Poly(adenosine diphosphate-ribose) Polymerase: The Distribution of a Chromosome-Associated Enzyme within the Chromatin Substructure†

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ABSTRACT: The distribution of a chromatin-bound, nuclear protein modifying enzyme, poly(adenosine diphosphate-ribose) polymerase, and its product, poly(ADP-ribose), among various fractions of sheared and nuclease-digested HeLa cell chromatin has been examined. Epichlorohydrin-tris(hydroxymethyl)aminomethane-cellulose and glycerol gradient fractionation of solubilized chromatin indicated that poly(ADP-ribose) polymerase activity was associated primarily with the template active regions (euchromatin), whereas the transcriptionally inert chromatin fractions were found to contain relatively low levels of ADP-ribosylating activity. When isolated HeLa cell nuclei were digested in situ with micrococcal nuclease and the resultant chromatin was fractionated into nucleosome monomers (ν bodies) and oligomers by sucrose gradient centrifugation, only material sedimenting faster than the 11S monomers was found to contain appreciable poly(ADP-ribose) polymerase activity. If, on the other hand, isolated HeLa cell nuclei were first incubated with labeled NAD, the substrate for poly(ADP-ribose) polymerase, prior to the preparation and fractionation of nuclease-digested chromatin, it was found that those chromatin fractions which possess significant poly(ADP-ribose) polymerase activity (nucleosome oligomers) are relatively deficient in the labeled product of this enzyme, and that a considerable portion of the homopolymeric product is ultimately associated with the 11S ν bodies. Additional evidence is presented which indicates that the absence of nucleosome monomer-associated poly(ADP-ribose) polymerase activity is not due to the absence of a suitable acceptor on these structures, and that the activity of this enzyme within the chromatin is most probably dependent upon the physical integrity of the oligomeric structures themselves.

Poly(ADP-ribose)[1] polymerase is a ubiquitous nuclear enzyme which, because of its extraordinarily tight association with eukaryotic chromatin (requiring 1 M salt for dissociation) (Sugimura, 1973), provides a unique probe with which to assess both the distribution of a chromosome-associated enzyme within the chromatin substructure, as well as to aid in the elucidation of the physiological importance of the apparent highly ordered structure of proteins along the chromosomal DNA. Although Keller et al. (1975) have recently shown that the chromosome-associated enzyme, protein kinase, is localized within the transcriptionally active regions of sheared chromatin, the present study represents the first time that the distribution of such an enzyme within nuclease-treated chromatin fractions has been examined.

Poly(ADP-ribose) polymerase catalyzes the formation of a homopolymer of ADP-ribose units linked by 1′-2′ glycosidic bonds. The substrate for the reaction is NAD, and in the presence of DNA, the enzyme successively adds ADP-ribose units onto an initial ADP-ribose residue which has been reported to be covalently attached to various nuclear proteins, including histones, nonhistone chromosomal proteins, and a Ca^{2+}-, Mg^{2+}-dependent endonuclease (Nishizuka et al., 1969; Otake et al., 1969; Burzio and Koide, 1972). Although considerable information about this tightly bound chromosomal enzyme has been obtained in recent years, little substantive progress has been made toward an understanding of what precise function its homopolymeric product, poly(ADP-ribose), has in chromatin. Several studies have implicated a role for poly(ADP-ribose) polymerase in the regulation of eukaryotic DNA replication or repair. Results from this laboratory, and others, have shown that the synthesis of poly(ADP-ribose) causes perturbations of chromatin structure which lead either to the stimulation (Roberts et al., 1973) or inhibition (Burzio and Koide, 1970, 1971; Nagao et al., 1972) of DNA synthesis, depending upon the source of tissue used.

In recent years, considerable progress has also been made toward the establishment of a new and experimentally consistent model of chromatin structure in the eukaryotic cell. Initial evidence for this new model was provided by the neutron diffraction studies of Bradbury and his co-workers (Baldwin et al., 1975), and by electron microscopy (Olins and Olins, 1974), which revealed that the chromatin fiber has a structure resembling regularly spaced beads on a string. Spherical nucleoprotein particles (ν bodies, nucleosomes), approximately 60–80 Å in diameter and connected by thin (15 Å) threads, could be seen in electron micrographs of dispersed chromatin. Support for this basic model has also been obtained through biochemical studies, which have shown that a variety of nucleases, both endogenous and exogenous, cleave chromatin at regularly spaced intervals, and that limit digests with these enzymes produce fragments of DNA with an average length of 200 base pairs (Hewish and Burgoyne, 1973; Burgoyne et al., 1974; Van Holde et al., 1974; Finch et al., 1975). Fur-

