Reconstitution of HeLa Cell Poly(adenosine diphosphate ribose) Polymerase with Purified Oligonucleosomal Chromatin†

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ABSTRACT: Purified poly(ADP-Rib) polymerase was found to be incapable of in vitro histone modification. The aim of the present study was to determine, via reconstitution of the pure enzyme with oligonucleosomes, the level of chromatin organization required for histone ADP-ribosylation. In preliminary studies to assess characteristics of chromatin most favorable to modification of histones, a unique relationship between poly-ADP-ribosylation and the size complexity of chromatin fragments, as generated by mild micrococcal nuclease digestion of HeLa nuclei, was revealed. Poly(ADP-Rib) polymerase specific activity increased with increasing oligonucleosome size. Factors found to influence poly(ADP-Rib) polymerase activity in native isolated chromatin fragments included (a) the stoichiometry of the enzyme associated with isolated chromatin fragments, (b) the distribution of nonhistone proteins in oligonucleosomes, and (c) the size of oligonucleosomes, i.e., the number of nucleosomes per oligomeric unit. Analysis of the distribution of protein-bound poly(ADP-Rib) from the in vitro enzyme reaction with oligonucleosomes indicated that 60–85% was covalently bound to the nuclear enzyme poly(ADP-Rib) polymerase. As the nucleosome size increased from 1 to 12 nucleosomes, the percentage of ADP-Rib bound to the enzyme decreased from 85 to 60%, while the percentage bound to histones (H3, H2a, H2b, and H1) increased from 8 to 25% in the same fragments. These data indicated that intramolecular interactions in oligonucleosomes significantly influenced modification of histones in vitro. The level of histone modification (percent of total protein-bound ADP-Rib) in isolated oligonucleosomes (>8 nucleosomes) was found to be comparable to that noted in intact nuclei, 25 and 30%, respectively, thus indicating that isolated oligonucleosomes might serve as models to study histone modification in vitro. Isolated oligonucleosomes (8 nucleosomes), stripped of endogenous poly(ADP-Rib) polymerase activity, were reconstituted with purified HeLa poly(ADP-Rib) polymerase. Characterization of these fragments revealed that the enzyme was bound to linker regions in reconstituted chromatin as previously found in native chromatin. The purified enzyme failed to ADP-ribosylate histones when exogenous histones and DNA were added to reaction mixtures. However, when the purified enzyme was reconstituted with oligonucleosomal chromatin, histone modification was restored. The fraction of protein-bound ADP-Rib found in nucleosomal histones (H3, H2b, and H2a) was 6.1 and 8.1% in reconstituted and native chromatin, respectively. The data suggest that there are strict steric requirements between histones and the enzyme in chromatin for effective modification of histones and that the reconstituted system can prove useful for future determination of these parameters.

Previous studies have shown a unique relationship between poly(ADP-Rib)[1] polymerase activity and the complexity of chromatin fragments generated by mild micrococcal nuclease digestion of HeLa nuclei (Butt et al., 1978). It was observed that enzyme activity (activity per microgram of chromatin DNA) successively increases as the nucleosome size increased from 1 to 8–10 nucleosomes. In fragments larger than 8–10 nucleosomes, enzyme activity remained constant, but at a value significantly lower than that noted in octanucleosomes. Subsequently, it was shown that micrococcal nuclease preferentially cleaved chromatin at a periodicity of 8 and 16 nucleosomes (Butt et al., 1979). Several suggestions were proposed to explain the former phenomena: (a) periodic distribution of poly(ADP-Rib) polymerase in chromatin; (b) nonrandom distribution of acceptor proteins; (c) dependence of enzyme activity on nucleosome complexity.

In order to gain greater insight into the factors responsible for the above observations, the present study was undertaken. Chromatin fragments of varying complexity were isolated and poly(ADP-Rib) polymerase activity was measured. A correlation has been found between the distribution of poly(ADP-Rib) covalently attached to specific acceptors in isolated chromatin fragments and the complexity of these fragments. Significant in vitro histone ADP-ribosylation was detected predominantly in larger polynucleosomes, and this information has been exploited in the present study to establish preliminary experimental conditions favoring histone modification by a purified preparation of poly(ADP-Rib) polymerase. The preceding report described the automodification of poly(ADP-Rib) polymerase (Jump & Smulson, 1980). This unusual property of the enzyme provided a unique opportunity for correlation of the presence of the enzyme with various chromatin fragments by both its activity and its automodification. It is our view that the modification of histones rather than enzyme automodification relates more to the biological function of this enzyme in modulating chromatin structure in vivo. However, this will be elucidated further in future experiments. A detailed study of the kind presented here has been required so that a model system can be developed to study poly-ADP-ribosylation of chromatin proteins under defined in vitro conditions.

The use of reconstitution of defined components isolated from multicomponent systems is not a novel approach but a practical one to understand the interaction of complex macromolecules in vitro and ultimately extend the information

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[1] Abbreviations used: ADP-Rib, adenosine diphosphate ribose; NAD, nicotinamide adenine dinucleotide; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; NHP, nonhistone protein; BSA, bovine serum albumin; $NaDodSO_4$, sodium dodecyl sulfate.

0006-2960/80/0419-1031$01.00/0

obtained to in vivo conditions. The preceding report described the isolation and characterization of HeLa poly(ADP-Rib) polymerase (Jump & Smulson, 1980). Upon purification, this enzyme was shown to lose catalytic capacity for histone modification. In the studies presented below, we have used this purified enzyme in reconstitution studies with oligonucleosomes. Oligonucleosomes stripped of endogenous enzyme activity can be reconstituted with purified enzyme to restore histone modification. The studies reported here represent preliminary characterization of this reconstituted enzyme–chromatin complex.

