Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 3423–3427, June 1982
Biochemistry

Poly(ADP-ribosyl)ation of polynucleosomes causes relaxation of chromatin structure

[nucleosome superstructure/electron microscopy/poly(ADP-ribose) polymerase/histone H1 modification]

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Communicated by Jean Dausset, February 22, 1982

ABSTRACT When rat pancreatic polynucleosomes were poly(ADP-ribosyl)ated with purified calf thymus poly(ADP-ribose) polymerase and examined by electron microscopy, a relaxation of their native zigzag structure was observed. At high ionic strengths control nucleosomes condensed into 250-Å-thick fibers, but poly(ADP-ribosyl)ated polynucleosomes did not; they showed a close resemblance to chromatin depleted of histone H1. The relaxed state of poly(ADP-ribosyl)ated polynucleosomes was also confirmed by sedimentation velocity analysis. Histone H1 was found to be the major histone acceptor of poly(ADP-ribose). Poly(ADP-ribose) linked to histone H1 did not seem to cause its dissociation from the chromatin, but it impaired significantly its effect on chromatin condensation.

Post-translational modifications of histones—e.g., phosphorylation (1), acetylation (2, 3), and poly(ADP-ribosyl)ation (4, 5) have been suggested as possible mechanisms to modulate the nucleosomal structure during DNA transcription and replication.

Poly(ADP-ribose) polymerase was suggested to be preferentially located at internucleosomal regions of HeLa cell nucleosomes (6, 7); its specific activity was shown to be highest at selected folded regions of 8–10 nucleosomes in higher-ordered chromatin (8). In addition, the poly(ADP-ribosyl)ation of several chromatin components, including all the histones, was demonstrated (9, 10). All these findings reinforced the suggestions of involvement of poly(ADP-ribosyl)ation in DNA replication, DNA repair, and gene expression through alteration of the chromatin structure.

In order to test this hypothesis, pancreatic polynucleosomes (20–40 nucleosomes) were poly(ADP-ribosyl)ated by using purified calf thymus poly(ADP-ribose) polymerase. Changes of nucleosome structure were examined by electron microscopy and by ultracentrifugation and the poly(ADP-ribosyl)ated histones were characterized.

MATERIALS AND METHODS

Preparation of Polynucleosomes. Rat pancreatic polynucleosomes were isolated as described (11). Nuclei were digested with micrococcal nuclease (Sigma) at 30°C for 2 min, with 0.5 unit of nuclease per mg of DNA. The polynucleosomes (20–40 nucleosomes) were isolated on linear 5–29% sucrose gradients by centrifugation at 260,000 × *g* for 150 min in a Beckman SW 41 rotor and were characterized by electron microscopy and circular dichroism.

Poly(ADP-ribosyl)ation of Polynucleosomes. Polynucleosomes were poly(ADP-ribosyl)ated by using purified calf thymus poly(ADP-ribose) polymerase at 25°C in an incubation medium of 500 μl containing 8 μg of DNA-independent enzyme (12, 13), 1 A_{260} unit of nucleosomes, 100 mM Tris·HCl at pH 7.8, 1 mM NAD (Sigma), 0.4 mM dithiothreitol, and 8 mM $MgCl_2$. Under these conditions the incubation medium is turbid, probably due to the size of nucleosomes used. For the characterization of poly(ADP-ribose) acceptors 62 μCi (1 Ci = 3.7 × 10^{10} becquerels) of [^{32}P]NAD (34.8 Ci/mol) (New England Nuclear) was added to the incubation medium. Control nucleosomes were incubated under the same conditions without NAD. After 15 min, the reaction mixture was cooled on ice and nicotinamide (Merck) was added (final concentration 10 mM). The nucleosomes were examined by electron microscopy or were dialyzed overnight in 5 mM triethanolamine·HCl, pH 7.4/0.2 mM EDTA/20 mM NaCl prior to the sedimentation velocity determinations.

