A Relationship between Nuclear Poly(adenosine diphosphate ribosylation) and Acetylation Posttranslational Modifications. 2. Histone Studies†

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ABSTRACT: In the accompanying paper [Malik, N., & Smulson, M. (1984) *Biochemistry* (preceding paper in this issue)], we report that certain acetylated domains of chromatin were selectively retained by an anti-poly(ADP-Rib) antibody column. In this paper, we describe investigations of this phenomenon at the molecular level of protein interactions. We observed that the majority of endogenously hyperacetylated histones have a high affinity toward the polymer antibody column. It is speculated that these proteins were bound to the column via endogenous poly(adenosine diphosphate ribose) [poly(ADP-Rib)] since the binding was reversed upon treatment of the histones with alkali prior to immunofractionation. In order to analyze the distribution of acetate and poly(ADP-Rib) on histone proteins, [^{3}H]acetylated nuclei were incubated in vitro with [^{32}P]NAD. Acetate was incorporated mainly into H3 and H4 while H1 was the major acceptor protein for poly(ADP-Rib). These results suggest that a correlation may exist in vivo between the two posttranslational modification processes and that identical histone molecules may be accessible to both modifications.

The chromatin-associated enzyme poly(ADP-Rib) polymerase catalyzes the posttranslational modification of histones. In an earlier work, antibody to poly(ADP-Rib)[1] was coupled to Sepharose, and the resultant immunoadsorbent was used to fractionate, specifically, histone H1 subpopulations undergoing this nuclear protein modification (Wong et al., 1983b). When this method of separation was used, it was additionally observed that poly(ADP-ribosylated) H1 species are highly accessible to in vitro phosphorylation by nuclear protein kinase. It was noted that phosphorylated H1 molecules were retained by the anti-poly(ADP-Rib)–Sepharose column due to the presence of endogenous poly(ADP-Rib) components. We concluded at that time that the same molecular species of histone H1 may be comodified by both phosphorylation and poly(ADP-ribosylation) (Wong et al., 1983b).

In the accompanying paper (Malik & Smulson, 1984), the relationship between poly(ADP-ribosylation) and the acetylation modification of chromatin was investigated. The data showed that pulse-labeled, acetylated domains of nucleosomal chromatin were selectively retained by anti-poly(ADP-Rib) IgG–Sepharose. It was of importance to ascertain whether this specific retention was due to a similarity in the general proximity of both posttranslational modifications within regions of chromatin and/or the actual dual modification of the same histone molecules within nucleosomes by both the acetylation- and poly(ADP-ribosylation)-modifying enzymes. The following work has studied this question by characterizing the immunoaffinity of purified histones modified by both acetylation and poly(ADP-ribosylation).

Materials and Methods

Labeling of Cells with [^{3}H]Acetate. Logarithmically growing HeLa S3 cells [(3–4) × 10^5 cells/mL] were labeled as described in the preceding paper (Malik & Smulson, 1984).

Preparation of Modified Histones for Poly(ADP-Rib) Antibody Column. Either hyperacetylated or nontreated nuclei, prepared according to Sporn et al. (1969), were incubated with 50 μM [^{32}P]NAD in an incubation mixture containing 50 mM Tris-HCl, pH 8.0, 2 mM DTT, and 2 mM $MgCl_2$ for 15 min at 25 °C. The reaction was terminated by the addition of acid to 0.4 N H_2SO_4. Acid-soluble proteins were precipitated with 20% Cl_3CCOOH. The precipitates were washed with cold acidified acetone/acetone and dried (Butt & Smulson, 1980). Subsequently, the acid-extracted proteins were loaded onto a polymer antibody column and analyzed as previously described (Malik et al., 1983).

Gel Electrophoresis. Histones were electrophoresed in Triton/acetic acid/urea–polyacrylamide gels containing 6 M urea and 0.3% Triton X-100 as described by Alfageme et al. (1974). Fluorographic analysis of the proteins was performed by the use of New England Nuclear's EN^{3}HANCE.

