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A Relationship between Nuclear Poly(adenosine diphosphate ribosylation) and Acetylation Posttranslational Modifications. 1. Nucleosome Studies†

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ABSTRACT: The chromatin-associated enzyme poly(ADP-Rib) polymerase catalyzes the posttranslational modifications of histones. Antibody to poly(adenosine diphosphate ribose) [poly(ADP-Rib)] has been coupled to Sepharose, and the resulting immunoadsorbant was used to fractionate, specifically, oligonucleosomes derived from cells pulse labeled for the acetylation modifications of chromatin by incubation with [^{3}H]acetate followed by treatment with sodium butyrate. Generally, about 50% of the histone H3 and H4 mass becomes acetylated under these conditions. Pulse-labeled acetylated regions of chromatin were selectively retained by the anti-poly(ADP-Rib)–Sepharose column due to the presence of endogenous poly(ADP-Rib) components. The data suggest that certain histone molecules might be mutally poly(ADP-ribosylated) and acetylated, and this phenomenon was further explored at the protein level in the accompanying paper [Wong, M., & Smulson, M. (1984) *Biochemistry* (following paper in this issue)].

Acetylation, phosphorylation, and poly(ADP-ribosylation) of histones are postsynthetic covalent modifications that may be involved in the modulation of chromatin structure and function. Major questions, still unanswered, are the temporal and topographic relationships between these modifications and how they work in concert to regulate various biological functions. Recently, we developed a technique that is capable of elucidating how these modifications may be structurally related. Antibody to poly(ADP-Rib)[1] was coupled to Sepharose, and the resultant immunoabsorbant was used to fractionate either subpopulations of poly(ADP-Rib) acceptor proteins (Wong et al., 1983a) or domains of polynucleosomes (Malik et al., 1983) undergoing this specific nuclear protein modification. By employment of immunofractionation with

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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.

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anti-poly(ADP-Rib), it was subsequently observed that poly(ADP-ribosylated) histone H1 species were highly accessible to in vitro phosphorylation by nuclear protein kinase (Wong et al., 1983a). Accordingly, phosphorylated H1 molecules were noted to be retained by the anti-poly(ADP-rib)–Sepharose column due to the presence of endogenous poly(ADP-Rib) components. This data prompted us to explore the possible topographic relationships between poly(ADP-ribosylated) and acetylated domains of chromain and, in the following paper (Wong & Smulson, 1984), acetylated histones.

Numerous correlations have been observed between an increase in histone acetylation and gene activation. For example, histone acetylation has been established to precede increases in RNA synthesis in lymphocytes following stimulation by mitogens and in liver following partial hepatectomy (Pogo et al., 1966, 1968). Some investigators have observed both increases and decreases of poly(ADP-ribosylation) with respect to gene expression (Berger & Sikorski, 1980; Bohr & Klenow, 1981; Althaus et al., 1982; Tanuma & Johnson, 1983). Moreover, acetylation of chromatin results in its increased susceptibility to DNase I (Vidali et al., 1978; Nelson et al., 1978), where the active genes are selectively digested by treatment of nuclei with DNase I (Garel & Axel, 1976; Weintraub & Groudine, 1976). Poly(ADP-ribosylated) domains exist in regions of chromatin highly accessible to micrococcal nuclease digestion (Jump et al., 1979); moreover, DNase I digestion of nuclei markedly enhances endogenous poly(ADP-Rib) activity (Miller, 1975).

In this and the following paper, we have utilized the above immunofractionation procedure, using antibody to the modified moiety, poly(ADP-Rib), to ascertain whether regions of chromatin proximal to poly(ADP-ribosylated) nucleosomes are also active in the acetylation modification and, subsequently in the following paper, whether the same histone molecules are mutually poly(ADP-ribosylated) and acetylated.

Materials and Methods

[^{3}H]Acetate and [^{32}P]NAD were obtained from New England Nuclear. $(NH_4)_2SO_4$-fractionated anti-poly(ADP-Rib) IgG, a gift from Dr. T. Sugimura, was obtained from rabbits as described (Kanai et al., 1974). CNBr-activated Sepharose 4B was purchased from Pharmacia. All chemicals used were high-purity grade.

