for native enzyme. Since these characteristics are originated in the initiation among discrete steps of RNA synthesis and since the rate of RNA chain elongation is indistinguishable between the reactions catalyzed by native and reconstituted enzymes, it appears that self-reactivated core enzyme is different from native enzyme in the specificity of transcription initiation (Table IV).

In concert with the difference in the transcription specificity, self-reactivated core enzyme gives a different shape of near-ultraviolet circular dichroism spectrum from that of native core enzyme (Ishihama et al., 1979). In a consequence of the present observations, it remains to be determined that such a partially reactivated enzyme is an obligatory intermediate in the process of active enzyme formation, because structured species not on the renaturation pathway may be formed during the renaturation of some enzymes (Ikai & Tanford, 1971; Teipel & Koshland, 1971). These observations, together with the previous finding that self-reactivation proceeds only at nonphysiological conditions, e.g., in the presence of high concentrations of salt or glycerol (Saitoh & Ishihama, 1976), might suggest that RNA polymerase whose structure and function are completely identical with that of native enzyme, can be formed via one or both of the other two maturation pathways, i.e., σ subunit promoted or DNA-promoted maturation. Study of the properties of the enzymes formed in the presence of these maturation-promoting factors should provide a critical test of the above hypothesis.

Acknowledgments

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Nuclear Protein Modification and Chromatin Substructure. 3. Relationship between Poly(adenosine diphosphate) Ribosylation and Different Functional Forms of Chromatin†

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ABSTRACT: The relationship between poly(adenosine diphosphate) ribosylation of nuclear proteins and functionally different forms of chromatin from mid-S-phase HeLa nuclei was investigated. The major observations emerging from this study were that unique nonhistone proteins were modified in mid-S-phase HeLa nuclei. The major acceptor for poly(adenosine diphosphate-ribose) [poly(ADP-Rib)] was an internucleosomal nonhistone protein (protein C; 125 000 molecular weight). Histones H3, H1, H2b, and H2a but not H4 were ADP-ribosylated in S-phase nuclei. Chromatin fragments preferentially released by micrococcal nuclease were enriched in nonhistone proteins, poly(ADP)-ribosylated nuclear proteins, poly(ADP-Rib) polymerase activity and nascent DNA from the DNA replicating fork. In extended forms of chromatin, contiguous to the DNA replicating fork, poly(ADP-Rib) polymerase was maximally active. However, in chromatin distal to the replicating fork (i.e., more condensed structures), nucleosomal histones and histone H1 were not significantly ADP-ribosylated, and poly(ADP-Rib) polymerase activity was depressed two- to threefold. The data suggest that a subset of nucleosomes in extended regions of chromatin is subject to extensive ADP ribosylation.

The objective of the present study was to ascertain the relationship between nuclear protein modification and different functional forms of chromatin, as selectively released during digestion by micrococcal nuclease. Recent advances in understanding the structure of chromatin have revealed that its basic subunit nature is stabilized by cooperative interactions between DNA and protein (Kornberg, 1977; Elgin & Weintraub, 1975; Felsenfeld, 1978). The higher ordered packaging of nucleosomes into condensed chromosomal structures is thought to involve protein–protein and pro-

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tein–nucleic acid interactions (Felsenfeld, 1978; Finch et al., 1977; Benyajati & Worcel, 1976). The covalent modification of proteins that participate in the organization of chromatin in higher ordered structures is likely to have a dynamic influence on the functional properties of chromatin (i.e., transcription and replication). Therefore, a knowledge of the to of these modified proteins in chromatin is essential to understanding both the structure and function of the nucleus.

Poly(ADP-Rib)[1] polymerase, a tightly bound chromatin enzyme, catalyzes the successive transfer of ADP-Rib units from NAD to nuclear proteins (see reviews by Hayaishi & Ueda, 1977; Smulson & Shall, 1976; Hilz & Stone, 1976). The products of the reaction are short poly(ADP-Rib) chains covalently attached to both histone and nonhistone chromosomal proteins. In previous reports, poly(ADP-Rib) polymerase was found to be associated with the internucleosomal regions of chromatin (Giri et al., 1978a,b; Mullins et al., 1977). Nevertheless, this internucleosomal location does not preclude enzymatic ADP ribosylation of both nucleosomal and internucleosomal proteins. Initial efforts to associate poly(ADP-Rib) polymerase with functional aspects of chromatin, either transcriptionally active, replicative, or condensed chromatin, have shown that enzyme activity is enriched in extended chromatin (Mullins et al., 1977), but not necessarily in transcriptionally active chromatin (Yukioka et al., 1978). These observations suggest an association of poly(ADP-Rib) polymerase with structures undergoing DNA synthesis, rather than RNA synthesis. Other studies have indicated that poly(ADP-Rib) functions in DNA replication or repair. These data include: (a) increase in poly(ADP-Rib) content and poly(ADP-Rib) polymerase activity in vivo in nuclei during S-phase (Kidwell & Mage, 1976; Colyer et al., 1973); (b) activation of poly(ADP-Rib) polymerase activity in either nuclei treated with DNase I (Miller, 1975) or in cells treated with agents that induce repair functions, e.g., methyl nitrosourea (Smulson et al., 1977) and bleomycin (Miller, 1976); (c) high NAD turnover in vivo during S phase (Rechsteiner et al., 1975); and (d) enhancement of DNA template-primer activity in HeLa nuclei incubated with NAD (Smulson et al., 1975).

Because of the purported involvement in DNA synthesis, we reasoned that proteins modified by poly(ADP-Rib) polymerase in nuclei isolated from mid-S-phase cells would reflect the preferred acceptors for poly(ADP-Rib) in vivo. The association of these proteins with replicating structures was tested with micrococcal nuclease which has been shown to preferentially digest chromatin at or near the DNA replicating fork (Hildebrand & Walters, 1976; Seale, 1976). Determination of the distribution of both poly(ADP-Rib) polymerase and its acceptors in functionally different forms of chromatin selectively generated by micrococcal nuclease will serve to provide considerable insight into the role poly(ADP-Rib) polymerase plays in the structural organization of chromatin undergoing DNA synthesis.

