Association of Poly(adenosine diphosphate ribosylated) Nucleosomes with Transcriptionally Active and Inactive Regions of Chromatin†

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ABSTRACT: We have investigated whether transcriptionally active or inactive gene sequences are associated in vivo with poly(adenosine diphosphate ribosylated) regions of chromatin. Soluble HeLa cell chromatin derived from nuclei treated either briefly or extensively with micrococcal nuclease was fractionated on an anti-poly(adenosine diphosphate ribose)–Sepharose column [Malik, N., Miwa, M., Sugimura, T., Thraves, P., & Smulson, M. E. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2554–2558] to obtain fractions that were enriched or depleted in poly(ADP-ribosylated) chromatin. DNA obtained from these fractions was then probed for active and inactive gene sequences with a cDNA probe made from total cell mRNA and a probe for the β-globin gene. Chromatin enriched in poly(ADP-ribosylated) nucleosomes contained both active and inactive gene sequences as detected by the probes and appeared to be more nuclease sensitive than that found in the fraction of chromatin depleted of poly(ADP-Rib). Poly(ADP-ribosylated) chromatin from nuclei digested briefly with nuclease showed an enrichment in both active and inactive genes while that treated extensively with nuclease showed either no enrichment or a depletion of active and inactive genes. Actively transcribed chromatin was digested at a rate several times that of the bulk or inactive chromatin. Nevertheless, the enrichment of active genes in poly(ADP-ribosylated) nucleosomes derived from brief nuclease digestion was greater than that of inactive genes. These results are interpreted as showing that some, but not all, of actively transcribed chromatin contains associated poly(ADP-ribosylated) proteins. However, since poly(ADP-ribosylated) proteins are also associated with inactive genes, the function of this modification cannot be assigned solely to transcription.

Poly(ADP-ribosylation) is a unique nuclear posttranscriptional modification of proteins, catalyzed by a ~116000-dalton non-histone protein, that involves the covalent attachment of ADP-Rib[1] units from NAD to nuclear acceptor proteins (Smulson & Sugimura, 1980; Hayaishi & Ueda, 1982). The function(s) for this nuclear protein modification has (have) not been well established. Both structural and regulatory roles have been suggested. The cross-linking of two H1 molecules has been demonstrated in vitro (Stone et al., 1977; Butt & Smulson, 1980) and in vivo (Wong et al., 1983) and may be involved in the maintenance of higher order chromatin structure.

Several protein acceptors of poly(ADP-Rib) in the nucleus have been identified. These include all five histone proteins, HMG 14 and 17 (Levy et al., 1977), possibly a DNA ligase (Creissen & Shall, 1982), a DNA topoisomerase (Ferro et al., 1983), a DNA endonuclease (Yoshihara et al., 1975), and poly(ADP-Rib) polymerase itself (Yoshihara et al., 1977; Jump et al., 1980). Enzyme activity in most cases is decreased by the modification, presumably resulting from a decrease in binding affinity for DNA. Histones modified by hyperacetylation (Wong & Smulson, 1984) or by phosphorylation (Wong et al., 1983b) also appear to be poly(ADP-ribosylated).

The involvement of poly(ADP-ribosylation) has also been suggested in DNA repair, where a rapid increase in polymer synthesis can be demonstrated when cells are treated with DNA damaging agents (Smulson et al., 1977; Durkacz et al., 1980; Juarez-Salinas et al., 1979; Berger et al., 1980). The signal for this rapid increase in poly(ADP-ribosylation) is apparently DNA strand breaks to which the poly(ADP-Rib) polymerase binds. In this context, it is of interest that Weisbrod (1982) has shown that topoisomerase I is enriched in transcriptionally active erythrocyte mononucleosomes. It was speculated that the DNA nicking activity of this enzyme may be involved in RNA synthesis. The transient DNA strand breaks caused by this enzyme may act as the signal for poly(ADP-ribosylation) in actively transcribed chromatin. The putative involvement of HMG 14 and 17, acetylated histones, and topiosmerase I in transcription suggested to us that poly(ADP-ribosylation) may also play a role in this process.

By use of anti-poly(ADP-Rib) coupled to Sepharose (Wong et al., 1983a), poly(ADP-ribosylated) proteins have been immunospecifically fractionated from total nuclear protein. In addition, oligonucleosomal forms of chromatin have been fractionated by this technique to enrich for those fragments of chromatin containing associated poly(ADP-ribosylated) proteins from the bulk of unmodified chromatin (Malik et al., 1983; Prieto-Soto et al., 1983; Malik & Smulson, 1984).

Having developed a method to examine chromatin domains proximal or distal from sites of poly(ADP-ribosylation), it seemed possible, then, to ask whether any of these chromatin fragments contained actively transcribed DNA sequences. To answer this question, we isolated chromatin from asynchronously growing, log-phase HeLa cells, fractionated the chromatin by anti-poly(ADP-Rib) affinity chromatography, and probed the DNA derived from these fractions for active genes with a cDNA probe made from total mRNA isolated from these cells.

Materials and Methods

Materials. The HeLa cells were a kind gift of Dr. Bernard Moss, NIH, Bethesda, MD. The cloned human β-globin gene was generously supplied by Dr. A. Neinhuis, NIH, Bethesda, MD.

Isolation of Chromatin: Methods I and II. HeLa cells at a density of 4.3×10^5 cells/mL were harvested, washed once with spinner salts and once with buffer A (1 mM potassium phosphate, pH 6.4, 1 mM $CaCl_2$, 0.3 M sucrose, 0.5 mM

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[1] Abbreviations: ADP-Rib, adenosine diphosphate ribose; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; DAPI, 4',6-diamidino-2-phenylindole; PMSF, phenylmethanesulfonyl fluoride; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; SDS, sodium dodecyl sulfate.

