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A nuclear protein-modifying enzyme is responsive to ordered chromatin structure

Tauseef R.Butt, Julia F.Brothers, Chandrakant P.Giri* and Mark E.Smulson

Department of Biochemistry, School of Medicine and Dentistry, Georgetown University, Washington, DC 20007, USA

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ABSTRACT

Poly (ADP-ribose) polymerase, a nuclear protein-modifying enzyme, binds to the internucleosomal linker region of chromatin, although it modifies certain core nucleosomal histones in addition to histone H1. The activity per unit of DNA chromatin changes with the nucleosome repeat number. It reaches a maximum on chromatin of 8-10 nucleosomes in length. As the complexity of chromatin with respect to nucleosome repeat number and compactness increases, a decline and stabilization of specific activity is noted. The difference in specific activity is maintained through resedimentation and dialysis of particles. It does not appear due to differences in polymer chain length or differential degradation of poly (ADP-ribose). The data suggest a relationship between ADP-ribosylation and chromatin organization and vice versa.

INTRODUCTION

Information on the composition and interrelationships between proteins that exist in core nucleosomes and in the linker regions in chromatin is of considerable importance for a better understanding of the structure and function of chromatin. Poly (ADP-ribose) polymerase, a nuclear protein-modifying enzyme, is firmly bound to chromatin. Accordingly, we have recently been able to begin to catalogue basic properties of this complex enzymatic nuclear protein modification with the nucleosomal structure of chromatin. Our initial data suggested that in micrococcal nuclease generated chromatin the enzyme was associated with internucleosomal linker DNA (1). This was more firmly established recently by demonstrating the enzyme activity on purified nucleosome core + linker particles, but absence of activity on core particles lacking linker DNA (2). However, in a study utilizing both native chromatin and simple nucleosome oligomers, it was shown that besides modifying linker-associated proteins, the enzyme is capable of modifying to a considerable extent core nucleosomal histones (3). Recent evidence by Kidwell and co-workers (4) suggests that one site of chromatin modification by the enzyme is histone H1, and a resulting dimer of H1 molecules is generated by such ADP-ribosylation. Furthermore, in sheared, soluble chromatin, it has been shown

that H1-poly (ADP-ribose) complex can be correlated with chromatin condensation as determined by light scattering techniques after addition of polyamine (5). This was of particular interest since we had observed that the specific activity for poly (ADP-ribose) tended to increase with simple nucleosome repeat number (1,2). These combined observations prompted the current investigation in which we sought to ascertain whether there is a correlation between the activity of poly (ADP-ribose) polymerase and progressively higher orders of chromatin structure.

MATERIALS AND METHODS

(Adenine-2-8-^{3}H)NAD, (10 Ci/mmole) was purchased from New England Nuclear. Micrococcal nuclease (14773 units/mg) was purchased from Worthington Biochemicals, and catalase was obtained from Schwarz/Mann. Phenylmethylsulfonyl fluoride was purchased from Sigma Chemical Co. ØX174 DNA was purchased from Bethesda Research Laboratories, and restriction enzyme Hae III was a gift from Dr. Jack Chirikjian's laboratory of this department.

HeLa S3 cells were grown at 37^{o}C in spinner flasks. Eagle's S-MEM (Grand Island) growth medium contained 10% fetal calf serum. Logarithmically growing cells (5-6 x 10^{5}/ml) were harvested by centrifugation and washed with spinner salt. Nuclei were isolated by Dounce homogenization according to the method of Sporn *et al*. (12).

Isolation of Chromatin

Chromatin fragments were prepared by modification of the method of Renz *et al*. (6). Nuclei were washed with buffer containing 0.2 M sucrose, 1 mM $CaCl_2$, 5 mM Tris-HCl (pH 7.5), and 80 mM NaCl and centrifuged at 3500 x g. The pellet was immediately suspended in the above buffer at 2 x 10^{8} nuclei/ml. Micrococcal nuclease was added (4 units/1 x 10^{6} nuclei), and the nuclease incubated at 0^{o}C for 30 minutes. The reaction was terminated by the addition of a solution of EDTA to a final concentration of 1 mM. The suspension was centrifuged at 2500 x g; the nuclear pellet was suspended at 0^{o}C in 1 mM EDTA solution and allowed to lyse for 30 minutes. Renz *et al* (6) lysed lymphocyte nuclei in buffer containing 80 mM NaCl, however, we were unable to do so with HeLa cell nuclei under these conditions. The suspension was centrifuged at 5000 x g, and the supernatant contained approximately 50% of the total chromatin in the form of fragments. Alternatively (Fig. 3), the nuclei were washed and resuspended in digestion buffer as above, the nuclei were incubated at 37^{o}C for 5 min and then digested with micrococcal nuclease (30 units/1 x 10^{8} nuclei) for 1 min and 45 seconds at 37^{o}. The reaction was terminated and

nuclei were lysed for 20 minutes as described above.