Materials and Methods

[*adenine*-2,8-^{3}H]NAD (3.3 Ci/mmol) was purchased from New England Nuclear. [^{32}P]NAD was synthesized from [^{32}P]ATP (350 Ci/mmol, Amersham) and NMN (Sigma) catalyzed by NAD pyrophosphorylase (Boehinger) (Wong et al., 1977). [^{32}P]NAD was purified by ion-exchange chromatography (Dowex AG-1, Bio-Rad) and analyzed for purity by phosphoethanolimine thin layer chromatography (98%). Micrococcal nuclease (6000 units/mg) was obtained from Worthington Biochemicals, phenylmethanesulfonyl fluoride ($PhCH_2SO_2F$) from Sigma, and Methotrexate from NCI, NIH. X-ray film (SB-5) was purchased from Kodak (Rochester, NY).

HeLa S_3 cells were maintained at 37 °C in spinner flasks as previously described (Mullins et al., 1977). For cell synchronization, cell growth was stopped at the G_1/S boundary by addition of methotrexate (230 μg/L) to the culture medium of asynchronously growing log phase cells (5×10^5 cell/mL) (Smulson et al., 1975). After 16 h, the metabolic block was released with the addition of thymidine to 10 μM, and cells were harvested 4–5 h later at mid-S phase. Triton X-100 washed nuclei were prepared according to Whitlock & Simpson (1976). All buffers were supplemented with $PhCH_2SO_2F$ (0.2 mM) as a protease inhibitor.

HeLa cell nuclei were incubated with 1 mM [^{3}H]NAD (2.5 Ci/mol) or 1 μM [^{32}P]NAD (750 Ci/mol) (8×10^7 nuclei/mL) in 0.25 M sucrose, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 M Tris, pH 7.2, 0.2 mM $PhCH_2SO_2F$, at 25 °C. Nucleosomes were prepared by micrococcal nuclease digestion (4 units of micrococcal nuclease/A_{260} unit) of HeLa nuclei (8×10^7 nuclei/mL) in 0.25 M sucrose, 0.1 mM $CaCl_2$, 1 mM Tris, pH 7.2, 0.2 mM $PhCH_2SO_2F$ at 25 °C for the indicated times. Digestion was terminated by addition of EDTA to 5 mM. Nuclei were lysed hypotonically and soluble chromatin separated from undigested chromatin by sedimentation (4 °C) at 3000*g* for 5 min. Nucleosomes were fractionated by sucrose gradient centrifugation: 5–20% sucrose, 5 mM Tris, pH 7.2, 1 mM EDTA, 0.2 mM $PhCH_2SO_2F$; SW 40, 15 h, 4 °C.

Gel Electrophoresis of Chromatin. Chromatin fragments were also resolved by electrophoresis in 4.5% acrylamide (acrylamide, *N,N'*-methyl bisacrylamide, 30:1) slab gels (20 °C) using 20 mM Tris-HCl (pH 7.5), 2 mM EDTA as the buffer (Varshavsky et al., 1976). Following electrophoresis at 200 V for 2–2.5 h, gels were stained with ethidium bromide (3 μg/mL) in electrophoresis buffer for 20 min at room temperature and photographed. Gels were dried as described by Giri et al. (1978a) for radioautography.

Gel Electrophoresis of Proteins. Chromatin proteins were precipitated with 3 volumes of absolute ethanol and stored at −20 °C overnight. Precipitated proteins were collected by centrifugation at 20000*g* for 20 min and resuspended in 10 mM $NaPO_4$, pH 7.0, 1% $NaDodSO_4$, 1% β-mercaptoethanol and boiled for 2 min prior to electrophoresis. Modification of a procedure initially described by Weber & Osborn (1969) was used for electrophoresis of protein samples in 5–22% acrylamide (acrylamide, *N,N'*-methylbisacrylamide, 200:1) slab gels with 0.1 M $NaPO_4$ (pH 7.0), 0.1% $NaDodSO_4$ as buffer. Following electrophoresis for 14 h (25 °C) at 80–100 mA/slag gel, gels were fixed and stained in 0.2% Coomassie Blue, 50% methanol, and 7% acetic acid for 6 h and destained in 20% methanol and 7% acetic acid. Destained gels were photographed and dried as before.

Autoradiography. Kodak SB-5 X-ray film was exposed to dried gels containing ^{32}P-labeled proteins in a Du Pont Cromex intensifier at −70 °C for an appropriate time. The autoradiograms were scanned by densitometer (ORTEC, Inc.).

Results

Poly(ADP) Ribosylation of Chromosomal Proteins in HeLa

[1] Abbreviations used: ADP-Rib, adenosine diphosphate-ribose; NAD, nicotinamide adenine dinucleotide; $NaDodSO_4$, sodium dodecyl sulfate; Cl_3AcOH, trichloroacetic acid; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; HMG, high mobility group proteins; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; NHP, nonhistone protein; NMN, nicotinamide mononucleotide; β-MSH, β-mercaptoethanol; Tdr, thymidine; BSA, bovine serum albumin.

