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Nucleosome periodicity in HeLa cell chromatin as probed by micrococcal nuclease

[chromatin structure/polynucleosomes/poly(ADP-ribose) polymerase/nuclear proteins]

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ABSTRACT **When HeLa cell nuclei were treated with micrococcal nuclease (nucleate 3-oligonucleotidohydrolase, EC 3.1.4.7), lysed, and centrifuged, the supernatant from early digests contained two predominant classes of polynucleosomes of repeat size 8*N* and 16*N*. With increasing digestion time, the 16*N* polynucleosome appeared to be cleaved to the 8*N* species and finally to the basic subunit of chromatin. The size of the polynucleosomes has been determined by DNA analysis and on polyacrylamide electrophoretic gels of native chromatin particles. The16*N* polynucleosome appears to be a unique higher ordered structural component of HeLa cell chromatin. Our recent report, showing that the nuclear protein-modifying enzyme poly(ADP-ribose) polymerase increases in specific activity progressively with increasing nucleosome repeat size up to 8–10*N*, has been extended in the present study. Activity was also elevated in the polynucleosomes of the 16*N* structure preferentially cleaved by micrococcal nuclease, although specific activity of the enzyme was highest in octanucleosomes. Acceptors for poly(ADP-ribose) have also been determined in these particles.**

Despite recent advancement in our understanding of the structure of the nucleosome core particle, similar information concerning the higher order structure of chromatin remains largely undetermined (1–3). At low ionic strength the nucleofilament with diameter of 100 Å is a single chain of nucleosomes (4–7). In the presence of monovalent and divalent cations, the diameter of the nucleofilament is increased to 250–300 Å (4, 5, 7). Based on electron micrographic data, Finch and Klug (4) postulated a solenoid model of chromatin.

Kinetic studies performed on the action of micrococcal nuclease (nucleate 3′-oligonucleotidohydrolase, EC 3.1.4.7) on interphase chromatin and metaphase chromosomes (8–10) have supported the theory of random cleavage by the enzyme at uniformly susceptible sites on linker regions of polynucleosomes. However, it has also been shown that newly replicated chromatin is relatively more susceptible to the action of micrococcal nuclease (11).

Using electron microscopic studies and sedimentation analysis on the chromatin produced after the digestion of lymphocyte nuclei by micrococcal nuclease, Hozier *et al.* (7) have postulated that chromatin is organized into superbeads containing six to ten nucleosomes. However, ambiguity about these structures still remains since Hozier *et al.* (7) were unable to isolate and resolve them on sucrose gradients. In kinetic studies performed in the work below, it has clearly been shown that micrococcal nuclease cleaves HeLa chromatin at a periodicity of eight nucleosomes. In addition to sedimentation analysis, we have been able to characterize these structures by (*i*) native chromatin gels, (*ii*) determination of the size of the DNA, and (*iii*) the presence of a full complement of nuclear histones.

These observations on nucleosome periodicity stemmed directly from our previous observation that the specific activity of a chromatin-bound enzyme, poly(ADP-ribose) polymerase, progressively increased with increasing nucleosome repeat number (12). This enzyme catalyzes the formation of a homopolymer of ADP-ribose units linked by 1′-2′ glycosidic bonds. NAD is the substrate for the enzyme which, in the presence of DNA, catalyzes the transfer of ADP-ribose units onto an initial ADP-ribose residue covalently attached to various nuclear proteins, including histones and nonhistone proteins (13, 14). We have shown that the enzyme is bound to the internucleosomal region of chromatin (15, 16). Apart from modifying histone H1 and nonhistone proteins, core histones can be extensively modified when chromatin is in its native conformation (13, 14).

Because of its stable association with eukaryotic chromatin, this enzyme provides a unique probe to assess the distribution of an enzyme within chromatin substructure and to elucidate the biological importance of higher orders of chromatin structure.