Results

Effect of Sodium Butyrate on Poly(ADP-Rib) Polymerase Activity. The acetylation modification of histones, as shown in the preceding paper (Malik & Smulson, 1984), can be conveniently studied by exposing cells to sodium butyrate, which causes a reversible accumulation of highly acetylated histones in their nuclei through an inhibition of histone deacetylase (Vidali et al., 1978; Cousens et al., 1979; Nelson et al., 1980; Covault & Chalkley, 1980). To establish first the effects of cellular treatment with butyrate on the incorporation of [^{32}P]ADP-Rib into histones and non-histone nuclear proteins, HeLa cells were exposed to 50 mM sodium butyrate for either 1 (hypoacetylation) or 18 h, the latter condition known to substantially increase the levels of acetylation of the nucleosomal core histones (Simpson, 1978; Sealy & Chalkley, 1978; Vidali et al., 1978, Cousens et al., 1979). Nuclei from control and butyrate-treated cells were isolated and assayed for the poly(ADP-Rib) polymerase activity (Table I). Although sodium butyrate inhibited the enzymatic activity by 12–25%, there was no significant alteration in the distribution of [^{32}P]ADP-ribosylation among the various acid-soluble protein acceptors, as determined by electrophoresis of isolated

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[1] Abbreviations: ADP-Rib, adenosine diphosphate ribose; NAD, nicotinamide adenine dinucleotide; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; KSCN, potassium thiocyanate; Cl_3CCOOH, trichloroacetic acid; DTT, dithiothreitol.

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Table I: Effect of Sodium Butyrate on Poly(ADP-ribosylation) of Nuclear Proteins[a]

butyrate concn (mM)	time of incubation (h)	poly-([^{32}P]ADP-Rib) cpm ($\times 10^{-4}$)	% inhibition
0	1	8.4	
50	1	7.4	12.6
0	18	8.3	
50	18	6.1	25.2

[a] HeLa cells were incubated with butyrate as indicated above, and then nuclei were prepared. Nuclei (2.5×10^6) were incubated with 0.4 μCi of [^{32}P]NAD (10 μM) at 25 °C for 10 min. The amount of radioactivity incorporated into the acid-insoluble fraction was determined as previously described (Mullins et al., 1977).

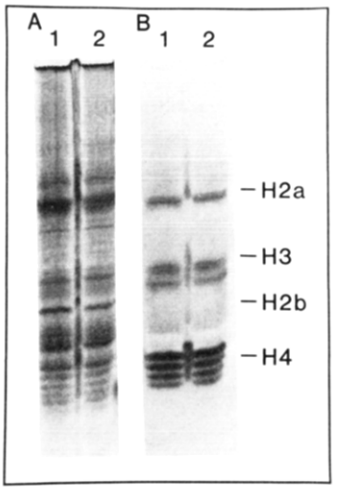

FIGURE 1: Fluorogram of acetylated acid-soluble nuclear proteins. Hyperacetylated nuclear proteins were extracted by 0.4 N H_2SO_4 from nuclei derived from [^{3}H]acetate-labeled cells as described in the text. The acid-soluble proteins were subsequently analyzed by Triton/acetic acid/urea gel electrophoresis and fluorography as described under Materials and Methods. Lanes 1 and 2 are duplicates. The assignment of the position of histones was based upon comparison with the data of Alfageme et al. (1974) and upon the migration of histone standards. (A) Amido black stain; (B) fluorogram.

nuclear proteins (data not shown).

Binding of Hyperacetylated and in Vitro Poly(ADP-ribosylated) Acid-Soluble Proteins to Anti-Poly(ADP-Rib) IgG–Sepharose. HeLa cells were pulse labeled for 30 min in vivo with [^{3}H]acetate and chased for 18 h with 50 mM sodium butyrate as described previously (Malik & Smulson, 1984). Subsequently, nuclei from the labeled cells were prepared, and the extent of acetylation of histones present in hyperacetylated nuclei was examined by electrophoresis of acid-soluble proteins on a Triton/acetic acid/urea gel (Figure 1). The fluorograph of the gel (see also Figure 5) shows that [^{3}H]acetate was incorporated predominantly into multimodified species of H3 and H4 and to a lesser extent into H2a, H2b, consistent with the observations of other laboratories (Simpson, 1978; Perry & Chalkley, 1982). These data, obtained with intact nuclei, differ slightly from the acetylation pattern noted for histones extracted from oligonucleosomes in the preceding paper (Malik & Smulson, 1984). In those experiments, amido black staining of gels indicated that less than 50% of histones H3 and H4 accumulated acetate in the presence of butyrate.

