Relationship between Nicotinamide Adenine Dinucleotide Concentration and in Vitro Synthesis of Poly(adenosine diphosphate ribose) on Purified Nucleosomes†

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ABSTRACT: When oligonucleosomes (8–10 N) were incubated under conditions favoring poly(ADP-ribosylation) with concentrations of [^{32}P]NAD 10 μM and higher, the labeled chromatin components migrated in 3–8% gradient native chromatin polyacrylamide gels, in positions of far greater size than the starting nucleosomes. Only a small fraction of chromatin and/or oligonucleosome components was found to be involved in this aggregation. This phenomenon could be demonstrated as well by the increased sedimentation of ADP-ribosylated chromatin components in velocity sucrose gradients. In contrast, at [^{32}P]NAD concentrations $\leq$1 μM, the modified nuclear proteins were found strictly associated with the original oligonucleosomes. The extent of aggregation was proportional to both substrate concentration and time of incubation with NAD. During this process, progressively longer chains of poly(ADP-Rib) (10–60 N) were generated on chromatin proteins correlating with the level of complex formation. Analysis of protein by either acid–urea or NaDodSO$_4$–gel electrophoresis indicated that at NAD concentrations favoring aggregation, poly(ADP–Rib) induced ADP-ribosylated complexes of histone H1, and possibly core histones were observed. Poly(ADP–Rib) polymerase requires both DNA and nuclear proteins for activity. The presence of this enzyme in the high molecular weight complexes was demonstrated on sucrose gradients and also by direct enzyme assays, in situ, in regions of 3–8% gradient chromatin gels containing the complexed chromatin components.

The nuclear enzyme poly(ADP-Rib)[1] polymerase alters the structure of chromatin by the generation of chains of NAD-derived poly(ADP-Rib) which are covalently bound to nuclear proteins. Histone H1 modification leads to a cross-linking of H1 to form a dimeric complex (Stone et al., 1977; Adamietz et al., 1978; Ring & Cole, 1979). It has been speculated that this natural biopolymer may well be a ubiquitous cross-linking agent in eukaryotic chromatin. It is possible that the poly(ADP-Rib) synthesizing and degrading activity of eukaryotic cell nuclei constitutes a system for affecting a transient and localized condensation of chromatin by permitting a reversible interaction of adjacent or neighboring nucleosomes within chromatin. It is therefore of interest that the enzymatic activity for this system responds to increasing complexity of polynucleosome size when the activity is measured in vitro (Butt et al., 1978, 1979).

Nolan et al. (1980) further showed that the poly(ADP-Rib)–histone H1 cross-linking reaction was favored in vitro in large nucleosome species. Agents such as spermine and calcium, which cause chromatin condensation, also have been shown to increase in vitro synthesis of H1–poly(ADP-Rib) complex formation (Byrne et al., 1978).

These data emphasize a possible relationship between condensed forms of chromatin and a potential cross-linking and/or condensing function mediated by poly(ADP-ribosylation). A second question, still unanswered, is whether once formed, will poly(ADP-ribosylated) nucleosomes result in a localized condensation of chromatin? However, support for this notion was recently provided by the observation of a T_m increase in chromatin following ADP-ribosylation (Janakidevi & States, 1980). However, it should be noted that nucleosomes released early during micrococcal nuclease digestion, in contrast to those released later, were found to possess high specific activity for ADP-ribosylation, although this represented a small proportion of the total labeled incorporation (Jump et al., 1979).

We recently observed that incubation of oligonucleosomes with NAD (50 μM) caused a slower mobility of the modified chromatin components on 3–8% gradient native chromatin polyacrylamide gels without affecting the electrophoretic properties of bulk nucleosomes. Since the domain of poly(ADP-ribosylation) in chromatin is quite limited, this aggregation was only observed by the poly(ADP-Rib) labeling of the reaction products. The studies below were designed to ascertain whether specific poly(ADP-Rib) high molecular weight complexes contain cross-linked chromatin components and/or nucleosomes.

Materials and Methods

Materials. [^{32}P]NAD was either purchased from New England Nuclear (specific activity 32–56 Ci/mmol) or synthesized by the procedure described earlier (Jump et al., 1979). [*adenine*-2,8-^{3}H]NAD (3.4 Ci/mmol) was also purchased from New England Nuclear. All the reagents were of analytical grade. Micrococcal nuclease was purchased from Worthington and Proteinase K from Merck. *Escherichia coli* ribosomal subunits were a gift from Dr. Zahid of the University of Toronto. Kodak SB5 X-ray film was used for autoradiography. HeLa S_3 cells were maintained in suspension cultures at 37 °C in Spinner flasks. All the cells used in the present studies were grown asynchronously to a midlog density [(5–8) × 10^5 cells/mL of culture medium].

Preparation of Chromatin from HeLa Nuclei. HeLa cell nuclei were prepared by the method of Sporn et al. (1969) and washed with Triton X-100 (0.3%) containing buffer at least

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[1] Abbreviations used: ADP-Rib, adenosine diphosphate ribose; NAD, nicotinamide adenine dinucleotide; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; NaDodSO$_4$, sodium dodecyl sulfate.

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3 times before nuclease digestion in buffer containing 0.25 M sucrose, 5 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, and 80 mM NaCl.

The nuclei were incubated with nuclease digestion buffer at 1×10^8 nuclei/mL and treated with micrococcal nuclease [30 units/(1×10^8 nuclei)] for 3–5 min at 37 °C. The reaction was terminated, and the nuclei were lysed with 1 mM EDTA as described previously (Butt et al., 1978). Alternatively, the nuclei were digested with micrococcal nuclease [400 units/(1×10^8 nuclei)] for 10–20 min at 4 °C. In both digestion conditions, 8–12% of the chromatin DNA was rendered acid soluble. Chromatin was separated on 10–30% linear sucrose gradients as described previously (Butt et al., 1978, 1979). An analysis of various chromatin fractions from a sucrose gradient has been shown by polyacrylamide gel electrophoresis (Figure 1).

