Characterization of a Putative Poly(adenosine diphosphate ribose)–Chromatin Complex†

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ABSTRACT: It was found in the accompanying manuscript [Butt, T. R., & Smulson, M. (1980) *Biochemistry* (preceding paper in this issue)] that the chromatin-associated enzyme poly(ADP-Rib) polymerase caused an NAD-dependent complex formation of a small but specific domain of chromatin and/or components of chromatin. A direct correlation between NAD concentration, the level of chromatin aggregation, and the length of poly(ADP-Rib) chains in the complex was detected. Direct evidence was provided suggesting that at the least, ADP ribosylated histones and poly(ADP-Rib) polymerase were present in the complexed material. Additional parameters have been measured here to further characterize the components and organization of this complex. Digestion studies of the complex with Proteinase K and phosphodiesterase indicated that the poly(ADP-Rib) polymer was bound to proteins and also was partially resistant to digestion within the complex. Studies with micrococcal nuclease, at both the nuclear and oligonucleosome level, demonstrated a significant resistance to digestion of domains of poly(ADP-ribosylated) chromatin as compared to unmodified regions of chromatin. However, the appearance of the characteristic nucleosome repeat cleavage products of chromatin could be demonstrated in the labeled complex after digestion with DNase I and micrococcal nuclease. A putative involvement of histone H1 and the poly(ADP-Rib)-induced cross-linking of this histone in complex formation was suggested. It was found in vitro that H1 poly(ADP-Rib) dimer formation occurred at those NAD concentrations favoring chromatin aggregation. Furthermore, the selective removal of histone H1 from oligonucleosomes by a gentle Dowex procedure abolished the poly(ADP-Rib)-promoted aggregation of these particles.

For several years we have been interested in chromatin structure and in the role that poly(ADP-ribosylation) plays in nucleoprotein organization. Although it is clear that core histones become covalently modified by this enzyme (Giri et al., 1978a; Jump et al., 1979) and that poly(ADP-ribosylation) of histone H1 leads to a complex cross-link of this protein (Stone et al., 1977), more information is needed about the resultant conformational changes in chromatin if we are to understand the role ADP-ribosylation plays in biological function. In seeking answers to these questions, we initially concentrated on the influence of the various levels of chromatin complexity on the activity and on the acceptors for poly(ADP-Rib).[1] For example, it was found that the enzyme is not bound to core nucleosomes but associated with internucleosomal regions of chromatin (Giri et al., 1978b). The nuclear proteins modified in simple nucleosomes (Giri et al., 1978a,b) or in more complicated structures (Jump et al., 1980a) were characterized in detail. A relationship between the polynucleosome chain length employed in an in vitro assay and the specific activity of the enzyme was observed (Butt et al., 1978). However, more recently we have been interested in the converse, that is, the effect of ADP-ribosylation on chromatin structure. For example, preliminary techniques have been developed to reconstitute purified polymerase with polynucleosomes depleted of the enzyme (Jump et al., 1980a) to aid in understanding the influence of this modification on chromatin.

The preceding manuscript (Butt & Smulson, 1980) was concerned with the structural changes induced in purified nucleosomes upon ADP-ribosylation. Of interest was the observation that an NAD concentration-dependent aggregation of chromatin, or at least components of chromatin, was observed by either electrophoresis or velocity sedimentation. Presumably only a small domain of chromatin contains polymerase (Jump et al., 1979, 1980a), and, accordingly, only a small fraction of nucleosomes participated in this complex formation. This facet made identification of whether organized nucleosomes existed in the complex difficult, and part of the work below addresses this question by use of more sensitive methods.

It is still not clear whether the reaction under study represents polynucleosome condensation. In the preceding paper (Butt & Smulson, 1980), NAD-dependent generation of long chains of poly(ADP-Rib) were correlated with complex formation, consistent, but not definitive, proof of a connection between the two phenomena. Micrococcal nuclease has been most useful in the past in elucidation of structural domains of nucleosomes. We have used this nuclease along with other enzymes to characterize the complex in more detail.

There is at least one well-characterized cross-linking reaction of poly(ADP-Rib), namely, the H1 poly(ADP-Rib) dimer (Stone et al., 1977), and new evidence (Adamietz et al., 1979) suggests that poly(ADP-Rib) may complex other histones and nonhistone proteins. A role for H1 histone has been advanced recently implicating this histone in the maintenance of higher-order structures of chromatin. With this in mind, and also in view of the ability of poly(ADP-Rib) to cross-link this histone, we have begun to develop techniques to study the possible involvement of H1 histone with the aggregation of chromatin promoted by poly(ADP-ribosylation).

Materials and Methods

Materials. [^{32}P]NAD (32–56 Ci/mmol) and [*adenine*-2,8-^{3}H]NAD (around 3–4 Ci/mmol) were purchased from New England Nuclear or synthesized as described by Jump et al. (1979). Venom phosphodiesterase, micrococcal nuclease, DNase I, and DNase II were purchased from Worthington.

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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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Proteinase K was a product of Merck. HeLa S_3 cells were grown logarithmically in suspension cultures. Cells were routinely harvested at a density of (5–8) × 10^5/mL.

Preparations of HeLa Nuclei and Chromatin. Nuclei were prepared by the method of Sporn et al. (1969) or, alternatively, cells were suspended (5 × 10^7/mL) in buffer A (10 mM Tris-HCl, pH 8.0, 0.25 M sucrose, 0.1 mM $CaCl_2$, 0.2 mM PMSF, and 0.5% Triton X-100) and dounce homogenized (20 strokes) at 4 °C. Nuclei were sedimented at 2000*g* for 10 min and resuspended to the same concentration in buffer A. The nuclei were homogenized and sedimented as above. They were then washed 2 times with buffer A without Triton. For results described in Figures 4 and 5, the chromatin was prepared as follows. Nuclei were suspended in nuclease digestion buffer and digested with 40 units of micrococcal nuclease at 37 °C for 2 min as described in the preceding paper (Butt & Smulson, 1980). The solubilized chromatin was separated on 5–20% linear sucrose gradient (80 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, and 0.2 mM PMSF). The centrifugation was performed at 4 °C in an SW40 rotor at 38 000 rpm for 13 h. The centrifugation procedure was adequate to resolve di- and trinucleosome peaks, which were pooled, incubated with [^{32}P]NAD and ADP ribosylation, and dialyzed. The oligonucleosome particles were then probed with 43 units of micrococcal nuclease/A_{260} for various periods of time, and the product was analyzed on 5–20% sucrose gradient, as above.