Isolated chromatin was separated on 10-30% (w/v) linear sucrose gradients containing 1 mM sodium phosphate (pH 6.8), 0.2 mM EDTA, and 80 mM NaCl. 0.4 ml of 80% glycerol (v/v) was present at the bottom of the gradient as a cushion. 10-20 A_{260} units (0.5 ml) of isolated chromatin were layered onto the gradients. The gradients were centrifuged in a Beckman SW40 rotor at 40,000 rpm for 4.5 hrs at 4^{o}.

For resedimentation studies (Fig. 3), nucleosomal dimer was prepared by digesting 2 x 10^{8} nuclei with micrococcal nuclease (200 units/1 x 10^{8} nuclei) for 2 min at 37^{o} as described above. The nuclei were lysed and centrifuged at 7.7 x g for 10', the nucleosomal preparation was recovered from the supernatant. The nucleosomal preparation was centrifuged in a 5-20% (w/v) linear sucrose gradient containing 1 mM sodium phosphate (pH 6.8), 0.2 mM EDTA and 80 mM NaCl. Centrifugation was performed in a Beckman SW40 rotor at 38,000 rpm for 11 hrs at 4^{o}. The dimer region of the gradient was pooled and used for further studies.

ADP-Ribosylation of Chromatin

Poly (ADP-ribose) polymerase assays were performed according to Mullins et al (1). Unless otherwise mentioned, the assays were performed at 20^{o} in a 0.2 ml volume containing 100 mM Tris-HCl pH 8.0, 1 mM dithiothreitol, 2 mM $MgCl_2$, 40 mM NaCl, 0.1 mM (adenine-2-8-^{3})NAD (0.5 µCi/assay), and isolated chromatin (1-10 µg DNA). The reaction was terminated by placing the tubes in ice and adding TCA (20% final concentration). The acid-insoluble material was filtered on glass fiber filters, and radioactivity was determined by a liquid scintillation spectrometer with 30% efficiency for ^{3}H counting.

Extraction of Nuclear Proteins and DNA

Total nuclear proteins from chromatin fractions were extracted by 20% trichloroacetic acid and precipitated by adding 10 vol of acetone. The precipitates were vacuum dried and dissolved in sample buffers (1% SDS 8 M urea, 1% mercaptoethanol and 10% sucrose). Proteins were separated by performing polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (13).

Chromatin fragments of various nucleosomal repeat numbers were analyzed by electrophoresis at 4^{o} using 3.5% acrylamide, 0.5% Agarose (acrylamide: N,N'-methylenebisacrylamide, 20:1) in a slab gel apparatus according to Todd and Garrard (14). 6 mM Tris-HCl pH 8.0, 3 mM sodium acetate, and 0.3 mM EDTA was used as the running buffer. Samples were taken directly from each sucrose gradient fraction and applied into the gel slots. The samples were electrophoresed

at 200 volts until the bromophenol blue was 2 cm from the bottom of the gel. Only 0.01 A_{260} units of chromatin were required to be able to see the bands in the ethidium bromide stain.

DNA fragments were isolated as described previously (1) and separated on Agarose gels as described by Sharp et al. (15). Fragments of ØX174 DNA cleaved with Hae III endonuclease were used as molecular weight markers (15).

RESULTS

Chromatin fragments were isolated from HeLa cell nuclei by action of micrococcal nuclease (see Materials and Methods). To generate forms of chromatin with differing nucleosome repeat number, the isolated chromatin was separated on 10-30% (w/v) linear sucrose gradients in the presence of 80 mM NaCl (Figure 1A) as described for lymphocyte nuclei by Renz et al. (6). DNA size analysis of regions in the gradient (insert) show that subfractions of chromatin have been separated by this technique ranging from chromatin of approximately 200-2000 base pairs of DNA. The optical density peak (fraction 18) contained approximately 1700 base pairs of DNA or around 8-9 nucleosomes.

To show that the procedure employed above yields chromatin with nucleosomes still intact, fractions were analyzed by polyacrylamide-agarose gel electrophoresis as described in Methods. As shown by the data in Figure 1B, reasonably pure preparations of nucleosome repeats of increasing complexity could be prepared by this method, although there was some overlap in oligomer resolution especially in the faster sedimenting particles.

The activity of poly (ADP-ribose) polymerase versus the concentration of chromatin assayed was determined from selected regions of the gradient (Fig. 1C). At least 3 concentrations of chromatin were assayed for each fraction. The incorporation of NAD into poly (ADP-ribose) was linear with increasing chromatin concentration for oligonucleosomes of all size ranges tested. When the incorporation per equal unit of chromatin was plotted against gradient fraction (Fig. 1A), a progressive increase in specific activity with increasing nucleosome repeat number was observed. This observation has been noted with nucleosomes prepared under different conditions of nuclease digestion (see Fig. 3); with only minor differences, a strict ordered increase in specific activity has been found. The data shows that maximal specific activity is observed in nucleosomes sedimenting in fractions 17-19, which represent nucleosome repeats of approximately 8-10. These particles also represent the highest proportion of nucleosome fragments generated in these experiments and therefore may represent preferred cleavage sites of micrococcal nuclease. Specific activity is lower on chromatin of higher nucleosome content, and

stabilizes at a constant value in chromatin in the faster sedimenting regions of the gradient.