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PMSF), and resuspended to 2×10^7 cells/mL in buffer A containing 0.3% (v/v) Triton N-101, 10 mM KF, 3 mM cAMP, and 1 mM nicotinamide. The cells were homogenized on ice in a loose-fitting Dounce homogenizer and centrifuged at 1000*g* for 5 min at 4 °C. The pellet was then washed once by resuspension and centrifugation as above in the same buffer containing Triton N-101 and inhibitors used above, once with buffer A without Triton N-101, and once with buffer R (3 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, 0.25 M sucrose, 80 mM NaCl, 1 mM nicotinamide, 0.5 mM PMSF). The resulting washed nuclei were resuspended in buffer R to 1×10^8 nuclei/mL, warmed for 1 min at 37 °C, and supplemented with 50 units/mL of micrococcal nuclease (Worthington). The digestion was carried out at 37 °C for the times indicated in the text and figure legends. To stop the digestion, either EDTA (method I) or EGTA (method II) was added to a concentration of 2 mM, and the nuclei were centrifuged for 5 min at 1000*g*. The supernatant of this centrifugation was saved. The pellet was resuspended in 1 mM EDTA/1 mM EGTA to 1×10^8 nuclei/mL and allowed to stand on ice for 30 min before being centrifuged again for 5 min at 3000*g*. In method I, the supernatants from the latter two centrifugations were pooled and frozen at −20 °C as soluble chromatin (S). In method II, only the first supernatant obtained after nuclease digestion contained appreciable amounts of DNA and was therefore not combined with the second supernatant. In both cases, the remaining pellet contained very few intact nuclei and much nuclear debris on inspection under the microscope. These pellets were resuspended in 6 M guanidinium hydrochloride to 2×10^6 nuclei/mL and frozen.

Isolation of Chromatin: Method III. Chromatin from 10^8 HeLa cells (S_2) was isolated as described by Thraves & Smulson (1982). The chromatin (30 A_{260} units) was then layered on 10–30% sucrose gradients and centrifuged at 38000*g* for 3 h essentially as previously described (Butt & Smulson, 1982). Fractions of 0.3 mL were collected, and selected fractions containing oligonucleosomes 1–7 units long were pooled, concentrated, and incubated for 5 min at 23 °C with [^{32}P]NAD (Malik et al., 1983).

Affinity Chromatography. Chromatography of chromatin on anti-poly(ADP-Rib)–Sepharose was performed as previously described (Malik et al., 1983) with the following modifications. Chromatin prepared by method II was applied to an antibody column that had been equilibrated with 2 mM EDTA, pH 7.5. The column was then washed with approximately 10 column volumes (30 mL) of this buffer before the bound material was eluted. Chromatin (obtained by method I or II) binding to the antibody column was eluted with 6 M guanidinium hydrochloride. Transmittance at 254 nm was continuously monitored by a Unicord II flow monitor (LKB).

DNA Extraction and Estimation. DNA of soluble or insoluble chromatin was precipitated from 6 M guanidinium hydrochloride/66% ethanol overnight at −20 °C. Precipitated nucleic acids were then redissolved in 5% SDS/2 mM 2-mercaptoethanol and incubated for 10 min at 60 °C. To this was added 2.5 M KCl to a final concentration of 0.425 M, and the tubes were centrifuged at 0 °C for 10 min at 7000*g*. The supernatants were then ethanol precipitated overnight at −20 °C.

The DNA was treated with 100 μg/mL RNase A (Sigma)/10 units/mL RNase T_1, for 1 h at 37 °C. The DNA samples were then phenol extracted twice and ethanol precipitated, washed with cold ethanol, dried in a vacuum, and redissolved in 10 mM Tris-HCl, pH 7.5/1 mM EDTA. DNA concentration was determined both by absorbance at 260 nm and by DAPI fluorometric assay (Brunk et al., 1979).

Blotting and Hybridization. For dot blots, 6.25 μg of each DNA sample was lyophilized, treated with 150 mM NaOH for 1 h at 65 °C, neutralized, and brought to a final concentration of 20× SSC. The composition of 1× SSC is 0.15 M NaCl and 0.015 M sodium citrate. The DNA was then applied in serial dilution to Schleicher & Schuell BA83 nitrocellulose soaked in 25× SSC with a manifold (BRL). For Southern blots, 10 μg of each DNA sample was electrophoresed on a 3 mm thick vertical 1.5% agarose gel. Ethidium bromide (2 μg/mL) was included in the gel. Subsequent preparation of the gel and transfer were performed essentially as described by Southern (1975) except that the transfer buffer was 25× SSC. Nitrocellulose membranes were baked immediately after blotting and subsequently prehybridized in 5× SSC, 50% formamide, 0.1% each of BSA, ficoll, and poly(vinylpyrrolidone), 1 mg/mL heat-denatured herring sperm DNA, and 100 μg/mL poly(A) for 4–8 h at 42 °C. Heat-denatured radioactive probe was then added to prehybridization buffer, and the membranes were incubated at 42 °C for 24–48 h. Membranes were washed twice for 10 min each at room temperature with 2× SSC/0.1% SDS and once with 1× SSC/0.1% SDS at 68 °C for 1 h before autoradiography on Kodak SB-5 film. To remove radioactivity remaining from previous hybridizations, nitrocellulose membranes were washed with distilled water at 68 °C for 1 h.

Preparation of Probes. Total HeLa cell mRNA was prepared essentially as described for DNA except that it was initially precipitated in 6 M guanidinium hydrochloride/33% ethanol. The poly(A^+) mRNA was obtained by chromatography over poly(U)–Sephadex (BRL) (Deeley et al., 1977). A labeled cDNA probe was then made from this RNA by published methods (Baer & Rhodes, 1983; Buell et al., 1978). The reaction mixture (0.1 mL) contained 1 mM each of ATP, dGTP, and dTTP, 0.1 mCi [^{32}P]dCTP (>2000 Ci/mmol), 50 mM KCl, 50 mM Tris-HCl, 5 mg/mL oligo(dT_{8-12}), 50 mg/mL actinomycin D, 20 mM DTT, 20 units of AMV reverse transcriptase (Beard, Life Sciences), and 5 μg of HeLa mRNA. The plasmid (pBR322) containing the *Pst*I fragment of the β-globin gene was nick translated by established procedures (Maniatis et al., 1975).