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[1] Abbreviations used are: (ADP-ribose), adenosine diphosphate ribose; Ado(P)-Rib-P and ψ-ADP-ribose, 2′-(5″-phosphoribosyl)-5′-AMP; NAD, nicotinamide adenine dinucleotide; $NaDodSO_4$, sodium dodecyl sulfate; Cl_3AcOH, trichloroacetic acid; S-MEM, spinner minimal essential medium; $PhCH_2SO_2F$, phenylmethylsulfonyl fluoride; ECTHAM, epichlorohydrin-tris(hydroxymethyl)aminomethane; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; UV, ultraviolet.

thermore, nucleoprotein particles isolated from nuclease-digested chromatin and examined by electron microscopy are seen to resemble the bead-like structures visible on intact, dispersed chromatin (Van Holde et al., 1974; Senior et al., 1975). More recently, studies employing a variety of cross-linking reagents have shown that the nuclease-resistant particles obtained from digested chromatin contain octomers of the four small molecular weight histones (Thomas and Kornberg, 1975). Although histone H1 appears to be absent from these structures, there is evidence to indicate that it is involved in the structural maintenance or functioning of the internucleosome regions of chromatin fibers (Honda et al., 1975).

In the living cell, chromatin appears to exist in two forms, euchromatin, which is transcriptionally active, and which typically comprises less than 10% of the total genetic material, and heterochromatin, which is relatively inert. Isolated chromatin can be separated, by a variety of techniques, into fractions which reflect these in vivo differences, as judged by relative template efficiency with exogenous RNA polymerase. Recently, workers have shown that chromosomal subunits isolated from transcriptionally active chromatin do differ qualitatively from those released from inactive chromatin by nuclease digestion (Gottesfeld et al., 1975). Thus, the nuclease-resistant structures of active chromatin appear to have a slightly higher sedimentation coefficient, due to the presence of a complex pattern of nonhistone chromosomal proteins and RNA not found in the ν bodies isolated from transcriptionally inert chromatin. However, chromatin subunits enriched in nonhistone chromosomal proteins cannot be distinguished from those which contain only histones on the basis of DNA sequences complementary to polysomal polyadenylated RNA molecules (Paul and Malcolm, 1976).

In view of this recent progress toward an understanding of the substructure of eukaryotic chromatin, it seemed of importance, particularly in view of its stable association with chromatin, to explore the distribution of poly(ADP-ribose) polymerase, and its product, in the various chromatin fragments released by endonucleolytic digestion. By comparing these results with similar studies involving chromatin which had been fractionated into transcriptionally active and inactive regions on ECTHAM-cellulose and glycerol gradients, a clearer picture of the structural and functional aspects of both chromatin and the chromatin-modifying enzyme, poly(ADP-ribose) polymerase, has been obtained.

Materials and Methods

[*adenine*-2,8-^{3}H]NAD (3.7 Ci/mM) was purchased from New England Nuclear. Micrococcal nuclease (6000 u/mg) and snake venom phosphodiesterase were obtained from Worthington Biochemicals, and the catalase and human IgG markers were purchased from Schwarz/Mann. Phenylmethylsulfonyl fluoride ($PhCH_2SO_2F$) was from Sigma. SV40 DNA and *Hin*d II and III restriction endonucleases were gifts from Dr. Jack Chirikjian of this department.

HeLa S3 cells were maintained at 37 °C in spinner flasks in Eagle's S-MEM (Grand Island) containing 10% fetal calf serum. Cells were harvested by centrifugation and washed with spinner salts and detergent-washed nuclei isolated by Dounce homogenization according to the method of Sporn et al. (1969).

Sonicated chromatin was prepared from HeLa cell nuclei according to the procedure of Marushige and Bonner (1966). Micrococcal nuclease digested chromatin was prepared from isolated HeLa cell nuclei essentially according to the procedure of Noll et al. (1975). Washed nuclei were suspended in buffer A (50 mM Tris-HCl, pH 7.4, 25 mM KCl, 1 mM $MgCl_2$, 0.25 M sucrose, and 15 mM β-mercaptoethanol) at 4×10^7/ml. This suspension (1 ml) was made 1 mM with respect to $CaCl_2$ and digested with 80–500 u/ml micrococcal nuclease for 2–10 min at 37 °C. Digestion was terminated by chilling in ice and addition of 20 μl of 100 mM NaEDTA, pH 7. The nuclei were centrifuged for 10 min at 4000g, lysed by vigorous resuspension in 1.0 ml of 0.2 mM NaEDTA, pH 7, and centrifuged again at 12 000g for 10 min to remove nuclear debris and undigested chromatin. The supernatant containing the nuclease-digested chromatin material was either used immediately or stored frozen at −80 °C.

ECTHAM-cellulose was prepared by the method of Peterson and Kuff (1969), and solubilized chromatin was fractionated on a 1.6 × 22 cm ECTHAM-cellulose column according to the method of Reeck et al. (1972).