For analysis of the action of various nucleases on poly(ADP-ribosylated) chromatin, polynucleosomes were prepared and ADP-ribosylated as described in the preceding paper (Butt & Smulson, 1980). After termination of the ADP-ribosylation reaction, the concentration of the components in the chromatin suspension was 60 mM Tris-HCl, pH 8.0, 80 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, 0.2 mM EDTA, 5 mM nicotinamide, and around 20% sucrose. The chromatin suspension was distributed into five equal aliquots and treated with different enzymes. (a) For micrococcal nuclease, the chromatin was made to 1.0 mM $CaCl_2$ and the reaction initiated by the addition of nuclease (10 units/0.15 A_{260}). Three equal samples (0.05 A_{260} each) were removed, and the reaction was terminated by 5 mM EDTA at 1, 3, and 7 min. (b) Proteinase K reaction was terminated by addition of PMSF (1.5 mM) and chilling the tubes in ice. (c) For DNase I, the pH of the suspension was adjusted to 6.8 by addition of HCl. The reaction was initiated with DNase I (1 unit/0.15 A_{260}) and terminated after 1, 3, and 7 min by the addition of EDTA (5 mM). (d) Venom phosphodiesterase was added to the chromatin (10 μg/0.15 A_{260}) and the reaction terminated as in (c). (e) For DNase II studies, the pH of the chromatin was adjusted to 6.8 and the reaction was initiated by addition of 10 units of DNase II. The reaction was terminated by chilling the tubes.

The samples were applied on to gradient chromatin gels (3–10%), and the electrophoresis was performed at 4 °C as described previously (Butt & Smulson, 1980). For better resolution of the bands on chromatin gels, it was desirable to apply a minimal volume (20–40 μL) per gel slot. After electrophoresis, the gel was stained with ethidium bromide, chromatin was fixed with acid, and the gel was processed for autoradiography as described earlier (Butt & Smulson, 1980).

Kinetics of Micrococcal Nuclease Action and Determination of Perchloric Acid Soluble Chromatin. The kinetics studies were performed according to Axel et al. (1973). An equal volume of cold 2 M $HClO_4$ and 2 M NaCl was added to a tube of digested nuclei. The nuclei were kept on ice for 60 min and the precipitate was pelleted at 10000*g* for 15 min. The absorbance at 260 nm of the supernatant was determined and corrected for scatter by reading at A_{350} nm. The absorbance of undigested nuclei treated in the same manner was subtracted from each value. The percentage of digestion was calculated as (A_{260} × 100/A_{260}) − (undigested × 1.67). The 1.67 corrects for hyperchromicity of nucleotides in acid (Sollner-Webb et al., 1976). The validity of this method was determined by trichloroacetic acid precipitation of uniformally labeled [^{3}H]DNA in nuclei.

Histone H1 was preferentially extracted by John's procedure as described by Stone et al. (1977). Acid/urea gels were performed according to Panyim & Chalkley (1969). Chromatin was stripped of histone H1 by Dowex 50W-X2 treatment (Thoma & Koller, 1977). All the procedures were performed at 1–4 °C. Chromatin particles were pooled from a sucrose gradient, and an equal volume of extraction buffer (40 mM NaCl, 100 mM $NaHSO_3$, and 100 mM NaH_2PO_4, pH 6.8) was added followed by an addition of 0.25 volume of Dowex 50W-X2 slurry. The samples were stirred for 30 min, and the chromatin was recovered by centrifugation. The recovery of the stripped chromatin was normally 70% of the total extracted.

Results

Enzyme Digestions To Characterize NAD-Induced Chromatin Complex. Oligonucleosomes (≤10 N) were incubated with 10 μM [^{32}P]NAD, conditions shown in the preceding paper (Butt & Smulson, 1980), favoring complex formation of poly(ADP-ribosylated) nucleosome components. For indirect characterization of this complex, the samples were subsequently treated with enzymes which either degrade poly(ADP-Rib) attached to nuclear proteins or, alternatively, cleave polynucleosomes to yield characteristic nucleosomal products. The effects of these treatments on bulk oligonucleosomes were followed by ethidium bromide staining of the chromatin after electrophoresis (Figure 1A); effects on the complex were followed by the radioautograms of the gels (Figure 1B).

Proteinase K. Little effect was noted on overall nucleosome structure by this enzyme under these experimental conditions (Figure 1A, lanes 1 vs. 5–7). In contrast, the highly reduced electrophoretic mobility of poly(ADP-ribosylated) components in the complex was abolished, confirming that ADP-Rib chains are associated with polynucleosomal proteins. In this connection it was recently shown, using purified poly(ADP-ribosylated) core nucleosomes, that 80% of the label was released by trypsin digestion (Jump et al., 1980b) with the expected minimal effect on nucleosome structure (Whitlock & Simpson, 1977).

Snake Venom Phosphodiesterase. This enzyme is known to cleave the pyrophosphate linkage of poly(ADP-Rib). It was noted to reduce the labeling in the complex by 50% (Figure 1B, lane 1 vs. lanes 11–13), indicating that the polymer is possibly in a buried form in the complex, relatively resistant to cleavage.

DNases I and II. Both enzymes cleaved bulk polynucleosomes to yield characteristic chromatin fragments (Figure 1A; cf. lanes 1 and 8–10; 14–16); however, ADP-ribosylated proteins were noted to be associated, to a limited extent, with DNase I degradation products (Figure 1B, lane 1 vs. lane 10).