Results

Isolation of Differing Nucleosomal Domains. The aim of these experiments was to isolate chromatin containing poly(ADP-ribosylated) proteins. Accordingly, nuclei from asynchronous, logarithmically growing HeLa cells were isolated in the presence of a phosphodiesterase inhibitor, KF, and a poly(ADP-Rib) glycohydrolase inhibitor, cAMP. An inhibitor of poly(ADP-Rib) polymerase, nicotinamide, was also employed to prevent artifactual poly(ADP-Rib) synthesis during the isolation procedures. Creation of strand breaks in DNA stimulates synthesis of the poly (Smulson et al., 1977; Juarez-Salinas et al., 1979) and could conceivably occur during the preparation of nuclei and the subsequent nuclease treatment. The nuclei were digested for various times with 50 units/mL micrococcal nuclease, since an earlier study had indicated the preferential release by this nuclease of chromatin subfractions enriched in poly(ADP-ribosylated) proteins and poly(ADP-Rib) polymerase activity (Jump et al., 1979).

We prepared chromatin by three different routes during the course of these experiments. After nuclease digestion, nuclei were lysed in 2 mM EDTA (method I) or in 2 mM EGTA (method II) and separated into soluble and insoluble chromatin fractions by centrifugation. In the latter method, the soluble fraction of chromatin contained a larger percentage of the total

cellular DNA (>70%), as determined by DAPI fluorescence, than that obtained in method I. In method III, nuclei were prepared by the method of Sporn et al. (1969), and the chromatin obtained from these nuclei sedimented through a 10–30% sucrose gradient. Oligonucleosomes 1–7 units long were pooled, concentrated, and labeled in vitro with [^{32}P]NAD.

In the data shown in Figure 1, soluble chromatin prepared by method I was applied to a poly(ADP-Rib) antibody column in phosphate-buffered saline and washed extensively. In earlier studies, nucleosomes were labeled in vitro with NAD prior to immunofractionation (Malik et al., 1983; Prieto-Soto et al., 1983). In most experiments in the present study, no in vitro synthesis of exogenous poly(ADP-Rib) was performed, and absorption to the antibody column relied upon the presence of in vivo synthesized poly(ADP-Rib) at the sites in chromatin where it presumably exists naturally. The presence of endogenous poly(ADP-Rib) in the bound, but not unbound material, has been verified by immunoblotting (to be published elsewhere). Additionally, this laboratory has recently shown the natural presence of 15 units of poly(ADP-Rib) attached to histone H1 isolated from intact, unlabeled cells by use of the same antibody (Wong et al., 1983).

Continuous monitoring of UV transmittance at 254 nm showed a considerable fraction of UV-absorbing material bound to the column in chromatin digested for a short time with nuclease (Figure 1A), and with increasing time of digestion, the total UV absorbance in the bound peak decreased. Thus, after 1 min of nuclease digestion, approximately 88% of the applied DNA showed binding to anti-poly(ADP-Rib), whereas only 27% of DNA digested for 20 min bound to the column. This latter level of binding corresponded to that obtained when chromatin was incubated with exogenous NAD prior to immunofractionation (Malik et al., 1983).

The inserts in Figure 1 show agarose gels of the DNA extracted from the chromatin that was unbound and bound to the anti-poly(ADP-Rib)–Sepharose column. As expected, the general size of oligonucleosomes obtained by this method decreased in chain length with increased digestion. Thus, in the short digest (Figure 1A) polynucleosomes exceeding 15 chain lengths were observed to be bound to the antibody column. At the longest period of nuclease digestion (Figure 1D), nucleosomal chain lengths in the bound fraction were extremely short with the average being mainly of mononucleosomal size. The decrease in binding of chromatin to the antibody column with increased nuclease digestion might thus be expected, since the probability that any chromatin fragment containing a poly(ADP-Rib) chain would increase with nucleosomal chain length. This overall concept will be explored in more detail below. The data also confirm earlier observations (Jump et al., 1979) that poly(ADP-ribosylated) regions of chromatin are nuclease sensitive. As nuclease digestion progressed, the ratio of limit digest product (i.e., mononucleosomes) in the bound vs. unbound fraction was noted to decrease. In the late digest (panel D, insert), the ADP-ribosylated chromatin showed reduced amounts of mononucleosomes compared to the unmodified chromatin, while the reverse was true in the earlier digests.

Since there was some possibility that 150 mM NaCl might cause some disassociation of poly(ADP-ribosylated) proteins from actively transcribed chromatin fragments, we also performed immunofractionation by utilizing method II. In this procedure, nuclei were lysed in EGTA and applied to the immunofractionation column in low salt (i.e., 2 mM EDTA) rather than phosphate-buffered saline (equivilent to 0.15 M NaCl). The same general pattern of binding was observed