[^{3}H]Acetate Labeling of the Cells. A total of 2×10^8 HeLa cells growing in suspension culture at a density of 2×10^5 cells/mL was pulse labeled with 1 mCi of [^{3}H]acetate (New England Nuclear, 4 Ci/mmol) for 30 min at 37 °C. The labeling was terminated by dilution with cold (4 °C) medium and centrifugation. Cell pellets were washed once with cold (4 °C) medium containing 50 mM sodium acetate and 50 mM sodium butyrate and subsequently incubated in 50 mM sodium butyrate for 1 or 18 h for hypo- or hyperacetylation, respectively (Perry & Chalkley, 1982).

Preparation of the Chromatin. Poly([^{32}P]ADP-ribosylation) was performed according to methods previously described (Malik et al., 1983).

Analysis of [^{3}H]Acetylated Histones. Total histones were extracted with 0.4 N H_2SO_4 (Butt & Smulson, 1980). The acid-soluble material was separated by 15% Triton/acetic acid/urea–polyacrylamide gel electrophoresis (Alfageme et al., 1974).

Immunofractionation of Acetylated Chromatin Particles. The coupling of anti-poly(ADP-Rib) antibodies to cyanogen bromide activated Sepharose 4B was as previously described (Malik et al., 1983). Hypo- or hyperacetylated oligonucleosomes modified in vivo and/or poly(ADP-ribosylated) in vitro were immunofractionated on anti-poly(ADP-Rib) IgG–Sepharose. The column running conditions were identical with those described previously (Malik et al., 1983). Immunofractionated fractions were trichloroacetic acid precipitated, and the acid-insoluble radioactivity of the individual fraction was determined (Malik et al., 1983).

Table I: Susceptibility of Acetylated Domains of Chromatin to Nuclease Digestion[a]

preparation	extent of digestion		acid-insoluble [^{3}H]acetylation	
	A_{260}	%	dpm × 10^{-5}	%
nuclei	50	100	30.8	100
S1	7.8	16	6.3	20
S2	11.6	23	9.3	30

[a] HeLa cells were labeled with [^{3}H]acetate and subsequently incubated for 18 h with 50 mM sodium butyrate as described under Materials and Methods. Nuclei were prepared as described previously (Butt et al., 1978; Malik et al., 1983) and were digested for 3 min with 60 units of micrococcal nuclease at 37 °C (Butt & Smulson, 1980; Malik et al., 1983). The digested nuclei were pelleted by centrifugation at 3600*g*. The supernatant (S1) was saved. The pelleted nuclei were subsequently lysed in 2 mM EDTA, pH 7.0. The solubilized oligonucleosomes (S2) were obtained by centrifugation of the lysed nuclei at 6800*g* for 10 min and characterized by electrophoresis (Figure 2). The A_{260} of the three samples as well as acid-precipitable radioactivity was determined.

Results and Discussion

Preparation of in Vivo Acetylated Polynucleosomes. HeLa cells were treated in vivo with [^{3}H]acetate for 30 min followed by incubation in the presence of 50 mM sodium butyrate, conditions reported to favor the hyperacetylation of chromatin domains (Perry & Chalkley, 1982). Sodium butyrate has been shown to induce profound changes upon cell growth and morphology (Boffa et al., 1981). Further, it has been observed that butyrate inhibits histone deacetylase, resulting in the accumulation of multiacetylated forms of histones in chromatin (Vidali et al., 1978a,b; Simpson, 1978; Candido et al., 1978; Sealy & Chalkley, 1978; Cousens et al., 1978; Boffa et al. 1978). Hyperacetylation of histones H3 and H4 at the ϵ-amino groups of specific internal lysine residues has been well established under these conditions (DeLange et al., 1975; Allfrey, 1977). In addition to its suppression of deacetylation, butyrate has also been shown to be a selective inhibitor of [^{32}P]phosphate incorporation into histones H1 and H2A in HeLa cells (Boffa et al., 1981).