Analysis of Labeled Proteins in Polyacrylamide Gels Containing NaDodSO$_4$. The poly(ADP-ribosylation) reaction was carried out on chromatin particles of various complexity. The total proteins were precipitated with 3 volumes of absolute ethanol at −20 °C for 12 h. The precipitated material was centrifuged at 8000*g* for 10 min, dissolved in 10 mM Tris-HCl, pH 6.8, 0.1% NaDodSO$_4$, 6 M urea, 1% 2-mercaptoethanol, and 20% glycerol, and boiled for 2 min prior to electrophoresis. Electrophoresis was performed in 10% polyacrylamide (acrylamide/*N*,*N*-methylenebisacrylamide, 200:1 w/v) slab gels with 50 mM sodium phosphate, pH 7.0, and 0.1% NaDodSO$_4$ as the gel buffer. The following proteins were used as molecular weight markers: myosin, M_r 200 000; β-galactosidase, M_r 116 000; phosphorylase, M_r 94 000; bovine serum albumin, M_r 68 000; albumin, M_r 43 000.

Native Chromatin Gels. A linear gradient of 3.5–8% (or as described in figure legends) of polyacrylamide (acrylamide/*N*,*N*-methylenebisacrylamide, 20:1 w/w) was established in slab glass plates. The gel buffer contained 89 mM Tris base, 89 mM boric acid, and 2.5 mM EDTA at pH 8.3. Preelectrophoresis was performed for about 2 h at 5 °C at 150 V/slab before the application of the samples. ^{32}P-Labeled poly(ADP-ribosylated) chromatin was applied on to the gels and electrophoresis performed for 8–10 h at 150 V. The nucleoproteins were fixed on the gel with 10% acetic acid, and the gel was processed for radioautography as described below.

Analysis of Acid-Soluble Proteins on Acetic Acid/Urea Gels. ADP-ribosylated proteins were extracted with 0.4 N H_2SO_4 for 30 min and precipitated with 20% trichloroacetic acid. The precipitates were washed with acidified acetone, dried, and dissolved in a solution containing 0.9 N acetic acid, 8 M urea, and 1% mercaptoethanol. The proteins extracted from chromatin samples incubated with concentrations of NAD higher than 10 μM were difficult to dissolve in sample buffer. Accordingly, the samples were vortexed and left at room temperature for up to 2 h to achieve total solubilization. Polyacrylamide (15%) gel electrophoresis was performed as described by Panyim & Chalkley (1969).

All the gels were fixed with 10% acetic acid and stained with Coomassie Blue. The gels were dried on a Bio-Rad Model 224 slab gel drier and exposed to X-ray film for an appropriate period.

Analysis of Chains of Poly(ADP-Rib) on Polyacrylamide Gels. For cleavage of poly(ADP-ribosyl) chains from nuclear proteins, ADP-ribosylated chromatin (0.2 A_{260} unit) was precipitated with 3 volumes of absolute ethanol (−20 °C overnight), and the samples were centrifuged. The pellet was dissolved in 0.25 mL of 0.1 M Tris base (pH 9.5) and incubated at 37 °C for 60 min to release the polymer from the proteins (Adamietz et al., 1978). The solution was brought to pH 7.00 by the addition of HCl, EDTA (5 mM), NaDodSO$_4$ (0.1%), and Proteinase K (5 μg) were added, and the suspension was incubated for another 60 min at 37 °C. The suspension was extracted with phenol/isoamyl alcohol/chloroform (24:24:1). The aqueous layer was reextracted with the phenol solution. Poly(ADP-Rib) was precipitated with 5 volumes of absolute ethanol (−20 °C, overnight) along with nucleic acids, and the pellet was washed with alcohol. The samples were dissolved in 30 μL of 8 M urea and 1% NaDodSO$_4$. The electrophoresis was performed in 20 15-cm slabs containing 20% polyacrylamide gel (acrylamide/*N*,*N*-methylenebisacrylamide, 20:1 w/w), 0.1% NaDodSO$_4$, and 8 M urea. The gel buffer contained 89 mM Tris, 89 mM boric acid, 5 mM EDTA, and 0.1% NaDodSO$_4$; the samples were electrophoresed until the bromophenol blue was 15 cm through the gel. The slab gel was exposed to X-ray film at −70 °C.

Poly(ADP-Ribosylation) of Chromatin. Chromatin fragments of different nucleosome repeat number were incubated with test mixture containing 50 mM Tris-HCl, pH 8.00, 2 mM $MgCl_2$, 1 mM DTT, and the desired concentration of NAD (0.02–0.3 mL of total volume). Since chromatin fragments were fractionated from sucrose gradients, the contribution of salts from the gradient buffer in the test mixture was the following: 10 mM NaCl, 0.02 mM EDTA, 0.1 mM sodium phosphate, pH 6.8, and about 0.7–2% sucrose. The reaction was carried out for 5 min at 20 °C and terminated by one of the following procedures: (A) addition of 5 mM nicotinamide followed by rapid chilling of the tubes on ice (for chromatin gels); (B) addition of 3 volumes of cold absolute ethanol (for analysis of protein on NaDodSO$_4$ gels); (C) addition of 5% trichloroacetic acid and 5 mM pyrophosphate (for enzyme assay). The poly(ADP-Rib) polymerase activity was determined, in situ, on gels as described previously (Giri et al., 1978).

Sedimentation Analysis of ADP-Ribosylated Chromatin. Chromatin fragments of 9–11 repeat number were incubated with test mixture containing 86.5 nM, 1 μM, 10 μM, and 100 μM NAD and 1 μCi of [^{3}H]NAD as described above. The reaction was terminated with nicotinamide, and samples were vacuum dialyzed in Schleicher and Schuell collodion bags with an approximate molecular weight cutoff of 25 000 for 3 h against 100 mM KCl, 10 mM Tris-HCl, pH 7.5, and 0.2 mM EDTA. Most of the unincorporated [^{3}H]NAD and free poly(ADP-Rib) was removed by this procedure (unpublished observations). After dialysis, the samples (≤0.3 mL) were applied on 10–30% linear sucrose gradients containing 100 mM KCl, 0.2 mM EDTA, and 10 mM Tris-HCl, pH 7.5. Ribosomal subunits (40 S and 60 S) were used as markers. Ribosome subunits were prepared from polysomes (Ragnotti et al., 1969) by treatment with 100 mM KCl and 1 mM EDTA for 5 min at 20 °C. The gradients were centrifuged for 2 h at 46 000 rpm in a Beckman SW 50-1 rotor at 4 °C. The fractions of the gradients were processed as described in the text.