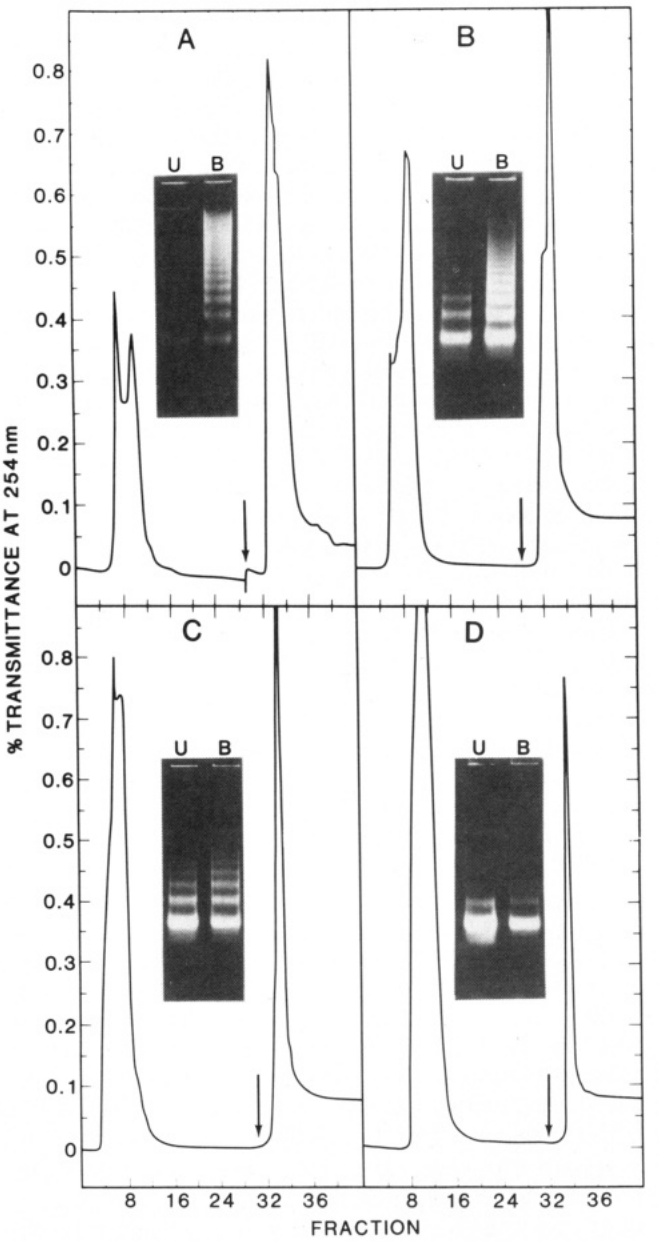

FIGURE 1: Immunofractionation of chromatin digested by micrococcal nuclease for various extents of time on an anti-poly(ADP-Rib)–Sepharose column. HeLa nucleic from 10^8 cells were digested with micrococcal nuclease (50 units/10^8 nuclei) at 37 °C for 1 (A), 5 (B), 10 (C), and 20 min (D). Soluble chromatin was subsequently prepared by method I as described under Materials and Methods and immunofractionated on an anti-poly(ADP-Rib)–Sepharose column. The columns were loaded and washed with phosphate-buffered saline. Transmittance at 254 nm was continuously monitored. When no further UV-absorbing material was eluted, 6 M guanidinium hydrochloride was added to elute bound material. The inserts in each panel represent $^1/_{50}$ of the DNA extracted from the pooled unbound (U) and bound (B) peak fractions electrophoresed on 1.5% agarose. The gel was stained with ethidium bromide.

with this procedure; the fraction and size of chromatin binding to the column decreased with digestion time. DNA was extracted from the pooled unbound and bound fractions obtained by method II and electrophoresed on an agarose gel (Figure 2). Corresponding to the larger amount of chromatin released by the use of EGTA to lyse nuclei (see above), nearly 91% of the chromatin was bound by the column after 2.5 min of digestion, whereas 73.6% was bound after 20 min of digestion.

Methods I and II differed in that a greater fraction of the total cell chromatin was extracted into soluble form in method II and that, as a consequence of the large size of method II

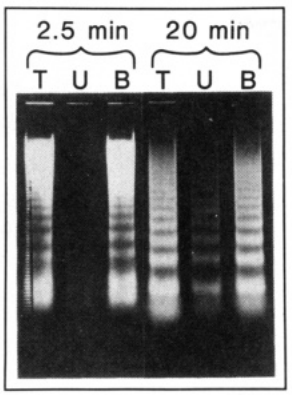

FIGURE 2: Chromatin immunofractionated in low salt. HeLa nuclei from 2 × 10^8 cells were digested with micrococcal nuclease (50 units/10^8 nuclei) at 37 °C for 2.5 and 20 min. Soluble chromatin was then prepared by method II as described under Materials and Methods and immunofractionated on an anti-poly(ADP-Rib)–Sepharose column. The columns were loaded and washed with 2 mM EDTA. One-fiftieth of the DNA extracted from unfractionated total chromatin (T), unbound (U), and bound (B) pooled peak fractions was electrophoresed on 1.5% agarose. The gel was stained with ethidium bromide.

chromatin, a greater percentage bound to the antibody column at every nuclease digestion time point.

Probing Nucleosomal Chromatin for Active Genes. It was of importance to establish hybridization conditions for detecting the presence or absence of transcriptionally active and inactive gene sequences in the immunofractionated chromatin samples shown in Figures 1 and 2. Accordingly, equal amounts of DNA extracted from soluble and insoluble chromatin prepared by method II were electrophoresed in 1.5% agarose gels and subsequently transferred to nitrocellulose under conditions reported to maximize binding of nucleosomal-sized DNA (Thomas, 1980; Albanese & Weintraub, 1980). Ethidium bromide staining of the gel (Figure 3A) showed the expected pattern of nucleosomal arrays in the soluble chromatin fraction but none in the insoluble fraction. The observed arrays showed an increased bias in the low molecular weight fragments (monomer, dimer, trimer, etc.) with time of nuclease digestion. The average molecular weight of the ethidium bromide staining material in the insoluble (P) fraction also decreased with time of digestion but migrated well below the position of the mononucleosome. Possible contamination of the DNA samples by RNA was eliminated by treatment for 1 h at 65 °C in 150 mM NaOH in the case of dot blots or by treatment for 1 h at 37 °C with 100 mg/mL RNase A/10 units/mL RNase T, followed by phenol extraction in the case of the Southern blots. The DNA from the gel was transferred to nitrocellulose by the method of Southern (1975). The filters were then probed for active genes as well as inactive human β-globin gene.