Micrococcal Nuclease. The generation of lower oligomers and ultimately mononucleosomes by this enzyme was essentially complete after 7 min (Figure 1A; cf. lanes 1–4). It was of interest that a small but significant portion of the labeled

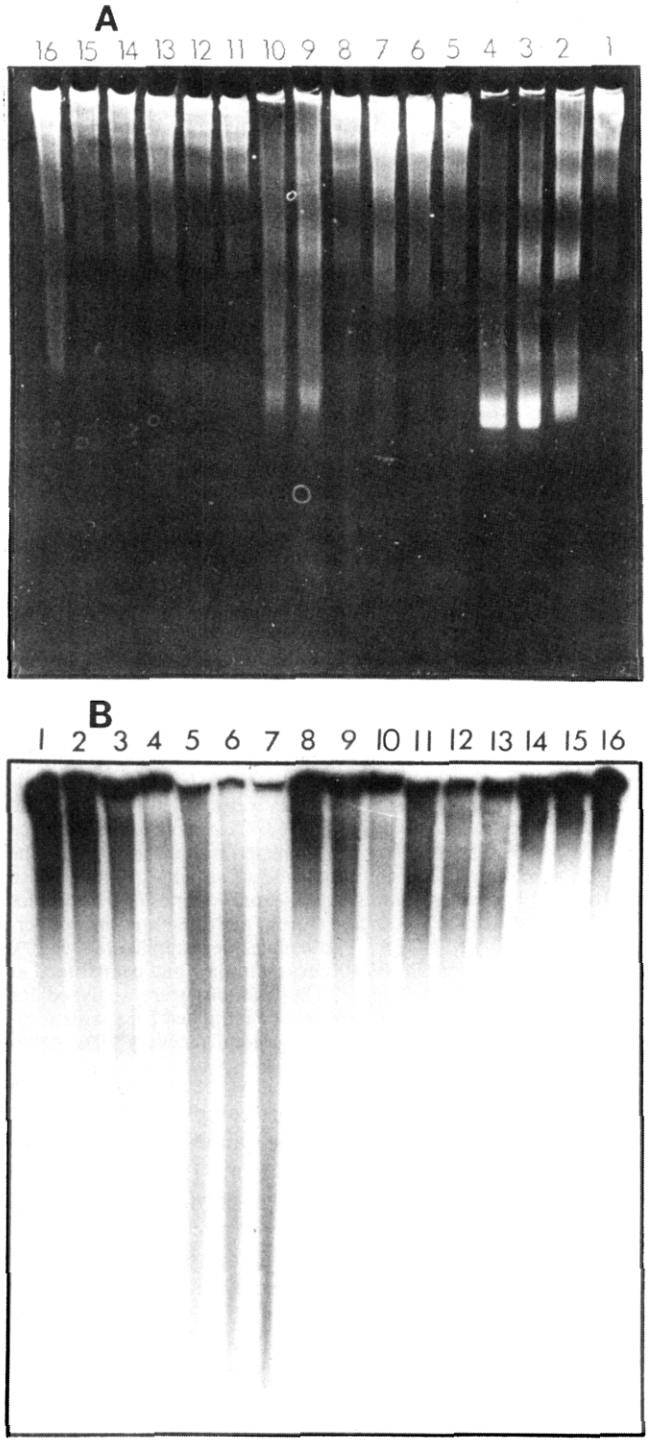

FIGURE 1: Action of various enzymes on poly(ADP-ribosylated) complexed chromatin and analysis on native chromatin gels. Chromatin (0.75 A_{260} unit; 10–12 N polynucleosomes) was incubated with 2 μCi of [^{32}P]NAD in the presence of 10 μM unlabeled NAD for 5 min at 20 °C under standard conditions. The reaction was terminated with nicotinamide (5 mM), the chromatin distributed into five equal aliquots, and each adjusted for conditions suitable for the respective enzyme treatment. Each aliquot was treated with the enzymes listed below, and the reactions were terminated by removing equal samples for electrophoresis at 1, 3, and 7 min as described under Materials and Methods. (Lane 1) Control (no enzymes added); (lanes 2–4) micrococcal nuclease; (lanes 5–7) Proteinase K; (lanes 8–10) DNase I; (lanes 11–13) snake venom phosphodiesterase; (lanes 14–16) DNase II. (A) Ethidium bromide stain; (B) autoradiograph.

complex was also cleaved to distinct lower sized nucleosomes. These data suggest, but are far from conclusive evidence, that chromatin components organized in nucleosomes are complexed by poly(ADP-Rib). It has been observed previously that SV40 chromatin, condensed by histone H1, becomes relatively resistant to cleavage by micrococcal nuclease (Varshavsky et al., 1977). The data obtained here on chromatin may also be related to these observations. On the basis of these results, a comparison was made on the susceptibility of regions of chromatin modified by poly(ADP-Rib) polymerase to micrococcal nuclease vs. the susceptibility of unmodified regions of chromatin.