Results

NAD Dependence of Complex Formation. Preparations containing predominantly 4–8 oligonucleosomes were used for most of the studies below, since it was previously determined that chromatin fragments of this size range appeared to have highest in vitro activity for poly(ADP-Rib) polymerase (Butt et al., 1978, 1979) and also maximal concentration of a 112 000-dalton protein, recently determined to be the automodified form of poly(ADP-Rib) polymerase (Jump & Smulson, 1980).

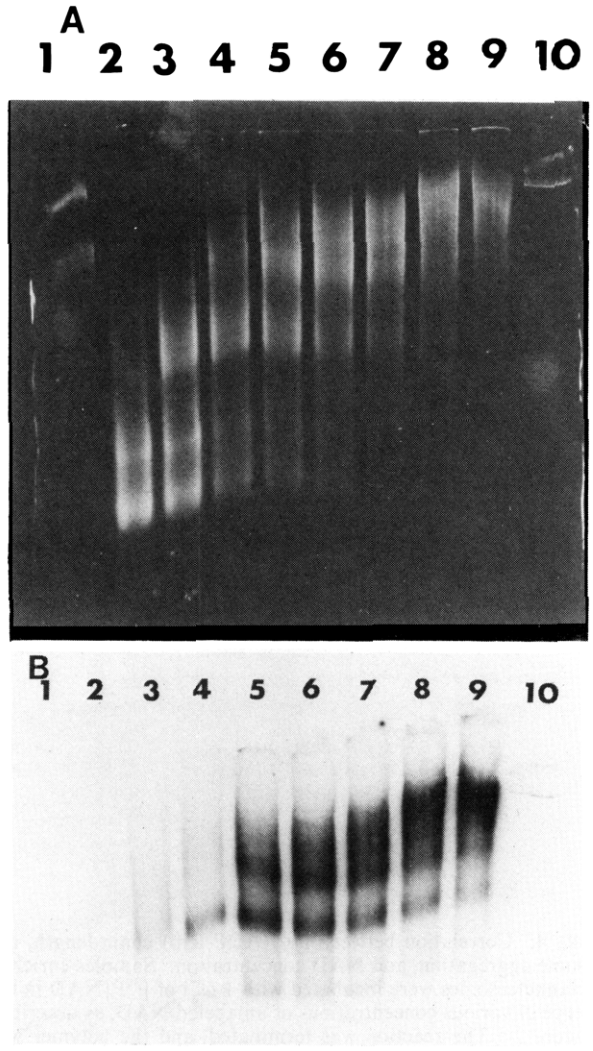

FIGURE 1: Modification of nuclear proteins in oligonucleosomes of differing repeat size with 0.1 μM [^{32}P]NAD under standard assay conditions. The samples were directly applied to a 2.5–8% gradient polyacrylamide gel after terminating the reaction with 5 mM nicotinamide. (A) Ethidium bromide stain; (B) autoradiograph. (Lane 1) *E. coli* ribosomal subunits; (lanes 2–9) chromatin samples from various *pooled* fractions from a sucrose gradient (Butt et al., 1978).

When oligonucleosomes of differing size were incubated with limiting substrate (0.1 μM [^{32}P]NAD), the labeled poly(ADP-Rib) was found directly associated with the same sized nucleosome particles used for the assay (Figure 1). It was established earlier that the chromatin proteins covalently ADP-ribosylated under these conditions are predominantly core histones, histone H1, and a few nonhistone proteins (Jump et al., 1979). The major acceptor of ADP-Rib (60–80% of incorporation) was an automodified form of poly(ADP-Rib) polymerase (Jump et al., 1980a).

Oligonucleosomes were incubated under conditions optimal for poly(ADP-Rib) polymerase activity with an increasing range of NAD from 25 nM to 1 mM (Figure 2). In the range of substrate concentration from 1 to 100 μM, a 100-fold increase in ADP-Rib synthesis was noted in these preparations (Table I). Analysis of the products on native chromatin gels (Figure 2A) indicated that NAD at these concentrations did not affect the overall mobility of the bulk of the oligonucleosomes in this gel system. However, radioautography of the incorporated ADP-ribosylated particles revealed a progressive increase in the size of the modified products, which was directly related to substrate concentration (Figure 2B). It was noted that when NAD concentrations were greater than