These relationships may be due to a number of possible situations. Since it is difficult to experimentally study the interaction of either enzymes or non-histone proteins (the polymerase) with chromatin, the observation has

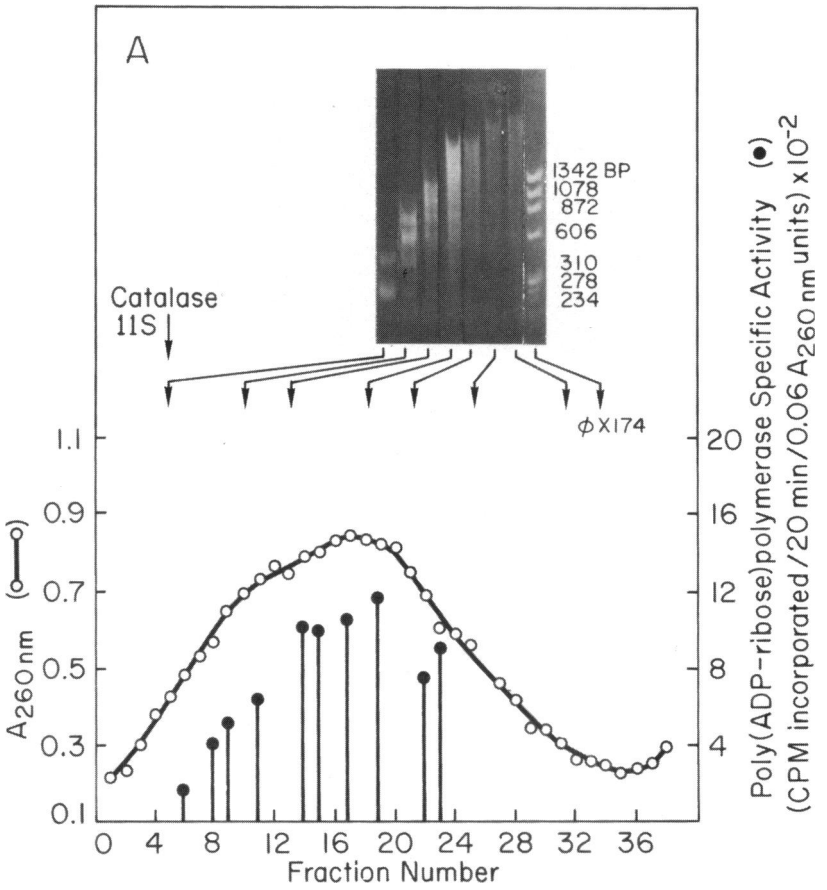

Figure 1A. Sucrose Gradient Analysis of Isolated Chromatin Fragments and Analysis of Poly (ADP-ribose) Polymerase Activity.
Chromatin was prepared from logarithmically growing cells as described in Materials and Methods. 0.5 ml of isolated chromatin (9 A_{260nm} units) was applied onto a 10-30% linear sucrose gradient, and centrifugation was performed at 40,000 rpm for 4.5 hours at 4° in a Beckman SW40 rotor. The top insert shows the size of the DNA as analyzed by Agarose gel electrophoresis. The arrows indicate the fraction used for DNA extraction. Hae III restriction fragments of ØX174 were used as known molecular weight markers. Various fractions from each gradient were assayed for poly (ADP-ribose) polymerase activity as described in Materials and Methods. The results are presented (vertical lines) as specific activity of the enzyme assayed.

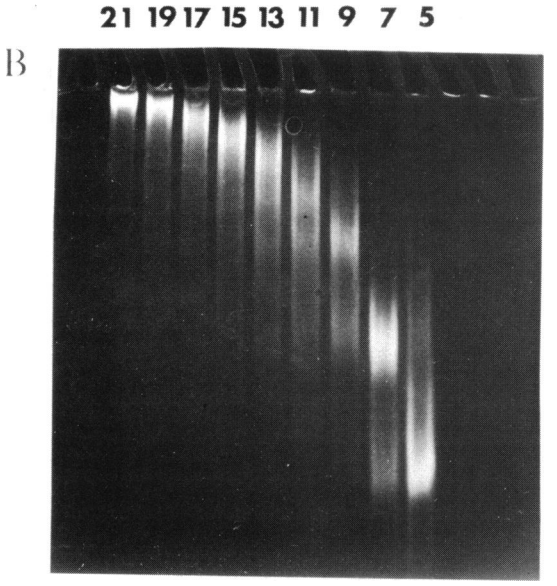

Figure 1B. Polyacrylamide-Agarose Gel Electrophoresis of Native Chromatin Particles.
An aliquot (0.03 A_{260nm} units) of different fractions of a comparable gradient to Fig. 1A were electrophoresed as described in Methods. The inserts, 5,7, 9,11,13,15,17,19 and 21 are the fraction numbers of the gradient (see Fig. 1A).