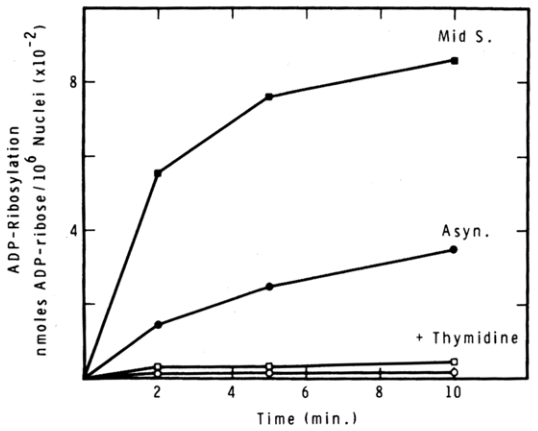

FIGURE 1: Kinetics of poly(ADP-Rib) polymerase activity in nuclei isolated from asynchronous and mid-S-phase cells. Isolated HeLa nuclei were incubated with 1 mM [^{3}H]NAD in the presence and absence of 5 mM thymidine at 37 °C. Aliquots were removed at the designated times and Cl_3AcOH-precipitable radioactivity was determined. Mid-S-phase nuclei, NAD (■—■); asynchronous nuclei, NAD (●—●); mid-S-phase nuclei, 1 mM NAD + 5 mM thymidine (□—□); asynchronous nuclei, 1 mM NAD + 5 mM thymidine (○—○).

Table I: Preliminary Physical Characteristics of ADP-Ribosylated Proteins in Mid-S-Phase Nuclei

designation[a]	mol wt (× 10^3)	soluble in[b] 0.4 N H_2SO_4	% of poly-(ADP-Rib) incorp into nuclei	tentative identification
A	280.0	−	1.2	
B	174.0	−	2.5	
C	125.0	−	22.7	poly(ADP-Rib) polymerase[d]
D	75.0	−	3.5	
E	69.2	−	4.4	
F	52.5	−	2.4	
G	38.0	−	2.5	
H	22.0	+	4.4	histone 1[e]
I	15.3	+	24.7	3
J	14.0	+	14.4	2b
K	13.8	+	14.1	2a

[a] ADP-ribosylated proteins in HeLa nuclei were given letter designations for reference in discussion in the text. [b] An aliquot of chromatin associated protein was extracted in 0.4 N H_2SO_4 as previously described (Giri et al., 1978a) and electrophoresed as in Figure 2. [c] Densitometry of a scan of the radioautograms of whole nuclei was used to determine the relative amount of label associated with each class of proteins. [d] See text. [e] Positive identification of the labeled histones was made by electrophoresis in a Triton–acetic acid–urea gel (Alfageme et al., 1974).

Nuclei. In order to clearly establish that nuclei from mid-S-phase HeLa cells more actively synthesize poly(ADP-Rib), a comparative study of the kinetics of incorporation of NAD (1 mM) into (ADP-Rib) in nuclei isolated from mid-S-phase and asynchronous cells was carried out. Figure 1 shows that poly(ADP-Rib) polymerase activity was two to three times greater in nuclei isolated from mid-S-phase cells than in nuclei isolated from asynchronous cultures. In each case, poly(ADP) ribosylation was 90–95% inhibited by a fivefold molar excess of thymidine added to the reaction mixture. This study essentially confirms previously published data that poly(ADP-Rib) polymerase is more active in nuclei isolated from S-phase cells (Smulson et al., 1975). Incorporation of NAD into Cl_3AcOH-insoluble material was also sensitive to thymidine, a standard inhibitor of poly(ADP-Rib) polymerase

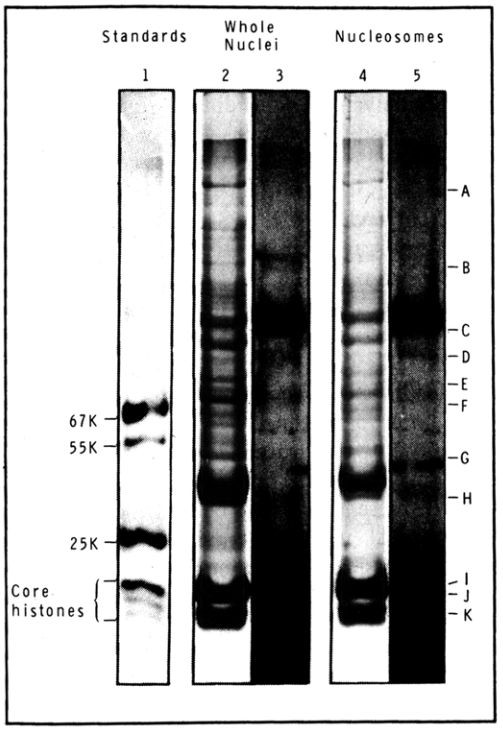

FIGURE 2: Comparison of ADP-ribosylated proteins in nuclei and isolated chromatin. Triton-treated nuclei from mid-S-phase cells were incubated (8×10^7 nuclei/mL) with 1 μM [^{32}P]NAD (750 Ci/mol) for 5 min at 25 °C. Nuclei washed free of unreacted [^{32}P]NAD were resuspended to 8×10^7 nuclei/mL, warmed to 25 °C, and digested for 10 min with micrococcal nuclease (8 units of enzyme/A_{260} unit of chromatin). Digestion was terminated by adjusting to 10 mM EDTA and cooled. No net loss in Cl_3AcOH-insoluble radioactivity was encountered by shearing DNA in this manner. An aliquot of the nuclear suspension was dialyzed against 1% $NaDodSO_4$. Chromatin was prepared by micrococcal nuclease digestion: 4 units of micrococcal nuclease/A_{260} of chromatin, for 2-min digestion at 25 °C. Digestion was stopped with EDTA (10 mM). Nuclei were lysed hypotonically and soluble chromatin was separated from undigested chromatin by sedimentation at 3000*g*. Soluble chromatin was further digested with micrococcal nuclease (8 units of micrococcal nuclease/A_{260} of chromatin) for 10 min. The reaction was stopped with EDTA to 10 mM, and the suspension was dialyzed against 1% $NaDodSO_4$. Nuclear and chromatin proteins (300 μg/well) were electrophoresed as described in Materials and Methods. After electrophoresis, slab gels were stained with Coomassie Blue (columns 2 and 4) and dried for radioautography (columns 3 and 5). Column 1 represents protein standards: BSA (67 000 mol wt), tubulin (55 000), chymotrypsinogen (25 000), core histones (H3, H2b, H2a, and H4). Columns 2 and 3 represent nuclear proteins, columns 4 and 5 chromatin-associated proteins. Protein bands labeled with radioactivity are indicated by capital letters (A–K).