Materials and Methods

Materials. [^{32}P]NAD (256 Ci/mmol) and [*adenine*-2,8-^{3}H]NAD (28 Ci/mmol) were synthesized as described in the preceding report (Jump & Smulson, 1980). Protein markers for $NaDodSO_4$–polyacrylamide gel electrophoresis were purchased from Bio-Rad, Inc.: myosin (M_r 200 000); β-galactosidase (M_r 116 000); phosphorylase *b* (M_r 94 000); bovine serum albumin (M_r 68 000); ovalbumin (M_r 43 000). HeLa nucleosomal histones (H3, H2b, H2a, and H4) were extracted from 0.3 M KCl washed HeLa chromatin with 0.4 N H_2SO_4 as previously described (Giri et al., 1978). HeLa DNA was isolated from HeLa nuclei and chromatin particles as described before (Butt et al., 1978).

Cultivation of HeLa Cells in Suspension. HeLa S_3 cells were maintained at 37 °C in a spinner flask as previously described (Mullins et al., 1977).

Preparation of HeLa Chromatin Fragments of Varying Complexity. Triton X-100 washed nuclei were prepared according to Whitlock & Simpson (1976). All buffers were supplemented with $PhCH_2SO_3F$ (0.2 mM) as a protease inhibitor.

Nucleosomes were prepared by micrococcal nuclease digestion of HeLa nuclei (1 unit of micrococcal nuclease per A_{260nm}; 5.0×10^7 nuclei/mL) in 0.25 M sucrose, 0.1 mM $CaCl_2$, 5 mM Tris, pH 8.0, and 80 mM NaCl at 37 °C for 1 min. Digestion was terminated by the addition of EDTA to 5 mM. Nuclei were lysed hypotonically and soluble chromatin was separated from undigested chromatin by sedimentation (3000*g*; 5 min; 4 °C). Nucleosomes were separated by sucrose gradient centrifugaton in 10–30% sucrose, 10 mM Tris, pH 8.0, 1 mM EDTA, and 80 mM NaCl for 7 h: SW 40; 38000*g*; 4 °C. Gradients were fractionated as previously described (Mullins et al., 1977).

Gel Electrophoresis of Chromatin Proteins. Proteins associated with various chromatin fractions were precipitated with 3 volumes of absolute ethanol and stored at −20 °C overnight. Precipitated proteins were electrophoresed in 7.5% acrylamide [acrylamide–*N*,*N*′-methylbis(acrylamide), 100:1] slab gels (0.15 × 15 × 22 cm) and prepared for radioautography as described in the preceding report (Jump & Smulson, 1980).

Gel Electrophoresis of Chromatin DNA. DNA extracted from various chromatin fractions, as described by Butt et al. (1979), was precipitated with 3 volumes of absolute ethanol and stored at −20 °C overnight. DNA (50 μg) was separated by electrophoresis in 7.5% acrylamide [acrylamide–*N*,*N*′-methylbis(acrylamide), 100:1] in 89 mM Tris–borate, pH 8.3, and 2.5 mM EDTA. The molecular weights of chromatin DNA fragments were compared to the mobility of restriction fragments: ϕX-174 cleaved by *Hae*III or cleaved by *Hpa*I. Gels were stained in ethidium bromide (3 μg/mL) and photographed as previously described.

Preparation of Stripped Chromatin. Oligonucleosomal chromatin of 8–10 nucleosomes was prepared by limited micrococcal nuclease digestion of HeLa nuclei as previously described (Butt et al., 1979). Oligonucleosomes were dialyzed against buffer A (10 mM Tris, pH 8; 1 mM EDTA; 0.1 mM $PhCH_2SO_2F$) plus 0.3 M KCl for 6–8 h and then layered over a discontinuous sucrose gradient: 10 mL of 10% sucrose; 2 mL of 40% sucrose in buffer A. Chromatin was sedimented for 6 h at 38000*g*, SW 40, and 4 °C. Chromatin was recovered from the 10–40% sucrose interface and dialyzed against buffer B (10 mM Tris, pH 8; 1 mM EDTA; 80 mM NaCl; 0.2 mM $PhCH_2SO_2F$) and kept at −20 °C until use. The A_{260}/A_{280} was 1.65, the DNA size was 750–1295 base pairs, and the core histones were present in the proper ratio. Histone H1 was lost during the stripping process.

Reconstitution of Poly(ADP-Rib) Polymerase with Stripped Chromatin. Purified poly(ADP-Rib) polymerase [post Sephadex G-200 step; see Jump & Smulson (1980)] was used for reconstitution. Enzyme in buffer C (see preceding paper) (0.8 unit; 7.2 μg of protein; 64 pmol of enzyme) was mixed with oligonucleosomal chromatin in buffer A plus 0.2 M KCl and 1 mM DTT (32 μg of DNA; 28 μg of protein; average nucleosome size, 6 nucleosomes; 42 pmol of hexanucleosomal chromatin). The stoichiometry of enzyme to chromatin fragments was 1.52. Chromatin plus enzyme (0.6 mL) was dialyzed against buffer B plus 0.2 M KCl and 1 mM DTT for 12 h at 4 °C. The buffer was then changed to buffer A plus 80 mM KCl and 1 mM DTT for an additional 6 h of dialysis. Reconstituted chromatin fragments were isolated by velocity sedimentation in 10–30% sucrose gradients in buffer A plus 80 mM KCl and 1 mM DTT: SW 40; 35000*g*; 12 h; 4 °C. Gradients were fractionated and assayed for chromatin and poly(ADP-Rib) polymerase activity as previously described (Mullins et al., 1977).