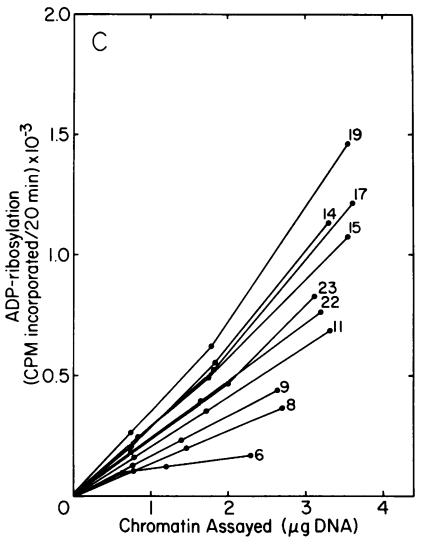

Figure 1C. The Effect of Increasing Concentrations of Chromatin of Varying Nucleosome Chain Length on Poly (ADP-ribose) Polymerase Activity.
The numbers refer to the sample numbers of the appropriate fractions from Figure 1A.

added significance. The conformation of a certain nucleosome repeat number might allow maximal interaction of enzyme and acceptor in vitro. Additionally, either enzyme and/or acceptors might be released during nuclease digestion and rebind to a preferred nucleosome repeat number. The enzyme and/or acceptors might be localized in a region of chromatin most susceptible to nuclease under these digestion conditions. Some, but not all of these possibilities are directly testable.

A number of control experiments were performed to verify the validity of the apparent relationship between nucleosome chain length and enzyme specific activity. To show that the marked changes in enzyme specific activity were not due to a shorter half-life of the poly (ADP-ribose) or the differential activity of degrading enzymes such as poly (ADP-ribose) glycohydrolase or phosphodiesterase, the following experiment was performed. Chromatin isolated from the upper, middle, and lower regions of the gradient was incubated with (adenine-8-^{3}H)NAD for 20 minutes, after which the incorporation was terminated by the addition of thymidine (inhibitor of the enzyme) at a final concentration of 5 mM. The loss of labeled polymer (acid-insoluble radioactivity) was subsequently monitored for the next 20 minutes under the assay conditions utilized to measure polymer synthesis. As shown in Figure 2, the breakdown of poly (ADP-ribose) was not significant in nucleosome repeats possessing high, medium, and low ADP-ribosylation activity. Thus, the endogenous ADP-ribosylation activity decline that occurs after 8-10 nucleosomes would not seem to be due to increasing degradation of the polymer.

To eliminate the possibility that the results on enzyme activity and different orders of nucleosome complexity described above might be caused by potential inhibitors, activators, or non-nucleosomal aggregated particles, the following experiment was performed. Two regions of the gradient possessing considerable differences in size and activity were pooled, dialyzed, concentrated and separately resedimented in 10-20% sucrose gradients (Fig. 3A and 3B). The data indicates that the chromatin samples maintain their nucleosome size complexity upon a second sedimentation. As shown by the polyacrylamide-agarose gels in Figure 4, the lower nucleosome repeat preparation is predominantly composed of nucleosome dimers. The oligomer preparation (composed of nucleosome repeats 6-8) showed some minimal degradation to smaller particles upon the two steps of concentration and recentrifugation. The three-fold difference in activity of polymerase was also maintained through dialysis and resedimentation of the two populations of particles although there was some loss of activity noted. This data reduces the likelyhood of the presence of

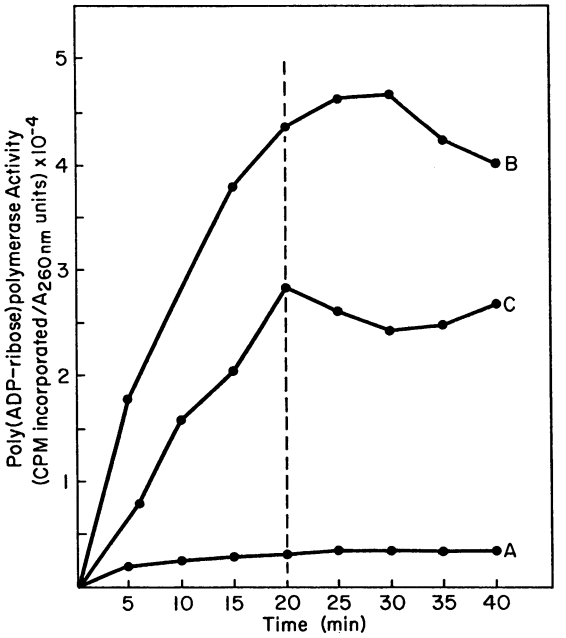

Figure 2. Turnover of Poly (ADP-ribose), Synthesized by Chromatin Fragments of Varying Chain Length.
Although a different sucrose gradient was used to isolate chromatin, the fraction numbers approximately correspond to those shown in Figure 1A. Chromatin from (A) fractions 5-8, (B) fractions 10-15, and (C) fractions 20-25 was assayed for poly (ADP-ribose) polymerase activity by incubating with ^{3}H-NAD under normal assay conditions. The reaction was terminated after 20 minutes by addition of 5 mM final concentration of thymidine.