To study the relationship between acetylation and poly(ADP-ribosylation) an immunoaffinity chromatography method was utilized (Malik & Smulson, 1984). Nuclei derived from ^{3}H-hyperacetylated cells were incubated with 50 μM [^{32}P]NAD in vitro, and subsequently, the nuclear proteins were

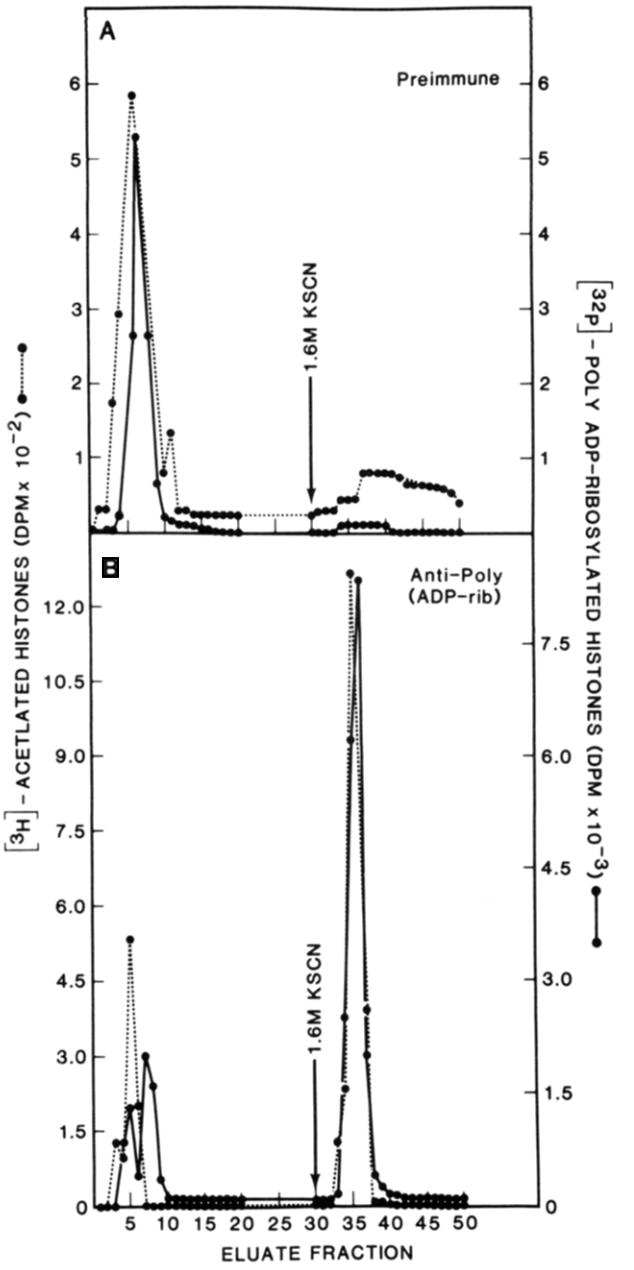

FIGURE 2: Immunofractionation of hyperacetylated and poly(ADP-ribosylated) acid-soluble proteins on anti-poly(ADP-Rib) antibody column. Nuclei labeled with [^{3}H]acetate as in Figure 1 were incubated with 50 μM [^{32}P]NAD under conditions optimal for poly(ADP-ribosylation) (Wong et al., 1983b). The nuclear proteins were subsequently extracted with 0.4 N H_2SO_4 as described under Materials and Methods. The sample was applied to either a preimmune IgG–Sepharose 4B column (A) or an anti-poly(ADP-Rib) IgG–Sepharose 4B column (B). Phosphate-buffered saline was applied to the column, and 1-mL fractions were collected. The acid-insoluble [^{3}H]acetate (--) and poly([^{32}P]ADP-Rib) (—) radioactive incorporation were monitored. When no further radioactivity eluted, an additional 10 fractions were collected. Subsequently, the bound material was eluted with 1.6 M KSCN.