Results

(A) Relationship between Poly(ADP-Rib) Polymerase and Chromatin Fragments of Varying Complexity. To establish the optimal size of polynucleosomes favoring histone ADP-ribosylation for subsequent reconstitution studies, we determined the acceptor populations for poly(ADP-Rib) in chromatin fragments of varying complexity obtained by mild micrococcal nuclease digestion of HeLa nuclei. In this study, all forms of chromatin solubilized by micrococcal nuclease are recovered and separated by velocity sedimentation (Figure 1). Previous studies have dealt only with chromatin retained by the nucleus after digestion and separated from undigested chromatin by nuclear lysis and low-speed sedimentation (Butt et al., 1978). Wittig & Wittig (1977) reported that chromatin fragments (1–4 nucleosomes) diffuse from nuclei during nuclease digestion and are not recovered when nuclei are pelleted for subsequent lysis to solubilize trapped chromatin. In our previous studies, these fragments were found to contain appreciable poly(ADP-Rib) polymerase activity and acceptors for poly(ADP-Rib) (Jump et al., 1979).

The separation of chromatin fragments by velocity sedimentation used for the subsequent studies is shown in Figure 1. The chromatin profile represents a 7.4% perchloric acid soluble digest, and 47% of total nuclear chromatin is recovered in the gradient. Figure 1 (insert) shows an electrophoretic separation of DNA isolated from the various pools of chromatin. No evidence of nucleosome aggregation was observed.

Poly(ADP-Rib) polymerase activity was measured in the pooled fractions as indicated in Figure 1. Each assay was adjusted to contain 50 μg of chromatin DNA (A_{260nm} = 1.0), so that the only variables between assays were the nucleosome size (insert, Figure 1) and the intrinsic protein composition of the chromatin (Figure 2A). The data show that poly-

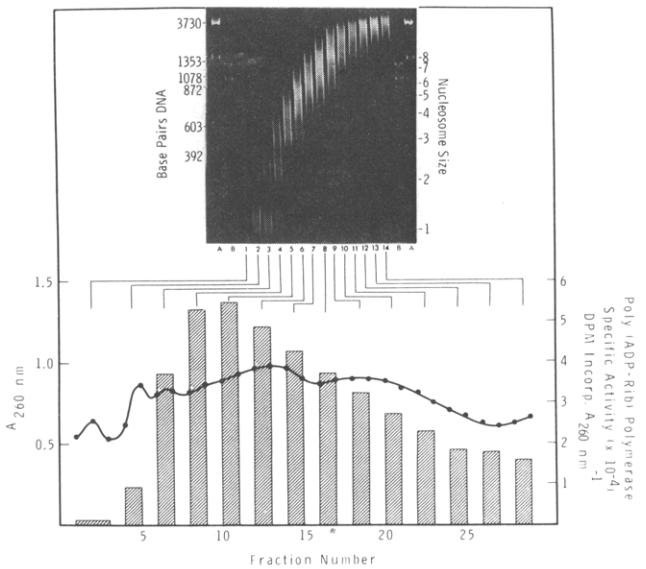

FIGURE 1: Velocity sedimentation of chromatin fragments of varying complexity. Chromatin was prepared by micrococcal nuclease digestion of HeLa nuclei and separated by velocity sedimentation as described under Materials and Methods. Absorbance at 260 nm was determined for individual gradient fractions (●). Various pooled fractions, as indicated (bars), were assayed for poly(ADP-Rib) polymerase as described under Materials and Methods. One absorbance unit (260 nm) of chromatin (50 μg of chromatin DNA) from the various pools was extracted for DNA as previously described (Butt et al., 1979) and electrophoresed in polyacrylamide gels (insert). Lanes A and B represent ϕX-174 cleaved by *Hpa*I and *Hae*III, respectively. Lanes 1–14 represent DNA isolated from the various chromatin pools 1–14, respectively.

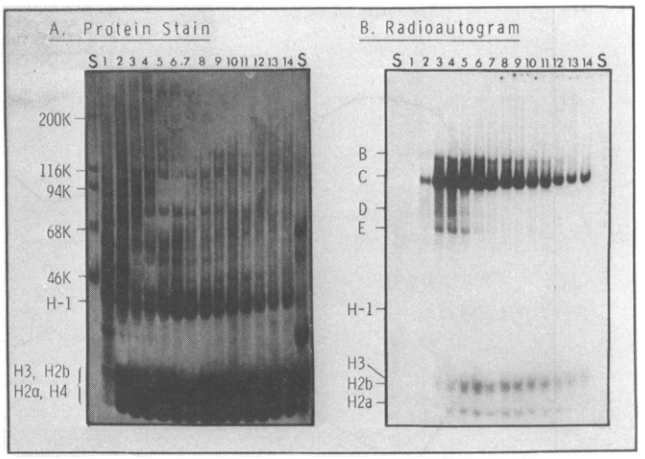

FIGURE 2: Relationship between oligonucleosome size and nuclear protein acceptors of ADP-ribosylation. Proteins were prepared from the [^{32}P]NAD assays of poly(ADP-Rib) polymerase of the various chromatin pools indicated in Figure 1 and electrophoresed as described under Materials and Methods (A). Radioautographic analysis (B) of the dried protein gel was as previously described (Jump et al., 1979). Lanes labeled with S represent protein standards. Lanes 1–14 represent the proteins associated with the various chromatin pools in Figure 1 (lanes 1–14, respectively).

(ADP-Rib) polymerase specific activity was not constant in the various chromatin fragments but was noted to reach a maximum in fractions containing approximately 4–6 nucleosomes. In larger chromatin fragments, enzyme activity decreased nearly 3.5-fold. Analysis of the DNA associated with the various chromatin fractions (Figure 1 insert) facilitated assigning an average nucleosome size to the various pools.

Previously, Butt et al. (1978) reported that the maximum enzyme specific activity was reached on octanucleosomes. However, as noted above, there were differences in digestion conditions between the two studies, as well as in distributions of histone H1/core histones (see below). These pooled polynucleosome fractions provided well-characterized chromatin fragments of increasing size and differing potential for ADP-Rib incorporation for the subsequent examination of the relationship between nucleosome size and histone and nonhistone protein modification.