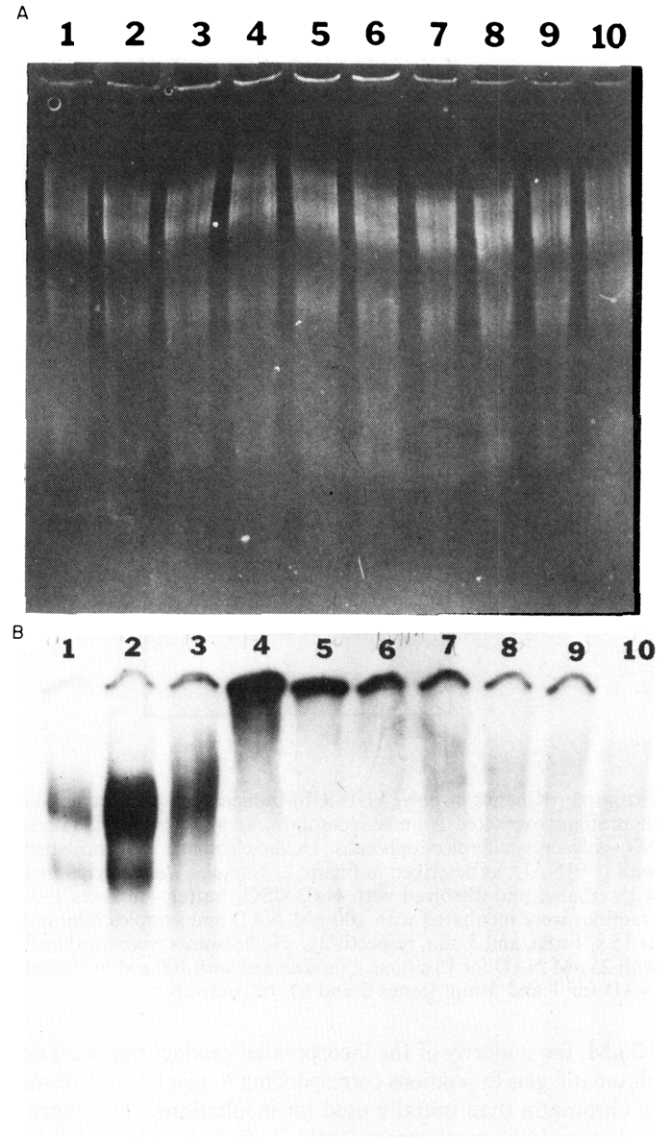

FIGURE 2: NAD concentration-dependent complex formation of polynucleosomes, as analyzed by chromatin gel electrophoresis. An aliquot of chromatin (0.07 A_{260} unit) from oligonucleosome, as analyzed above (lane 6, Figure 1), was incubated with various concentrations of NAD at 20 °C for 5 min while[^{32}P]NAD was maintained constant at 0.25 μCi per assay. The reaction was terminated by placing the tubes on ice and by the addition of nicotinamide to a final concentration of 5 mM. The samples were subjected to electrophoresis on 3–8% gradient polyacrylamide gels as described under Materials and Methods. (A) Ethidium bromide stain; (B) autoradiograph. (Lane 1) Chromatin incubated with 25 nM NAD for 5 min at 5 °C. (Lanes 2–6) Chromatin incubated at 20 °C with 25 nM, 1 μM, 10 μM, and 1 mM NAD, respectively. In lanes 7–10, the samples were incubated with 100 μM NAD for 5 min and terminated with nicotinamide, and 10 μg of proteinase K was added; the samples were incubated for an additional 1, 5, 10, and 20 min at 20 °C, respectively.

Table I: Relationship between NAD Concentration and Poly(ADP-Rib) Synthesis in Octanucleosomes[a]

NAD concn in test mixture	*n* mol of ADP-Rib synthesized/A_{260}
226 nM	0.024
1 μM	0.092
10 μM	1.049
100 μM	3.641
1 mM	8.362

[a] Polynucleosomes (8–9 N) were incubated with 0.5 μCi of [^{3}H]NAD for 5 min as described under Materials and Methods.

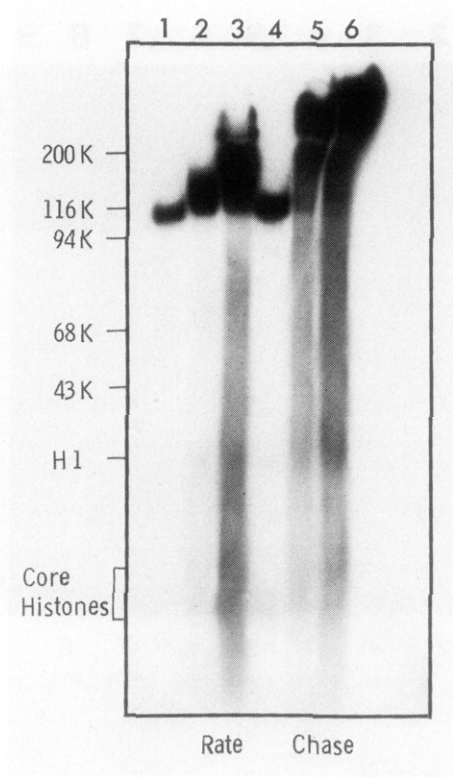

FIGURE 3: Kinetics of poly(ADP-Rib)-induced complex formation of proteins extracted from nucleosomes, as analyzed by NaDodSO$_4$–polyacrylamide electrophoresis. Octanucleosomes were incubated with [^{32}P]NAD, as described in Figure 2. Samples were precipitated with ethanol and dissolved with NaDodSO$_4$ buffer. In lanes 1–3, fractions were incubated with 100 μM NAD and samples removed at 15 s, 1 min, and 5 min, respectively. Nucleosomes were incubated with 25 nM NAD for 15 s (lane 4) and chased with 100 μM unlabeled NAD for 1 and 5 min (lanes 5 and 6), respectively.

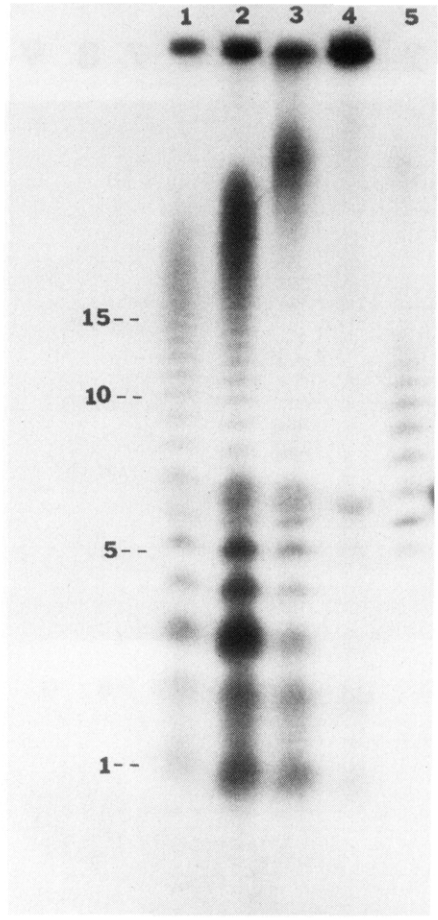

FIGURE 4: Correlation between poly(ADP-Rib) chain length, nucleosome aggregation, and NAD concentration. Samples enriched in octanucleosomes were incubated with 1 μCi of [^{32}P]NAD in the presence of various concentrations of unlabeled NAD, as described in Figure 2. The reaction was terminated, and the polymer was released by incubation in 100 mM Tris, pH 9.5, and purified as described under Materials and Methods. Lanes 1–4 represent polymer from chromatin incubated with 25 nM, 1 μM, 10 μM, and 100 μM NAD, respectively, and lane 5, marker polymer with average chain length of 10 N.

10 μM, the majority of the incorporated product migrated on chromatin gels to positions corresponding to much larger forms of chromatin than initially used for incubations. The aggregation could be most conveniently studied at 100 μM NAD. At higher concentrations (lane 6), the labeled reaction product was so large as to preclude its entrance into 3% polyacrylamide gels. The aggregation was noted to be progressive with successive increases in substrate concentration and also occurred in discrete steps when analyzed with respect to time of incubation with a fixed concentration of substrate on NaDodSO$_4$ gel (Figure 7).