Electron Microscopy. Chromatin samples diluted to 0.5 μg/ml (expressed as DNA concentration) with a buffer containing 5 mM triethanolamine at pH 7.4, 0.2 mM EDTA, and the appropriate concentration of NaCl (see figure legends) were fixed in 0.1% glutaraldehyde (Balzers, Lichtenstein) for 1 hr at room temperature. Adsorption of the specimens onto positively charged carbon-coated grids (400 mesh) was performed as described (14). The specimens were stained with uranyl acetate [2% (wt/vol) in H_2O], rinsed in H_2O, and air dried. Finally, the grids were rotary shadowed with carbon/platinum at an angle of 7° in an Edwards evaporator equipped with an electron gun (EVM 052, Balzers). The thickness of the metal deposition was monitored on a quartz thin crystal monitor (QSG 201 D, Balzers). The grids were examined in a Siemens Elmiskop 101. The magnification was calibrated by using a carbon grating replica (Fullam, Schenectady, NY).

Sedimentation Velocity. Ultracentrifugation was performed in a model E Spinco ultracentrifuge equipped with a digital scanner (15) at 28,500 rpm.

Characterization of Histone Acceptors of Poly(ADP-ribose). After poly(ADP-ribosyl)ation the reaction mixture was dialyzed overnight against 10 mM Tris·HCl, pH 7.4/1 mM EDTA/20 mM NaCl. Samples were then layered on linear 5–29% sucrose gradients. The gradient fractions 2–8 (peak I) and 11–16 (peak II) (see Fig. 4) were separately pooled, dialyzed against water, and lyophilized. Total histones from peak I (nucleosomal fraction) were extracted with 0.4 M HCl/10 mM dithiothreitol/8 M urea. Electrophoresis was performed on acetic acid/urea/15% polyacrylamide gels (16). After staining with Coomassie blue and destaining, the gels were dried and autoradiographed, using Fuji x-ray films. For selective extraction of histone H1 and core histones, the nucleosomes were precipitated from the

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poly(ADP-ribosyl)ation mixture by the addition of 2 vol of magnesium acetate buffer to final concentrations of 10 mM $MgCl_2$ and 50 mM sodium acetate, pH 5.0. Histone H1 was extracted selectively from the nucleosomal pellet with 5% perchloric acid for 1 hr (17, 18) and purified from high mobility group proteins by selective precipitation with acetone (19). Core histones were extracted with 0.4 M HCl/8 M urea overnight. Electrophoresis was performed as described above. For the characterization of histone acceptors from peak II, following the dialyses and lyophilization as for peak I, samples were incubated in 0.3 M NaOH for 1 hr at 37°C to hydrolyze the linkages between poly(ADP-ribose) and acceptor proteins. Histone H1 and core histones were then selectively extracted and separated by electrophoresis with acid/urea/Triton X-100 gels (16) as described above.

RESULTS

Fig. 1 *A* and *C* shows electron micrographs of native pancreatic chromatin incubated in the poly(ADP-ribosyl)ation medium without NAD in the presence of poly(ADP-ribose) polymerase (control). The zigzag open structure (Fig. 1*C*) that is a characteristic feature of the morphology of native chromatin (20, 21) is observed.