(Hayashi & Ueda, 1977). In addition, the two- to threefold increase in labeling kinetics (Figure 1) was similar to the increase in micrococcal nuclease digestion kinetics in nuclei from differing phases of the cell cycle (Figure 3).

Since we were interested in determining the preferred acceptors for poly(ADP-Rib) in S-phase chromatin, nuclei were routinely modified with [^{32}P]NAD (1 μM) for 5 min (25 °C). The use of [^{32}P]NAD enabled detection of proteins that served as acceptors for poly(ADP-Rib). The covalent linkage between poly(ADP-Rib) and protein has been reported to be unstable in alkaline buffers (Hilz & Stone, 1976). A modification of a gel electrophoresis system initially described by Weber & Osborn (1969) was therefore used to resolve nuclear and chromatin associated proteins, while maintaining the covalent linkage between poly(ADP-Rib) and protein (Figure 2). All proteins modified by poly(ADP-Rib) corresponded to chromatin-associated proteins. A tabulation of the physical

properties of the ADP-ribosylated proteins is given in Table I.

In a previous study, we demonstrated that the covalent attachment of poly(ADP-Rib) did not significantly influence the mobility of modified proteins (Giri et al., 1978a). Hence, identification of labeled proteins was not complicated. Figure 2 and Table I show that nucleosomal histones are major acceptors of poly(ADP-Rib) in S-phase nuclei. A second gel system, Triton–urea–acetic acid (Alfageme et al., 1974), was employed to examine the distribution of poly(ADP-Rib) among nucleosomal histones (data not shown). Histones H3, H2b, and H2a, but not H4, were modified. Giri et al. (1978a) reported that H1 was the major acceptor for (ADP-Rib) among the histones in asynchronous HeLa nuclei. However, ADP ribosylation of histone H1 in S-phase nuclei was considerably less compared with that seen for the nucleosomal histones (Figure 2). The difference between our previous results and the present data may be explained by the preferred association of H1 with condensed rather than extended chromatin. A plausible explanation for this observation is that, during S-phase, chromatin is not as condensed as in asynchronous nuclei. H1, then, may not be in a location that permits ADP ribosylation.

Several nonhistone proteins are acceptors for poly(ADP-Rib) in S-phase HeLa nuclei. The molecular weights of these proteins, as determined by $NaDodSO_4$–polyacrylamide gel electrophoresis, range from 37 000 to 298 000 (Table I). Approximately 40% of the poly(ADP-Rib) covalently linked to protein was associated with nonhistone proteins. Similar observations have been made by Okayama et al. (1978a) on the distribution of ADP-Rib linked to protein in rat liver nuclei. An acid-insoluble protein (protein C; 125 000 mol wt) was seen as the major nonhistone protein acceptor for poly(ADP-Rib). Poly(ADP-Rib) covalently attached to protein C represented nearly 23% of the total poly(ADP-Rib) linked to protein in HeLa nuclei (Table I). Determination of the identity and function of protein C is being actively pursued in our laboratory. However, it should be noted that purified poly(ADP-Rib) polymerase from calf thymus (130 000 mol wt) has been reported to catalyze its automodification under in vitro conditions (Yoshihara et al., 1977). Any suggestion that protein C represents the HeLa poly(ADP-Rib) polymerase must remain tentative.

Kinetics of Digestion of Chromatin at the DNA Replicating Fork with Micrococcal Nuclease. A number of studies have suggested a role for poly(ADP-Rib) polymerase in DNA replication and repair. It would seem reasonable to expect that regions of chromatin active in DNA synthesis would be enriched in poly(ADP-Rib) polymerase or its acceptor proteins. In order to test this possibility, experimental conditions were developed using micrococcal nuclease to isolate nucleosomal fragments enriched in nascent DNA from the DNA replicative fork. In the experiment, asynchronously growing cells uniformly labeled with [^{14}C]Tdr were pulse labeled with [^{3}H]Tdr for 1 min. Nuclei were prepared and digested for 2 min with micrococcal nuclease. Nucleosome fragments fractionated by velocity sedimentation were analyzed for the distribution of [^{14}C]TMP and [^{3}H]TMP incorporated into DNA. Figure 3 shows that the specific activity of the ^{3}H-pulsed label is highest in mono- and dinucleosomes cleaved preferentially by micrococcal nuclease, whereas the ^{14}C-labeled chromatin was digested more slowly along with the bulk chromatin. These data extend the previous observations made by Hildebrand & Walters (1976) by showing that chromatin subunits near the replicating fork are more susceptible to nuclease attack than