acid extracted with 0.4 N H_2SO_4. The acid-soluble proteins were loaded onto either a preimmune IgG–Sepharose (Figure 2A) or an anti-poly(ADP-Rib) IgG–Sepharose column (Figure 2B). The conditions for fractionating poly(ADP-ribosylated) histones by immunofractionation columns were established previously (Wong et al., 1983b) and are essentially similar to the methods utilized in the preceding study on the fractionation of chromatin particles (Malik & Smulson, 1984). As shown in Figure 2B, the majority of the trichloroacetic acid precipitable [^{32}P]ADP-Rib and pulse-labeled [^{3}H]acetate radioac-

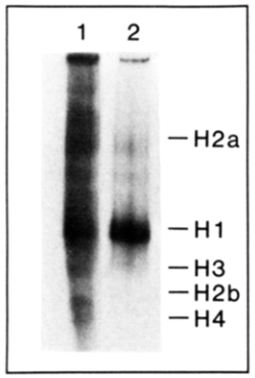

FIGURE 3: Analysis of poly(ADP-ribosylated) histone species bound by anti-poly(ADP-Rib) IgG–Sepharose 4B immunofractionation. Samples from unfractionated double-labeled histones (lane 1) and the bound material (lane 2) from the experiment of Figure 2 were precipitated with 20% Cl_3CCOOH and washed with acidified acetone/acetone. Electrophoresis of the samples on a Triton/acetic acid/urea–polyacrylamide gel and ^{32}P autoradiography were performed as described under Material and Methods.

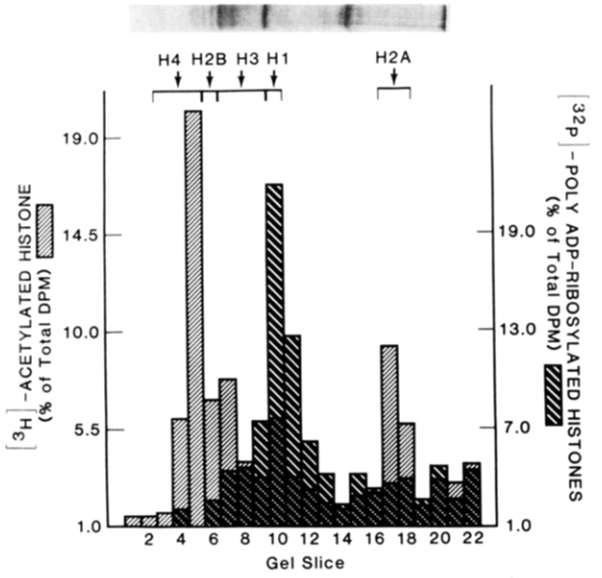

FIGURE 4: Analysis of double-labeled hyperacetylated and poly(ADP-ribosylated) histones retained on the anti-poly(ADP-Rib) IgG–Sepharose 4B column. Lane 2 of Figure 3 was sliced into different fractions according to its staining and ^{32}P-labeled patterns. The gel slices were solubilized, and the radioactivity for both ^3H and ^{32}P was quantified. In both cases, the data are expressed in each fraction as a percentage of the total dpm measured in all gel slices. [^3H]-Acetylated histones as percent of total dpm (light-hatched bars); poly([^{32}P]ADP-ribosylated) histones as percent of total dpm (dark-hatched bars). The dotted-hatched regions represent overlapping areas. The insert at the top is a representative (not to scale) stain of the unfractionated histones.

tivity was retained by the polymer antibody column and subsequently released by KSCN. When a preimmune IgG–Sepharose column was used (Figure 2A), essentially all of the poly(ADP-ribosylated) and acetylated histones passed directly through the column. In addition, removal of poly(ADP-Rib) from histones abolished binding of these proteins to the anti-poly(ADP-Rib) column (see below). These observations, obtained with semipurified nuclear proteins, are consistent with a similar experiment (Figure 3; Malik & Smulson, 1984) performed with intact chromatin and suggest that certain species of soluble nuclear proteins containing poly(ADP-Rib) may also be acetylated.