Fig. 1 *B* and *D* shows the poly(ADP-ribosyl)ated sample under the same spreading conditions. There is a relaxation of the native structure, the molecule occupying a greater area as compared to the control experiment. In addition it is clear that, after poly(ADP-ribosyl)ation, the native zigzag structure of chromatin has been converted to a beads-on-a-string structure, which is characteristic of H1-depleted chromatin (21). Similar results were obtained when the amount of poly(ADP-ribose) polymerase was reduced to 1/10th. The time course of the effect of poly(ADP-ribosyl)ation was investigated. The initiation of relaxation of the native structure was observed after 1 min of incubation, and a plateau was reached after 7 min (data not shown). It was previously shown by Thoma *et al.* (21) that when the ionic strength is increased native chromatin folds toward the solenoid conformation, whereas H1-depleted chromatin does not fold into any regular definite fiber. Therefore we analyzed the poly(ADP-ribosyl)ated chromatin under conditions of higher ionic strength to further investigate its similarity to H1-depleted chromatin. Fig. 2 *A* and *B* shows the control and poly(ADP-ribosyl)ated nucleosomes, respectively, which were fixed with fixation buffer containing 20 mM NaCl. No condensation of poly(ADP-ribosyl)ated chromatin was observed as compared to native chromatin. The same striking difference was also obtained at 75 mM NaCl, as shown in Fig. 2*C*, where the native and poly(ADP-ribosyl)ated chromatin were layered on the same grid. The identification of both treated and untreated chromatin on the same grid shows that the relaxation of the chromatin was not due to a spreading artefact but was a consequence of a biochemical modification. Finally, Fig. 3 shows control (*A*) and poly(ADP-ribosyl)ated (*B*) chromatin that were fixed directly in the poly(ADP-ribosyl)ation medium in the presence of 8 mM Mg^{2+}. The relaxed state of poly(ADP-ribosyl)ated chromatin was also observed after these fixation conditions. Similar results were obtained when glutaraldehyde fix-

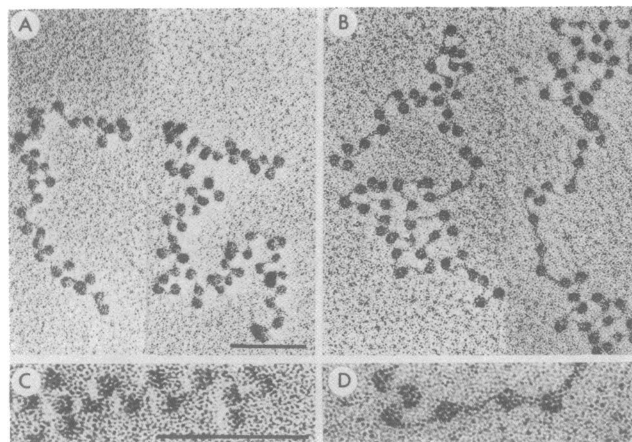

FIG. 1. Effect of poly(ADP-ribosyl)ation upon the structure of chromatin at low ionic strength. (*A* and *C*) Native pancreatic chromatin (control), (*B* and *D*) poly(ADP-ribosyl)ated chromatin. The specimens were fixed with 0.1% glutaraldehyde in a buffer containing 5 mM triethanolamine at pH 7.4 and 0.2 mM EDTA. The bars indicate 1,000 Å.

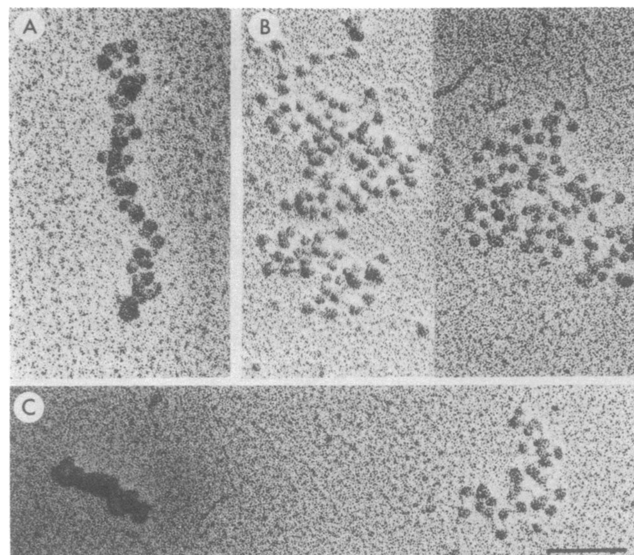

FIG. 2. Appearance of the chromatin samples at higher ionic strength. The specimens were fixed with 0.1% glutaraldehyde in a buffer containing 5 mM triethanolamine at pH 7.4, 0.2 mM EDTA, and 20 mM NaCl (*A*, *B*) or 75 mM NaCl (*C*). (*A*) Native pancreatic chromatin (control), (*B*) poly(ADP-ribosyl)ated chromatin, (*C*) cospreading of native and poly(ADP-ribosyl)ated chromatin fixed in 75 mM NaCl. The bar indicates 1,000 Å.

