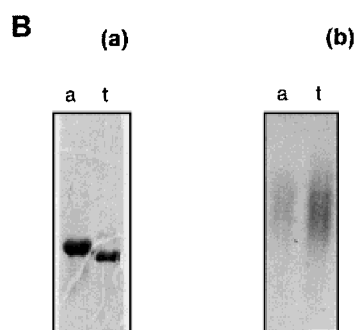


Fig. 2. Poly(ADP-ribosyl)ated H1s from [^{32}P]-labeled rat testis nuclei. **A:** RP-high-performance liquid chromatography (HPLC) of the 0.5 M NaCl extract from labeled nuclei. The elution system is described in Materials and Methods. Flow rate 1 ml/min; 0.5 ml/fraction. The fractions corresponding to the H1s were individually collected. The recovery was in the H1 ratio a:d-e:c:t, 4:1:3:2 (w/w/w/w). **B:** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (a) and autoradiography (b) of the poly(ADP-ribosyl)ated H1a and H1t fractionated by RP-HPLC; 5-μg proteins were loaded.

ADP-R chains were associated with the enzyme fraction (Fig. 3, lanes 6,8), whereas a shorter, but heterogeneous, poly(ADP-R) derived from the H1 variants (Fig. 3, lanes 7,9). This finding confirms that the enzyme is highly activated by H1s and that most polymers of ADP-ribose modify poly(ADP-ribose)polymerase itself.

The polymers detached from H1a and H1t appeared to lack branches. The maximum ADP-R chain length was slightly greater than 20 residues for H1t (Fig. 3, lane 7). The poly-(ADP-R) covalently bound to H1a was not longer than 8–10 U (Fig. 3, lane 9).

Analogous results were obtained by analyzing the poly(ADP-R) covalently bound to the H1s modified by incubation of nuclei with [^{32}P] NAD (Fig. 3, lanes 10,11). The length of polymers was slightly reduced, probably because of an active poly(ADP-R) turnover in the nuclei.

Large-Scale Preparation of Poly(ADP-ribosyl)ated H1s

In a typical experiment, the H1 variant under analysis (50 μg) was incubated with purified PARP and [^{32}P]NAD in a final volume of 1.25 ml. Table I shows the recovery of the poly-(ADP-ribosyl)ated H1a and H1t proteins, eluted from PB-agarose and separated from the automodified PARP by PCA extraction. The recovery of the modified histones was 2–3% of the total proteins used in the assays. The ADP-R pmoles calculated per H1 pmoles gave further evidence of a more extensive modification of H1t. The yield of the poly(ADP-ribosyl)ated variants from [^{32}P]NAD incubated nuclei, after PBA chromatography, was very similar. The poly-(ADP-ribosyl)ated subtypes recovered from several experiments were individually pooled to be used in the following analyses. The structure of each subfraction, in the native or modified form, was first analyzed by circular dichroism either before or after renaturation treatment (unpublished data).

Reconstitution of RP-HPLC-Fractionated H1s With H1-Depleted Chromatin

Circular dichroism analyses. The native chromatin (Fig. 4A, 2) gave a typical spectrum,