Acceptors of Poly(ADP-Rib) in Various Chromatin Fractions. A portion of the fractions assayed with [^{32}P]NAD in the previous study which represented oligonucleosomes of increasing complexity was precipitated with ethanol, and the associated protein was analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis and radioautography. Examination of Figure 2A reveals that the mass of nucleosomal histones (stain) is fairly constant in the various chromatin fractions, substantiating that equal amounts of chromatin from each pooled fraction were analyzed. The ratio of histone H1 to nucleosomal histones was variable under these conditions with the highest H1/core histone ratio found in hexanucleosomes through octanucleosomes (lanes 5–7). Renz et al. (1977) previously reported on the preferred association of H1 with octanucleosomes. Under different digestion conditions than those utilized in the current study, we found reasonably equal H1 distribution in oligonucleosomes containing 1–16 nucleosomes (Butt et al., 1978, 1979).

A myriad of nonhistone proteins (20 000–500 000 M_r) was found to be associated with the various chromatin fragments (Figure 2A). Many of these proteins were distributed nonrandomly. This was most apparent for nonhistone proteins from 60 000 to 95 000 M_r. In general, there appeared to be a higher concentration of nonhistone proteins in smaller chromatin fragments, i.e., those regions of chromatin preferentially susceptible to nuclease digestion.

Radioautographic analysis of the protein acceptors of ADP-Rib is shown in Figure 2B. Examination of the radioautogram shows that the predominant acceptor in each chromatin particle is a 112 000 M_r nonhistone protein, protein C. Butt et al. (1979) also observed that protein C represented the major protein acceptor in octanucleosomes. The accompanying report presents considerable evidence suggesting that protein C represents the ADP-ribosylated form of poly(ADP-Rib) polymerase (Jump & Smulson, 1980). If the stoichiometry of covalently bound ADP-Rib to the enzyme is reasonably constant in the various fractions, then the relative distribution of the enzyme in the different chromatin particles could be determined. The data show that the enzyme is extensively automodified in chromatin fragments (2–9 nucleosomes), suggesting a preferred association with chromatin preferentially cleaved by micrococcal nuclease as compared to more condensed chromatin. At this time, it is technically difficult to quantitate enzyme mass and chain length of oligo(ADP-Rib). This is an area that is currently being developed with an antibody specific to the enzyme. However, we have tentatively suggested that this enzyme might be bound in the midregion of superbeaded regions (16 nucleosomes) of chromatin (Butt et al., 1979).

Other nonhistone proteins were also noted to be acceptors. In particular, a 70 000 M_r nonhistone protein (protein E) is seen as a prominent acceptor with a nonrandom distribution in the various chromatin fragments. This acceptor was also detected previously in intact nuclei [Butt et al. (1979), Figure 5].

Nucleosomal histones were found to be significant acceptors of ADP-Rib in the in vitro analysis especially in larger polynucleosomes. Histone H1, however, was not noted to be ex-

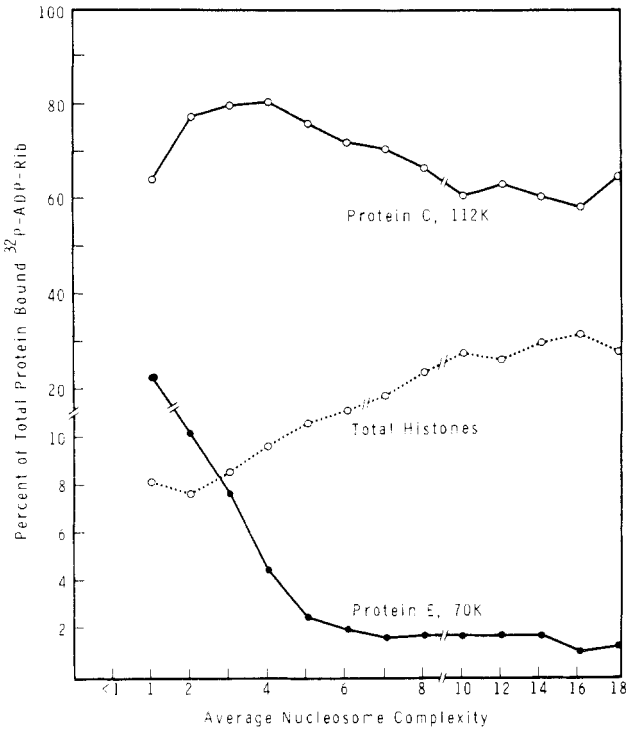

FIGURE 3: Percent protein-bound [^{32}P]poly(ADP-Rib) associated with protein C, protein E, and total histones in chromatin fragments of varying complexity. Total [^{32}P]ADP-Rib covalently bound to protein was recovered from the protein gel (Figure 2) by excising each labeled region from each lane and solubilizing in 0.4 mL of 30% hydrogen peroxide–0.2 mL of 70% perchloric acid overnight at 60 °C. Total disintegrations per minute were determined by liquid scintillation counting. The fraction of the total protein-bound [^{32}P]ADP-Rib bound to protein C, protein E, and total histones was determined and plotted. The average nucleosome size (1–18 nucleosomes; pools 1–14, respectively) was determined from the DNA gel in Figure 1 (insert).

tensively modified in chromatin fragments prepared by this present procedure. These in vitro studies indicate that essentially the same proteins that were modified in the various chromatin fragments are also modified in intact nuclei; however, they were not necessarily modified to the same extent. Therefore, the association of the enzyme with these acceptor proteins appears to be preserved after disruption of ordered chromatin conformation by micrococcal nuclease.