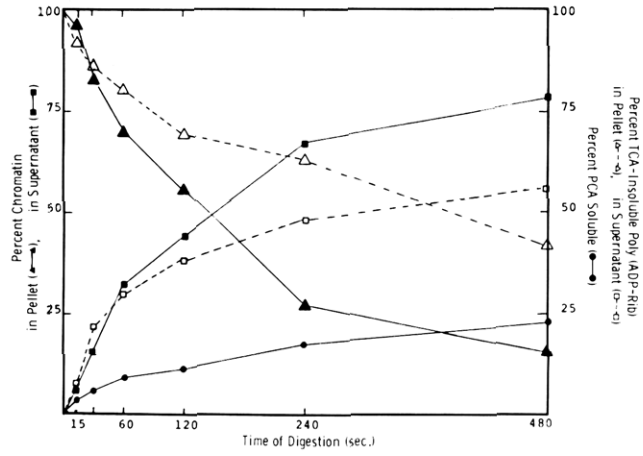

FIGURE 2: Micrococcal nuclease digestion of ADP-ribosylated HeLa nuclei. HeLa nuclei (5×10^7/mL) were modified with 1 mM [^{3}H]NAD (5 Ci/mol) for 10 min at 25 °C. Nuclei were washed in buffer and warmed to 37 °C for micrococcal nuclease digestion. Nuclei (5×10^7/mL) were digested for the times indicated with micrococcal nuclease (2 units/A_{260}) at 37 °C. Samples (0.7 mL) were removed and the reactions stopped by the addition of 0.07 mL of 0.1 M EDTA and cooled on ice. A portion (0.05 mL) was taken for measuring the fraction perchloric acid soluble (●—●). Soluble chromatin was separated from insoluble chromatin via hypotonic lysis of nuclei and sedimentation at 5000g for 10 min (4 °C). Pellets were resuspended in buffer. Chromatin DNA in the supernatant (■—■) and pellet fractions (▼—▼) was quantitated by absorbance at 260 nm. The supernatant (soluble) chromatin was corrected for hyperchromicity due to the nuclease solubilized nucleotides. Trichloracetic acid precipitable radioactivity associated with the supernatant fraction (□---□) and pellet (▽---▽) was determined.

Resistance of ADP-Ribosylated Oligonucleosomes to Micrococcal Nuclease. Figure 2 shows micrococcal nuclease digestion of HeLa nuclei previously incubated with 1 mM [^{3}H]NAD. The liberation of soluble ADP-ribosylated and bulk chromatin from ADP-ribosylated nuclei was measured. The data show that initially (15–60 s) bulk and ADP-ribosylated chromatins are released at similar rates. However, after 1 min of digestion, bulk chromatin in liberated more rapidly than modified chromatin.

In a second study to determine whether poly(ADP-ribosylation) of nuclear proteins had an effect on nuclease digestion, nuclei modified with 1 mM NAD or 1 mM NAD plus 10 mM thymidine [an inhibitor of poly(ADP-Rib) polymerase] were digested with nuclease. The results in Figure 3 show little if any effect on digestion kinetics with nuclease at 4 units/A_{260} of chromatin. However, at 1 unit/A_{260}, a significant resistance to nuclease digestion by prior ADP-ribosylation of HeLa nuclei was noted. Note also that the inhibitory effect on digestion could be reversed by including thymidine in the modification milieu.

It is likely that ADP-ribosylation is playing multiple roles in the cell nucleus by eliciting structural changes at different domains of the chromatin. One of these domains is more susceptible to the action of micrococcal nuclease, and thus it is interesting to note that the *specific activity* of ADP-ribosylation is high in the small fraction of labeled nucleosomes

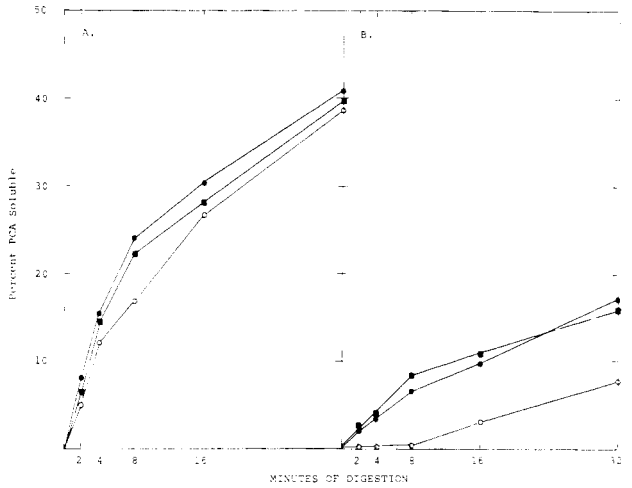

FIGURE 3: Effect of ADP-ribosylation of HeLa nuclei on the kinetics of different concentrations of micrococcal nuclease. HeLa nuclei (7 × 10^7/mL) were incubated in the presence of 1 mM NAD (○—○), 1 mM NAD + 10 mM Tdr (■—■), or no addition (●—●) for 5 min at 25 °C in modification buffer. Nuclei were washed free of the buffer and resuspended in micrococcal nuclease digestion buffer to 5 × 10^7/mL. Nuclei were digested for the indicated times with micrococcal nuclease at 4 (A) or 1 unit/A_{260} (B). Extent of digestion was determined by measuring the fraction of chromatin soluble in perchloric acid (see Materials and Methods).

which is released preferentially by action of the nuclease (Jump et al., 1979, and also Figure 2). However, the bulk of ADP-ribosylation occurs in the domain of chromatin which is relatively resistant to micrococcal nuclease (Figure 2). These results would suggest that ADP-ribosylation can induce structural changes, causing decreased accessibility of DNA to nuclease.

The effects seen in Figures 2 and 3 may be accomplished by compaction of nucleosomes into higher ordered structures mediated by electrostatic interaction between ADP-Rib and the positively charged residues on histones. The apparent resistance to digestion conferred upon chromatin by poly(ADP-ribosylation) was further investigated at the subunit level with purified nucleosomes (Figure 4). Chromatin enriched in trinucleosomes was prelabeled with [^{32}P]NAD, subjected to limited micrococcal nuclease digestion (0.5–5 min), and analyzed by velocity sucrose sedimentation (Figure 4). The ADP-ribosylated trinucleosome co-sedimented with the bulk of chromatin in the absence of digestion (A). During nuclease digestion, bulk chromatin (indicated by the A_{260} profile) was rapidly cleaved to monomers initially and to material by 3.0 min, sedimenting mainly at the top of the gradient. In contrast, the ADP-Rib-containing particles were found to be resistant to nuclease compared to unmodified trinucleosomes. After 5 min, a labeled particle sedimenting within the range of dimer was the remaining product. A similar experiment was performed with poly(ADP-ribosylated) dinucleosomes. By 3 min, after approximately 95% of the unmodified dimer was digested (data not shown), a resistant particle similar to that in Figure 4D was noted. These combined results appear to be comparable to the nuclease resistance of SV40-complexed minichromatin containing H1 (Varshavsky et al., 1977). In this system, chromatin is more compact, and the linker region is highly resistant to micrococcal nuclease.