MATERIALS AND METHODS

Micrococcal nuclease was obtained from Worthington. [^{32}P]-NAD was synthesized as described (13).

Nuclease Digestion. HeLa cell nuclei were prepared by the method of Sporn *et al.* (17). All the solutions contained 0.5 mM phenyl methyl sulfonyl fluoride. Nuclei were washed with 0.2 M sucrose/1 mM $CaCl_2$/5 mM Tris·HCl, pH 7.5/80 mM NaCl and centrifuged at 3500 × *g*. The pellet was immediately suspended in the above buffer at 1×10^8 nuclei per ml. Micrococcal nuclease was added (30 units/1×10^8 nuclei), and the nuclei were incubated for the desired period of time at 37°C. The reaction was terminated by addition of EDTA to a final concentration of 1 mM. The suspension was centrifuged at 3500 × *g*; the nuclear pellet was suspended at 0°C in 1 mM EDTA solution (2×10^8 nuclei per ml) and allowed to lyse for 20 min. The suspension was centrifuged for 8.5 min at 5000 × *g*; the supernatant contained released chromatin. In a typical experiment, 20–80% of the chromatin was released during the time course (Fig. 1), as determined by absorbance at 260 nm and diphenylamine test for DNA estimation. During the digestion period from 30 to 180 sec, 5–11% of the material with absorbance at 260 nm was rendered acid soluble.

ADP-Ribosylation and Gel Electrophoresis. Poly(ADP-ribose) polymerase was assayed as described (13). Proteins containing poly(ADP-ribose) were precipitated with 3 vol of absolute ethanol at −20°C for 10 hr. The precipitates were collected by centrifugation at 20,000 × *g* for 20 min, dissolved in 10 mM sodium phosphate, pH 7.0/1% sodium dodecyl sulfate ($NaDodSO_4$)/1% 2-mercaptoethanol, and boiled for 2 min prior to electrophoresis. Electrophoresis was performed in 5–22% linear gradient, polyacrylamide (acrylamide/*N*,*N*-methylenebisacrylamide, 200:1, wt/wt) slab gels with 0.1 M

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Abbreviation: $NaDodSO_4$, sodium dodecyl sulfate.

sodium phosphate, pH 7.0/0.1% $NaDodSO_4$ as gel buffer. For total nuclear protein gel electrophoresis, the proteins were precipitated as above and electrophoresed according to Laemmli (18).

DNA Size Determination. Appropriate fractions from the sucrose gradient (5–3 μg of DNA) were adjusted to 0.1 M NaCl/0.1% $NaDodSO_4$/5 mM EDTA. Proteinase K (10–5 μg) was added to the preparations; incubation at 37°C for 1–2 hr was followed by DNA extraction with phenol/isoamyl alcohol/chloroform, 24:1:24 (vol/vol). The aqueous layer was recovered, dialyzed overnight in 0.2 mM EDTA solution at 4°C, and freeze dried. Electrophoresis was performed in 4% polyacrylamide slab gels (19). Fragments of φX174 DNA cleaved by *Hae* III (19), *Hpa* I, and *Hpa* II (20) were used as molecular weight markers.

Chromatin Gel. A solution of chromatin particles was analyzed by slab gel electrophoresis on 2.5% polyacrylamide/0.5% agarose (acrylamide/*N,N'*-methylenebisacrylamide, 20:1). The gel buffer was 89 mM Tris base/89 mM boric acid/2.5 mM EDTA, at pH 8.3. Electrophoresis was performed for about 1 hr before application of samples. Samples were applied immediately after sedimentation analysis; electrophoresis was performed at 4°C for 8–10 hr at 150 V.