putative small molecular weight inhibitors or activators influencing results described above. This was also indicated by mixing experiments on these purified fractions; neither heat inactivated preparations nor small concentrations of each fraction influenced the respective activities when assayed alone (data not shown). It is possible that since poly (ADP-ribose) polymerase requires DNA for activity, the DNA composition of oligonucleosomes is an optimal size for maximal enzyme activity. Unfortunately, it was impractical to directly test this hypothesis without purified enzyme. Addition of exogenous DNA to nucleosomal assays generally either has no effect or on inhibitory effect on chromatin.

Average chain length determinations of the poly (ADP-ribose) synthesized by nucleosomal preparations of dimer and oligomer were performed as described by Sugimura and coworkers (7). There is a linear relationship between the chain length of poly (ADP-ribose) eluted from hydroxylapatite with concentration

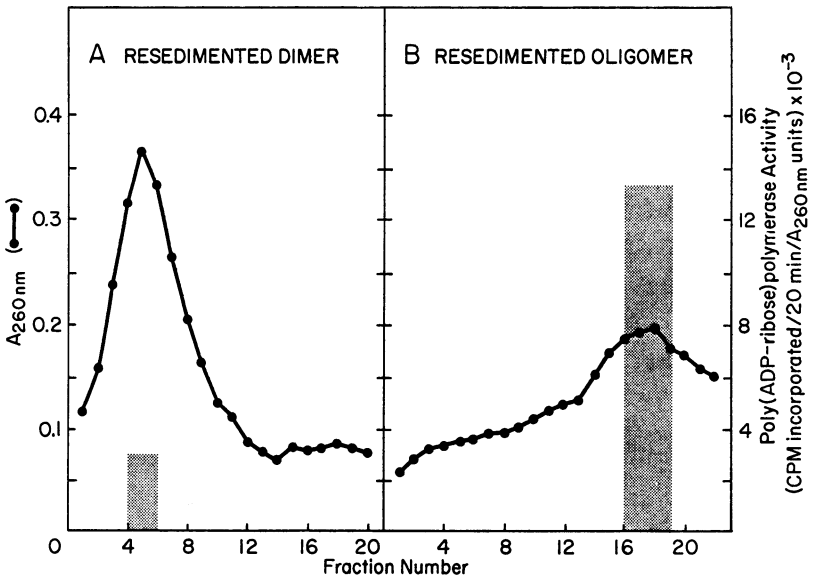

Figure 3. Specific Activity of Poly (ADP-ribose) Polymerase of Nucleosomal Dimer and Oligomer Fractions after Resedimentation.
A nucleosomal dimer was prepared as described in Methods. Nucleosome oligomer of corresponding fractions 15-18 of a gradient such as shown in Figure 1A were pooled. The two preparations were dialyzed in the sucrose gradient buffer and concentrated; 0.255 and 0.587 A_{260nm} units of dimer and oligomer, respectively, were recentrifuged in separate 10-20% linear sucrose gradients for 3 hrs at 50,000 rpm in Beckman SW50.1 rotor. Designated fractions were dialyzed, concentrated and assayed for enzyme activity as before. The direction of sedimentation is from left to right and the solid bars represent the enzyme activity assayed.

of phosphate buffer (7). The overall elution pattern of the radioactivity was the same in both preparations (Figure 5). The peak oligo-ADP-ribose eluted at 0.22 M phosphate in both cases representing an average chain of approximately 13 units. It seems probable therefore that the increased enzyme activity observed in Figure 1A and 1C might be due to the number of ADP-ribosylated sites rather than the chain lengths synthesized.

It has been reported that under certain conditions nucleosomal proteins rearrange during micrococcal nuclease digestion. Histone H1 has been reported to be preferentially associated with chromatin fragments consisting of greater than 6 nucleosomes (6). However, as indicated by the staining patterns of SDS electrophoresis of total nuclear proteins (Figure 6), the ratio of histone H1 to core histones is the same in nucleosome repeat from 2-10 under the digestion and isolation conditions employed. Histone H1 was absent from mononucleosomes