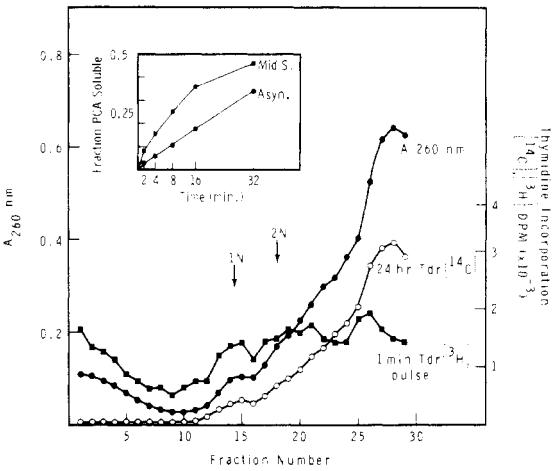

FIGURE 3: Kinetics of micrococcal nuclease digestion of nuclei isolated from asynchronous and mid-S-phase cells. Asynchronous cells (3.5 × 10^5/mL) were uniformly labeled by two consecutive 24-h treatments with 0.005 μCi/mL [^{14}C]thymidine (NEN, 40 mCi/mmol) followed by a 2-h chase in label-free medium. Cells were pulse labeled with 40 μCi/mL [^{3}H]thymidine (NEN, 40 Ci/mmol) for 1 min. The pulse labeling was stopped by pouring the cell suspension over crushed ice. Nuclei were prepared and digested for 2 min with micrococcal nuclease (2 units/A_{260}). Chromatin fragments were separated in 10–30% sucrose velocity gradients: SW 40, 38K, 6 h, 4 °C. Direction of sedimentation is from left to right. Gradients were fractionated as previously described (Giri et al., 1978a). Absorbance was at 260 nm (●—●), and Cl_3AcOH-insoluble radioactivity was determined for each fraction: ^{14}C (○—○); ^{3}H (■—■). 1N, mononucleosome; 2N, dinucleosome. Insert: Nuclei were prepared from mid-S-phase cells (■—■) and asynchronous cells (●—●). Nuclei were resuspended in digestion buffer (see Materials and Methods) to 5 × 10^7/mL and warmed to 37 °C for 4 min. Micrococcal nuclease was added to 2 units of enzyme/A_{260} of chromatin. Aliquots were removed at the designated times and percent perchloric acid soluble chromatin was determined (Cech & Pardue, 1977).

subunits in nonreplicating chromatin. In addition, a comparative study of the digestion kinetics of chromatin in nuclei isolated from mid-S phase and asynchronous cells was carried out. Figure 3 (insert) shows that micrococcal nuclease digests chromatin from mid-S phase nuclei two to three times more rapidly than chromatin from asynchronous nuclei. This more rapid digestion may reflect the more relaxed or extended nature of chromatin in nuclei during S phase. Note also that the increased digestion kinetics seen in this study are similar to the increase in ADP ribosylation observed in S-phase nuclei (Figure 1). Hence, the organization of chromatin within the nucleus may be a factor in regulating poly(ADP-Rib) polymerase activity in vivo.

If nuclei were first incubated with NAD (1 μM) under conditions for ADP ribosylation of chromosomal proteins and then digested with micrococcal nuclease, no significant change in the kinetics of digestion were noted (data not shown). Because the amount of poly(ADP-Rib) incorporated into protein in nuclei is a function of the NAD concentration during incubation (data not shown), the low levels of NAD used above may not be sufficient to initiate an effect on chromatin structure that could be detected by micrococcal nuclease digestion. The protein modification conditions employed here were not complicated by gross structural perturbations that may have been induced by poly(ADP) ribosylation of chromosomal proteins. However, this is an area that is currently under active investigation.

Interrelationship of Functionally Diverse Forms of Chromatin Selectively Released by Micrococcal Nuclease. Having established conditions to isolate chromatin subunits enriched in nascent DNA, we continued by isolating chromatin

fragments from S-phase nuclei preincubated with [^{32}P]NAD. Three digestion times were employed in order to isolate chromatin fragments representing chromatin proximal, intermediate, and distal to the DNA replicating fork. Under the conditions employed in this study, poly(ADP-Rib) glycohydrolase activity was not observed (data not shown). The ADP-ribosylated proteins associated with specific chromatin fragments were subsequently analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis.

The distributions of nucleosomal particles in sucrose gradients (Figure 4) and native chromatin gels (inserts, Figure 4) display nucleosome subunit profiles typical of those reported previously (Mullins et al., 1977). For example, as digestion time is increased from 30 s (Figure 4A) to 16 min (Figure 4C) (i.e., 3.5 and 24% perchloric acid soluble, respectively), mononucleosomes increase at the expense of larger oligonucleosomes (see insert I, each panel). The nuclease digestion pattern reported here shows that chromatin in HeLa nuclei isolated from S-phase cells retains the basic subunit structure described for other systems (Lohr et al., 1977; Felsenfeld, 1978; Kornberg, 1977).

In general, the distribution of radioactivity in sucrose gradients was found to be associated with the nucleosomal fragments (≥ mononucleosomes). The specific activity of poly(ADP-Rib) associated with specific nucleosomal fragments (1N–3N) decreases as digestion time increases. This was demonstrated both by sucrose gradients and by the specific activity of incorporation of poly(ADP-Rib) in specific chromatin fragments resolved in native chromatin gels (insert II, Figure 4). The chromatin most accessible to micrococcal nuclease represents a subset of chromatin with a unique acceptor population not randomly distributed in chromatin. The radioactivity profile revealed that the distribution of ^{32}P was not entirely coincident with the chromatin profile (A_{260nm}). This observation indicated that this form of chromatin had as one unique feature a protein composition that was extensively ADP ribosylated, and that the association of these proteins with chromatin subunits influenced sedimentation characteristics.

Analysis of the protein composition in the areas indicated (1–4, Figure 4) revealed the association of protein C with nucleosomal fragments that sediment faster than the core mononucleosome. Previous reports from our laboratory have shown that the distribution of poly(ADP-Rib) polymerase activity in chromatin fragments (Mullins et al., 1977; Giri et al., 1978b) has essentially the same distribution as protein C. The smallest chromatin fragment found to retain polymerase activity had a composition of a heavy mononucleosome (160–185-bp DNA and 2 each H2a, H2b, H3, H4), i.e., a mononucleosome contiguous with internucleosomal (linker) DNA (Mullins et al., 1977; Giri et al., 1978b). Although characterization of this particle has not been attempted in this study, association of protein C with linker DNA of nucleosomal fragments would be expected to have a significant influence on sedimentation rates of chromatin subunits.