RESULTS

Preferential polynucleosome cleavage sites

During preliminary control experiments required for our earlier study on the relationship between poly ADP-ribosylation and higher forms of chromatin structure (12), two distinct peaks (representing differing nucleosome repeat sizes) were occasionally observed in sucrose gradients used to prepare oligonucleosomes. A study of micrococcal nuclease early digestion kinetics of HeLa cell nuclei has revealed that these structures represent important structural cleavage sites of chromatin (Fig. 1). HeLa cell nuclei were digested with micrococcal nuclease (30 units of enzyme per 1×10^8 nuclei) for varying lengths of time. The chromatin preparations were separated on linear 10–30% sucrose gradients designed to separate nucleosomes up to approximately 30*N*. In agreement with our earlier studies (12), by 3 min of digestion, one major polynucleosome species (peak 1) was evident in the gradient. Lower nucleosome repeats (1*N*–5*N*) also appeared as distinct secondary cleavage products. At 30 sec under these digestion conditions, micrococcal nuclease cleaved the chromatin such that two major classes of polynucleosome species could be clearly detected by sedimentation analysis. About 50 and 80% of the total chromatin applied on the gradient (as determined by A_{260} and DNA estimation) was recovered in 30-sec and 3-min digests, respectively. Of the chromatin recovered on the gradient, 30 and 35% was accounted for in peak 1 and peak 2 respectively, for the 30-sec digest and 34 and 24% for the 3-min digest. Results identical to those in Fig. 1 were obtained when nuclease digestion and sedimentation analysis or both were performed at low salt concentrations (5 mM NaCl). Under these conditions, the two major peaks migrated slightly more slowly (data not shown). This suggests that nucleosome aggregation was not a complication in our analysis. In addition, Renz *et al.* (21) have already studied in detail the effect of salt on sedimentation properties of higher order chromatin from lymphocytes.

The DNA was isolated from peak tubes of the 30-, 90-, and 180-sec digests and analyzed on polyacrylamide gels (Fig. 2*A*). DNA extracted from a region of the sucrose gradient (fraction 10, 180 sec) containing predominantly 4–5*N* nucleosomes was used as an oligonucleosome marker (slot 7). The top band of this preparation represents pentanucleosome DNA. Assuming an average repeat length for HeLa chromatin of 183 base pairs

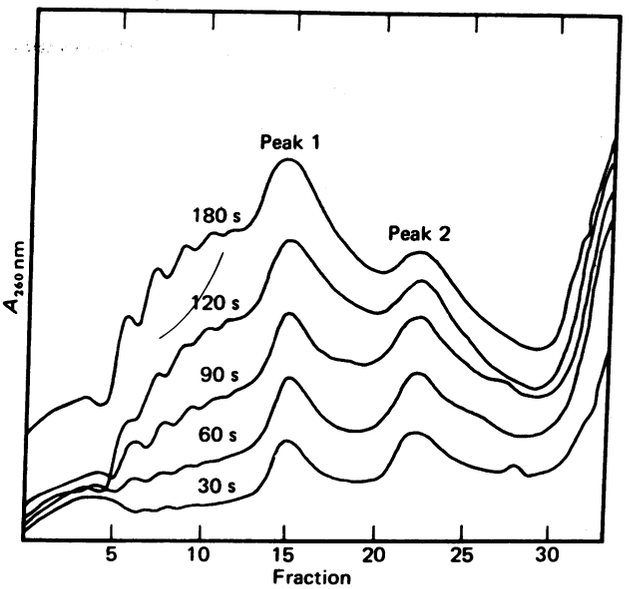

FIG. 1. Higher order chromatin forms preferentially cleaved by micrococcal nuclease. HeLa cell nuclei (1×10^8/ml) were incubated in 1.0 ml of nuclease buffer (12) for 5 min at 37°C. Micrococcal nuclease (30 units/10^8 nuclei) was added and samples were made 1 mM in EDTA at 30 (3×10^8 nuclei), 60 (2×10^8), 90 (2×10^8), 120 (2×10^8), and 180 (1×10^8) sec. The nuclei were centrifuged and lysed in 1 ml of 1 mM EDTA for 20 min at 5°C. Twenty to eighty percent of the total chromatin was solubilized during 30–180 sec of digestion. The suspension was subsequently centrifuged for 8.5 min at $5000 \times g$. The supernatant, containing between 4 and 15 $A_{260\,nm}$ units was layered on a 12-ml, 10–30% linear sucrose gradient containing 0.2 mM EDTA, 1 mM sodium phosphate (pH 6.8), and 80 mM NaCl and centrifuged in an SW 40 rotor at 40,000 rpm at 4°C for 4.5 hr. Gradients were monitored for $A_{259\,nm}$ with an ISCO UV monitor; 0.33-ml fractions were collected. Direction of sedimentation is from left to right.