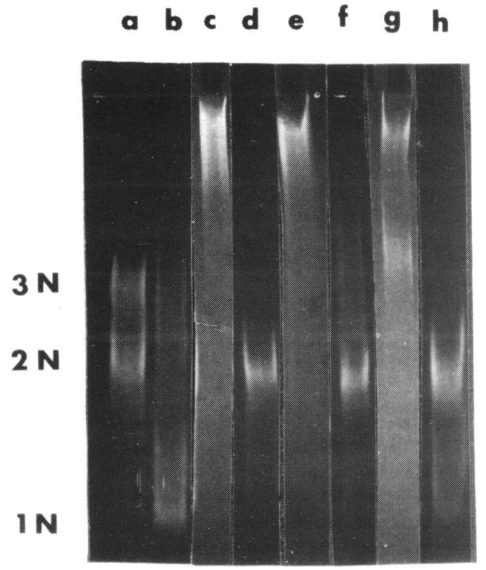

Figure 4. Agarose-Polyacrylamide Gel Electrophoresis of Chromatin Particles from Experiment Described in Figure 3.
Slot (a), markers of nucleosomal dimers and trimer; slot (b), marker nucleosomal monomer; slots (c) and (d) represent pooled oligomer and dimer before resedimentation, respectively. Slots (e) and (f) are the same fractions after dialysis and concentration. Slots (g) and (h) are pooled oligomer and dimer fractions after recentrifugation, dialysis and concentration as described in Figure 3.

(fraction 6). Minor differences in certain non-histones can be observed. We have recently described (3) analysis of poly (ADP-ribose) labelled acceptors in purified nucleosomal dimer, trimer and oligomers (4-6 N). When assayed under in vitro conditions the oligomer particles showed increased modification of histone H1 and certain HMG non-histone proteins. Such an observation could partially explain the results described in present studies.

DISCUSSION

The novel pattern of a seemingly cooperative relationship between nucleosome number and ADP-ribosylating activity which has emerged is summarized in the data of Figure 7, which has been normalized in order to include data from a number of independent experiments. Specific activity rises in a progressive fashion. Maximal specific activity is reached at approximately 9 nucleosomes. The slight decline and increase in activity between 9 and 11 nucleosomes may be due to experimental error; however, with slight variation, they were noted in a number of independent experiments. Poly (ADP-ribose) polymerase activity

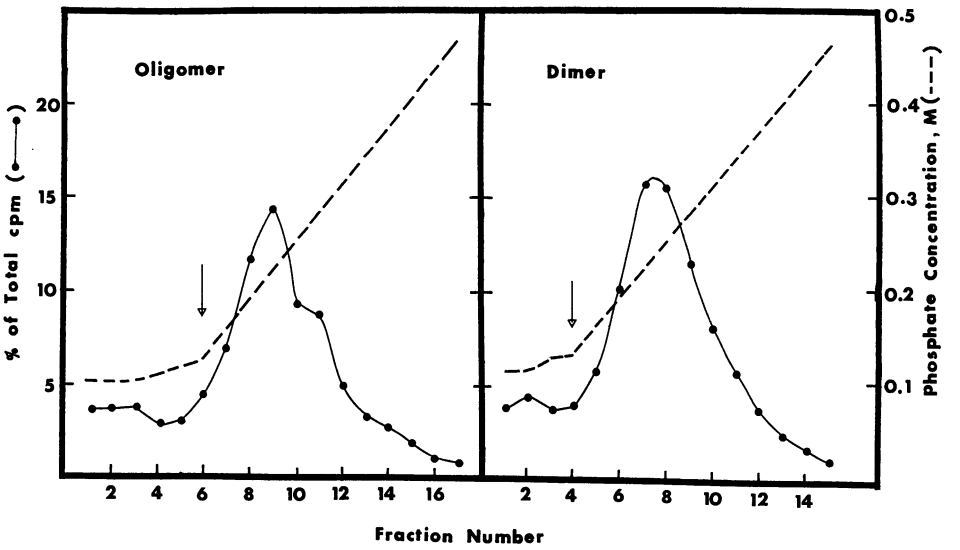

Figure 5. Average Chain Lengths of Poly (ADP-ribose) Synthesized by Nucleosomes of Differing Repeat Numbers.
The labeled Poly (ADP-ribose) was synthesized from nucleosome dimer and oligomer fractions of chromatin as described in Figure 1. Poly (ADP-ribose) was isolated and chromatographed on hydroxylapatite columns as described by Sugimura et al. (7). 8,000 cpm and 5,000 cpm were applied on columns for dimer and oligomer isolated polymer, respectively. The columns were washed with 0.1 M phosphate buffer until all small molecular weight radioactive material which did not bind to the column was eluted. (This also includes poly (ADP-ribose) shorter than 4.5 (7).) The elution of poly (ADP-ribose) was started as indicated by an arrow with a linear gradient of 0.1 M to 0.5 M phosphate buffer. Over 50% of the total radioactive material bound to the column. The results were plotted as percentage of the total cpm recovered.