Since presumably all the acetylated histones bound to the anti-poly(ADP-Rib) column contain some level of poly(ADP-ribosylation), it was of subsequent interest to determine the relative extent of each modification on the various histones of the immunopurified, bound fractions. Immunofractionated samples from the bound fraction of Figure 2B were analyzed by Triton/acetic acid/urea–polyacrylamide gel electrophoresis and subsequent double-label quantitation. Figure 3 shows the ^{32}P autoradiography of the unfractionated (lane 1) and bound (lane 2) samples. Amongst the histones, H1 was noted to be the major poly(ADP-Rib) acceptor although other histones were also modified. The gel was then sliced into different sections according to its staining and ^{32}P-labeled profile. When the ^3H and ^{32}P radioactivity in each gel slice was quantitated as a percent of the total radioactivity of proteins on the gel (Figure 4), a marked difference in the distribution of the acceptor proteins for [^3H]acetate and poly([^{32}P]ADP-Rib) was noted. [^3H]Acetate was incorporated mainly into histones H3 and H4 while histone H1 was the major acceptor protein noted for poly(ADP-Rib). Accordingly, those acetylated species of inner core histones are not necessarily the most highly ADP-ribosylated subpopulations of these proteins.

Analysis of Hyperacetylated Histones Containing Endogenous Poly(ADP-Rib) on Anti-Poly(ADP-Rib) Antibody Column. In the studies above, nuclei were incubated with NAD in vitro, prior to extraction of histones for immunofractionation. However, we have previously described the endogenous presence of poly(ADP-ribosylated) histones in HeLa cells (Wong et al., 1983a). Additionally, in the preceding paper (Malik & Smulson, 1984), considerable amounts of pulse-labeled acetylated forms of oligonucleosomes were noted to bind to anti-poly(ADP-Rib)–Sepharose (but not to preimmune IgG–Sepharose) due to *endogenous* levels of polymer. Accordingly, acid-soluble proteins were extracted directly from nuclei containing hyperacetylated histones and immunofractionated (Figure 5A). From the elution profile, approximately 80% of the [^3H]acetylated proteins were absorbed to the column. In order to confirm that these hyperacetylated proteins were retained on the column due to an endogenous poly(ADP-Rib) linkage, a duplicate unfractionated sample was treated with alkali (Tris-HCl, pH 9.5) prior to rechromatography (Figure 5B). The bond between protein acceptors and poly(ADP-Rib) has been established to be alkali labile (Nishizuka et al., 1968; Riquelme et al., 1979) while the N-acetylated histone linkage is not susceptible to this reagent (Figure 5B, insert). In contrast to the earlier observation, essentially all of the ^3H-hyperacetylated histones from the alkali-treated sample were noted to elute directly through the column (Figure 5B).

It was of interest that in a number of experiments where only endogenous levels of poly(ADP-Rib) were investigated, not only were the poly(ADP-ribosylated) histones and a large percentage of the pulse-labeled acetylated histones retained by the anti-poly(ADP-Rib) columns but, in addition, was a majority of the histone protein mass. This was noted only when cells were treated for 18 h with 50 mM butyrate, and the histone binding was clearly reversed when poly(ADP-Rib) was cleaved from histones by alkali treatment. We have noted earlier that only approximately 10% of chromatin [albeit, nearly 100% of the poly(ADP-ribosylated) species] is retained by the anti-poly(ADP-Rib) IgG–Sepharose (Malik et al., 1983). Although no effect on in vitro polymerase activity was noted by the 18-h treatment of cells with butyrate (Table I), the observations described above suggest that butyrate may cause a large accumulation of poly(ADP-Rib) on acceptors in intact cells similar to its effect on acetylation levels. Poly ADP-ribosylation is stimulated in vivo by DNA strand breaks (Smulson et al., 1977; Sudhakar et al., 1979; Benjamin & Gill,