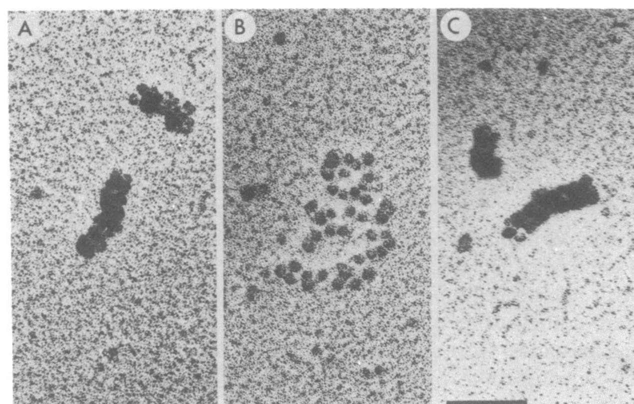

FIG. 3. Visualization of the chromatin samples directly fixed with 0.1% glutaraldehyde in the poly(ADP-ribosyl)ation medium. After fixation, samples were diluted with the buffer described in *Materials and Methods* containing 75 mM NaCl. (*A*) Native pancreatic chromatin (control), (*B*) poly(ADP-ribosyl)ated chromatin, (*C*) native pancreatic chromatin mixed with automodified poly(ADP-ribose) polymerase. The bar indicates 1,000 Å.

ation was omitted. Fig. 3*C* shows the results of a control experiment in which autopoly(ADP-ribosyl)ated enzyme (22) was incubated with native chromatin in the presence of 10 mM nicotinamide as described in *Materials and Methods*. Under these conditions, in which poly(ADP-ribosyl)ation of chromatin is inhibited, preformed poly(ADP-ribose) did not cause any relaxation of chromatin. These results were also confirmed by incubation of native chromatin with isolated poly(ADP-ribose) (data not shown).

Sedimentation velocity analysis in 20 mM NaCl of control and poly(ADP-ribosyl)ated nucleosomes gave sedimentation coefficients ($s^0_{20,w}$) of 59.4 ± 1.5 S for control nucleosomes and of 50.6 ± 0.3 S for poly(ADP-ribosyl)ated nucleosomes. These results further indicate the relaxed state of the poly(ADP-ribosyl)ated nucleosomes as compared to the control nucleosomes, which are in partly condensed form at this ionic strength.

After the striking structural differences between control and poly(ADP-ribosyl)ated chromatin were observed, the following problems were analyzed: (*i*) What are the histone acceptors of poly(ADP-ribose)? (*ii*) Are there any nucleosomal components, particularly H1, that are detached from the chromatin after poly(ADP-ribosyl)ation and thus give rise to the observed relaxed appearance?

To answer these questions, the poly(ADP-ribosyl)ated material was first analyzed by sucrose gradient centrifugation. As shown in Fig. 4, the [^{32}P]NAD was measured together with the absorbance in each fraction. Two major peaks were observed: I, in the area of nucleosomal fraction, and II, at a density of about 12% sucrose, representing presumably unbound material.

When total histones were extracted from the material of peak I and analyzed on acid/urea gels by autoradiography (Fig. 5*A*), a band on top of the gel and a second band migrating more slowly than histones were strongly labeled. Moreover, when H1 was selectively extracted from poly(ADP-ribosyl)ated nucleosomes (Fig. 5*B*) a similar labeled pattern as for total histones was observed. When the stained gel of this autoradiogram was analyzed a major band at histone H1 level and an additional slowly migrating band of lower intensity (which was shown to be highly labeled in Fig. 5*B*) were observed (data not shown). The intensity of the major H1 band was decreased, as compared to its intensity when extracted from control nucleosomes. The additional band was not observed in control nucleosomes (data not shown). We therefore identify the labeled bands as different species of H1–poly(ADP-ribose) complexes (23, 24). On the basis of the intensity of staining at H1 level, which decreased when nucleosomes were poly(ADP-ribosyl)ated, we estimate the amount of poly(ADP-ribosyl)ated H1 as 20–30%. The remaining core histones after H1 extraction were not labeled (data not shown).