Other ADP-ribosylated nonhistone proteins (viz., D, E, F, and G; see Figures 4 and 5) appear to have an internucleosomal location similar to protein C. However, these proteins are infrequently represented in the population of modified proteins and are not easily detected in our present system. Nucleosomal histones and histone H1 were also seen to be ADP ribosylated and to be associated with discrete chromatin fragments released by micrococcal nuclease.

In seeking an explanation for the decrease in ADP ribosylation in specific forms of chromatin as nuclease digestion increased (Figure 4), a second, more defined study was performed. ADP-ribosylated S-phase HeLa nuclei were digested with micrococcal nuclease to generate dinucleosomes characteristic of various extents of digestion (1–64 min, 2–40% perchloric acid soluble). Dinucleosomal fragments were isolated by velocity sedimentation and ADP-ribosylated proteins analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis as in Figure 4.

The data in Figure 5 clearly show that dinucleosomes rapidly released from nuclei by micrococcal nuclease (1 min) are enriched in nonhistone proteins and ADP-ribosylated proteins (both histone and nonhistone). Dinucleosomes isolated from later digests are seen to have less histone modification and ADP-ribosylated protein C. The specific activity of modification of dinucleosomes generated after 1 and 64 min of digestion was 21 and 7 mmol of ADP-Rib/dinucleosome, respectively. This reveals two interesting points: (a) ADP ribosylation of nuclear proteins, particularly nucleosomal histones, is not randomly distributed throughout chromatin; and (b) a subpopulation of nucleosomes in extended regions of chromatin is subject to extensive ADP ribosylation. It should also be apparent that dinucleosomes generated late in digestion, arising from more nuclease resistant or condensed forms of chromatin, contain relatively little histone modification.

An alternative explanation for the decrease in specific activity of ADP ribosylation in dinucleosomes (Figure 5) may have been the presence of poly(ADP-Rib) glycohydrolase activity during nuclease digestion. Evidence for this contaminating activity would be revealed by monitoring the extent of poly(ADP-Rib) hydrolysis during the micrococcal nuclease digestion period. Little change in Cl_3AcOH-insoluble [^{32}P]poly(ADP-Rib) was indicated. A novel enzymatic activity detected in rat liver cytosol has been reported to cleave the linkage between poly(ADP-Rib) and histone H2b (Okayama et al., 1978b). This activity has not been investigated in the above study. The results reported in Figure 5 suggest that the selective modification of various regions of chromatin was determined by chromatin conformation.

Distribution of Poly(ADP-Rib) Polymerase in Chromatin. Since micrococcal nuclease preferentially digests chromatin at the DNA replicating fork (Figure 3) (Hildebrand & Walters, 1976), it was of interest to correlate the distribution of poly(ADP-Rib) polymerase activity in chromatin with the distribution of acceptors for poly(ADP-Rib). HeLa nuclei from S-phase cells were digested with micrococcal nuclease for varying times. Nucleosomal fragments were separated as before; mono-, di-, and trinucleosome regions of the various gradients were pooled separately and assayed in vitro for poly(ADP-Rib) polymerase activity. The results in Table II show that the specific activity of poly(ADP-Rib) polymerase increases from monomer to trimer, which is in agreement with our previous observations (Giri et al., 1978a). However, poly(ADP-Rib) polymerase activity decreases in specific nucleosomal fragments as digest time increases. These observations correlate well with the distribution of acceptors for poly(ADP-Rib) in chromatin. These data suggest that chromatin preferentially liberated by micrococcal nuclease is enriched in both poly(ADP-Rib) polymerase activity (Table II) and poly(ADP) ribosylated proteins (Figure 5). In addition, the acceptors for poly(ADP-Rib) in the in vitro assays in this study were similar to those reported earlier, viz., H3, H1, and an HMG protein (data not shown).