Correlation of the Distribution of Protein-Bound ADP-Rib in Chromatin Fragments and in Intact Nuclei. In order to obtain more data on the distribution of ADP-Rib among various key acceptors, we quantitated total [^{32}P]ADP-ribosylated proteins generated in the different oligonucleosome fractions by direct radioactive determination of total protein-bound radioactivity. The fraction associated with protein C, the 70000 M_r nonhistone protein (protein E), and total histones is presented in Figure 3. The major portion (60–80%) of protein-bound poly(ADP-Rib) was clearly found to be associated with protein C. However, enzyme (protein C) automodification appeared to be dependent on the nucleosome size. In general, as the nucleosome size increased, less enzyme automodification and, more significantly, less nonhistone protein modification were noted. This observation was exploited below where conditions favoring histone modification were studied with purified polymerase reconstituted with large fragments of chromatin. Previous reports from our laboratory have stressed the importance of nucleosome compaction into superbeaded or selenoidal structures in nuclei in order to obtain histone ADP-ribosylation (Mullins et al., 1977; Giri et al., 1978; Butt et al., 1979). Such intramolecular folding is highly likely and more favored as nucleosome size increases. In this regard, it is also of interest to note that the in vitro synthesis of the poly(ADP-Rib) cross-linked dimer of histone H1 (Stone et al., 1978) has been recently found to be favored in 16-nucleosome polynucleosomes over 8-nucleosome and lower species (N. Nolan and M. Smulson, unpublished results).

Table I: Distribution of in Vitro Synthesized Poly(ADP-Rib) among Various HeLa Nuclear Proteins[a]

protein fraction	protein-bound ADP-Rib: pmol of ADP-Rib[a]	protein-bound ADP-Rib: % of total
poly(ADP-Rib) polymerase	0.57	43.5
other nonhistone proteins	0.21	16.0
histone H1	0.15	11.5
core histones	0.38	29.0

[a] HeLa nuclei (5×10^7/mL) were incubated with [^{32}P]NAD (1 μM, 1 Ci/mmol) for 10 min at 25 °C. The reaction was terminated by the addition of thymidine to 50 μM, and micrococcal nuclease was added to 200 units/mL; nuclei were digested for 5 min at 37 °C, and proteins were precipitated in ethanol as described under Materials and Methods. Proteins were separated by $NaDodSO_4$-polyacrylamide gel electrophoresis. Protein-bound [^{32}P]ADP-Rib was quantitated by excising labeled proteins from the gel, followed by liquid scintillation counting as previously described (Jump & Smulson, 1980). Total protein applied to the acrylamide gels was 318 μg; 1.31 pmol of [^{32}P]ADP-Rib was recovered.

An illustration of the nonrandom distribution of a nonhistone protein acceptor is illustrated by the decreased fraction of poly(ADP-Rib) covalently bound to protein E (70000 M_r) with increasing nucleosome size (Figures 2 and 3). This phenomenon might be due to (a) the absence of protein E in larger oligonucleosomal structures, (b) nucleosome interference with the acceptor site on the protein for modification, or (c) noninteraction of protein E with the enzyme. Current efforts are directed at identifying protein E. It is of interest that as protein E in vitro ADP-ribosylation decreases, the modification of histones increases.

As noted earlier, the relative distribution of poly(ADP-Rib) in the various protein fractions in isolated chromatin is not similar to that detected in intact nuclei. When these distributions in Figure 3 and Table I are compared, it can be seen that histone modification is more favored in intact nuclei than in isolated chromatin fragments. The relative distribution of ADP-Rib in nuclei between histones and nonhistone proteins is similar to that reported previously (Jump et al., 1979; Okayama et al., 1978). The data in Figure 3 show that large oligonucleosomal fragments (8 nucleosomes) have a greater portion of the protein-bound ADP-Rib in histones when compared to smaller nucleosomal structures, 30 and 8%, respectively. In the intact nucleus, 40% of the protein-bound ADP-Rib is bound to histones. This is partitioned between core histones (29%) and histone H1 (12%).

It is our current view that histone ADP-ribosylation plays a significant role in modulating chromatin structure. Therefore, structures having significant potential for histone modification will better serve as model systems to study poly-ADP-ribosylation in vitro. The difficulties in elucidating the role of poly(ADP-Rib) polymerase in modulating chromatin structure are apparent. Reconstitution of purified poly(ADP-Rib) polymerase with oligonucleosomes was carried out in an effort to simplify this system. The study discussed above shows that oligonucleosomes larger than 8 nucleosomes when incubated with [^{32}P]NAD under the appropriate conditions result in 25–30% of the protein-bound ADP-Rib being

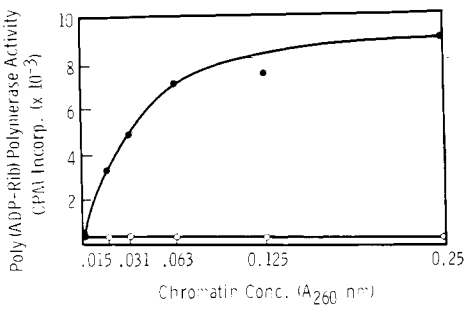

FIGURE 4: Poly(ADP-Rib) polymerase activity using stripped chromatin as the substrate. Increasing concentration of HeLa oligonucleosomal chromatin stripped of endogenous enzyme as described under Materials and Methods was incubated in the presence (●) and absence (○) of purified poly(ADP-Rib) polymerase (0.85 μg/mL) under the standard assay conditions (minus calf thymus histones and DNA). The reaction was terminated after 10 min and trichloroacetic acid insoluble radioactivity was determined.

covalently attached to histones. Accordingly, since the overall objective of these studies was to ascertain conditions favoring histone modification, we reasoned that the use of oligonucleosomal structures stripped of endogenous poly(ADP-Rib) would yield the greatest chance of restoration of histone modification.