Protease studies (Figure 2, lanes 7–10) showed that the labeled poly(ADP-Rib) was associated with nucleosomal proteins (also see below). Accordingly, when labeled proteins were analyzed on NaDodSO$_4$–polyacrylamide gels with respect to time of complex formation with 100 μM NAD (Figure 3, lanes 1–3) or under chase conditions with nonradioactive NAD (lanes 4–6), a progressive increase in size of product was noted. However, it was not clear at this level of analysis whether the complexed labeled material (Figure 2B) represented modified chromatin per se or, alternatively, components of chromatin (i.e., automodified polymerase, cross-linked histones, etc.). No obvious increase in ethidium bromide staining material was detected at the labeled positions in the chromatin gels at the higher substrate concentrations (Figure 2A). Retarded mobility in this gel system must be a consequence of one or both of the following: (a) change in size or conformation and/or (b) a net charge on the particles. Although long poly(ADP-Rib) chain lengths are generated under these conditions, data presented below (Figure 5) would tend to exclude charge as the only determinant for the retarded mobility.

Complex Formation and Poly(ADP-Rib) Chain Lengths. Poly(ADP-Rib) chain lengths were determined in native and highly complexed samples of chromatin, generated by increasing NAD concentrations. The polymer was cleaved from the chromatin acceptor proteins with Tris buffer (pH 9.5) and subsequently electrophoresed. Under these relatively mild conditions, not all of the poly(ADP-Rib) is quantitatively released from proteins (Adamietz et al., 1978). The data (Figure 4) reveal a broad size range of poly(ADP-Rib) chains ranging from mono(ADP-Rib) to chains 60–90 residues in length. It was of considerable interest to note that the length of the most highly radioactive chains, within each series, was also NAD concentration dependent. Under conditions of maximal aggregation (100 μM NAD), the poly(ADP-Rib) barely entered the gels. On the basis of the correlation between NAD concentration and both polymer chain lengths and extents of aggregation, it is likely that poly(ADP-Rib) association with nucleosomal proteins may be causing the major contribution to the aggregation phenomenon noted above (Figures 2 and 3).

NAD Levels and Oligonucleosome Sedimentation. Octanucleosomes were incubated with increasing concentrations of NAD in the presence of constant [^{3}H]NAD and subsequently subjected to centrifugation on linear sucrose gradients

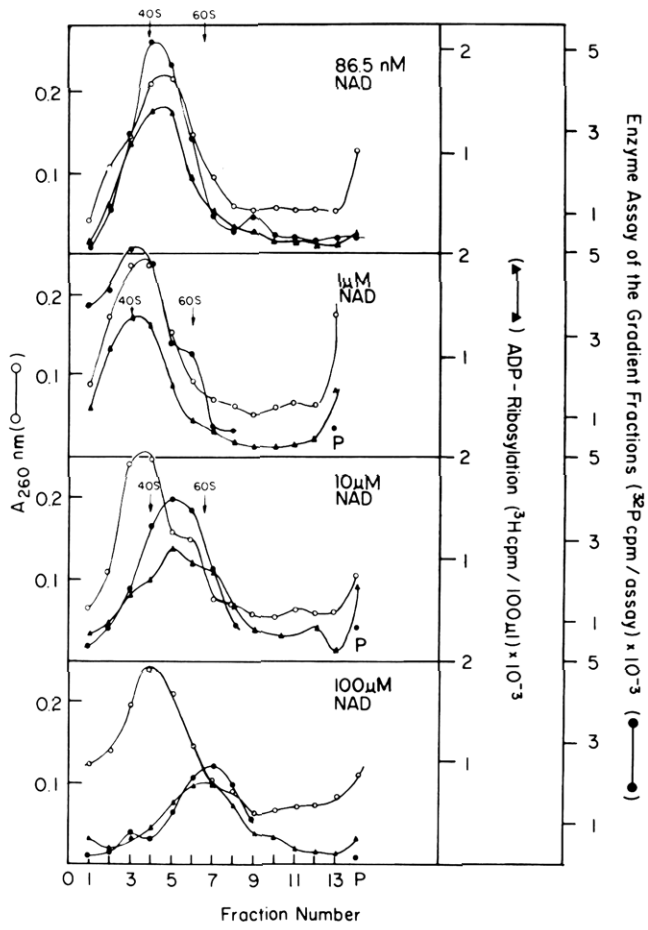

FIGURE 5: NAD concentration-dependent aggregation of polynucleosomes and polymerase activity as analyzed by sedimentation velocity. Chromatin fragments of 9–11 repeat length were pooled from the fractions of the gradient (Figure 1, lanes 8 and 9) and incubated as in Figure 2. Polynucleosomes (0.7 A_{260} unit) were incubated with 86.5 nM, 1 μM, 10 μM and 100 μM unlabeled NAD in the presence 1 μCi of [^{3}H]NAD. The reactions were terminated with nicotinamide, and samples were dialyzed for 3 h as described under Materials and Methods and applied onto 10–30% sucrose gradients. The gradients were centrifuged for 2 h at 45 000 rpm in a Beckman SW50-1 rotor at 4 °C. The gradients were fractionated into 13 fractions (0.32 mL each), and the A_{260} was read manually (O—O). After fractionation of the gradient, the bottom of the tube was scraped and washed with 10% sucrose gradient buffer (0.32 mL) and is represented as pellet P. A portion of each fraction (100 μL) was precipitated with 5% trichloroacetic acid to determine [^{3}H]-poly(ADP-ribosylation) (▲—▲). A 50-μL sample of selected fractions were subsequently assayed for poly(ADP-Rib) polymerase activity by using [^{32}P]NAD (0.5 μCi/μM per assay) (●—●). HeLa ribosomal subunits (1.6 A_{260} units) were centrifuged as markers in a separate gradient.

(Figure 5). The gradients were fractionated, and the A_{260} and [^{3}H]ADP-ribosylated chromatin were determined for each fraction. Subsequently, each fraction was assayed for poly-(ADP-Rib) polymerase activity by utilizing [^{32}P]NAD. In agreement with the electrophoretic analysis (Figure 2A), the A_{260} profiles of the fractionated chromatin indicate that the sedimentation properties of the bulk of the nucleosomes were not affected by NAD concentrations up to 100 μM. Also, in accordance with the electrophoretic data (Figure 2B), a progressive increase in the sedimentation rate of poly(ADP-ribosylated) material (^{3}H containing) was noted with increasing substrate concentration. Under the conditions of these experiments, the aggregated poly(ADP-ribosylated) chromatin complex had an *s* value of approximately 65 S while that of unmodified octanucleosomes was 40 S.