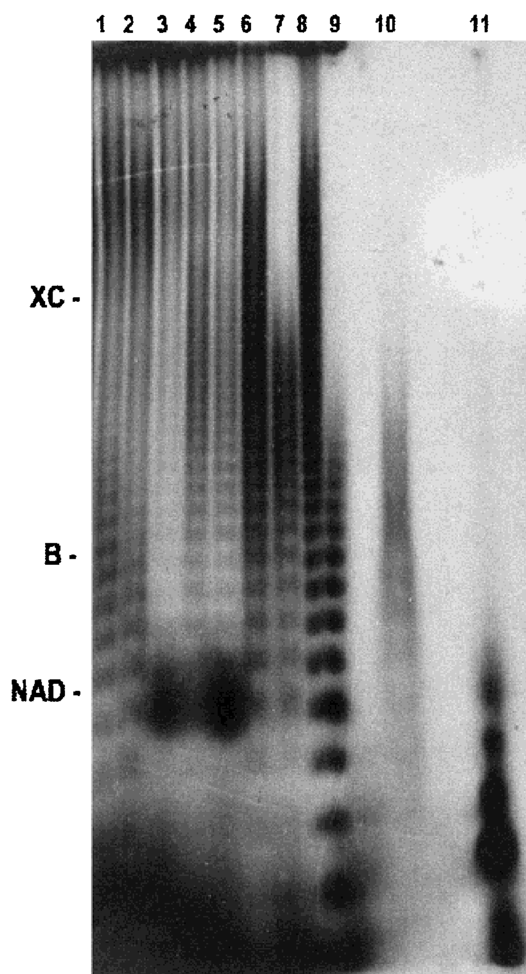


Fig. 3. Autoradiography of the electrophoresed [^{32}P] poly(ADP-ribose) associated with the H1 variants. **Lanes 1–5**, patterns of polymers synthesized with Method I. The PB agarose ^{32}P-eluates (3,500 cpm) in the absence (1) and presence of H1a (2), H1d-e (3), H1c (4), and H1t (5) were analyzed. **Lanes 6–9**, ADP-R polymers after extraction of the PB agarose ^{32}P-eluates with 5% (v/v) perchloric acid (Method I). Lanes 7 and 9 correspond to the products from the PCA extracts (3,000 cpm) containing H1t and H1a, respectively. **Lanes 6,8**, patterns of products associated with the precipitates (3,000 cpm), which probably contain the enzyme and the longest and branched polymers remain bound. **Lanes 10,11**, ^{32}P-polymers from the labeled H1t (**lane 10**, 2,000 cpm) and H1a (**lane 11**, 3,000 cpm) after incubation of nuclei with [^{32}P] NAD (Method II).

within the range 245–300 nm, with a molar ellipticity much lower than the values measured for the H1-depleted oligonucleosomes (Fig. 4A, 1). The rat testis H1-depleted chromatin (spectrum 1) was reconstituted individually with the modified and unmodified H1 subtype (H1a, H1t), at different H1/oligonucleosome ratios (from 0.2 to 2.0 H1 molecules per nucleosome) in 5 mM Tris-HCl (pH 7.4)/60 mM NaCl. The CD spectra of the native variants/H1-depleted

TABLE I. Yield of Poly(ADP-Ribosyl)ated H1s After PB-Agarose Chromatography*

Histone	ADP-ribosylated H1 (μg/ml)	ADPR/H1 (pmol/pmol)	*ADPR-H1* % native H1
Total H1	3.5	19.0	2.3
H1a	1.8	27.0	1.8
H1t	1.5	30.0	1.5

*The poly(ADP-ribosyl)ated histones were prepared incubating 50 μg H1s under the conditions described under Materials and Methods. The percentage of the modified histones was calculated, taking 50 μg as the total.

oligonucleosome complexes reproduced those described before [De Lucia et al., 1994]: The positive ellipticity decreased at different extents upon addition of H1a (Fig. 4B, 3) and H1t (Fig. 4C, 3), with the highest reduction in the presence of H1a. This variant was able to contract the stripped chromatin to give Q values below those measured for the native oligonucleosomes (Fig. 4B, 1).

The same variants, modified by poly(ADP-R) at the extent shown in a previous section, behaved in an opposite manner. Upon addition of poly(ADP-ribosyl)ated H1t to the H1-depleted chromatin, the spectrum resembled that determined for the native chromatin (Fig. 4C, 4). On the contrary, the stripped oligonucleosomes were only slightly affected by the modified H1a (Fig. 4B, 4). These results were better evidenced when the chromatin/H1 ratio was increased to 1:1 (Fig. 5). A direct comparison of Q values at 274, 282, and 296 nm clearly showed that the DNA-binding ability of the native H1a and H1t is completely reversed after modification with poly(ADP-R). In a control experiment, the poly(ADP-R) with the same length and at the same amount found associated with the H1s, had no effect on the CD spectrum of the stripped chromatin (data not shown).

DISCUSSION

H1 variants can be regarded as potential modulators of chromatin structure and function. The effect of their microheterogeneity is further enhanced by their post-translational modifications as phosphorylation [Roth and Allis, 1992] and poly(ADP-ribosyl)ation [Poirer et al., 1982; Quesada et al., 1989; De Lucia et al., 1996].