(B) Reconstitution of HeLa Poly(ADP-Rib) Polymerase with Stripped Oligonucleosomes. Characteristics of Stripped and Reconstituted Chromatin. Poly(ADP-Rib) polymerase could be quantitatively removed from chromatin, as described under Materials and Methods, without significant alteration in the subunit structure of oligosomes. One disadvantage of this technique, however, is the concomitant removal of H1 and some nonhistone proteins. In order to test for the absence of endogenous enzyme and the capacity of the stripped chromatin to support poly(ADP-Rib) synthesis, we performed an assay with increasing chromatin concentration in the presence and absence of purified enzyme. No endogenous poly(ADP-Rib) polymerase was found associated with stripped chromatin (Figure 4). However, after addition of a precise amount of purified enzyme, this form of stripped chromatin was found to be a competent substrate for synthesis of poly(ADP-Rib) (Figure 4). When comparisons were carried out by using amounts of chromatin comparable to the DNA–histone used in the standard assay, a twofold stimulation in enzyme activity was noted [Table II of Jump & Smulson (1980)]. Note that no exogenous DNA or histones were added to the reaction and that chromatin in Figure 4, which represents the proper ratio of DNA to histones, supported the reaction completely. The enzyme had an apparent K_m for chromatin DNA of 1.2 μg/mL DNA (calculated from Figure 4). Ito et al. (1979) reported that the calf thymus enzyme has an apparent K_m for DNA of 2.5 μg/mL when exogenous DNA and histones are used as binding substrates.

In order to determine if poly(ADP-Rib) polymerase was bound to oligonucleosomes in a nonrandom fashion, two studies were carried out. Initially, the sedimentation of reconstituted oligonucleosomes (approximately 8 nucleosomes) was compared to the sedimentation of original native oligonucleosomes (Figure 5). It was observed that the reconstituted chromatin sedimented slower in gradients than native chromatin. This may be due to the absence of H1 in the stripped chromatin since this histone has been implicated in chromatin condensation and concomitant sedimentation characteristics, as discussed by Stradling (1978). To determine whether binding of poly(ADP-Rib) polymerase with stripped oligonucleosomes had occurred during the reconstitution, we performed enzyme

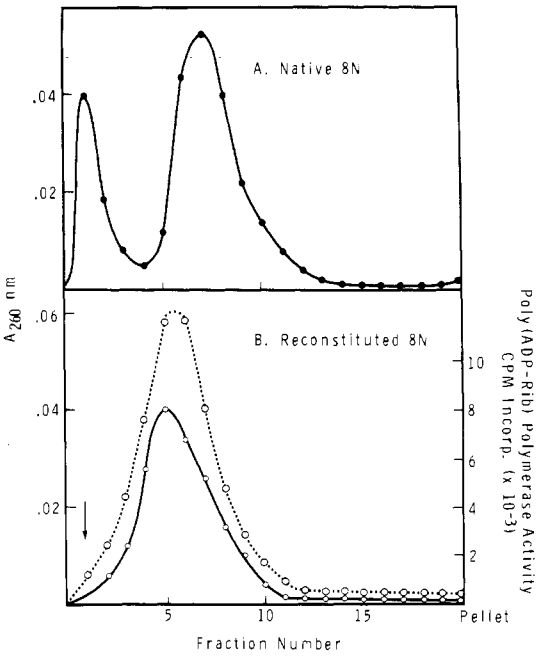

FIGURE 5: Velocity sedimentation of native and reconstituted HeLa chromatin. Native (A) and reconstituted (B) HeLa chromatin (see Materials and Methods) were sedimented in 10–30% sucrose gradients (10 mM Tris, pH 8; 80 mM NaCl; 1 mM EDTA; 1 mM DTT; 0.2 mM $PhCH_2SO_2F$) for 6 h at 38000*g*, SW 40. Gradients were fractionated and analyzed for absorbance at A_{260nm} (●–●, ○–○) and poly(ADP-Rib) polymerase activity (○--○) as previously described (Mullins et al., 1977).

assays throughout the sucrose gradient fractions (Figure 5B). The enzyme assay was conducted to detect only chromatin-associated poly(ADP-Rib) polymerase since no exogenous DNA or histones were added. The position marked by the arrow indicates the position at which nonchromatin-bound enzyme would migrate (5.2 S) (Jump & Smulson, 1980). The data show that enzyme activity sedimented coincident with that of the reconstituted oligonucleosomes.

Previous studies have shown that chromatin-bound poly(ADP-Rib) polymerase is not bound to core nucleosomes but to the linker regions of chromatin (Giri et al., 1978). Figure 6A shows the migration of native mononucleosomes in 10–30% sucrose gradients. Native oligonucleosomes sedimented considerably faster and are seen to have associated enzyme activity (Figure 6B). Reconstituted oligonucleosomes were digested with micrococcal nuclease and analyzed by sedimentation velocity centrifugation (Figure 6C). The low levels of chromatin applied to the sucrose gradient precluded obtaining an A_{260nm} profile. However, the enzyme assays indicate that when the reconstituted particles were cleaved to mononucleosomes, enzyme activity was found associated with heavy monomers, in a fashion similar to that demonstrated with the native particles (Figure 6A). These data suggest that reconstitution of the enzyme has been nonrandom on chromatin fragments and that the enzyme has a preference for reassociation with the linker region of chromatin.