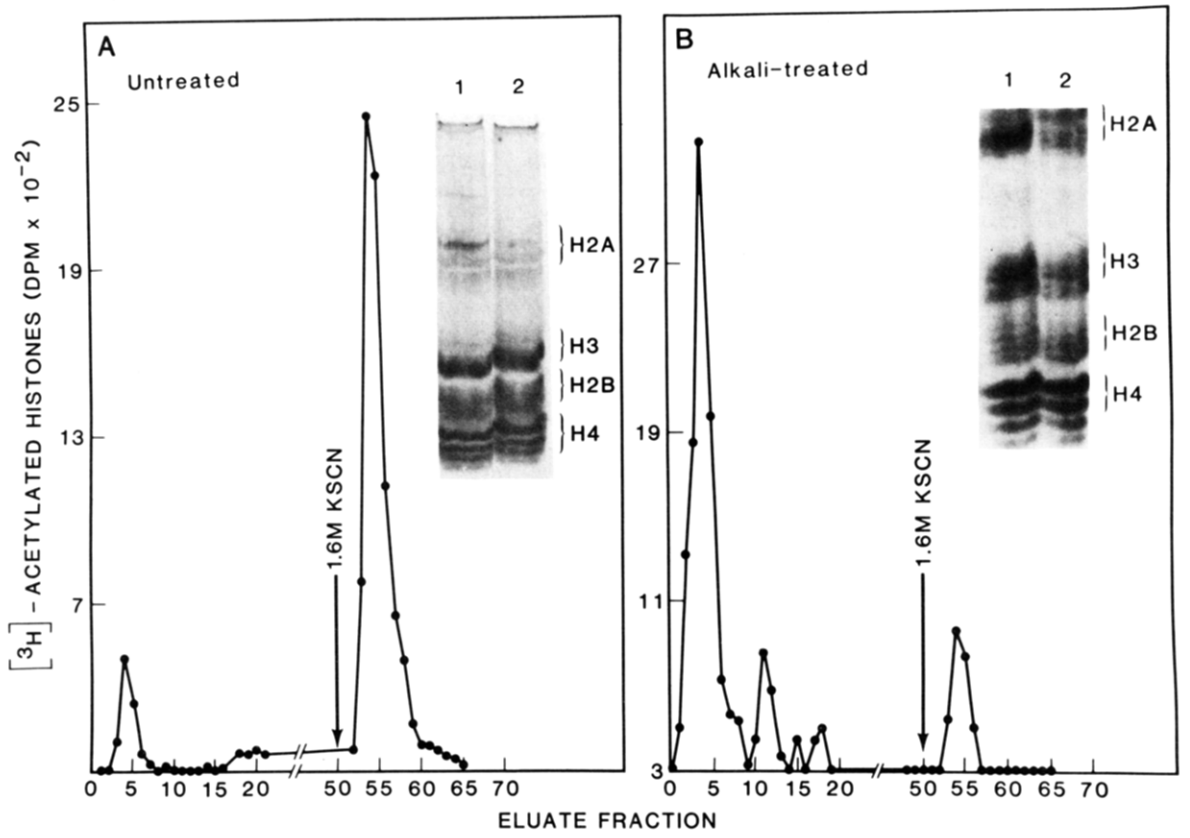

FIGURE 5: Isolation of endogenous poly(ADP-ribosylated) and acetylated histones by immunofractionation with anti-poly(ADP-Rib) antibody. Histones were extracted directly from [^{3}H]hyperacetylated nuclei with 0.4 N H_2SO_4 and precipitated with 20% Cl_3CCOOH prior to chromatography. (A) Chromatography on anti-poly(ADP-Rib) IgG–Sepharose was performed as described in Figure 2. The data represent acid-insoluble radioactivity. Fluorogram of original sample (lane 1) and bound (lane 2) proteins on a Triton/acetic acid/urea gel (see insert). (B) A histone sample, similar to above, was treated with 0.1 M Tris base (pH 9.5) for 1 h at 37 °C to release poly(ADP-Rib) (Butt et al., 1980); the reaction was terminated by the addition of HCl to pH 7.0. The chromatography on the anti-poly(ADP-Rib) column was performed as above. Fluorogram of the original sample (lane 1) and unbound (lane 2) proteins on a Triton/acetic acid/urea gel (see insert).

1980) and is thought to play a role in DNA repair (Shall et al., 1980; Thraves & Smulson, 1982). Accordingly, the above phenomenon may be related to the observations of Smerdon et al. (1982), which indicated that butyrate stimulates the rate of excision repair in cells. Additionally, Scher & Friend (1978) have observed that butyrate causes the accumulation of DNA strand breaks in erythroleukemic cells.