The probe for active genes was prepared by extracting total RNA from asynchronous, logarithmically growing HeLa cells, purifying the polyadenylated RNA by poly(U)–Sephadex chromatography, and reverse transcribing with AMV reverse transcriptase in the presence of ^{32}P-labeled dCTP. Alkaline agarose gel electrophoresis of the labeled probe showed it to have an average length of 300–500 bases, although significant ^{32}P incorporation was found in cDNA species as large as 3000 nucleotides. In a recent paper, Baer & Rhodes (1983) have used a similar type of cDNA probe made from total cytoplasmic poly(A$^+$)-containing mRNA and have shown that it displayed significant hybridization to nucleosomal cores preferentially bound by RNA polymerase II. The abundance and size distribution of active genes detected by this probe in

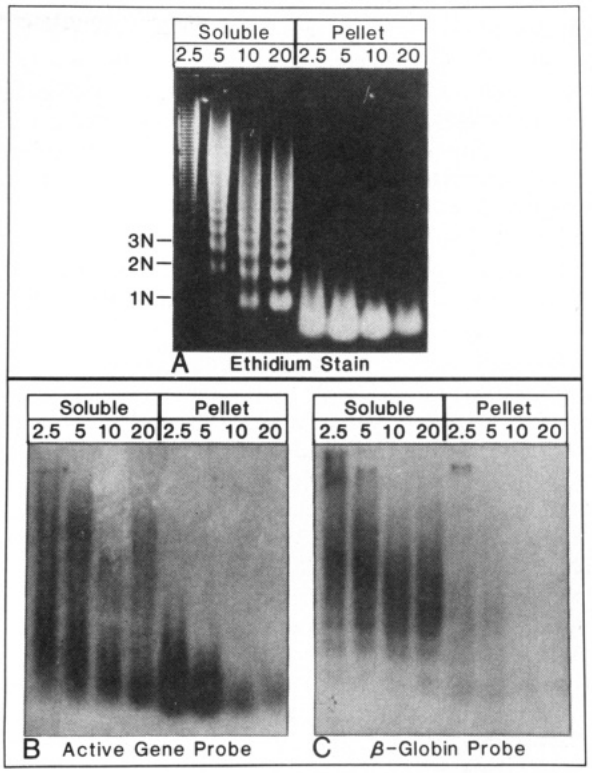

FIGURE 3: Southern blot analysis for active and inactive genes of soluble and insoluble chromatin fractions. HeLa cell nuclei from 2 × 10^8 cells were digested with micrococcal nuclease (50 units/10^8 nuclei) at 37 °C for 2.5, 5, 10, and 20 min. Soluble chromatin was then prepared by method II as described under Materials and Methods. DNA was extracted from the soluble chromatin (S) as well as insoluble chromatin pellet (P) and treated with ribonuclease to remove RNA as described under Materials and Methods. Equal amounts (10 μg) of each DNA sample were electrophoresed on 1.5% agarose and blotted to nitrocellulose. After being baked and prehybridized, the nitrocellulose was probed with a labeled cDNA probe made to total HeLa cell mRNA and autoradiographed. The paper was washed to remove radioactivity and reprobed for β-globin gene sequences as described under Materials and Methods. The ethidium bromide stain of the agarose gel is shown in (A). Autoradiographs of the nitrocellulose blot probed for active genes and β-globin gene are shown in (B) and (C), respectively.

the DNA samples are shown in Figure 3B. Several observations are apparent. First, active genes occurred primarily as a smear in the DNA samples, even though the majority of the DNA can be seen by ethidium bromide staining (and by inactive gene hybridization, see below) to be in nucleosomal arrays. These results are consistent with the recent observations of Davis et al. (1983), who showed that the DNA of the active ribosomal gene in cultured cell lines and mouse liver does not exist in a distinct nucleosomal structure. Furthermore, Lohr (1983) has shown that when the galactokinase gene is transcribed in yeast, its DNA appears after nuclease digestion and appropriate hybridization as a smear in the region of nucleosomal repeats. Spinelli et al. (1982) showed a similar lack of definition of active genes in chromatin of *Paracentrotus lividus* cells.

Second, Figure 3B shows that active genes were digested several times more rapidly than the bulk DNA. The size of fragments hybridizing to the probe extended below the size of a mononucleosome core, and there was no apparent accumulation of DNA as a mononucleosomal band. The total amount of hybridization in each lane decreased with time of nuclease digestion, suggesting a progressive loss of hybridizable DNA as the fragments became shorter. The average fragment size of the DNA detected by the probe is significantly smaller

than that of the ethidium bromide staining bulk DNA. A portion of the DNA detected by the active probe was apparently resistant to nuclease digestion and persisted as high molecular weight fragments as late as 20 min into the nuclease digestion. This DNA shows faint but distinct nucleosomal banding apparent in later digestion times where the nuclease-sensitive DNA no longer obscures it.

Third, the insoluble chromatin fractions (P) appeared to be highly enriched in active genes. Davis et al. (1983) also have recently reported an enrichment of active ribosomal gene sequences in insoluble chromatin.

Chromatin Structure and an Inactive Gene. As a control experiment, we rehybridized the same Southern blot, washed free of radioactivity, with a probe for the β-globin gene that is not transcribed in HeLa cell cultures. The abundance and distribution of the inactive gene, β-globin, are shown in Figure 3C. The β-globin probe was prepared by nick-translating plasmid pBR322 into which was inserted the 4.4-kb *Pst*I β-globin gene fragment cloned in *Escherichia coli* (Lawn et al., 1980). In contrast to the hybridization with the probe to active genes (Figure 3B), the presence of the β-globin gene coincided with that of the bulk nucleosomal array of DNA. Hybridization to a sharp, uncharacterized high molecular weight band was observed in some of the samples. Second, the chromatin of the inactive gene was digested at the same rate as the bulk of the chromatin (cf. Figure 3A,C). Finally, the insoluble chromatin (P) contained very little inactive gene compared to the presence of active genes in this fraction.