NAD Concentration Dependence of Histone H1 Cross-Linking. A cross-linked complex consisting of two molecules of histone H1 and a poly(ADP-Rib) chain of 15–16 units has been described by Stone et al. (1977) and Adamietz et al. (1978) and more recently discussed by Ring & Cole (1979).

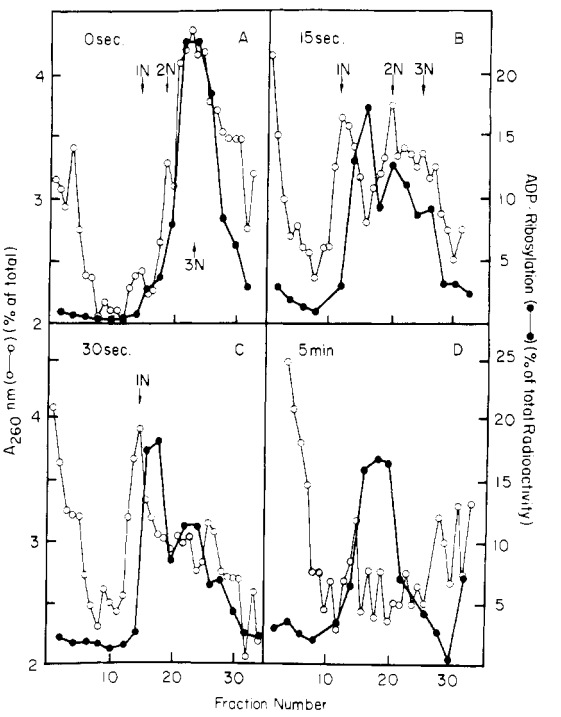

FIGURE 4: Comparative rate of digestion of modified and unmodified trinucleosomes by micrococcal nuclease. A preparation of chromatin enriched in trinucleosomes was isolated as described under Materials and Methods. The chromatin particles were pooled (5.6 A_{260}) and incubated with 2 μCi of [^{32}P]NAD (56.0 Ci/mmol) in the presence of 1 μM NAD. The reaction was terminated with nicotinamide. The samples were dialyzed, concentrated, and subsequently digested with micrococcal nuclease (50 units/A_{260}); equal volumes of the samples were removed after 0.5, 1.0, and 5 min, and the reaction was terminated with 10 mM EDTA. The samples were applied to a 5–20% linear sucrose gradient in SW40 Beckman rotor and centrifuged at 4 °C for 11 h at 38 000 rpm. The fractions were analyzed for acid-precipitable radioactivity (30 000 cpm applied per gradient recovery was around 80%) and for A_{260} (total recovery 70%–90%).

Agents which condense chromatin have been shown to stimulate the in vitro formation of this complex (Byrne et al., 1978). We have recently studied the synthesis of this complex in isolated polynucleosomes (Nolan et al., 1980). Fifteen H1 intermediates with progressively longer poly(ADP-Rib) chains were noted, and in vitro, larger polynucleosomes favored synthesis of this complex over a smaller structure. Naturally occurring H1 dimers and chemically cross-linked heterodimers of high mobility group proteins 14 and 17 with H1 and core histones have recently been described by Ring & Cole (1979), and even more complex potential H1 interactions with itself or other nuclear proteins were suggested by various workers (Chalkley & Hunter, 1975; Boulikas et al., 1980). In addition, histone H1 has been implicated in condensation of the spacer regions of DNA between core particles and in stabilizing higher ordered chromatin structures (Renz et al., 1977; Worcel, 1978). Because of the marked influence of NAD concentration on chromatin aggregation noted in this and the preceding paper (Butt & Smulson, 1980), it was of interest to study H1 complex formation under the same substrate ranges.

In the experiment shown in Figure 5, intact nuclei were incubated with 2 nM, 0.1 μM, and 100 μM [^{32}P]NAD, subsequently selectively extracted for histone H1, and electrophoresed on 18% polyacrylamide gels. At the two lower concentrations of NAD, where chromatin complex formation is not observed, no in vitro biosynthesis of histone H1 complex was found; instead mono-ADP-ribosylated histone H1 appeared to be the modified species (lane 5, Figure 5). In

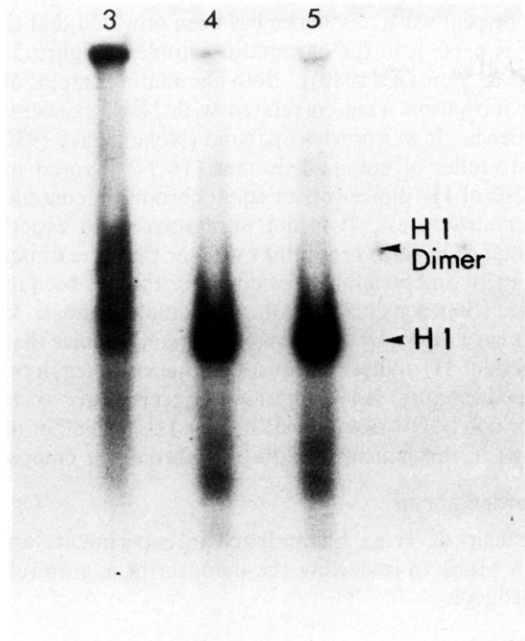

FIGURE 5: Effect of substrate (NAD) concentration on poly(ADP-Rib)–histone H1 complex formation. Nuclei (3×10^7) were incubated under optimal conditions for poly(ADP-Rib) polymerase with 0.5 μCi of [^{32}P]NAD in a volume of 0.5 mL at NAD concentrations of (lane 5) 2 nM, (lane 4) 0.1 μM, and (lane 3) 100 μM. H1 histone was selectively extracted from each sample as described by Stone et al. (1977) and electrophoresed in acetic acid/urea polyacrylamide slab gels, and the gels were exposed for radioautography as described under Materials and Methods.