After incubation of the cells with [^{3}H]acetate, nuclei were prepared from the cells and digested with micrococcal nuclease under well-characterized conditions previously established in the laboratory (Butt et al., 1978; Jump et al., 1979, 1980) in order to prepare polynucleosomes as a source of chromatin for further fractionation studies. Approximately 23% of the nuclear chromatin and 30% of the acetylated radioactivity were digested and released into a soluble form (S2) by micrococcal nuclease by this procedure (Table I). Native polyacrylamide gel electrophoresis of these particles was performed (Figure 1), and this is discussed below. The specific activity (i.e., dpm per absorbance unit) of the oligonucleosomal preparation (S2) was 803 compared to 616 for unfractionated whole nuclei. Accordingly, hyperacetylated domains of chromatin did not appear to be preferentially susceptible to nuclease digestion. This is consistent with the observations of Perry & Chalkley (1981), who noted increased susceptibility for DNase I but no specificity for micrococcal nuclease toward hyperacetylated regions of chromatin.

The ^{3}H-labeled polynucleosomes, characterized by native polyacrylamide electrophoresis (Figure 1) were noted to range

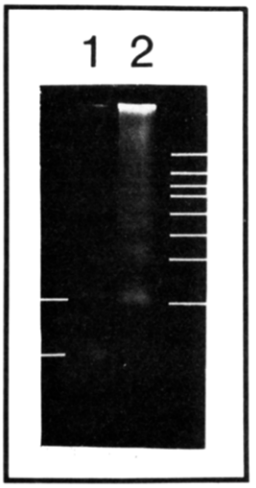

FIGURE 1: Polyacrylamide–agarose gel electrophoresis of acetylated native chromatin particles. An aliquot (0.03 A_{260}) of S1 (lane 1) or S2 (lane 2) as described in Table I and under Materials and Methods was electrophoresed on a 3% acrylamide–0.5% agarose gel with 89 mM Tris–borate, pH 8.3, containing 2.5 mM EDTA as running buffer and stained with ethidium bromide as previously described (Malik et al., 1982).

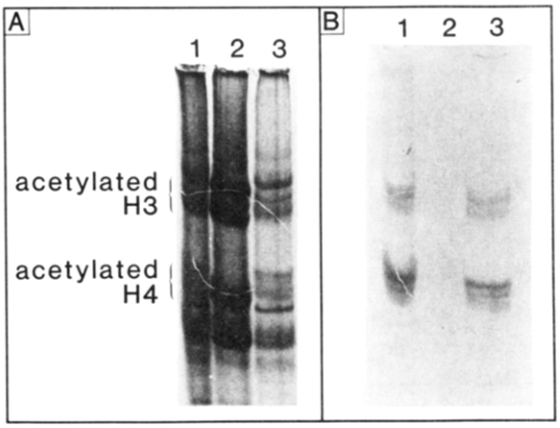

FIGURE 2: Analysis of acetylated histones present in unfractionated and immunofractionated oligonucleosomes. Acetylated nucleosomal chromatin was prepared as described in Table I and immunofractionated as previously described (Malik et al., 1983) and as noted in Figure 3. The samples of chromatin were pooled and dialyzed, and total histones were extracted with 0.4 N H_2SO_4 (Butt & Smulson, 1980). The samples were dissoved in pyronin Y containing buffer and separated on a 15% Triton/acetic acid/urea–polyacrylamide gel (Alfageme et al., 1974). After electrophoresis, the gel was treated with EN^{3}HANCE (New England Nuclear) and fluorographed. Lanes 1–3 represent the unfractionated sample and unbound and bound fractions, respectively. (A) Amido black stain; (B) autoradiography.

in chain size from one to eight nucleosomes. Exposure of the gel to fluorography (not shown) indicated [^{3}H]acetate incorporation roughly corresponded to the ethidium bromide staining of nucleosomes shown in Figure 1.