(22), the range of the base pairs in the top band of this sample (855–918) confirms the identity of five nucleosomes. DNA extracted from the material in peak 1 (Fig. 1) is shown in slots 2, 5, and 8. No significant size difference was noted in the DNA of this class of polynucleosomes during the various digestion time periods. The leading edge of this peak contained approximately 1425 base pairs (Fig. 2*B*). Probably because of breaks in DNA exposed after protein extraction, nucleosome repeat-size fragments were observed, especially in the sample in slot 8, and these repeats could be conveniently counted. From these data it is clear that peak 1 represents polynucleosomes of repeat size 8*N*. These polynucleosomes contained repeat lengths ranging approximately from 178 to 183 base pairs; we used Fig. 2*B* for the calculation below.

The DNA of peak 2 (slots 3, 6, and 9) had a range of base pairs of approximately 2550–3500. The middle of these bands was 3110 base pairs. Because of the precision of the DNA analysis of the material in peak 1, we estimate that the faster sedimenting nucleosomes represent 16*N* repeat lengths and that micrococcal nuclease preferentially cleaved HeLa cell chromatin at a periodicity of 8 and 16 nucleosomes.

To further show that polynucleosomes of peak 2 resolved on sucrose gradients were distinct oligomeric particles and not aggregates of material from peak 1 or smaller nucleosomes, we analyzed appropriate chromatin samples by native polyacrylamide gel electrophoresis (Fig. 3). Mobilities of the samples (slots A and B contained material from peaks 1 and 2, respectively) demonstrate that particles were clearly resolved by this technique. The material from peak 2 (16*N*) migrated more slowly than that from peak 1 (8*N*).

The major 8*N* polynucleosomal particles were a preferred digestion product of micrococcal nuclease and appeared to be