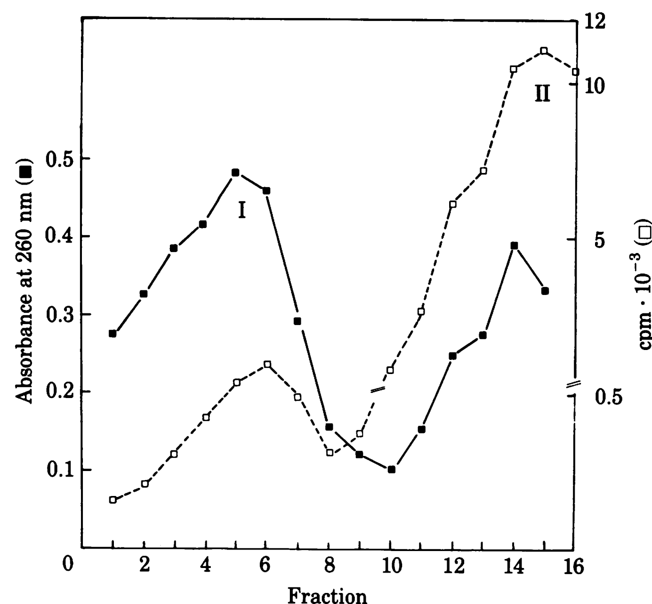

FIG. 4. Analyses of the poly(ADP-ribosyl)ated material on a sucrose gradient. Five A_{260} units of nucleosomes were poly(ADP-ribosyl)ated with [^{32}P]NAD, using 16 μg of purified calf thymus poly(ADP-ribose) polymerase as described in *Materials and Methods*. The reaction mixture was layered on a linear 5–29% sucrose gradient after dialysis. Absorbance at 260 nm (■) was measured in parallel to ^{32}P label (□) in all fractions of the gradient. Peak I represents the nucleosomal fraction. Peak II represents the unbound material at about 12% sucrose.

From these results we conclude that histone H1 is the major histone poly(ADP-ribose) acceptor and all the poly(ADP-ribosyl)ated histone H1 is in histone H1 complex form. Note that purified calf thymus poly(ADP-ribose) polymerase has preferentially poly(ADP-ribosyl)ated histone H1, as previously observed with the endogenous pancreatic enzyme (11, 25).

To investigate whether some chromatin components are detached from nucleosomes after poly(ADP-ribosyl)ation, we first analyzed the material of peak II on sodium dodecyl sulfate/polyacrylamide gels (26) by autoradiography (Fig. 5*C*). A highly labeled material apparently too large to enter the gel was detected on top of the gel. The nature of this material was investigated.

The presence of poly(ADP-ribose) polymerase in this poly(ADP-ribosyl)ated product was confirmed by enzyme activity measurements and by protein blotting analysis (27, 28) using a specific antiserum against calf thymus poly(ADP-ribose) polymerase (data not shown).

The presence of histone H1 and other histones in these complexes was further analyzed. The poly(ADP-ribose)-protein linkages were hydrolyzed prior to the extraction of histone H1 and core histones from the material of peak II in order to assure complete extraction (which may be difficult to perform directly from the complex). No proteins were observed when the extracted material was analyzed in acid/urea/Triton gels (Fig. 5*D*). It is important to note that 0.5 μg of histone H1 can be detected easily on these gels when stained under the same conditions (data not shown). This corresponds to 2% of the approximate amount of total histone H1 present in the nucleosomes used for the above tests. Thus, it appears that histone H1 (up to 2% of total) and other histones are not detached from the chromatin after poly(ADP-ribosyl)ation and that the major component of peak II is the poly(ADP-ribosyl)ated enzyme, which seems to have formed complexes as described by Ogata *et al.* (29, 30).