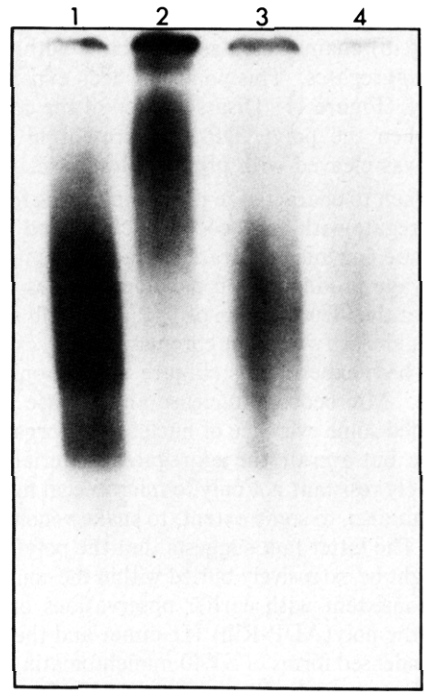

FIGURE 6: Effect of histone H1 depletion on ADP-Rib-induced chromatin complex formation. Polynucleosomes (8–10 N) were prepared as described under Materials and Methods and treated with Dowex 50W-X2 to selectively remove histone H1. An aliquot of polynucleosomes (0.07 A_{260} unit) with H1 (lanes 1 and 2) or without H1 (lanes 3 and 4) was incubated with 1 μM [^{32}P]NAD (lanes 1 and 3) or 100 μM [^{32}P]NAD (lanes 2 and 4). The reactions were terminated by placing the tubes on ice and by the addition of nicotinamide to 5 mM final concentration. The samples were electrophoresed on 3–8% gradient polyacrylamide gels and exposed for radioautography as described under Materials and Methods.

contrast, under conditions of chromatin aggregation (100 μM NAD), considerable histone H1 poly(ADP-Rib) dimer synthesis was observed (lane 3, Figure 5). Pulse (1 μM [^{32}P]-NAD) and chase (100 μM nonradioactive NAD) experiments presented in the preceding paper [Figure 8, lanes 4 and 6; Butt & Smulson (1980)] demonstrated the substrate concentration dependency of H1 complex formation in isolated nucleosomes during a pulse–chase experiment. In this context, it is of interest that homopolymers of H1 higher than dimer, due to poly(ADP-ribosylation), were suggested by gel analysis of 5% perchloric acid extracts of products synthesized by large polynucleosomes (Smulson et al., 1980). In this regard, Gaubatz & Sires (1978) have observed that a network of "poly H1", prepared by chemically cross-linking chromatin, can be extracted by the selective perchloric acid method for H1 isolation.

Histone H1 and Chromatin Complex Formation. As an initial approach to study the potential influence of the poly(ADP-Rib) polymerase catalyzed cross-linking of histone H1 on chromatin structure, the aggregation phenomenon described above was studied by using purified oligonucleosomes containing a normal complement of H1 and in the same particles, specifically depleted of histone H1. This approach depends on selectively removing histone H1 from polynucleosomes under conditions whereby nucleosome stability is maintained and little nonhistone proteins, especially poly(ADP-Rib) polymerase, are removed. Thoma & Koller (1977) have described a gentle procedure for the removal of histone H1 from polynucleosomes. It has been established that total histone H1 could be removed without any loss of core histones. Recently, Nelson et al. (1979) (using Dowex 50W-X2) have employed this procedure for selective removal of histone H1.

Octanucleosomes were prepared and incubated identically in the presence and absence of Dowex. Total nuclear proteins remaining in the two samples were analyzed by polyacrylamide electrophoresis. Under these conditions greater than 95% of H1 was removed from the chromatin (data not shown); the nonhistone protein complement of H1-depleted chromatin was the same in both samples. A 40% loss in poly(ADP-Rib) polymerase activity was noted; however, it is not clear whether this was reflective of the removal of H1 per se or the removal of polymerase. In spite of these complications, it was of interest that the H1-depleted nucleosomes were found to be incapable of complex formation (Figure 6), while nucleosomes treated under identical conditions but containing histone H1 clearly responded to the elevated NAD levels by forming complex material. We have recently reported on the successful reconstitution of purified poly(ADP-Rib) polymerase with polymerase-depleted polynucleosomes (Jump et al., 1980b). However, attempts to restore NAD-promoted aggregation of chromatin by reconstitution of depleted chromatin with either stoichiometric levels of H1 and/or exogenous purified polymerase (Jump et al., 1980a) have been unsuccessful thus far. These preliminary results, coupled with the earlier data do, however, suggest that ADP-ribosylation of histone H1 plays a role in the chromatin complex reaction.

Discussion

An NAD concentration dependent complexation of all or some components of a small domain of polynucleosomes was described in the preceding paper (Butt & Smulson, 1980). The in vitro concentration of NAD (50–100 μM) required to generate the complex formation is consistent with the level of NAD reported to exist in the eukaryotic nuclei (Rechsteiner et al., 1976). Another feature of this reaction was an apparent correlation between the chain lengths of poly(ADP-Rib) covalently attached to nuclear protein acceptors and the extent of complex formation. At 100 μM NAD, the chain length of

polymer was 60 or longer. It was assumed that the long poly(ADP-Rib) chains acted as connectors within the large chromatin aggregates. This point has been expanded in the present work (Figure 1). Disassociation of the complex was observed when the poly(ADP-Rib) present in complexed chromatin was cleaved with phosphodiesterase.