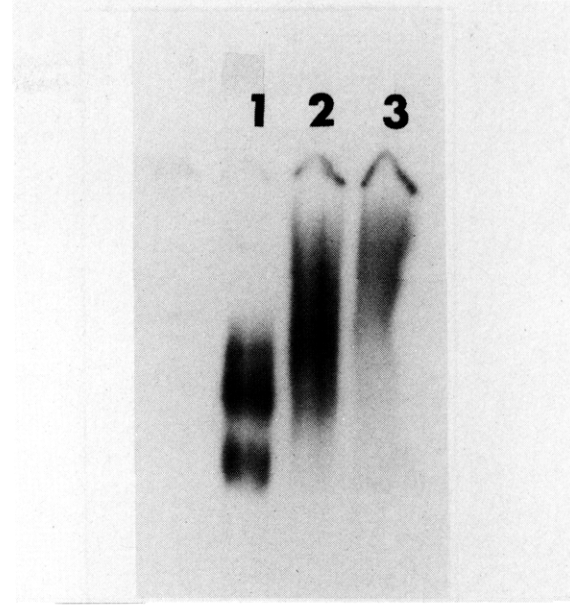

FIGURE 6: Presence of poly(ADP-Rib) polymerase on aggregated forms of chromatin by in situ enzyme analysis performed directly on polyacrylamide gels. Octanucleosomes (0.1 A_{260} unit) were either preincubated in the absence of NAD (lane 1) or in the presence of 10 μM and 100 μM *unlabeled* NAD (lanes 2 and 3). The reaction was terminated with nicotinamide, and the samples were applied to a 2.5–8% gradient polyacrylamide gel. The samples were electrophoresed for 8 h. The intact slab gel was subsequently incubated with a polymerase assay mixture containing [^{32}P]NAD, and the reaction was terminated and processed for autoradiography as described earlier (Giri et al., 1978). (Lane 1) Preincubation in the absence of NAD; (lane 2) preincubation with 10 μM NAD; (lane 3) preincubation with 100 μM NAD.

Purified poly(ADP-Rib) polymerase has been shown to possess a strict requirement for both DNA and nuclear protein acceptors for enzymatic activity (Jump et al., 1980a). Accordingly, in vitro assays were performed with [^{32}P]NAD across the various fractions of the sucrose gradients shown in Figure 5. Enzyme activity coincided with the chromatin absorbance profile in octanucleosomes incubated with 86 nM and 1 μM NAD. However, at higher NAD levels, the enzymatic activity associated with octanucleosomes cosedimented with the aggregated chromatin material. The data suggest that components of chromatin migrating in fractions 6–9 of Figure 5 (lower panel) with an *s* value of 65 S not only possessed [^{3}H]poly(ADP-ribosylated) material with very long chain lengths (see Figure 4) but also, in addition, catalytically active enzyme and presumably DNA. However, because of the overlap in the separation of chromatin species on the gradient and since the enzyme can undergo automodification in the absence of histones (Jump & Smulson, 1980), the above data, alone, do not support the presence of histones, DNA, or actual nucleosomes in these heavier fractions. However, the sedimentation coefficient of this material makes it very likely that a complexed form of chromatin is present in fractions 6–8 of the gradient.

Further support for the above observation was obtained by the experiment shown in Figure 6. It has previously been shown (Giri et al., 1978) that poly(ADP-Rib) polymerase activity can be conveniently assayed, via radioautographs, directly on nucleosomal particles separated on native polyacrylamide gels and that this in situ activity can be abolished by polymerase inhibitors.

In the experiment in Figure 6, chromatin aggregation was progressively accomplished by incubation of oligonucleosomes with increasing concentrations of nonradioactive NAD and

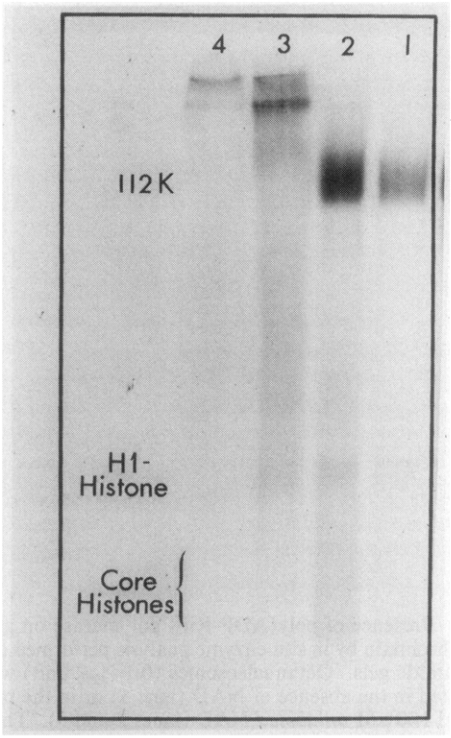

FIGURE 7: Identification of poly(ADP-Rib)–protein aggregates of different complexity on $NaDodSO_4$ gels. Polynucleosomes of 16 N (0.4 A_{260} unit) were incubated under standard conditions of poly(ADP-ribosylation) with 0.18 μCi of [^{32}P]NAD in the presence of 25 nM (lane 1), 1 μM (lane 2), 10 μM (lane 3), and 100 μM (lane 4) nonradioactive NAD. Denatured proteins were applied to 10% polyacrylamide gels containing 0.1% $NaDodSO_4$ and subjected to electrophoresis as described under Materials and Methods.

subsequently subjected to electrophoresis in native gels as described in Figure 2. An in situ enzyme assay was then performed directly on the gel by using [^{32}P]NAD. No alteration was detected in the mobility of nucleosomes incubated with nonradioactive NAD as detected by ethidium stain (data not shown). In the absence of preincubation with NAD, in situ enzymatic activity coincided with the main-band nucleosomes (Figure 6, lane 1). In contrast, there was a progressive decrease in mobility of in situ activity noted in lanes 2 and 3. The activity moved on the gels as apparent distinct particles. This radioautograph supported the sedimentation data and suggested the presence of distinct high-molecular-weight complexes possessing poly(ADP-Rib) polymerase activity and possibly its associated DNA and acceptor proteins.

Relationship of NAD Concentration to Protein Modifications. Since earlier data (Figure 4) had indicated progressively longer chains of poly(ADP-Rib) associated with nuclear proteins as a function of NAD concentration in the assay, it was of importance to examine the status of modification to proteins.