DISCUSSION

We have demonstrated that poly(ADP-ribosyl)ation of pancreatic polynucleosomes with purified poly(ADP-ribose) polymerase results in a relaxation of the overall chromatin structure. At low ionic strength poly(ADP-ribosyl)ation converts the native zigzag chromatin structure (20, 21) into a beads-on-a-string structure. The relaxation phenomenon is more apparent at higher ionic strengths (20 and 75 mM NaCl), in which control nucleosomes undergo a gradual condensation toward the 250- to 300-Å fibers and in which poly(ADP-ribosyl)ated nucleosomes maintain an open structure. The transition of the chromatin structure from a zigzag to a compact form under high ionic strength was described to be dependent upon the presence of histone H1 (21, 31). The pictures we obtained of poly(ADP-ribosyl)ated chromatin show a close resemblance to H1-depleted chromatin. The determination of histone acceptors of poly(ADP-

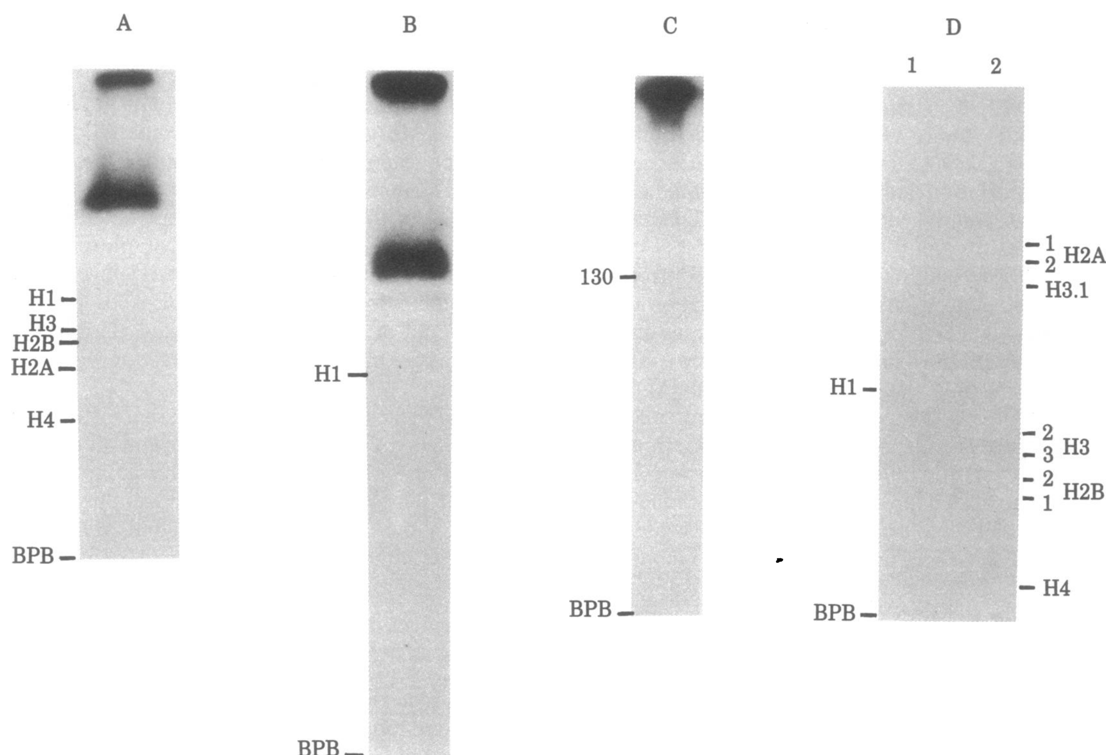

FIG. 5. Characterization of the [^{32}P]poly(ADP-ribose) histone acceptors in peak I and in peak II derived from the sucrose gradient illustrated in Fig. 4. (*A*) Autoradiogram of acid/urea gel of the [^{32}P]poly(ADP-ribosyl)ated histones extracted from peak I. (*B*) Autoradiogram of acid/urea gel of [^{32}P]poly(ADP-ribosyl)ated histone H1 extracted from modified nucleosomes precipitated from the reaction mixture. (*C*) Autoradiography of sodium dodecyl sulfate/7% polyacrylamide gels on which the material of peak II was layered. (*D*) Lanes 1 and 2, acid/urea/Triton gels of histone H1 and core histones, respectively, extracted from peak II after hydrolyses of poly(ADP-ribose)-protein linkages. BPB, bromophenol blue; 130, 130-kilodaltons.

ribose) showed that histone H1 was the major poly(ADP-ribosyl)ated histone. The possibility that poly(ADP-ribosyl)ation of histone H1 detaches these molecules from the chromatin is unlikely because under our experimental conditions no histone H1 was detected on top of the sucrose gradient onto which a poly(ADP-ribosyl)ated nucleosome mixture had been loaded (see Figs. 4 and 5). Therefore, it appears that poly(ADP-ribosyl)ation alters the effect of histone H1 on chromatin structure without detaching it from the chromatin. Studies of the reversibility of the relaxation phenomenon should clarify this point. We do not know the mechanism by which poly(ADP-ribosyl)ation would alter the involvement of H1 in the nucleosomal organization and cause a relaxation of the nucleosomal structure. However, it is conceivable that poly(ADP-ribose) linked to histone H1 affects the H1–DNA binding, either by a charge reduction of histone H1 or by modification of its conformation. In fact, Burzio *et al.* (32) demonstrated that poly(ADP-ribosyl)ated H1 has a diminished affinity for DNA and suggested that ADP-ribosylation of H1 would diminish the cross-linking ability of the protein and consequently cause a relaxation of the chromatin fiber.