An approach to characterize the complex was to digest the labeled aggregate with micrococcal nuclease and related nucleases. These enzymes generate characteristic nucleosome-repeat cleavage products from polynucleosomes, and it was reasoned that the identification of [^{32}P]ADP-Rib nucleosome subunits would clarify whether chromatin per se existed in the complex. These experiments (Figure 1) were only partially informative. Micrococcal nuclease and DNase I digestion studies yielded some evidence of nucleosomal organization in the complex, but overall, the aggregated material was noted to be relatively resistant not only to micrococcal nuclease and DNase II but also, to some extent, to snake venom phosphodiesterase. The latter fact suggests that the poly(ADP-Rib) polymer might be extensively buried within the complex. This would be consistent with earlier observations on both the stability of the poly(ADP-Rib) H1 dimer and the resistance of highly condensed forms of SV40 minichromatin to nuclease digestion. For example, Stone et al. (1978) found that the poly(ADP-Rib) glycohydrolase catalyzed hydrolysis of the polymer in the H1 complex was only one-ninth that of the free polymer. Experimental conditions which tended to denature the H1 complex allowed hydrolysis of polymer to occur more rapidly, suggesting that the polymer is exposed under these conditions. If ADP-ribosylated nuclear proteins on octanucleosomes were similarly buried in the complex described in the present work, they presumably would also be inaccessible to micrococcal nuclease, as in fact was observed (Figure 1). Accordingly, a detailed study was performed of the relative nuclease susceptibility of ADP-ribosylated trimer nucleosomes, compared with the unmodified species of these particles (Figure 4). Poly(ADP-ribosylated) nuclear complexes were found to be cleaved at a reduced rate.

Along these lines, Varshavsky et al. (1977) have described two H1-containing forms of SV40 minichromosomes, "extended" and "compact" structures. The extended form undergoes compaction upon an increase in ionic strength with a concomitant increase in sedimentation properties. It is of interest that the compact form of SV40 minichromosome was also found to be highly resistant to micrococcal nuclease digestion. It is also of relevance to the present work that the structural transition of SV40 minichromosome is critically dependent upon the presence of histone H1. In H1-depleted chromatin, no compaction was noted (Varshavsky et al., 1977). Analogous results were obtained in the present work (Figure 6). The poly(ADP-Rib)-promoted aggregation of polynucleosomes was found to be abolished when histone H1 was specifically removed by a gentle Dowex method. However, these results are not unambiguous since some polymerase "activity" was lost, and complex formation during reconstitution of stripped nucleosomes with H1 and purified polymerase has, as yet, not been demonstrated. Clearly, there is much support for an important role of histone H1 in chromatin condensation. One linkage of the poly(ADP-Rib)–H1 complex is probably via a modification of glutamic acid 6 from the amino terminal end (Burzio et al., 1979).

Because of the probable role of H1 in chromatin condensation, the ability of poly(ADP-Rib) polymerase to catalyze a complex formation of H1 into dimers (Stone et al., 1977) is an attractive hypothesis to explain the phenomenon noted in the present work. Evidence has been provided that this H1 dimer is present in the chromatin complex [Figure 5 and 8 of Butt & Smulson (1980)]. Both chromatin complex and H1 dimer formation were correlated with NAD concentration dependence. It was previously found (Nolan et al., 1980) that long stretches of polynucleosomes (16 N) favored in vitro synthesis of H1 dimer (on an equal chromatin content) over smaller structures. It is not unreasonable to expect that H1–poly(ADP-Rib) cross-links greater than the dimer exist biologically, and preliminary evidence for this has been recently provided (Smulson et al., 1980). In addition, Gaubatz & Sires (1978) have shown by chemical cross-linking studies that large networks of H1 molecules must be adjacent to each other in native chromatin. It is tempting, but premature, to assign a role for poly(ADP-ribosylated) histone H1 with other nuclear proteins in mechanisms leading to chromatin compaction.

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References

Adamietz, P., Bredehorst, R., & Hilz, H. (1978) *Eur. J. Biochem. 91*, 317–326.

Adamietz, P., Klapproth, K., & Hilz, H. (1979) *Biochem. Biophys. Res. Commun. 91*, 1232–1238.

Axel, R., Cedar, H., & Felsenfeld, G. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2029–2032.

Boulikas, T., Wiseman, J. M., & Garrard, W. T. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 127–131.

Burzio, L. O., Riquelme, P. T., & Koide, S. S. (1979) *J. Biol. Chem. 254*, 3029–3037.

Butt, T. R., & Smulson, M. (1980) *Biochemistry* (preceding paper in this issue).

Butt, T. R., Brothers, J. F., Giri, C. P., & Smulson, M. (1978) *Nucleic Acids Res. 5*, 2775–2788.

Byrne, R. H., Stone, P. R., & Kidwell, W. R. (1978) *Exp. Cell Res. 115*, 277–283.

Chalkley, R., & Hunter, C. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1304–1308.

Gaubatz, J., & Sires, B. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1464.

Giri, C. P., West, M. H. P., & Smulson, M. (1978a) *Biochemistry 17*, 3495.

Giri, C. P., West, M. H. P., Ramirez, M. L., & Smulson, M. (1978b) *Biochemistry 17*, 3501.

Jump, D. B., Butt, T. R., & Smulson, M. (1979) *Biochemistry 18*, 983–990.

Jump, D. B., Butt, T. R., & Smulson, M. (1980a) *Biochemistry 19*, 1031–1037.

Jump, D. B., Sudhakar, S., Tew, K. D., & Smulson, M. (1980b) *Chem.-Biol. Interact. 30*, 35–51.

Nelson, P. P., Albright, S. C., Wiseman, J. M., & Garrard, W. T. (1979) *J. Biol. Chem. 254*, 11 751–11 760.

Nolan, N. L., Butt, T. R., Wong, M., Lambrianidou, A., & Smulson, M. (1980) *Eur. J. Biochem.* (in press).

Panyim, S., & Chalkley, R. (1969) *Arch. Biochem. Biophys. 130*, 337–346.