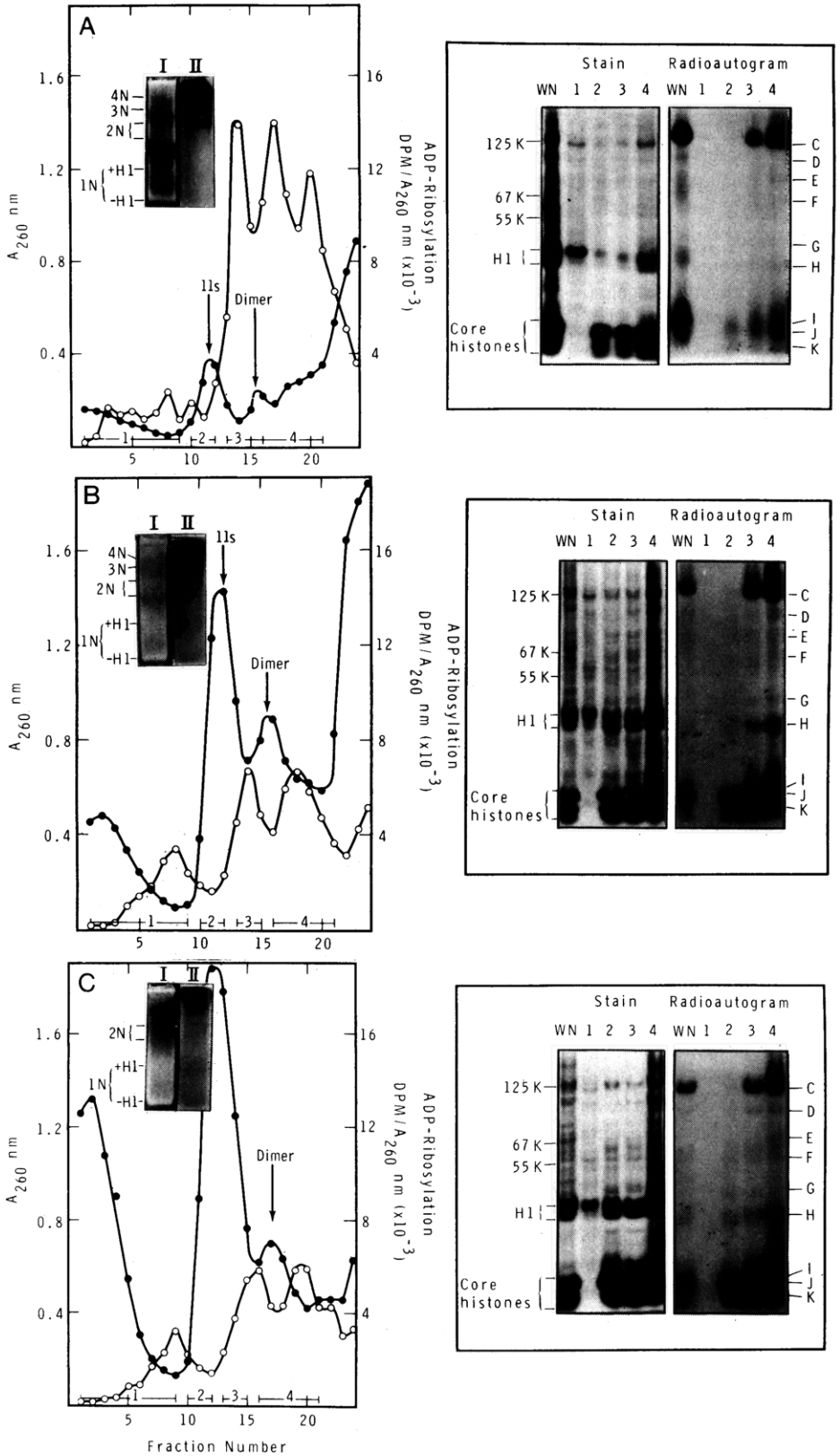

FIGURE 4: Sedimentation and electrophoretic analysis of the distribution of ADP-ribosylated chromatin proteins. Nuclei were isolated from mid-S-phase cells and modified with [^{32}P]NAD as in Figure 2. Modified nuclei were digested with micrococcal nuclease (4 units of enzyme/A_{260} unit of chromatin) at 25 °C for 30 s (panel A), 4 min (panel B), and 16 min (panel C) representing 3.5, 11, and 24% perchloric acid soluble digest, respectively. The soluble chromatin from each digest represents 20, 80, and 80% of total Cl_3AcOH-insoluble radioactivity and 20, 90, and 90% of total chromatin protein, respectively. Soluble chromatin samples were applied to native chromatin gels (see Materials and Methods): insert I represents a photograph of an ethidium bromide stained gel, insert II, a radioautogram of the corresponding gel. Soluble chromatin samples were also applied to 5–20% linear sucrose gradients. Direction of sedimentation is from left to right: A_{260nm} (●—●) and Cl_3AcOH-insoluble radioactivity (○—○) were determined for each fraction. Chromatin fractions from regions 1, 2, 3, and 4 of the gradients were pooled separately; proteins were precipitated and electrophoresed (see Materials and Methods). After electrophoresis, gels were stained with Coomassie Blue, dried and exposed for radioautography. 11S, catalase marker; 1N, mononucleosome; H1, histone H1; 2N (dimer), dinucleosome; 3N, trinucleosome; 4N, tetranucleosome; WN, whole nuclei.

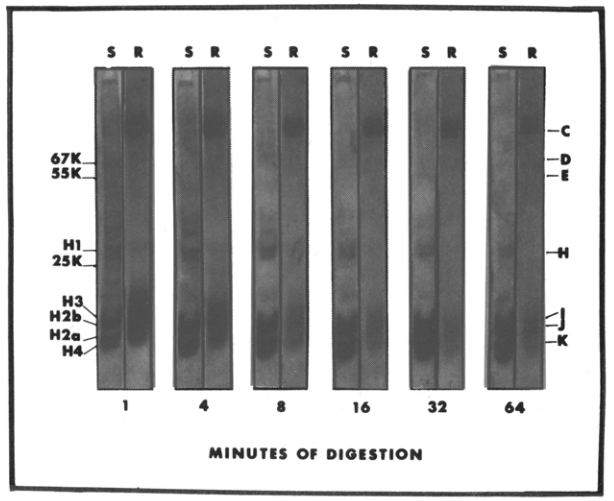

FIGURE 5: Electrophoretic analysis of ADP-ribosylated chromatin proteins associated with dinucleosomes from various extents of micrococcal nuclease digestion. ADP-ribosylated S-phase nuclei were digested with micrococcal nuclease for varying periods of time (1–64 min) as described in Figure 4. Dinucleosomes were separated via velocity sedimentation and proteins prepared for electrophoresis as described in Figures 2 and 4. These gels contained 7% acrylamide instead of the 5–22% acrylamide used previously. All other conditions were maintained. The dinucleosomes from the various digestion times (1–64 min) are characteristic of 2–40% perchloric acid soluble digest. Proteins were stained (S) with Coomassie Blue and dried for radioautography (R).

Table II: Poly(ADP-Rib) Polymerase Activity Associated with Mono-, Di-, and Trinucleosomal Fragments from Different Chromatin Digests[a]

% perchloric acid digest	poly(ADP-Rib) polymerase sp act. ($\times 10^{-5}$) (cpm incorp $A_{260\ nm}^{-1}$ (15 min)$^{-1}$)		
	mono-nucleosome	di-nucleosome	tri-nucleosome
3	0.53	2.87	4.00
11	0.35	1.68	2.30
17	0.21	1.50	1.67

[a] Micrococcal nuclease digested chromatin was prepared and fractionated as described in Figure 4, except nuclei (mid-S phase) were not previously modified with NAD. The resultant mono-, di-, and trinucleosome regions of the gradients were analyzed for poly(ADP-Rib) polymerase activity (Mullins et al., 1977).