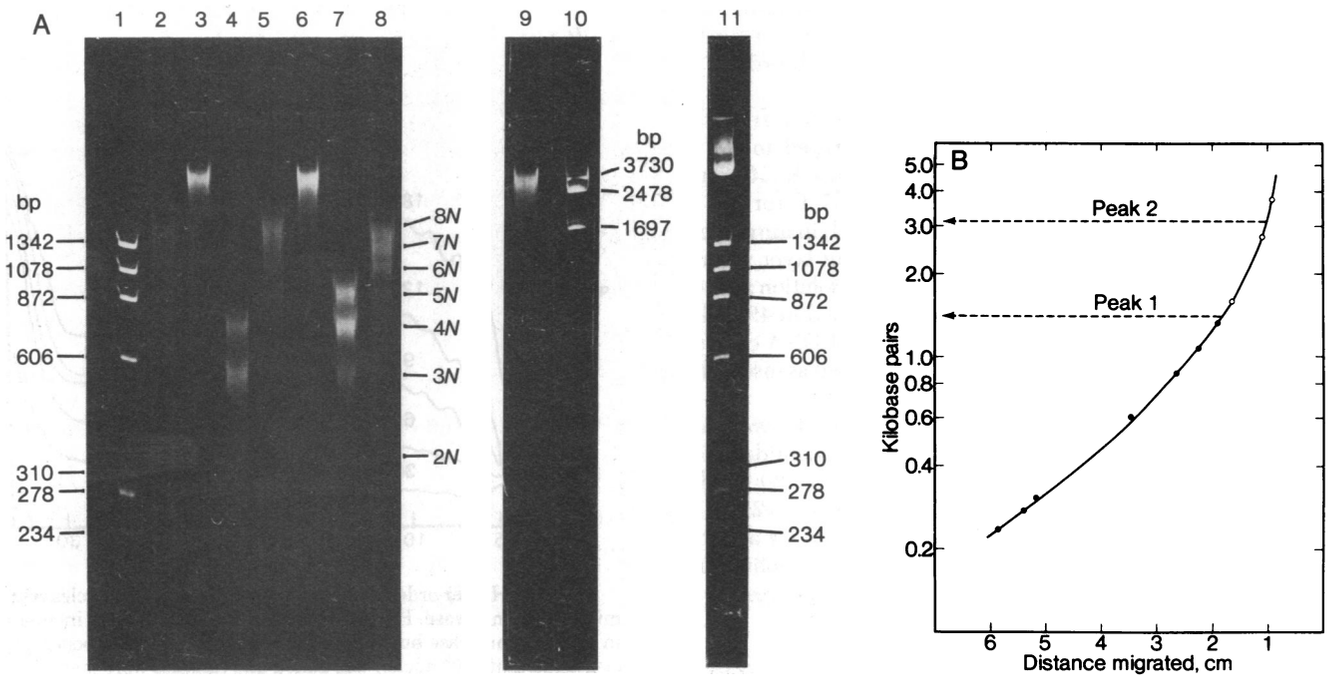

FIG. 2. Gel electrophoresis patterns of the DNA of polynucleosomes released during various periods of micrococcal nuclease digestion. (*A*) DNA was extracted from ≈0.10 $A_{260\ nm}$ unit of nucleosomal preparations from Fig. 1 and electrophoresed on 4% polyacrylamide slab gels. Slot 1, *Hae* III restriction cuts of φX174 (19); slot 2, peak 1, 30-sec digestion; slot 3, peak 2, 30-sec digestion; slot 4, fraction 10, 90-sec digestion; slot 5, peak 1, 90-sec digestion; slot 6, peak 2, 90-sec digestion; slot 7, fraction 10, 180-sec digestion; slot 8, peak 1, 180-sec digestion; slot 9, peak 2, 180-sec digestion; slot 10, *Hpa* I and *Hpa* II restriction cuts of φX174 (20); slot 11, *Hae* III restriction cuts of φX174 (19). (*B*) Calculated mobilities and base pairs of φX174 restriction fragments and DNA from peaks 1 and 2. ●, *Hae* III digestion; ○, *Hpa* I and *Hae* III digestion.

initially derived from the 16*N* particles and perhaps even a larger multiple of 8*N* periodicity. For example, equal amounts of the 8 and 16*N* polynucleosomes were present during early digestion periods (Fig. 1). By 2.5 min, and even more evident by 3 min of digestion, the 16*N* polynucleosomes were cleaved by micrococcal nuclease with a concomitant increase in 8*N* particles. However, precursor–product relationships cannot be conclusively judged from these experiments. There was also significant cleavage of the 8*N* particles to 1–6*N* nucleosomes at 3 min.

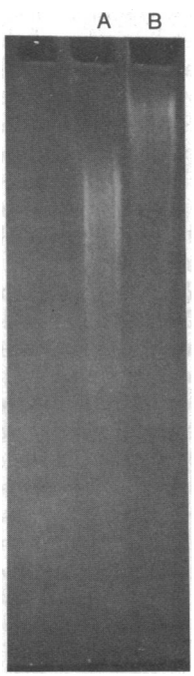

FIG. 3. Chromatin gel electrophoresis of nucleosomes and polynucleosomes. The gel was 2.5% polyacrylamide/0.5% Agarose; 0.05 $A_{260\ nm}$ unit of material from peak 1 (slot A) and peak 2 (slot B) was applied to the gel.