declines and stabilizes at a constant value at approximately 13 nucleosomes. The resolution of our preparations on sucrose gradients is limited for higher chromatin preparations, however, we are currently developing techniques to further explore this relationship in higher order chromatin structures. The apparent decrease and stabilization of poly (ADP-ribose) polymerase activity observed in approximately 13 or more nucleosome oligomers could be explained as follows. A chromatin structure with an increasing number of nucleosomes would be expected to attain a compact structure restricting the accessibility of the substrate to the enzyme and its acceptor (at least under the in vitro assay conditions employed). It is conceivable that in intact chromatin fragments of varying chain length, the activity of the enzyme might be regulated by its microenvironment (i.e., DNA, chromosomal proteins, and apparent

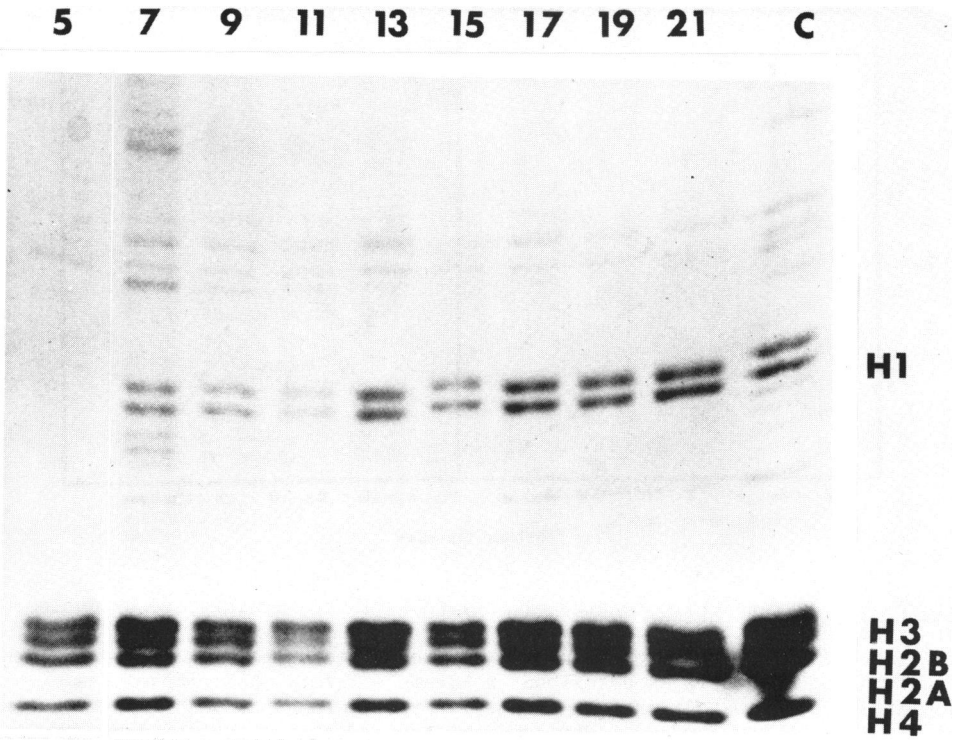

Figure 6. SDS-Polyacrylamide Gel Electrophoresis of Total Nuclear Protein of Chromatin Fragments Isolated from Sucrose Gradients.
Proteins were extracted from appropriate fractions of a sucrose gradients and electrophoresed as described in Materials and Methods. The inserts 5,7,9, 11,13,15,17,19 and 21 are the fraction numbers of a gradient similar to that shown in Figure 1A. C is the chromatin preparation before centrifugation.

size and number of linker regions). The chromatin fragments of 8-10 nucleosomes may represent a preferred structure for poly (ADP-ribose) polymerase.

Finch and Klug (8), showed that nucleosomes are organized in solenoids with approximately 6-9 nucleosomes per helical turn. It is also possible that poly (ADP-ribose) polymerase preferentially binds to the same number of nucleosomes. If this were so, it would explain the observations described in this paper. However, accurate determination of this would require experimental techniques (antibody to the enzyme) not currently feasible. It is of interest that under the nuclease digestion conditions employed in these experiments, nucleosomes of this approximate size range (8-10 nucleosome repeat number) are generated to the highest extent by micrococcal nuclease. This suggests that there is a unique structure of chromatin at this periodicity. It is also possible that poly (ADP-ribose) polymerase and/or its acceptors, preferentially