Probing Immunofractionated Poly(ADP-ribosylated) Chromatin for Active Genes. As noted earlier, immunofractionation of chromatin on anti-poly(ADP-Rib)–Sepharose allowed us to characterize those nucleosomes that were sites of poly(ADP-ribosylation) (Malik et al., 1983, 1984; Prieto-Soto et al., 1983). It was shown that the eluted chromatin components contained poly(ADP-ribosylated) histones as well as automodified poly(ADP-Rib) polymerase. We found that poly(ADP-ribosylated) histones, attached to stretches of oligonucleosomes bound to the column, were 6-fold more enriched in this modification compared to the histones of the unbound, unmodified chromatin. We interpreted this as evidence that non-poly(ADP-ribosylated) nucleosomes adjacent to the modified regions are copurified by this procedure. This was confirmed in a different manner by the data of Figure 1 (inserts). Both Weisbrod (1982) and Baer & Rhodes (1983) utilized core mononucleosomes to purify transcriptionally active genes. However, we have previously shown that poly(ADP-Rib) polymerase does not occur on trimmed core particles (Giri et al., 1978a,b) and thus have used larger sized oligonucleosomes in these experiments. Both active and inactive DNA sequences may occur on the same chromatin chain. Thus, a fraction of chromatin binding to the antibody column would not be expected to show a total enrichment compared to unfractionated chromatin in DNA sequences that are associated with poly(ADP-ribosylated) proteins.

Dot Blot Analysis. Two different approaches were taken to obtain a representative view of the relative abundance of active and inactive genes in poly(ADP-ribosylated) and unmodified chromatin by dot blot hybridization analysis of DNA derived from bound or unbound material. In the first, we compared nucleosomal DNA prepared by either method I or method II. These immunofractionated nucleosomes were obtained from either short or long digests of micrococcal nuclease. No in vitro poly(ADP-ribosylation) was performed with these nucleosomal preparations prior to immunofractionation. In the second approach, nucleosomes resolved according to chain length on a sucrose gradient (method III) were incubated in vitro with exogenous NAD and immunofractionated.

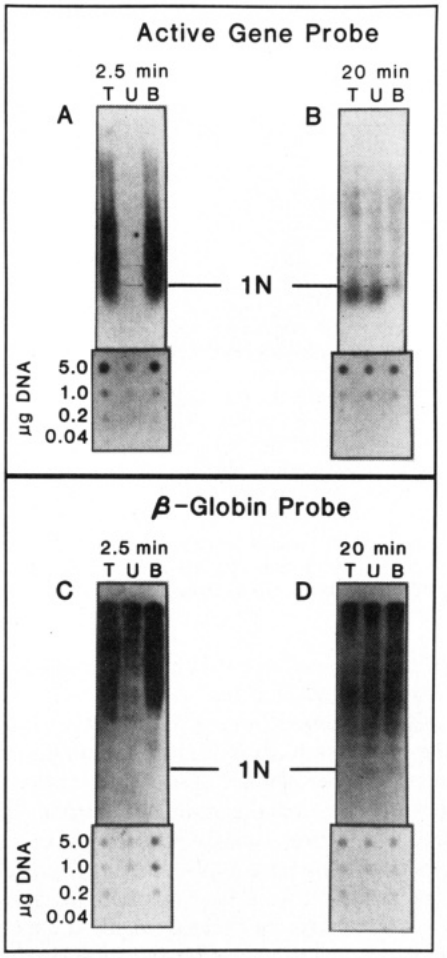

FIGURE 4: Dot blot and Southern blot analyses of immunofractionated chromatin. The DNA extracted from immunofractionated chromatin prepared as described in Figure 2 was electrophoresed on 1.5% agarose and blotted to nitrocellulose by the method of Southern (1975) or spotted directly to nitrocellulose with a manifold (BRL). Samples of 10 μg (except in the second lane in panel A, to which 2 μg was applied) were used in Southern transfers. DNA samples of 5, 1, 0.2, and 0.04 μg were spotted in dot blots. The membranes were probed for active (A and B) and inactive gene (C and D) sequences as described in Figure 3. Unfractionated total (T), unbound (U), and bound (B) antibody column fractions are shown for DNA derived from chromatin digested for 2.5 (A and C) and 20 min (B and D) with micrococcal nuclease as described in Figure 2.

DNA was extracted from the unfractionated (total) and unbound or bound nucleosomes, and equal concentrations of the respective DNAs, as determined by both A_{260} and DAPI fluorescence assay (Materials and Methods), were dot blotted in serial dilution to nitrocellulose (Figure 4, dot-blots; Figure 5). The nitrocellulose sheets were hybridized with the ^{32}P-labeled cDNA to total active genes and subsequently rehybridized to the ^{32}P-labeled β-globin probe. In most cases, there was reasonable correlation between the hybridization signal and the amount of DNA applied to the nitrocellulose within a dilution series.

Active genes and β-globin gene occurred in both non-(ADP-ribosylated) and ADP-ribosylated antibody column fractions. Both active and inactive gene sequences derived from chromatin digested for 20 min with micrococcal nuclease appeared to partition nearly equally between the unbound and bound antibody column fractions. This is especially evident by the dot blot data shown in Figures 4B,D and Figure 5B,E

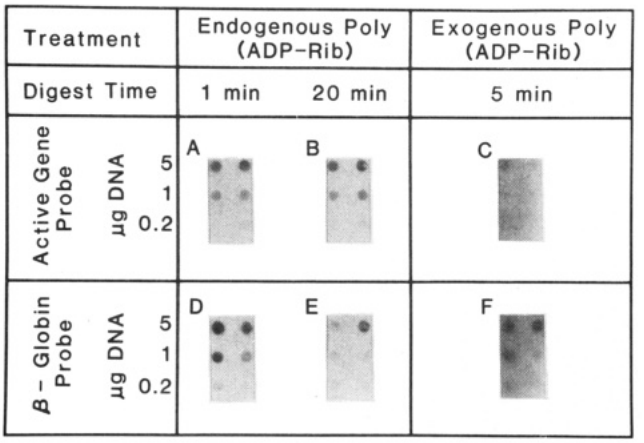

FIGURE 5: Dot blot analysis of immunofractionated chromatin. DNA, extracted from chromatin prepared by methods I and III and immunofractionated on anti-poly(ADP-Rib)–Sepharose, was blotted to nitrocellulose and probed for active (A–C) and inactive (D–F) genes as described in Figures 3 and 4. Samples of 5, 1, and 0.2 μg were spotted for unbound (left column) and bound (right column) antibody column fractions. Chromatin was prepared by method I with 1-min (A and D) and 20-min (B and E) digestion with micrococcal nuclease as described in Figure 1. Chromatin was prepared by method III with 5-min (C and F) digestion.