To assess the extent of histone acetylation in the inner histones of these particles, the total histones were acid extracted and subsequently analyzed on Triton/acetic acid/urea–polyacrylamide gels (Figure 2). The stain of the total histones (Figure 2A, lane 1) indicates that approximately 50% or less of histones H3 and H4 were acetylated under our conditions of butyrate treatment. The data (Figure 2B, lane 1) also show that the pulse-labeled acetylation occurred into multiacetylated families of histones H3 and H4 by the in vivo incubation. Negligible incorporation was noted into the other histones that were present on the gel as demonstrated by their staining properties. When total nuclear proteins from these preparations of oligonucleosomes were subjected to SDS electrophoresis, the majority of the [^{3}H]acetylated proteins were detected in the histone range of the gel (not shown). Therefore, minimal acetylation of non-histone proteins was present in the oligonucleosome preparations to be used subsequently for immunofractionation.

Separation of Acetylated and Poly(ADP-ribosylated) Chromatin by Immunoaffinity Chromatography. The anti-poly(ADP-Rib) IgG–Sepharose used in the subsequent studies has previously been shown to be specific for immunofractionation of poly(ADP-ribosylated) nucleosomes (Malik et al., 1983). Approximately 12% of unfractionated oligonucleosomes were noted to be bound to the antibody column; however, these represented essentially 100% of the original poly(ADP-ribosylated) nucleosomal species in an unfractionated chromatin sample (Malik et al., 1983). Past biochemical characterizations of this fraction were performed with oligonucleosomes that had been incubated in vitro with [^{32}P]NAD prior to immunochromatography. The poly(ADP-ribosylated) chromatin was found not to be retained by preimmune IgG columns. The eluted chromatin components were shown to contain poly(ADP-ribosylated) histones, as well as automodified poly(ADP-Rib) polymerase. Non-poly(ADP-ribosylated) nucleosomes, connected and proximal to the modified regions, were shown to copurify during this procedure, and the bound nucleosomes were found to be enzymatically active for the polymerase reaction after elution from the antibody column (Malik et al., 1983).

The data in Figure 1 show a native polyacrylamide gel of the nucleosomes to be utilized for immunofractionation. The preparation contained chains of oligomers from 1 to 8 units in length. Data not shown indicated that [^{3}H]acetate incorporation performed in vivo and [^{32}P]NAD incorporation performed in vitro (see below) corresponded to the ethidium bromide stain of the particles as shown in Figure 1, lane 2.

Pulse-labeled [^{3}H]acetylated oligonucleosomes were poly(ADP-ribosylated) in vitro with [^{32}P]NAD (10 μM) and immunofractionated, first on a preimmune IgG–Sepharose column as a control to monitor nonspecific interaction of material with the immunofractionation matrix (Figure 3, top). Approximately 95% of the [^{3}H]acetylated and poly([^{32}P]-ADP-ribosylated) nucleosomes were eluted during the washing of the column with phosphate-buffered saline, whereas only 5% of the radioactive nucleosomes were absorbed by the column. A similar sample of oligonucleosomes was applied to an anti-poly(ADP-Rib) IgG–Sepharose column (Figure 3, bottom). The data indicate that 87% of the poly([^{32}P]ADP-ribosylated) oligonucleosomes were specifically bound to the polymer antibody column, consistent with previous experiments (Malik et al., 1983). Approximately 13% of the poly(ADP-ribosylated) chromatin was noted to pass directly through the column and elute with the buffered saline. Past experiments indicate that these forms of chromatin may represent mono-(ADP-ribosylated) species of nucleosomes, which are poorly recognized by the anti-poly(ADP-Rib) antibody (Wong et al., 1983a).

The data also demonstrate that 69% of the pulse-labeled acetylated forms of polynucleosomes were retained by the anti-poly(ADP-Rib) IgG–Sepharose column. Although hyperacetylation conditions were utilized, it should be noted that only those domains of chromatin undergoing acetylation during the brief labeling period (30 min) are actually being examined, and these must be accessible to poly(ADP-ribosylation). By the use of [^{3}H]lysine- and [^{3}H]arginine-labeled chromatin, it was earlier shown that the poly(ADP-ribosylated) histones, attached to stretches of oligonucleosomes bound to the column,