Radioautograms (Figure 2B) demonstrated that while minimally modified nuclear proteins ($\leq$1 μM NAD) remained associated with oligonucleosomes used initially for the assays, proteins labeled with NAD >10 μM migrated entirely with higher molecular complexes. Therefore, all radioactive nuclear proteins isolated under these conditions must be derived from these complexes. The influence of NAD concentration on the poly(ADP-ribosylation) of total nuclear proteins was examined by $NaDodSO_4$–polyacrylamide electrophoresis (Figure 7). The data, using low concentrations of NAD of high specific activity (lanes 1 and 2), confirm previous observations from our laboratory on the specificity of nuclear protein ADP ribosylation (Jump et al., 1980a). Approximately 20% of the radioactivity was incorporated into core histones and histone H1, whereas the majority of the modification was detected in a broad band of molecular weight 112 000 representing the automodified forms of poly(ADP-Rib) polymerase. In contrast, the major labeled product obtained with higher concentration of NAD (lanes 3 and 4) migrated on $NaDodSO_4$ gels as a high-molecular-weight aggregated product, although some histone modification is still evident at 10 μM NAD (see also Figure 3). On the basis of the histone H1 cross-linking capacity of poly(ADP-Rib) (Stone et al., 1977), it is possible that the aggregated proteins might represent cross-linked nuclear proteins, perhaps still maintaining polynucleosomal structure (Butt et al., 1980). Less ADP-ribosylated histones and histones H1 were detected in the sample incubated with 100 μM NAD (lane 4). It is possible that these histones are complexed to the major high molecular weight band, resistant to $NaDodSO_4$ treatment, in the aggregated sample (see below). This is totally consistent with recent observations of Adamietz et al. (1979). In this study, only ADP-ribosylated nuclear proteins were retained on boronate cellulose columns. These modified proteins were found to migrate on $NaDodSO_4$ gels as high molecular weight complexes. However, after phosphodiesterase cleavage of poly(ADP-Rib) from these samples, considerable histones were detected, suggesting that they were initially cross-linked in a higher protein complex.

However, since the 112 000-dalton protein is the major ADP-ribosylated nuclear protein under these conditions, it must be assumed that the major radioactivity shown in Figure 2 is associated with this aggregated 112 000-dalton protein.

Pulse Chase of Modified Histones. The fate of ADP-ribosylated histones during complex formation was further investigated in a pulse–chase study where chromatin was incubated at low concentrations of [^{32}P]NAD for 30 s followed by a chase with nonradioactive 100 μM NAD for 1 and 3.5 min. Total histones were acid extracted, and histone H1 was selectively extracted and examined on 15% acid/urea polyacrylamide gels (Figure 8). A considerable amount of labeled material did not enter the gel in this experiment. The labeled proteins isolated after the 100 μM NAD chase would represent proteins associated solely with low mobility complexes (see Figure 2B). When total histones were examined (Figure 8, lanes 1–3), the most prominent changes noted during the chase with nonradioactive 100 μM NAD were the following: (1) the apparent loss of labeled histones from their original positions on the gel; (2) the appearance of new, higher molecular weight bands; and (3) the gradual change from mono(ADP-ribosylated) histone H1 to the higher molecular weight "H1 complex" (Stone et al., 1977). The alteration in the level of H1 modification was more clearly evident when this histone was selectively extracted (lanes 4–6). In low NAD (lane 4), the majority of the modification represented mono(ADP-ribosylation) of H1. During the chase with 100 μM nonradioactive NAD, the short chains were elongated, as shown by the 12–15 intermediate bands (lane 5) in the final construction of the "H1 complex" (lane 6). The H1 complex has been shown to migrate in $NaDodSO_4$ gels at a position corresponding to a molecular weight twice that of H1, while the intermediates migrate close to the unmodified H1 species (Nolan et al., 1980). The possible relationship of this NAD concentration-dependent reaction and the aggregation of nucleosomes and/or chromatin components is further investigated in the accompanying manuscript (Butt et al., 1980).

Discussion

The aggregation of chromatin by poly(ADP-Rib) would imply the interaction of the enzyme(s) with local, or distal,