A third, small but reproducible, population of oligonucleosomes, which we estimate to consist of particles of a repeat size 8*N* higher than the material in peak 2 (i.e., ≈24*N*) can be seen as a faster sedimenting peak in absorbance tracings in the 30-sec and 1.5-min gradients. However, the 8*N* polynucleosome remained the predominant species (*i*) even after extensive digestion times, (*ii*) in the presence of 5 mM NaCl (data not shown), and (*iii*) under different digestion conditions (12).

$NaDodSO_4$ gel electrophoresis was performed on the total chromatin-associated proteins of the 8*N* and 16*N* nucleosomes released early in digestion by micrococcal nuclease and compared with unfractionated HeLa cell chromatin proteins (Fig. 4). Both structures contained histones and nonhistone proteins. Some differences in the presence of nonhistone proteins could be observed. The ratio of histone H1 to core histones in both structures was approximately stoichiometric.

Poly ADP-ribosylation and higher nucleosomal forms

Previously we showed that the specific activity of poly(ADP-ribose) polymerase progressively increased with simple nucleosome repeat size (12). Activity reached a maximum value at 8–10*N* (probably 8*N*, in view of the present findings) and then declined and stabilized at a constant specific activity. Because of the high degree of resolution of these two forms of oligonucleosomes, it was of importance to assess the extent of ADP-ribosylation in these structures. Such data might conceivably be related to the approximate frequency and periodicity of association of this enzyme along higher folded regions of chromatin.

Polynucleosomes of 8 and 16*N* from early and late nuclease digestion were assayed for poly(ADP-ribose) polymerase ac-

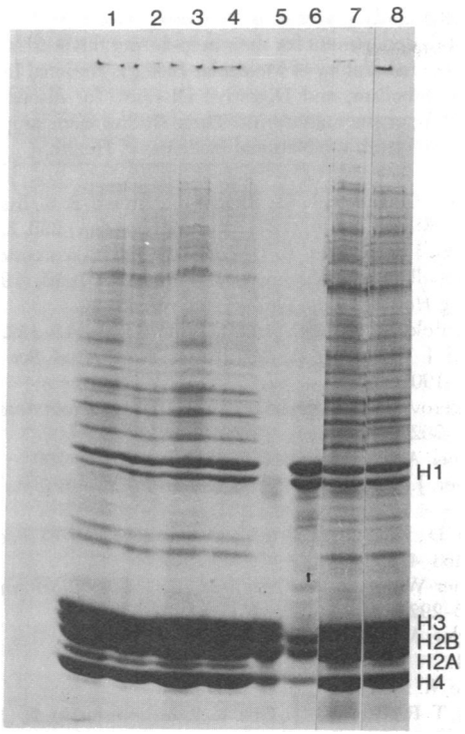

FIG. 4. $NaDodSO_4$ gel electrophoresis of proteins isolated from whole chromatin and oligonucleosomes preferentially cleaved by micrococcal nuclease. Proteins were prepared and electrophoresed. Proteins isolated from: slot 1, peak 1 of 30-sec digest; slot 2, peak 2, 30-sec digest; slot 3, peak 1, 180-sec digest; slot 4, peak 2, 180-sec digest. Slot 5, isolated core histone markers; slot 6, acid-extracted histone; slot 7, total unfractionated chromatin from a 30-sec digest; slot 8, total unfractional chromatin from a 180-sec digest.

tivity (Table 1). The reactions were linear with time and chromatin concentration in the assay. The data confirm the high specific activity of poly ADP-ribosylation in nucleosomes of 8*N* repeat size (12). It was surprising that the specific activity of the enzymatic reaction in 16*N* nucleosomes was lower than that in 8*N*, although the earlier data had indicated a decline in specific activity after 8–10*N* oligonucleosomes (12). This could indicate that as the 16*N* nucleosomes are further digested, proteins are released that tend to restrict activity, at least *in vitro*. It was of interest that the activity of polynucleosomes was higher in particles obtained from late micrococcal nuclease digests than in the same particle generated early. Although it is difficult to quantitate this type of data, taking into account that there are approximately 183 base pairs of DNA per nucleosome and efficiency of the counting is 30%, we estimate that 59 and 115 molecules of ADP-ribose residues are present per fractionated octanucleosome in the 30-sec and 180-sec digest, respectively.