To characterize further the *endogenously* poly(ADP-ribosylated) and acetylated material retained on the antibody column, the proteins were analyzed by Triton/acetic acid/urea–polyacrylamide gel electrophoresis (Figure 5, inserts). The [^{3}H]acetylated proteins, separated by gel electrophoresis, were detected by fluorography. The fluorographs in Figure 5B show that the inner core histones were mutually acetylated and poly(ADP-ribosylated).

Discussion

The work presented in this and the preceding paper (Malik & Smulson, 1984), coupled with earlier observations on the concurrent phosphorylation and poly(ADP-ribosylation) of histone H1 (Wong et al., 1983b) and the mutual acetylation and phosphorylation of histone H3 as described by Whitlock et al. (1980), suggests the interesting possibility that similar domains of chromatin may be jointly accessible to various posttranslational modifications of histones. Using the novel immunofractionation matrix of anti-poly(ADP-Rib), we found that poly(ADP-ribosylated) chains of nucleosomes appear to be proximal to other nucleosomes or possible the same nucleosomes that are accessible to acetylation (Malik & Smulson, 1984). However, when these studies were performed with chains of oligonucleosomes, it was not clear whether the same molecule of histone was modified by both poly(ADP-ribosylation) and acetylation. Accordingly, as presented above, we have fractionated purified, endogenously acetylated, proteins on the immunofractionation matrix. A large percentage of hyperacetylated inner core histones were found to be retained by the anti-poly(ADP-Rib) IgG–Sepharose column. This was subsequently shown to be due to the presence of endogenous poly(ADP-Rib) bound to these histone molecules, since the removal of endogenous poly(ADP-Rib) with alkali eliminated the retention of these molecules to the antibody column. With chromatin (Figure 2A in preceding paper), only about 10% of the histone mass was retained by the column; this represented acetylated histone mass as well as 100% of the radioactive label.

Chromatin undergoes a transitional alteration in structure during the cell cycle, and microscopic changes are evident during periods of transcription and DNA replication. In addition, nucleosomes are heterogeneous in structure due to the existence of a variety of posttranslational modifications and sequence variations of the histones (Isenberg, 1979). Thus, a high level of histone acetylation has been found to be associated with transcriptionally active regions of chromatin (Levy-Wilson et al., 1979; Davie & Candido, 1980). An increase in the acetylation of histones presumably alters the relationship between DNA and protein interactions within nucleosomes ultimately allowing a reaction with RNA polymerases and other factors required for transcription. The direct involvement of the poly(ADP-ribosylation) modification with transcription at this point is still only very indirect. It

is, however, of relevance that Weisbrod (1982) has recently shown the association of topisomerase with affinity-purified, transcriptionally active nucleosomes. It was speculated that the breaking and closing of DNA may be a necessary requirement for reducing the supercoil of DNA in transcriptionally active nucleosomes. Therefore, it is of interest that the poly(ADP-ribosylation) modification has been shown by numerous approaches in vivo to be activated by the presence of single-strand breaks in DNA (Thraves & Smulson, 1982). Poly(ADP-Rib) polymerase alters the structure of nucleosomes by generating long chains of poly(ADP-Rib) that are covalently attached to various nuclear proteins. It was demonstrated earlier (Butt & Smulson, 1980) that NAD causes a concentration-dependant condensation of those nucleosomes undergoing poly(ADP-ribosylation) as evidenced by the retardation of the mobility of these modified particles during gel electrophoresis and an increased sedimentation during sucrose gradient analysis. It is conceivable that the cross-linking of nuclear proteins via poly(ADP-Rib) may be an important step in stabilizing regions of chromatin containing single-stranded breaks during transcription. The same region of chromatin and, in fact, the same molecule of histones may also be modified by the acetylation modification. Using previously described techniques (Malik & Smulson, 1984), we have initiated a series of experiments to isolate the domains of chromatin proximal to the poly(ADP-ribosylation) modification and to compare the DNA sequences in these nucleosomes with respect to those regions of chromatin distal to this type of modification for the presence of active genes.

Acknowledgments

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