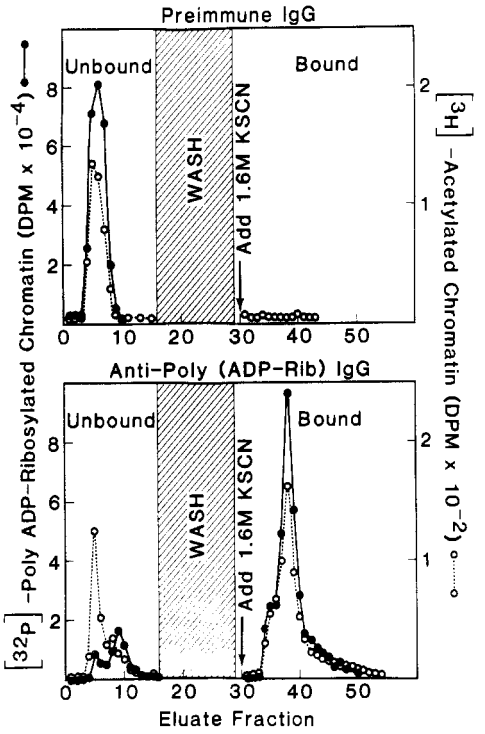

FIGURE 3: Fractionation of acetylated oligonucleosomes on preimmune IgG–Sepharose or anti-poly(ADP-Rib) IgG–Sepharose: HeLa cells labeled in vivo with [^{3}H]acetate (Materials and Methods) were harvested, and nucleosomes were prepared as described in Table I. The in vivo acetylated nucleosomes were poly(ADP-ribosylated) in vitro with [^{32}P]NAD (Materials and Methods). Immunofractionation was performed as described in detail previously (Malik et al., 1983). The top graph shows the fractionation on preimmune IgG–Sepharose, and the bottom graph shows the immunofractionation on anti-poly(ADP-Rib) IgG–Sepharose. (●) and (○) represents the acid-insoluble poly(ADP-ribosylated) or acetylated radioactivity, respectively.

had only a 6-fold enrichment of the modification compared to histones of unfractionated chromatin (Malik et al., 1983). This indicated that *non*-poly(ADP-ribosylated) nucleosomes, connected and proximal to the modified regions, were copurified by this immunofractionation procedure (Malik et al., 1983). Accordingly, the data in Figure 3 would suggest that acetylated regions of chromatin are in close association with the same limited domains of chromatin that are undergoing poly(ADP-ribosylation), indicating a possible functional correlation between these two covalent modifications of chromatin.

Effects of Endogenous Poly(ADP-ribosylation). The experiments described above were performed with in vivo hyperacetylated and in vitro poly(ADP-ribosylated) preparations of chromatin. It was of importance to investigate the immunofractionation properties of hyperacetylated chromatin not treated in vitro with labeled NAD [i.e., only those poly(ADP-ribosylated) domains modified endogenously].

The results of a number of experiments are tabulated in Table II. The binding of hypo- and hyperacetylated chromatin was more or less analogous. A considerable amount of retention by anti-poly(ADP-Rib) was noted in samples that were not treated with NAD, presumably due to the presence of small quantities of endogenous poly(ADP-Rib) in the nucleosomes (Malik et al., 1983). This is consistent with past observations on the presence of antigenic reactive quantities of endogenous poly(ADP-Rib) existing in both in vivo (Wong et al., 1983b) and in vitro preparations (Wong et al., 1983a).

The ^{3}H incorporation into bound and unbound nucleosomes was further characterized by acid extraction and electrophoresis (Figure 2, lanes 2 and 3). Radioactivity in the bound fractions was noted to reside predominantly in the multi-

Table II: Anti-Poly(ADP-Rib) Immunofractionation of Acetylation Domains of Chromatin: Influence of in Vitro ADP-Ribosylation[a]

in vivo and in vitro pretreatment of nucleosomes	[^{3}H] acetylation (%)		poly-([^{32}P]ADP-ribosylation) (%)	
	bound	unbound	bound	unbound
hyperacetylated	74	26		
hyperacetylated and poly(ADP-ribosylated) (in vitro)	69	31	87	13
hypoacetylated	79	21		
hypoacetylated and poly(ADP-ribosylated) (in vitro)	76	24	99	1