Table 1. Specific activity of poly(ADP-ribose) polymerase in oligonucleosomes of 8 and 16*N* periodicity differentially released from HeLa cell nuclei by micrococcal nuclease

Nuclease digestion time, sec	ADP-ribosylation,* cpm × 10^{-4}	
	8*N*	16*N*
30	54.67	25.98
180	105.44	35.87

Peak fractions (0.08–0.15 A_{260} unit) from the respective samples from Fig. 1 were incubated *in vitro* with [^{3}H]NAD as described (12).

* cpm ADP-ribose incorporated per A_{260} unit.

ADP-ribose acceptors in higher order structures

Acceptors for poly(ADP-ribose) in nuclei (*in situ*) were compared with those obtained from an incubation *in vitro* of purified octanucleosomes with [^{32}P]NAD. Chromatin proteins were prepared, electrophoresed on 5–22% linear polyacrylamide slab gels, stained for protein (Fig. 5*A*), and exposed to x-ray film (Fig. 5*B*). There were several significant differences in the selectivity of the enzyme, at least *in vitro*, for nuclear protein acceptors in octanucleosomes in contrast to those in whole nuclei. We have recently provided more extensive data on these nuclear protein acceptors modified by this enzyme in isolated lower nucleosomes and compared these data with the modification when performed in nuclei (13, 14). A nonhistone protein, which was the major acceptor for poly(ADP-ribose), was present only in nucleosomes greater than 2*N*. This protein is shown as the heavily labeled protein of $M_r \approx 125{,}000$ (Fig. 5*B*, slots 1 and 2). A protein of M_r 75,000, various nonhistone proteins, and high mobility group proteins, as well as histone H1 and core histones (slot 2), were also the predominantly modified species in nuclei. More detailed characterization of these modifications has recently been provided (14).

Similarly, core histones, histone H1, and a number of HMG and nonhistone proteins served as acceptors for ADP-ribosylation in octanucleosomes generated by preferential micrococcal nuclease cleavage (Fig. 5*B*, slot 1). The nuclear protein of M_r 75,000 did not appear to be an acceptor or to exist as a structural element in these particles. In contrast to whole nuclei, greater than 90% of the *in vitro* incorporation occurred in the protein of M_r 125,000 in octanucleosomes. The high rate of *in vitro* poly ADP-ribosylation in the latter structures could thus be partially explained by the association of such an acceptor protein with these particles. The *in vitro* acceptor analysis in nucleosomes lower than 8*N* showed essentially the same pattern with progressively less protein C modification. Yoshihara *et al.*