(lower DNA concentrations). This observation was consistent irrespective of the method of preparation of nucleosomes.

Second, poly(ADP-ribosylated) nucleosomal domains, obtained from short micrococcal nuclease digestions, appeared to possess an enrichment in active genes sequences. For example, a 4-fold greater hybridization, averaged over the dilution series, of active genes was noted in the DNA in the bound fraction in Figure 4A and to a lesser extent (using method I) in Figure 5A.

Significant hybridization of the inactive gene representative, β-globin, was observed in the poly(ADP-ribosylated) nucleosomes, obtained by all three methods (Figure 4C; Figure 5D–F). Thus, by dot blot analysis of chromatin, it appeared that probes for both active genes and one inactive gene hybridized most strongly with the bound nucleosomes. The incubation of nucleosomes (obtained by method III) with NAD in vitro (Figure 5C) appeared to give contrary results for reasons not understood at this time.

Nucleosomal DNA Blots. In Southern transfers the same observations were apparent. Equal amounts of unfractionated, unbound, and bound DNA from each digest time point were electrophoresed on 1.5% agarose, blotted onto nitrocellulose, and probed for active or inactive genes. (For lack of sufficient DNA, the lane of unbound DNA in the 2.5-min digest contained one-fifth the DNA of the other lanes.) Active genes, as detected by the cDNA probe, decreased in size and quantity with time as these genes were degraded. Also with time, the relative proportion of remaining actively transcribed DNA that bound as chromatin to the antibody column decreased. As observed by dot blot hybridization, both active and inactive DNA sequences were enriched in the bound antibody column fraction in the early digestion. While the average size of DNA fragments hybridizing to the β-globin probe in this sample (Figure 4C) was very large, the majority of actively (Figure 4A) transcribed sequences were small, ranging from the size of a mononucleosome to that of about a trinucleosome.

In the later digestion, hybridization of the active probe was diminished overall, and the majority of hybridizing DNA fragments were as small or smaller than the size of a mononucleosome. Overall hybridization in the unbound and bound antibody column fractions was roughly the same, though greater hybridization occurred in the unbound fraction below the size of a mononucleosome. Hybridization of the active gene probe to the bound fraction in this size range did not occur significantly below the level of the mononucleosome.

Discussion

Poly(ADP-Rib) polymerase activity has been shown to be activated in vivo and in vitro by agents that cause DNA strand breaks (Smulson et al., 1977; Durkaez et al., 1980; Juarez-Salinas et al., 1979; Berger et al., 1980), and inhibitors of poly(ADP-ribosylation) delay the rejoining of these breaks (Smulson et al., 1977; Durkacz et al., 1981). Weisbrod (1982) has shown that topoisomerase I activity copurifies with transcriptionally active mononucleosomes by HMG protein affinity chromatography. He speculated that the nicking–closing activity of this enzyme might be important for transcription by RNA polymerase. It is conceivable that topoisomerase, by introducing single-strand nicks into DNA, acts as one biological signal for poly(ADP-ribosylation). The possible involvement of topoisomerase in the process of transcription thus suggested that we might find poly(ADP-Rib) associated with actively transcribed chromatin.

In this study, the binding of poly(ADP-Rib) to an anti-poly(ADP-Rib) column has been used to isolate chromatin fragments that contain poly(ADP-ribosylated) nuclear proteins. The DNA extracted from these chromatin fragments was then probed for transcriptionally active and inactive DNA sequences. By comparing the relative abundance of these sequences in bound and unbound anti-poly(ADP-Rib) column fractions, we hoped to assess the association of poly(ADP-Rib) with these domains of chromatin.

In two of the three experimental approaches taken, we attempted to fractionate chromatin, by using the antibody column, on the basis of endogenous poly(ADP-Rib) alone. To minimize degradation of endogenous polymer during isolation of chromatin, inhibitors of polymer degradation were used. However, since none of these inhibitors are completely effective (Miwa et al., 1974), we cannot rule out the possibility that some of the endogenously poly(ADP-ribosylated) chromatin was not recovered by immunofractionation. To prevent possible loss or rearrangement of poly(ADP-ribosylated) nuclear proteins, the column fractionation was performed at low ionic strength. Chromatin preparation method II differed only slightly from method I experimentally but resulted in a large increase in the amount of high molecular weight chromatin in the soluble fraction. This may explain why a larger fraction of the method II soluble chromatin bound to the antibody column.

The ability of the anti-poly(ADP-Rib) column to bind the polymer has been abundantly demonstrated (Malik et al., 1983). The specificity of the column has been characterized by showing that chromatin (Malik et al., 1983; Prieto-Soto et al., 1983; Malik & Smulson, 1984) or nuclear proteins (Wong et al., 1983a,b) from which the polymer has been cleaved by snake venom phosphodiesterase or alkali no longer bind to the column. Binding of labeled poly(ADP-Rib), either free or covalently bound to protein, to the anti-poly(ADP-Rib) column can be demonstrated for chains as small as 3 units long (Wong et al., 1983), though binding of chains is increased at low ionic strength (below 150 mM NaCl, unpublished data). Mono- and di-(ADP-ribosylated) proteins do not bind to the column.

As a probe for transcriptionally active DNA sequences, we prepared cDNA to total poly(A^+) RNA from mid-log HeLa cells. An advantage of using a general probe for actively transcribed genes is that it provided us with an overall view

of active chromatin that one specific gene could not. Concern for how a specific gene partitioned between soluble and insoluble chromatin fractions was also circumvented. A disadvantage of using this probe, however, is the possible detection of members of multigene families and pseudogenes that may not be transcribed. At present, it is not known whether these represent a significant fraction of the hybridization signal.