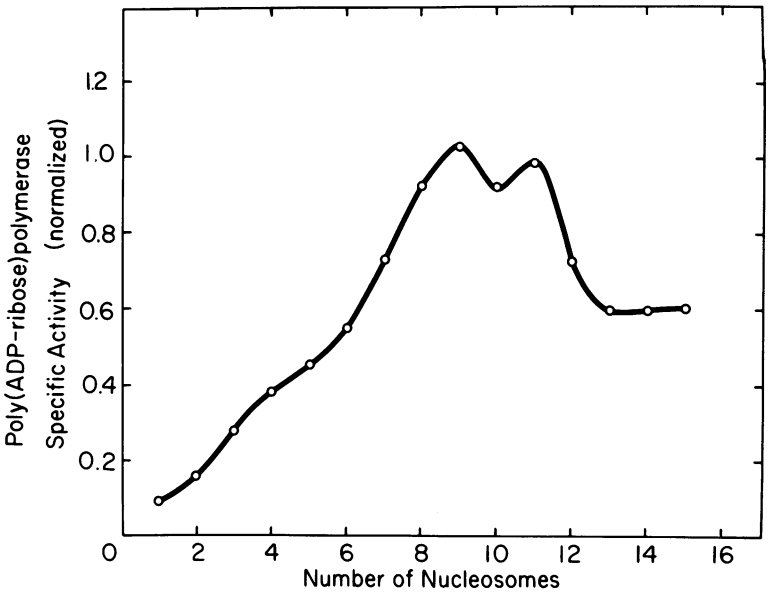

Figure 7. The Relationship Between Poly (ADP-ribose) Polymerase Specific Activity and the Number of Nucleosomes on a Chromatin Fragment.
The specific activity of poly (ADP-ribose) polymerase was normalized for three separate experiments. The average specific activity at normalized 1.00 was 40,000 cpm incorporated/OD_{260nm}/20 minutes. The approximate number of nucleosomes in fractions was estimated from the maximal DNA size in the fraction as shown by the insert in Figure 1.

binds to the same number of nucleosomes. This might occur in native chromatin or during the purification proceduresuired to isolate these repeats.

The following observations further support the notion that poly ADP-ribosylation of chromatin may be somehow involved in its condensation during interphase. (i) The amount of polymer synthesized is maximum in the S to G_2 phase transition of the cell cycle as estimated by immunoassay. The specific activity of poly (ADP-ribose) polymerase is also increased at the boundary of the S and G_2 phase of the cell cycle (17). (ii) Histone H1 is one of the major acceptors of poly (ADP-ribose) *in vivo* (9) and *in vitro* (3,10). (iii) It is becoming increasingly clear that histone H1, is involved in the higher orders of chromatin structure (11).

It is possible that the main function of poly ADP-ribosylation of chromatin might be to act as a natural crosslinking agent. Indeed a dimer of histone H1 connected by a chain of 15 ADP-ribose units has been isolated from eukaryotic cell nuclei (4). In soluble chromatin studies, Byrne *et al.* (5) have shown that there is a correlation between chromatin condensation induced by poly-

amines and poly (ADP-ribose) histone H1 dimer complex formation. These experiments, utilizing sheared chromatin are supportive of the observations obtained in the present study utilizing nucleosomes of defined repeat numbers. We have recently noted an increased modification of histone H1 in simple nucleosome oligomers (3) which might, in part, help explain the observations presented above. However, further experimentation is required to fully understand the significance of these observations. In an extension of these studies we have recently isolated poly (ADP-ribose) linked dimer of histone H1 in nucleosome preparations (unpublished observation).

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*Present address: Department of Biology, University of Rochester, Rochester, NY 14627, USA

REFERENCES

1. Mullins, D.W., Giri, C.P. and Smulson, M.E. (1977) Biochemistry 16, 506-513.
2. Giri, C.P., West, M.H.P., Ramirez, M.L. and Smulson, M.E. (1978) Biochemistry (in press).
3. Giri, C.P., West, M.H.P. and Smulson, M.E. (1978) Biochemistry (in press).
4. Stone, P.R., Lorimer, W.S., Kidwell, W.R. (1977) Eur. J. Biochem. 81, 9-18.
5. Byrne, R.H., Stone, P.R. and Kidwell, W.R. (1978) Exp. Cell. Res. (in press).
6. Renz, M., Nehls, P. and Hozier, J. (1977) Proc. Nat. Acad. Sci. USA 74, 1879-1883.
7. Sugimura, T., Yoshimura, N., Miwa, M., Nagai, H. and Nagao, M. (1971) Arch. Biochem. Biophys. 147, 660-665.
8. Finch, J.T. and Klug, A. (1976) Proc. Nat. Acad. Sci. USA 73, 1897-1901.
9. Ueda, K., Omachi, A., Kawaichi, M. and Hayaishi, O. (1975) Proc. Nat. Acad. Sci. USA 72, 205-209.
10. Wong, N.C.W., Poirier, G.G. and Dixon, G.H. (1977) Eur. J. Biochem. 77, 11-21.
11. Worcel, A. and Benyajati, C. (1977) Cell 12, 83-100.
12. Sporn, M.B., Berkowitz, D.M., Glinki, R.P., Ash, A.B. and Steven, C.L. (1969) Science 164, 1408-1410.
13. Laemmli, U.K. (1970) Nature 227, 680-685.
14. Todd, R.D. and Garrard, W.T. (1977) J. Biol. Chem. 252, 13, 4729-4738.
15. Sharp, P.A., Sugden, B. and Sambrook, J. (1973) Biochemistry 12, 3055-3063.
16. Blakesley, R.W. and Wells, R.D. (1975) Nature 257, 421-422.
17. Kidwell, W.R. and Mage, M. (1976) Biochemistry 15, 1213-1217.