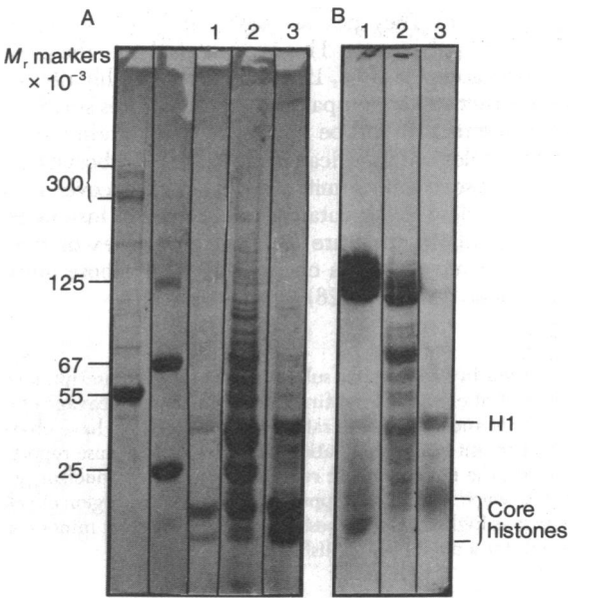

FIG. 5. Identification of *in vitro* acceptors for poly(ADP-ribose) polymerase in octanucleosomes. Purified octanucleosomes (1.0 $A_{260\ nm}$ unit/ml) and HeLa nuclei (8×10^7/ml) were incubated with [^{32}P]NAD (1 μCi/ml, 113 Ci/mol, 1 Ci = 3.7×10^{10} becquerels) under conditions optimal for poly ADP-ribosylation (13). Proteins were isolated and electrophoresed on a 5–22% linear polyacrylamide slab gel. After electrophoresis, gels were stained with Coomassie blue (*A*), dried, and exposed for radioautography (*B*). Slot 1, octanucleosomes; slot 2, nuclei-associated proteins; slot 3, proteins from slot 2 extracted with 0.2 M H_2SO_4.

(23) have reported that poly(ADP-ribose) polymerase isolated from calf thymus nuclei has a M_r of 130,000. They showed that in an *in vitro* reaction, the enzyme was capable of self-modification. Since most of the *in vitro* activity appears to represent ADP-ribosylation of this protein, one might speculate that the enzyme itself might be bound to chromatin at a periodicity of 8 nucleosomes or perhaps in the midregion of a 16 nucleosome structure.

DISCUSSION

Numerous reports to support a helical arrangement of nucleosomes in the higher ordered structure of chromatin have been made that use mainly physical techniques—namely, x-ray diffraction, neutron scattering, and electron microscopy (4, 24–27). The number of nucleosomes per turn has not been precisely determined for this structure. Finch and Klug (4) have described a solenoidal model for superstructure in chromatin. The nucleofilaments have been reported to be folded into supercoils containing six to nine nucleosomes per turn with a pitch of about 110 Å. Recently, Worcel and Benyajati (6) have proposed, by model building, that changes in chromatin repeat length should lead to variations in the number of nucleosomes per helical turn in higher order chromatin structure.

We feel that the chromatin structures isolated in the present work might be related to the number of nucleosomes per helical turn in HeLa cell chromatin. The characteristics of these structures are given briefly. (*i*) They are preferentially cleaved by micrococcal nuclease in both 5 mM and 80 mM NaCl. (*ii*) They occur with a repeat size of 8*N* and 16*N*. Sedimentation analysis of the chromatin cleaved by micrococcal nuclease under different conditions has not resolved the question of whether the 8 nucleosome periodicity extends to even higher structures. However, when DNA is extracted from whole chromatin prior to sedimentation analysis and electrophoresed on polyacrylamide gels, it is possible to resolve 8*N*, 16*N*, 24*N*, and up to 32*N* structures from each other. (*iii*) The 16*N* particle might be a preferred cleavage product and the precursor of the 8*N* particle. (*iv*) They possess high specific activity for poly(ADP-ribose) polymerase. This property is due to the presence of a protein acceptor of M_r 125,000, which is highly ribosylated in these structures as compared to the structures smaller than octanucleosomes. It will be important to ascertain more precisely the biological significance of high ADP-ribosylation activity and these structural units of chromatin. Moreover, it seems relevant, in view of the putative importance of histone H1 in higher chromatin structure (6), that a complex of two H1 molecules connected by a chain of 15 ADP-ribose units has recently been described (28).

Note Added in Proof. After submission of this manuscript, a report appeared that essentially confirms the preferential cleavage of chromatin at 8*N* and 16*N* sites in rat liver nuclei (29). We have observed, under different nuclei incubation conditions from those reported in this paper, the nonenzymatic release of nuclear ribonuclear protein particles, which sediment in approximately the same region of velocity gradients as higher polynucleosomes. These represent minor components of HeLa nuclei (unpublished data).

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