The partially digested chromatin immunofractionated in this study contained significant amounts of polynucleosomes. Since polynucleosomes bound by the antibody column may contain both poly(ADP-ribosylated) and non-poly(ADP-ribosylated) regions (Malik et al., 1983), our data must be interpreted with caution. Clearly, the chance that any chromatin fragment contains both modified and unmodified regions would decrease with size until the fragment reaches the size of a mononucleosomal repeat. To a first approximation, this fragment size may be considered the smallest unit of modification by poly(ADP-ribosylation), since many of the proteins involved in nucleosome structure are acceptors. Thus, in order to accurately assess the relative abundance of poly(ADP-ribosylated) chromatin from bound and unbound antibody column fractions, these fractions should be examined at the mononucleosomal level. This was accomplished in the current study by electrophoresis on agarose and Southern blot analysis.

Early in nuclease digestion, the majority of chromatin detected by ethidium bromide staining of agarose gels existed as high molecular weight polynucleosomes. It was, therefore, not surprising that most of this chromatin bound to the antibody column. With time of digestion, the amount of DNA increased in the unbound antibody column fraction (Figure 1, inserts; Figure 4). Also, during the course of digestion, ethidium bromide staining fragments of mononucleosomal size accumulated in both fractions, presumably due to the relative nuclease resistance of core particles. In a limit digest, one would expect that, provided appreciable digestion of mononucleosomes and core particles does not occur, the ratio of DNA in the bound and unbound antibody column fractions would represent the ratio of modified and unmodified nucleosomes. At any point in the digestion, in fact, the ratio of bound and unbound mononucleosomes should reflect the relative frequency of modified nucleosomes in a random digest since the probability that a mononucleosome contains poly(ADP-ribosylated) protein is constant in a random digest.

In ethidium bromide staining chromatin, however, the limit digest product did not appear in bound and unbound antibody column fractions as a constant ratio during nuclease digestion (Figure 1). Early in the digestion, bulk chromatin mononucleosomes were enriched in the bound fraction. As the digestion proceeded, the relative amount of mononucleosomal-length DNA is increased in the unbound fraction. By 20 min of digestion, where most of the chromatin had reached mononucleosomal size, approximately one in four mononucleosomes bound to the antibody column. Thus, in confirmation of earlier data (Jump et al., 1979), poly(ADP-ribosylated) nucleosomes appear to be digested by micrococcal nuclease at a slightly higher rate than unmodified chromatin. This higher nuclease sensitivity appears to be conferred without detectible alteration of the relative nuclease resistance of the core particle, since distinct nucleosomal banding of bulk chromatin was observed.

Actively transcribed DNA sequences followed a course of digestion very different from that of bulk chromatin. We found, as have others (Davis et al., 1983; Lohr, 1983; Spinelli et al., 1982), that actively transcribed chromatin was rapidly digested into fragments that did not band as nucleosomal repeats. The digestion of these sequences proceeded beyond the level of the mononucleosome, and an accumulation of actively transcribed DNA did not occur at the mononucleosomal level. Rather, an overall loss of hybridizable DNA occurred with time as this DNA became too small to bind to nitrocellulose. As a result, the small fragments present in early digestion samples were missing in later digestion samples, and the actively transcribed DNA fragments remaining in the later digestion sample were those that were more nuclease resistant. Thus, as the nuclease digestion proceeded, the ratio of mononucleosomal DNA hybridizing to the active gene probe in bound and unbound antibody column fractions reflected the relative numbers of poly(ADP-ribosylated) nucleosomes in the progressively more nuclease-resistant active genes.

Early in the digestion of chromatin, we observed a large enrichment of actively transcribed DNA sequences in the bound antibody column fraction. Since the majority of these sequences were of relatively short length, they were less likely to contain unmodified nucleosomes. After 20 min of digestion, most of the actively transcribed DNA present in the early digestion sample had disappeared. The majority of those fragments remaining were found in greater abundance in the unbound antibody column fraction. Thus, active nucleosomes less sensitive to nuclease contained less poly(ADP-ribosylated) protein than did more highly sensitive nucleosomes. We also noted that the majority of the active gene hybridization signal, and thus the most abundant genes and transcripts (Baer & Rhodes, 1983), were associated with the most nuclease-sensitive chromatin, seen only in the early digestion sample. This would imply that the abundance of poly(ADP-ribosylated) nucleosomes as well as degree of nuclease sensitivity might correlate with levels of transcriptional activity.

The possibility that nuclease sensitivity of chromatin can be correlated with its modification by poly(ADP-Rib) in both actively and inactively transcribed chromatin suggests that nuclease sensitivity may be, at least in part, a result of this modification. The site and degree of modification could determine the type and level of nuclease sensitivity. For example, poly(ADP-ribosylation) of H1 might lead to increased sensitivity of the internucleosomal linker region of higher ordered chromatin fibers, while modification of core histones might result in increased sensitivity of core particles.

Our results indicate that poly(ADP-Rib) is present in actively transcribed chromatin in HeLa cells. Some actively transcribed chromatin, however, does not appear to be modified, and conversely, there is much poly(ADP-ribosylated) chromatin that is not actively transcribed. We have observed some correlation between the presence of this modification and nuclease sensitivity in both transcriptionally inactive and active chromatin. The occurrence of poly(ADP-Rib) of nucleosomes clearly not involved in transcription suggests that this nuclear protein modification alone is not a sufficient condition for transcriptional activity. Indeed, since much the same could be said for histone acetylation (Perry & Chalkley, 1982), the presence of HMG 14 or 17 (Seale et al., 1983), or DNA methylation (van der Ploeg & Flavell, 1980), transcription may require one or more combinations of conditions. Recently, multiple types of modification on the same histone protein have been demonstrated (Wong & Smulson, 1984; Sterner & Allfrey, 1983). From our observations, one might be led to suspect that poly(ADP-ribosylation) has more than one function in the nucleus.

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