The results from both the experimental approaches described in the present paper con-

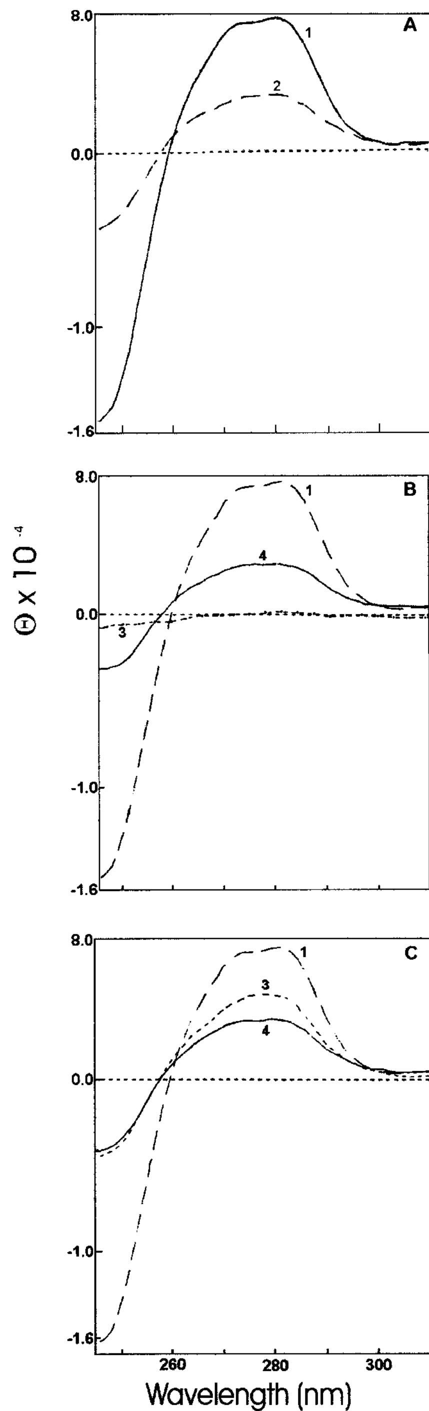


Fig. 4. Circular dichroism spectra of rat testis chromatin. **A:** H1-depleted (1) and native (2) oligonucleosomes. The H1-depleted chromatin (1) was reconstituted with H1a **(B)** and H1t **(C)** in the unmodified (3) and ADP-R modified (4) forms. The results obtained with a chromatin/H1 ratio 1:0.5 (nucleosome/histone molecule) are shown.

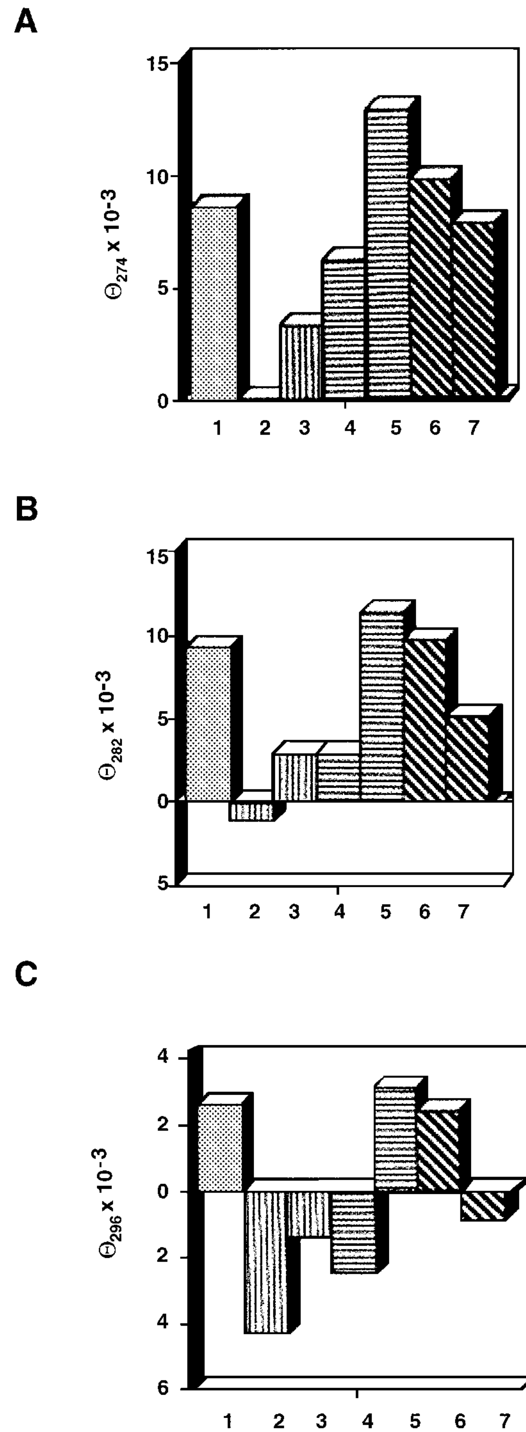


Fig. 5. Molar ellipticity of H1/chromatin complexes at different wavelengths. Molar ellipticities at 274 nm **(A)**, 282 nm **(B)**, 296 nm **(C)** were measured for H1-depleted oligonucleosomes (0.5–2.5 kbp) before (1) and after reconstitution with total H1 (2), total ADP-R-H1 (3), H1a (4), ADP-R-H1a (5), H1t (6), and ADP-R-H1t (7). The results obtained with a chromatin/H1 ratio of 1:1 (nucleosome/histone molecule) are shown.