[a] The immunofractionation on anti-poly(ADP-Rib)–Sepharose and radioactive incorporation of ^{3}H derived from in vivo labeling or ^{32}P derived from in vitro incubation of unfractionated samples of chromatin with [^{32}P]NAD prior to fractionation were performed as shown in Figure 3. Hyper- and hypoacetylation conditions of cells are described under Materials and Methods.

acetylated species of the inner core histones H3 and H4. The data in Figure 2B show that labeled acetylated histones are found only in the bound nucleosomes, while considerable unmodified H3 and H4 (by stain) were in the unbound chromatin (Figure 2A). The intensity of the stains in lanes 2 and 3 (Figure 2) are representative of the mass of chromatin unbound and bound on the column. Accordingly, approximately 10% of the chromatin mass was retained by the column. Inspection of lanes 2 and 3 clearly indicates that stainable acetylated histones from nucleosomes were retained by the column. However, since labeling only occurred for 30 min, while butyrate incubation progressed for 18 h, not all the mass of the arginine-rich histones would be expected to be radioactive.

Snake venom phosphodiesterase digestion of polymer attached to nucleosomes (Butt et al., 1980) or purified acceptors (Wong et al., 1983a) has been found to be only partially effective at cleaving poly(ADP-Rib) on these preparations; nevertheless, a loss of 20–30% of the binding capacity of acetylated nucleosomes was achieved by treating a sample of [^{3}H]nucleosomes with this enzyme. Furthermore, acetylated chromatin had been previously noted (Figure 3) not to be retained by a preimmune IgG–Sepharose column. These data suggest that endogenous poly(ADP-ribosylated) domains of chromatin, in close proximity to the acetylated regions, caused the retention of the latter (Table II) to the column.

We were interested in investigating whether the poly(ADP-ribosylated) fractionated forms of oligonucleosomes are enriched in transcribed gene sequences, since there is considerable information on the relationships between acetylated domains of chromatin and actively transcribed sequences (Levy-Wilson et al., 1979; Gorovsky et al., 1973; Gottesfeld & Butler, 1977; Weisbrod & Weintraub, 1981). High levels of acetylated histones and HMG 14 and 17 have been found to be associated with transcriptionally active regions of chromatin. These latter proteins are also ADP-ribosylated (Levy-Wilson et al., 1979; Wong et al., 1983a). Furthermore, DNase I preferentially cleaves transcriptionally active DNA sequences within chromatin (Garel & Axel, 1976; Weintraub & Groudine, 1976). We used Cedar's (Gazit & Cedar, 1980) method to DNase I digest, and subsequently to label with DNA polymerase I and [^{32}P]dCTP, those regions of nuclei that are sensitive to DNase I. Nucleosomes were prepared and immunofractionated on the anti-poly(ADP-Rib)–Sepharose column as described above. Approximately 70–75% of

the dCTP-labeled regions of chromatin, representing around 10% of the applied chromatin, were retained by the Sepharose. However, since the radioactivity bound to the preimmune columns (i.e., Figure 3, top) varied from 32 to 50% in these experiments, it is not clear at this time whether enrichment of transcriptionally active oligonucleosomes has been achieved in these experiments. Experiments utilizing anti-HMG 17–Sepharose have already been reported subsequently (Malik et al., 1984).

The immunofractionation procedures developed here with nucleosomes should allow more detailed studies to be performed on the possible biological relationships between various posttranslational modifications of chromosomal proteins and those regions of nucleosomes bounded by these modification systems. Both histones H3 and, to a lesser extent, H4 (the predominant acetylation nuclear targets) have also been shown to be acceptors for the poly(ADP-ribosylation) modification (Jump et al., 1980). One potential implication of the experiments described above is that the same molecular species of these histones may be mutually poly(ADP-ribosylated) and acetylated. A similar type of relationship has been described by Whitlock et al. (1980) for the acetylation and phosphorylation of H3 and H4 and also for poly(ADP-ribosylation) and phosphorylation of histone H1 (Wong et al., 1983a). Accordingly, in the following paper (Wong & Smulson, 1984) acetylated histones, purified from nuclei, have been characterized by immunofractionation to asssess the putative relationships between these modifications at the molecular rather than at the nucleosomal level of chromatin organization.

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