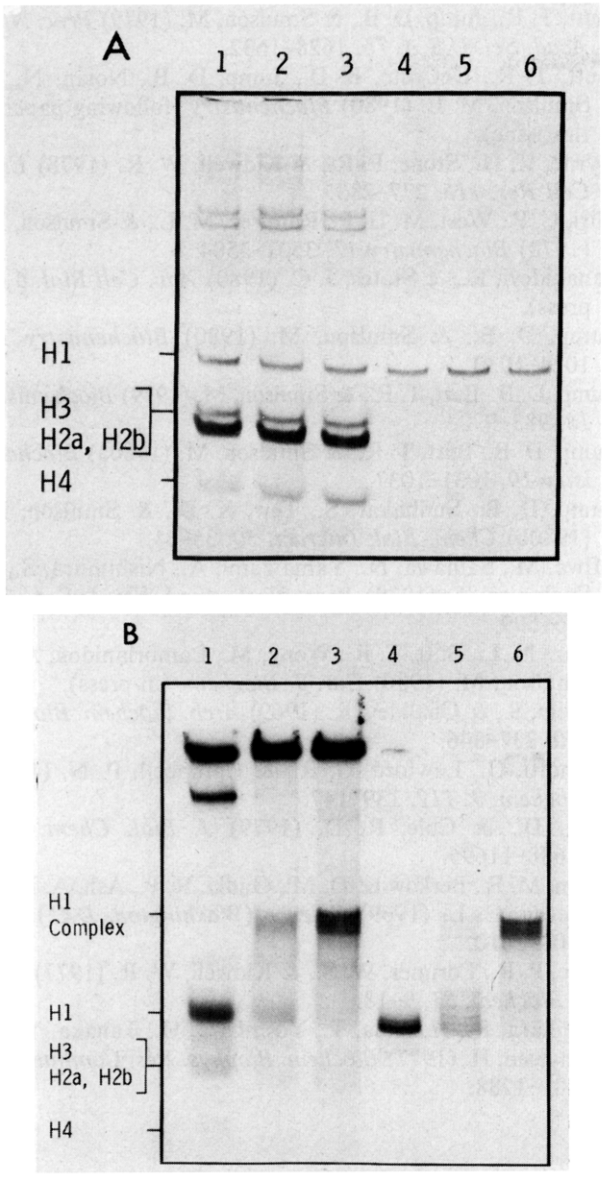

FIGURE 8: Analysis of acid-soluble proteins and histone H1 during a chase with 100 μM NAD. Chromatin fragments of 8–12 nucleosome repeat length (2.0 A_{260} units) were incubated under standard conditions. The reaction was initiated by the addition of 6 μCi of [^{32}P]NAD (25 nM), and after 30 s at 20 °C, two equal aliquots were removed (lanes 1 and 4). The rest of the samples were chased with NAD to final concentration of 100 μM for 1 min (lanes 2 and 5) and 3.5 min (lanes 3 and 6). In one series of samples (lanes 1–3), the reaction was terminated with 0.5 N H_2SO_4 while in the other series (lanes 4–6), the reactions were terminated with 5% perchloric acid and the samples subsequently extracted for total histones and histone H1, as described under Materials and Methods. In all cases, 0.5 A_{260} unit of nuclease-soluble chromatin was used as the carrier during the extraction procedures. The extracted histone samples were electrophoresed on 15% acid/urea gels for 10 h. (A) Stained gel; (B) autoradiograph.

stretches of polynucleosomes within chromatin. The progressively longer chain lengths of the polymer which appeared to correlate with levels of chromatin complexation (Figure 4) would argue for such networks of interactions. It has recently been shown by trypsin digestion studies that the ADP-ribosylation of cores histones is predominantly at the hydrophilic amino-terminal regions of these proteins (Burzio et al., 1979; Jump et al., 1980b). In addition, a branched poly(ADP-Rib) structure has been described (Miwa et al., 1979). Both of these observations would be consistent with a mechanism whereby chains of poly(ADP-Rib) could connect stretches of nucleosomes, assuming the enzyme is strategically located along the chromatin fibril. The ability of poly(ADP-Rib) to cross-link histone H1 (Stone et al., 1977, Figure 8) makes a nucleosome network hypothesis more attractive. NAD could activate such networks while poly(ADP-Rib) glycohydrolase could release such systems.

Consistent with a chromatin ligation reaction due to poly(ADP-Rib) is the in vitro observation that larger polynucleosomes favor the synthesis of the H1 cross-link over smaller particles (Nolan et al., 1980). In addition, small nucleosomes favor automodification of the polymerase itself, whereas histone ADP-ribosylation requires reasonably larger (8–10 N) polynucleosomes (Jump et al., 1980a). The data shown in the $NaDodSO_4$ gels of ADP-ribosylated proteins (Figure 7) are consistent with higher orders of protein connection by networks of longer and longer (Figure 4) NAD-dependent connectors. The levels of protein aggregation, just as the chromatin complex formation itself (Figure 2), were highly dependent on NAD concentration. This was most clearly emphasized when nucleosomes were "pulsed" with low levels of [^{32}P]NAD (Figure 8) (where only short chains of polymer are generated) and then "chased" with the high level of NAD required for complex formation. A marked aggregation of nuclear protein in these experiments was noted.

Further support for the hypothesis that poly(ADP-Rib)-connecting elements caused the marked effects on chromatin or chromatin components noted in this study has been provided by experiments (Byrne et al., 1978) showing that polyamines and Ca^{2+}, in the course of condensing soluble chromatin, also stimulated the synthesis of the poly(ADP-Rib) H1 dimer. The converse questions have been addressed, in a preliminary fashion, in the present work. Can a putative association of poly(ADP-Rib) with nucleosomal histones, in a highly specific (but quite limited) domain of chromatin, cause condensation of these structures?

Two new pieces of data lend strong support to the data presented in this and the accompanying manuscript. Janakidevi & States (1980) showed that the thermal denaturation of large chromatin (but not smaller) structures incubated with NAD was extended significantly over control chromatin at temperatures above 90 °C, suggesting that poly(ADP-ribosylation) was stabilizing a specific domain of chromatin. While whole chromatin analysis is quite complicated, it might be expected that the aggregated components in gels such as that shown in Figure 2 would possess enhanced stability to thermal denaturation. Adamietz et al. (1979) have developed methods which appear to segregate highly aggregated and/or cross-linked poly(ADP-ribosylated) nuclear proteins from nonmodified chromatin proteins by an affinity boronate procedure. These proteins migrate on $NaDodSO_4$ gels in size ranges of 200 000 daltons. It is noteworthy that when poly(ADP-Rib) is released from these high molecular weight proteins, electrophoretic analysis clearly demonstrates the generation of a significant quantity of core histones and histone H1. It must be concluded that these latter proteins were initially present as poly(ADP-Rib) complexes.

One potential complication in these studies is the automodification of the polymerase itself (Yoshihara et al., 1977; Jump & Smulson, 1980). In many instances this appears to be a major incorporation product of the NAD reaction. The significance of the reaction is obscure at present; however, it is possible that this process might bestow upon the polymerase itself multiple aggregation or cross-linking properties.

We have previously provided data showing that polymerase specific activity increases in vitro progressively with increasing nucleosome repeat size, reaching a maximum at approximately

8–10 nucleosomes (Butt et al., 1978). Further analysis of the polymerase automodification (Jump et al., 1980a) suggested that the above enzyme activity data might be related to a periodic distribution of the enzyme protein itself in certain domains of chromatin. If this is so, it might place the enzymatic poly(ADP-ribosylation) capacity in strategic positions for connecting and releasing (via glycohydrolase) folded regions within chromatin higher ordered structure.

It is, of course, possible that histones containing long chains of poly(ADP-Rib) might become released from nucleosomes and self-aggregate independent of chromatin. A major question thus is whether highly aggregated proteins *not* associated with chromatin particles would be of such size or possess macromolecular properties to sediment around 65 S (Figure 5) in sucrose density gradients or to allow migration in low percentage gels to positions corresponding to huge complexes (Figure 2). This seems unlikely; however, in the present work only the polymerase itself and modified histones have been *directly* detected in the complex; DNA has been detected only indirectly. The difficulty in analysis of the complex is complicated by the very limited domain of polynucleosomes which, at least in vitro, appear to participate in this reaction. Accordingly, in the accompanying manuscript more sensitive methods, including nuclease digestion studies, have been employed to characterize the components of the poly(ADP-Rib)-induced complex. A significant contribution of histone H1 in aggregation formation is suggested by these data.

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