verged toward different extents of covalent poly-(ADP-ribosyl)ation of H1s, to which specific polymer patterns were associated. Although the two modified variants, H1a and H1t, lacked branched and long polymers, ADP-R chains of specific lengths were found associated with each protein. Thus, the type and the number of polymers modifying the histone proteins appeared to be intrinsically determined by each variant, following different activation of the poly(ADP-ribose)polymerase. H1t stimulated the enzyme activity more than the other variants and resulted mainly poly(ADP-ribosyl)ated in vitro. The meaning of the preferential modification of the testis-specific subfraction must still be investigated and might correlate with the histone role during spermatogenesis.

The possible special function of H1t and of its poly(ADP-ribosyl)ation was further evidenced by circular dichroism of H1/stripped chromatin complexes, which confirmed the different affinities of H1s to DNA. As expected, the negative charges associated with the poly(ADP-ribosyl)ated H1a were able to alter the H1/DNA interactions, as shown by the stripped chromatin-like CD spectrum (Fig. 4B). By contrast, poly(ADP-R)-H1t induced a high dose-dependent compaction of the chromatin, as opposite to the native variant, which was described as a poor DNA-condensing protein, expecially after acid extraction [De Lucia et al., 1994; Khadake and Rao, 1995]. With the length of the polymer associated with H1t almost two to three times that modifying H1a, one would have expected that the longest ADP-R chains linked to H1t would further reduce the already low DNA-binding ability of the testis-specific protein. The CD spectrum measured for the modified H1t tended toward the values determined for the native chromatin and suggested that the ability of H1t to interact with chromatin increases upon modification by poly(ADP-ribose). The reproducible behavior of the modified testis variant let hypothize that the effect of poly(ADP-R) associated with H1t might correlate with structural features of the protein.

It was reported that the C-terminal domains of H1s, which make extensive contacts with DNA, may display variant-specific differences in their interactions with the nucleic acid [Khadake and Rao, 1995; Wellman, 1996; Khadake and Rao, 1997]. These domains are also flexible and extended in solution, but they may fold upon binding to DNA. DNA/protein interaction and the correct folding of the involved proteins are simoultaneous processes, and the conformational changes occurring are important in recognition of the specific DNA binding sites [Khadake and Rao, 1995, Wellman, 1996].

The main difference in the C-terminal amino acid sequences of H1a and H1t is a larger number of arginines in the testis-specific protein. Arginine side chains are more often in ordered regions of proteins than are lysines. Perhaps the Arg-rich C-terminus of H1t can be forced to fold by simply reducing electrostatic repulsion. In this regard, it is known that phosphate ions bind to H1s with high affinity, inducing protein folding [De Petrocellis et al., 1986), and prefer Arg-rich macromolecules.

These observations and the evidence that the C-terminus of H1t lacks most of the DNA-binding motifs [Khadake and Rao, 1995] strongly suggest that the contribution of poly-(ADP-ribose) to the DNA-condensing property of the testis-specific variant might consist in driving the folding of this domain, to gain the correct DNA-binding conformation.

This hypothesis might explain the apparent paradox that the poly(ADP-ribosyl)ation of H1t induces compaction of chromatin, whereas one would expect the phosphates of the polyanion to weaken the histone/chromatin interactions.

This controversial situation was already proposed when phosphorylation of H1 associated with chromatin condensation at mitosis was described [Roth and Allis, 1992].

The steric hindrance of single phosphates is certainly less than that of the largest poly(ADP-R). The question remains as to how the polynucleotide might act on H1t without interfering with DNA/histone or even histone-proteins interactions.

Studies are under way in an attempt to define both the ADP-ribose modification sites in each variant and the conformation assumed by the single histone upon association with poly-(ADP-R). Determination of whether the observed effect is a consequence of the partial unfolding of the acid-extracted histones or of intrinsic features of such a protein is under investigation by analyzing linker histone variants prepared under nondenaturing conditions.

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