We used the purified calf thymus poly(ADP-ribose) polymerase in our experiments in order to get an extensive modification of nucleosomes. Although we identified the purified enzyme as the most poly(ADP-ribosyl)ated component of our system on top of the sucrose gradient and we know that the purified enzyme is DNA independent (13), we do not presently know if a fraction of the enzyme is linked to the nucleosomal DNA for poly(ADP-ribosyl)ation of the chromatin and contributes to the relaxation phenomenon by an unknown mechanism.

Butt *et al.* (33, 34) reported that poly(ADP-ribosyl)ation of HeLa cell oligonucleosomes by their intrinsic enzyme favored the formation of nucleosomal aggregates, which were suggested to be correlated with the formation of poly(ADP-ribose)–H1 complexes. Although we identified the poly(ADP-ribose)–H1 complexes in the nucleosomal fraction of our system, we did not observe any aggregation of nucleosomes. This would imply that the presence of these complexes does not necessarily give rise to the internucleosomal network formation and their occurrence may be dependent upon the ratio of the amounts of poly(ADP-ribose) polymerase and chromatin components. Nonetheless, these apparent discrepancies do not exclude the possibility that relaxed domains of chromatin structure induced by poly(ADP-ribosyl)ation could be present within the HeLa cell nucleosomal aggregates.

The relaxation of the chromatin organization by poly(ADP-ribosyl)ation could be one of the mechanisms by which nuclear events such as DNA replication, DNA repair, and transcription are facilitated. In fact, Jump *et al.* (10) found that ADP-ribosylation mainly occurred in regions of chromatin undergoing DNA synthesis. Finally, several authors observed a great increase of poly(ADP-ribose) polymerase activity upon the induction of DNA repair (35–37). It is tempting to suggest that this massive poly(ADP-ribosyl)ation would produce a relaxation phenomenon that would render the damaged DNA more accessible to repair enzymes.

We thank Dr. Jean Pouyet for performing the sedimentation velocity analysis, Drs. M. Daune, M. E. Ittel, and M. Champagne for many stimulating discussions, and Mr. F. Hog for skilful technical assistance.

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