Comparison of Acceptors of ADP-Rib in Native and Reconstituted Chromatin. In order to determine if poly-ADP-ribosylation of proteins in reconstituted particles resembled that noted in native chromatin particles, we carried out an acceptor assay (Figure 7) of four samples: (a) native oligonucleosome (lanes 1 and 2); (b) enzyme plus exogenous calf thymus DNA and histones (lane 3 and 4); (c) enzyme plus exogenous HeLa DNA and histones (lanes 5 and 6); (d) reconstituted oligonucleosome (lanes 7 and 8). The data show

Table II: Distribution of [^{32}P]ADP-Rib in Various Protein Fractions of Purified Enzyme and Native and Reconstituted Chromatin[a]

assay conditions[b]	total recovered (dpm)	protein fractions: 112 000 M_r dpm	% of total	NHP < 112 000 M_r dpm	% of total	histone H1 dpm	% of total	nucleosomal histones dpm	% of total
native chromatin	2360	1779	75.4	166	7.0	224	9.5	191	8.1
purified enzyme, exogenous calf thymus DNA, and histones	2472	2107	86.2	238	12.9	27	1.1	54	
purified enzyme, exogenous HeLa DNA, and histones	2519	2339	92.1	259	6.3			20	
reconstituted chromatin	4735	3048	64.4	1403	29.6			286	6.1

[a] Regions corresponding to the specific protein fractions (see Figure 2) were excised from the NaDodSO$_4$–polyacrylamide gel and quantitated by liquid scintillation counting. [b] Details of the assay conditions are in the legend for Figure 7.

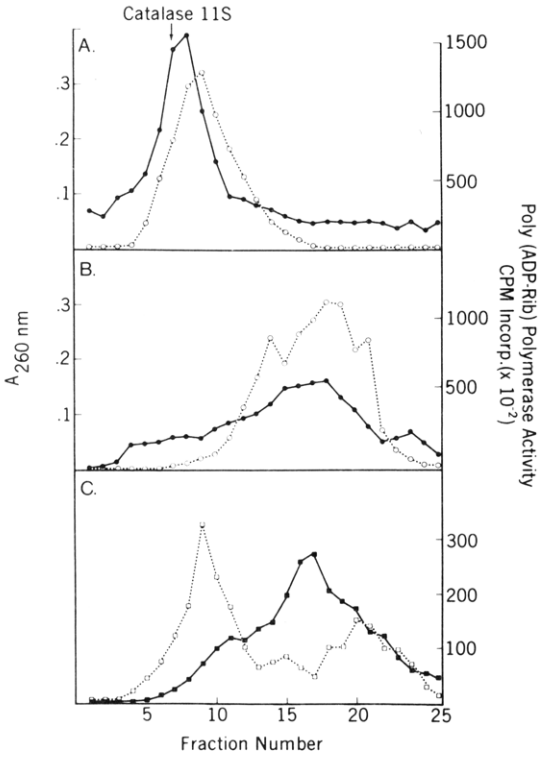

FIGURE 6: Micrococcal nuclease digestion of reconstituted chromatin. Velocity sedimentation of mononucleosome (A) and oligonucleosome (B) in sucrose gradients as described above was for 12 h at 35000g, SW 40, and 4 °C. Gradients were analyzed for absorbance at A_{260nm} (●) and poly(ADP-Rib) polymerase activity (○) as previously described (Mullins et al., 1977). (C) Velocity sedimentation of reconstituted chromatin digested (□) and undigested (■) with micrococcal nuclease (10 units of nuclease per A_{260nm} of chromatin; 1 min; 37 °C; digestion was terminated by the addition of 5 mM EDTA). Fractions were assayed for poly(ADP-Rib) polymerase activity as described above.

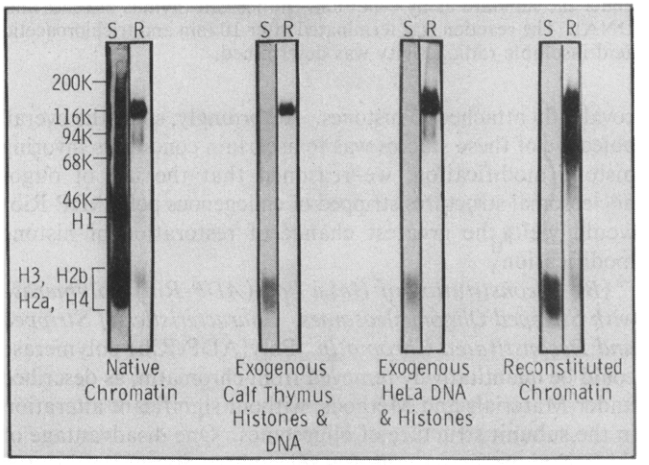

FIGURE 7: Acceptor analysis of purified enzyme and native and reconstituted oligonucleosomal chromatin. Native (0.10 unit of A_{260nm}) and reconstituted (0.05 A_{260nm}) chromatin was incubated in the standard acceptor assay (minus calf thymus histones and DNA) for 10 min at 25 °C. Samples were precipitated with ethanol and analyzed by NaDodSO$_4$–polyacrylamide gel electrophoresis. Purified poly(ADP-Rib) polymerase (0.3 μg/0.2 mL) was incubated with either calf thymus histones and DNA (12.5 μg/mL each) or HeLa histones and DNA (12.5 μg/mL each) in the standard acceptor assay for 10 min at 25 °C (Jump & Smulson, 1980). Acceptors were analyzed and described in the legend of Figure 2.

that histone modification was absent when either exogenous calf thymus or HeLa DNA and histones are added to the reaction with purified enzyme. However, histone modification was evident in both the native and reconstituted chromatin fragments. Quantitation of the ^{32}P-label distribution is provided in Table II. In two cases examined, 61 and 75% of the protein bound radioactivity were incorporated into the enzyme in reconstituted and native chromatin, respectively. Labeling of nucleosomal histones represented 8.1% in native and 6.1% in reconstituted chromatin.