In our previous report, the acceptor population of (ADP-Rib) in nuclei (in situ) and in isolated chromatin fragments (in vitro) was not entirely similar (Giri et al., 1978a). A HMG (high mobility group protein) (ca. 25 000 mol wt) was an acceptor for ADP-Rib in isolated chromatin fragments. The modification of these proteins in vitro reflects the wider spectrum of proteins made available as a result of nuclease digestion. Similar observations have been made for phosphorylation of chromatin proteins (Bohm et al., 1977). The significance of these proteins as in vivo acceptors in highly organized chromatin is still unexplained and may reflect their proximity to the poly(ADP-Rib) polymerase during in vitro incubation. Others have reported that certain HMG proteins serve as major acceptors in highly specialized cells, i.e., trout testis (Levy et al., 1978). The fact that these proteins are not major acceptors for poly(ADP-Rib) in nuclei from replicating HeLa cells may reflect the structural differences in the two forms of chromatin.

Discussion

A major observation concerning chromatin complexity and poly(ADP) ribosylation that emerged from this study was the preferential release by micrococcal nuclease of chromatin subfractions enriched in poly(ADP)-ribosylated proteins (both histone and nonhistone proteins), poly(ADP-Rib) polymerase activity, and nascent DNA from the DNA replicating fork. A number of reports have indicated that newly synthesized DNA in cells (in vivo), or in nuclei (in vitro), is rapidly packaged into chromatin subunits (Shelton et al., 1978; Hildebrand & Walters, 1976; Seale, 1976; Hancock, 1978). Micrococcal nuclease has been shown to selectively digest chromatin near the DNA replicating fork (Figure 2) (Hildebrand & Walters, 1976; Seale, 1976). In this report, histones in a subset of nucleosomes proximal to the site of initial attack by micrococcal nuclease were found to be extensively ADP ribosylated (Figure 5). This observation has three possible explanations: (a) ADP ribosylation of nucleosomal histones is a post-replicative event that occurs after nucleosome formation and near the DNA-replicating fork to promote refolding of chromatin after replication; (b) accessibility of modified chromatin to digestion is increased as a result of ADP ribosylation; or (c) modification of nucleosomal histones is a prereplicative event where modification of histones is required to relax closely packaged nucleosomes to increase accessibility of the DNA to the replicating complex. We have not observed significant changes in chromatin digestibility by nuclease as a result of ADP ribosylation (data not shown). However, if ADP ribosylation is restricted to a small fraction of the genome as the data suggest, a change in chromatin structure would probably not be detected by micrococcal nuclease digestion kinetics. Earlier studies reported by Smulson et al. (1975) showed increased template availability to *Escherichia coli* DNA polymerase I as a result of ADP ribosylation.

Recent data (Jump et al., manuscript in preparation) has shown that ADP-Rib is covalently linked to amino acid residues near the NH_2 termini of nucleosomal histones. Since the basic amino acid residues can potentially interact with DNA (Whitlock & Simpson, 1977), this peripheral location of poly(ADP-Rib) on nucleosomes permits two types of interactions: (a) repulsion of the ADP-ribosylated NH_2 termini of the histones from DNA; or (b) a change in the histone–histone interactions through electrostatic interaction between poly(ADP-Rib) and amino acid residues near the NH_2 termini of adjacent nucleosomes. In each case, poly(ADP-Rib) can be seen to potentially influence chromatin structure at the nucleosomal level and in higher ordered levels where nucleosomal histones and DNA interact to stabilize more compact structures.

It is highly likely that extended regions of the genome are characterized by several functional events that occur simultaneously but are restricted to separate domains. For example, recent studies by Simpson (1978) have shown that hyperacetylated chromatin is very labile to micrococcal nuclease digestion. Reversible acetylation of histones, particularly H3 and H4, is thought to be restricted to that region of the genome undergoing RNA synthesis (Simpson, 1978).

However, others have reported that poly(ADP-Rib) polymerase is not preferentially associated with transcriptionally active chromatin using DNase II to segregate active from inactive chromatin (Yukioka et al., 1978). Using similar techniques, we have not found acceptors for poly(ADP-Rib) in transcriptionally active chromatin (data not shown). Smerdon & Lieberman (1978) recently reported that regions of chromatin undergoing DNA repair are rapidly digested by micrococcal nuclease. The data presented here suggest that ADP ribosylation has an apparent association with regions of

chromatin undergoing DNA synthesis, either replication or repair. Correlative studies between DNA repair and poly(ADP-Rib) polymerase activity have been previously reported (Smulson et al. 1977; Miller, 1976). We suggest that poly(ADP) ribosylation of chromatin proteins functions to influence higher ordered chromatin for the synthesis of DNA during replication or repair. The mechanisms for this complicated process remain to be determined. It is of significance to note that Stone et al. (1977) have described the in vitro presence of a dimer of histone H1 covalently cross-linked by a 15-unit chain of poly(ADP-Rib). The synthesis of this complex is markedly enhanced in soluble chromatin by agents such as polyamines that induce chromatin condensation (Byrne et al., 1978). We have reported that poly(ADP-Rib) polymerase specific activity increases progressively with nucleosome repeat size (Butt et al., 1978). Maximal activity was found on polynucleosomes of repeat size 8N. More recently, it has been found that a poly(ADP-Rib)–H1 complex could be synthesized in larger polynucleosomal structures (16N, data not shown).

In conclusion, the distribution of a chromatin modifying enzyme, poly(ADP-Rib) polymerase, has been correlated with extended forms of chromatin undergoing replication. The type of detailed analysis developed for this study has illustrated the close relationship between structure and function of specific nuclear events. It is not unreasonable to expect that other nuclear modification processes respond to or cause structural changes in chromatin that occur during the cell cycle. Therefore, this is an area that merits further in-depth study.

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