Rechsteiner, M., Hillyard, D., & Olivera, B. M. (1976) *Nature (London) 259*, 695–696.

Renz, M., Nehls, P., & Hozier, J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1879–1883.

Ring, D., & Cole, R. D. (1979) *J. Biol. Chem. 254*, 11688–11695.

Smulson, M., Butt, T., Nolan, N., Jump, D., & DeCoste, B.

(1980) *Adv. Cell Biol. 6*, 59–70.
Sollner-Webb, B., Camerini-Otero, R. D., & Felsenfeld, G. (1976) *Cell 9*, 179–193.
Sporn, M. B., Berkowitz, D. M., Glinki, R. P., Ash, A. B., & Stevens, C. L. (1969) *Science (Washington, D.C.) 164*, 1408–1410.
Stone, P. R., Lorimer, W. S., & Kidwell, W. R. (1977) *Eur. J. Biochem. 81*, 9–18.
Stone, P. R., Lorimer, W. S., III, Ramchalis, J., Danley, M., & Kidwell, W. R. (1978) *Nucleic Acids Res. 5*, 173–184.
Thoma, F., & Koller, T. (1977) *Cell 12*, 101–107.
Varshavsky, A. J., Nedospasov, S. A., Schmatchenko, V. V., Bakayev, V. V., Chumackov, P. M., & Georgier, G. P. (1977) *Nucleic Acids Res. 4*, 3303–3325.
Whitlock, J. P., Jr., & Simpson, R. T. (1977) *J. Biol. Chem. 252*, 6515–6520.
Worcel, A. (1978) *Cold Spring Harbor Symp. Quant. Biol. 42*, 313–324.

Wheat Germ Protein Kinase Affects the Translation of Brome Mosaic Virus Ribonucleic Acid in Vitro†

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ABSTRACT: Wheat germ protein kinase inhibits in vitro translation of Brome Mosaic virus (BMV) RNA 1 and 2, without affecting the translation of RNA 4. Inhibition of formation of BMV polypeptides 1a and 2a is due to the arrest of initiation of polypeptide synthesis. It was found that protein kinase inhibits the formation of the 80S initiation complex with BMV RNA 1 and 2, without affecting the formation of the initiation complex with BMV RNA 4. Inhibition of protein synthesis by wheat germ protein kinase is accompanied by the phosphorylation of two ribosome-associated polypeptides, with molecular weights of 32 000 and 76 000, respectively. Both polypeptides are readily dephosphorylated by the enzyme(s) present in the cell-free extract. Their dephosphorylation is accompanied by restoration of the translational capacity of the system.

Recently, we isolated cAMP-independent protein kinase[1] from wheat germ (Rychlik & Zagórski, 1980), which exerts an inhibitory effect on translation of some natural messengers in vitro. Here we describe in detail the effect of purified protein kinase on in vitro protein synthesis. The experiments were carried out in a wheat germ cell-free system which is homologous for the enzyme. BMV template was used since wheat is one of the natural hosts of this virus.

BMV is a multicomponent virus, and its total RNA consists of four monocistronic messengers, called in the order of their decreasing length RNA 1, 2, 3, and 4 (Lane, 1974). These messengers can be separately translated in vitro into three nonstructural polypeptides, designated 1a, 2a, 3a, and the coat protein, referred to as polypeptide 4a (Shih & Kaesberg, 1976). A mixture of all BMV RNAs, total BMV RNA, also induces in vitro the translation of these polypeptides. In this case, however, the messengers are translated with different efficiencies (Zagórski, 1978a).

In the system primed with total BMV RNA as well as in that directed by separate BMV RNAs, we observed a different expression of viral genes in the presence of wheat germ protein kinase. It is known that protein kinases (ATP:protein phosphotransferases, EC 2.7.1.37) control a variety of cellular processes [for review, see Rubin & Rosen (1975)] and are also involved in the regulation of protein synthesis. This regulatory activity depends on the phosphorylation of specific proteins. Therefore, in order to understand the mechanism by which protein kinase affects the synthesis of some BMV polypeptides, we studied the phosphorylation of various components of the protein-synthesizing machinery in the presence of enzyme.

It is known that the reticulocyte system contains a cAMP-independent protein kinase, called the hemin-controlled repressor, which inhibits translation of natural messages by phosphorylating the small subunit of the initiation factor eIF-2 [for review, see Safer & Anderson (1978)]. This leads to a diminished formation of the 40S initiation complex. Analyzing the formation of initiation complexes in our system, we found that wheat germ protein kinase arrests the formation of the 80S initiation complex in the presence of mRNAs for nonvirion proteins (BMV RNAs 1, 2, and 3), without inhibiting the formation of the 40S complex. These results allow the supposition that the enzyme acts differently than the hemin-controlled repressor. The observed inhibition of initiation complex formation with RNAs 1, 2, and 3 but not with RNA 4 results in a differential in vitro expression of viral genes.

Materials and Methods

General. The methods of isolation of Brome Mosaic virus, BMV RNA, wheat germ cell-free extract, wheat germ tRNAs, and wheat germ protein kinase and the conditions for translation of BMV RNA as well as for aminoacylation of tRNA were described previously (Rychlik & Zagórski, 1980; Zagórski, 1978a,b). Wheat germ initiation factor eIF-2 was kindly given by Dr. A. Legocki, University of Agriculture, Poznań. Subcellular fractions were incubated with [γ-^{32}P]ATP under salt conditions optimal for translation in the presence as well as in the absence of protein kinase under conditions described previously (Rychlik & Zagórski, 1980). Dodecyl

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[1] Abbreviations used: BMV, Brome Mosaic virus; DTT, dithiothreitol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; eIF, eukaryotic initiation factor. Enzymes: protein kinase or ATP:protein phosphotransferase, EC 2.7.1.37; creatine kinase or ATP:creatine *N*-phosphotransferase, EC 2.7.3.2.

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