The data indicate that purified poly(ADP-Rib) polymerase, when reconstituted with nucleosomes, catalyzed a reaction whereby ADP-Rib was covalently bound to nucleosomal histones. However, we have not distinguished between terminal addition of chains initiated in vivo from initiation of nascent chains of poly(ADP-Rib) on histones. Ueda et al. (1979) reported that the rat liver enzyme could elongate chains of poly(ADP-Rib) on histones H1 that was chemically primed with ADP-Rib. When HeLa histones and DNA were substituted for calf thymus histones and DNA in the exogenous acceptor assay, no histone modification was observed. Therefore, these results stress the importance of proper orientation of histones in a chromatin complex with respect to the enzyme to achieve histone modification. A beginning in our understanding of the interaction of this enzyme with the multiple components of simple oligonucleosomes through reconstitution should aid in a better appreciation of the function of this modification in influencing chromatin structure.

Discussion

Previous studies (Butt et al., 1978) had indicated a unique relationship between poly(ADP-Rib) polymerase and orders of chromatin structure. The present study confirms and significantly extends these previous studies. Little detailed information is as yet available on the interaction of other nuclear enzymes with polynucleosomal structures of chromatin. Detailed analysis of the distribution of protein-bound poly(ADP-Rib) reveals that no single explanation for the increase

in poly(ADP-Rib) polymerase with increase in size of chromatin fragment can be made. Factors that are seen to influence the amount of protein-bound ADP-Rib include (1) the stoichiometry of enzyme in chromatin fragments, (b) the nonhistone protein distribution, and (c) the size of the oligonucleosome. The data presented above emphasized that "activity" measurements in chromatin fragments basically titrate the extent of automodification of poly(ADP-Rib) polymerase (protein C) (Figure 2). Whether this represents the actual mass of the enzyme associated with these various chromatin fragments remains to be determined.

The size of the chromatin used for in vitro assays had a significant influence on the distribution of poly(ADP-Rib) in the various protein fractions examined (Figure 3). Generally, as the chromatin particle increased in size, histone modification increased to a level observed in intact nuclei. In the largest structures (10–18 nucleosomes) examined in this study, 30% of the protein-bound polymer was found associated with histones. In nuclei, 40% of protein-bound ADP-Rib was bound to histones (Table I). Therefore, the use of oligonucleosomes to study histone modification appears to approximate the in vivo condition.

As mentioned earlier, histone ADP-ribosylation is thought to have significant but local effects on chromatin structure (Jump et al., 1979). Poly(ADP-Rib) polymerase is known to be nonrandomly distributed within chromatin (Jump et al., 1979; Mullins et al., 1977). The work above has extended these previous studies by showing that poly(ADP-Rib) polymerase has a preferred association with moderately extended forms of chromatin (Figures 1 and 3) near sites in chromatin initially cleaved by micrococcal nuclease. In these studies, the enzyme has been noted to undergo automodification in isolated chromatin fragments (Figure 2) and in isolated nuclei [Figure 2 of Jump & Smulson (1980)]. The function of automodification is not understood, nor is it known whether the apparent automodification of the enzyme is inter- or intramolecular. However, the data in Figure 3 provide important new information concerning the relationships of those levels of chromatin complexity favoring in vitro histone modification over enzyme automodification. In oligonucleosomal structures, where intramolecular folding would tend to promote enzyme interaction with histone H1 and nucleosomal histones, enzyme automodification was found to be reduced 20–25%. In these same structures, histone modification was seen to increase from 8 to 30%. In smaller structures where such intramolecular interaction is not favored, histone modification was considerably reduced. The situation is somewhat analogous to our recent observations (N. Nolan and M. Smulson, unpublished results) on the in vitro synthesis of the poly(ADP-Rib)–histone H1 dimer (Stone et al., 1978). The biosynthesis of this cross-linked histone was studied in both purified 8- and 16-nucleosome oligonucleosomes and found to be considerably higher in the larger structures.

The predominant automodification of protein C in small oligonucleosomes (2–4 nucleosomes) may reflect the absence of the appropriate histone acceptors proximal to the enzyme active site. Automodification thus may function in generating free or protein-bound poly(ADP-Rib) in regions of chromatin too extended for histone modification. Data obtained recently suggest that enzyme automodification may function to promote oligonucleosome condensation (unpublished observations). The above observations suggest that the enzyme must interact specifically with histones for modification. This interaction occurs in regions of chromatin where the linker-associated enzyme, through chromatin folding, interacts specifically with histone acceptors. In the absence of this interaction, the enzyme presumably assumes an acceptor-like activity and becomes automodified. This unique property of the enzyme may thus function to modulate chromatin structure in extended regions of chromatin where the enzyme–nucleosomal histone interaction may be restricted. More detailed studies will be required to further elucidate this interesting phenomenon.

Simple model systems must be developed to gain a greater understanding of the function of poly-ADP-ribosylation of chromatin proteins. This study has thus served to characterize one such system. We have reconstituted purified HeLa cell poly(ADP-Rib) polymerase with defined oligonucleosomal fragments, and this has led to a restoration of histone modification (Figure 7). In addition, the enzyme appears to reassociate with linker regions in oligonucleosomal structures (Figure 6). This location was previously demonstrated for the enzyme in native chromatin (Giri et al., 1978). The distribution of [^{32}P]ADP-Rib among the various protein fractions generated with the reconstituted chromatin, however, was not identical with that observed in native chromatin. This was particularly evident in the modification of nonhistone proteins (50 000–95 000 M_r). This may reflect the improper ratio of enzyme to chromatin subunit used in this study. The enzyme does not reside on every linker in chromatin, but it is presumably distributed according to the functional properties of nontranscribing chromatin. Studies are currently underway to quantitate this nonrandom distribution of poly(ADP-Rib) polymerase and to better define the domain in which it functions.

Acknowledgments

The authors thank Beverly DeCoste for excellent technical assistance and Dr. Nancy Nolan for critical discussions concerning this work.

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