Micrococcal nuclease digested chromatin was fractionated by centrifugation on either linear (5–20%) or isokinetic (C_t = 5%, C_r = 28.8%, V_m = 33 ml, Beckman SW 27 rotor) sucrose gradients containing 10 mM Tris-HCl, pH 7, 1 mM NaEDTA, and 0.1 mM $PhCH_2SO_2F$. The linear gradients were run either in a Beckman SW 50.1 rotor (5.3 ml) or a Beckman SW 40Ti rotor (13.5 ml), as detailed in each experiment. Except where noted, the gradients were fractionated with a Densi-Flow IIc (Searle), and the absorbance was monitored at 254 nm with an ISCO flow cell and UA-5 recorder.

The poly(ADP-ribose) polymerase assay contained, usually in a volume of 1.0 ml: 100 μmol of Tris-HCl, pH 7.4, 1.0 μmol of dithiothreitol, 2.0 μmol of $MgCl_2$, 0.5 μCi of [*adenine*-2,8-^{3}H]NAD, and either HeLa cell nuclei, sonicated chromatin, or nuclease digested chromatin, as indicated in each experiment. The assays were performed at room temperature and terminated either by the addition of 1.0 ml of ice-cold 20% trichloroacetic acid (Cl_3AcOH) containing 5 mM sodium pyrophosphate, or by the addition of 1.0 ml of ice-cold 0.32 M sucrose–2 mM $MgCl_2$–1 mM KH_2PO_4, pH 6.8, and rapid chilling. In the former case, insoluble material was collected on Whatman GF/C filter discs and the radioactivity determined in a Packard Model 3320 liquid scintillation spectrometer. In the latter case, incubated nuclei were recovered for further study by centrifugation in the cold and washing.

The endogenous glycohydrolase assay was performed at 37 °C with pooled samples of ADP-ribosylated, nuclease-digested chromatin which had been fractionated by sucrose gradient centrifugation. Each assay contained, in a volume of 1.0 ml, chromatin, 25 μmol of KH_2PO_4, pH 7, and 10 μmol of β-mercaptoethanol. The incubations were halted by the addition of 1.0 ml of ice-cold 20% Cl_3AcOH and chilling.

Polyacrylamide–sodium dodecyl sulfate gel electrophoresis was carried out by the method of Laemmli (1970) on samples of nuclease-digested HeLa cell chromatin fractionated by sucrose gradient centrifugation. Aliquots of the fractionated nucleosome preparations were first lyophilized and then treated with successive washes in 20% Cl_3AcOH, ethanol–ether (1:1), and absolute ethanol before drying in vacuo. The dried samples were then solubilized in a buffer containing 1% $NaDodSO_4$, 1% β-mercaptoethanol, 0.25 M sucrose, and 10 mM KH_2PO_4, pH 6.8, and heated in a boiling water bath for 5 min before being applied to the gels. After staining with Coomassie blue, the gels were scanned on a Gilford Model 240 UV spectrophotometer at 590 nm.

DNA was isolated from pooled nucleosome monomer and oligomer sucrose gradient fractions by the method of Noll et al. (1975), and subjected to electrophoresis in 1.5% agarose

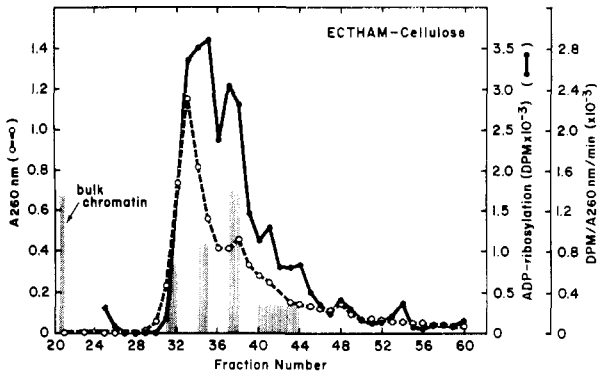

FIGURE 1: ECTHAM-cellulose chromatographic fractionation of sonicated HeLa cell chromatin and associated poly(ADP-ribose) polymerase activity. A sample of sonicated chromatin, prepared from isolated HeLa cell nuclei as described in Materials and Methods, was applied to a 1.6 × 22 cm ECTHAM-cellulose column and eluted with 0.01 M Tris base–0.01 M NaCl at 12 ml/h, 4 °C, as described by Reeck et al. (1972). Rate measurements of poly(ADP-ribose) polymerase activity were performed as described in Materials and Methods on individual fractions, and on pooled regions, as indicated by the hatched bars.

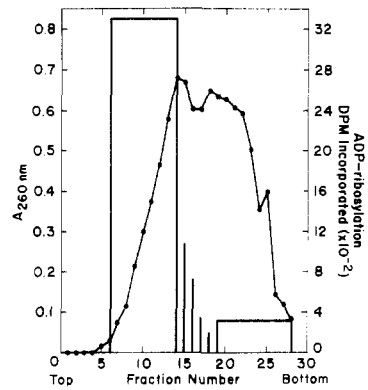

FIGURE 2: Glycerol gradient fractionation of sonicated HeLa cell chromatin and associated poly(ADP-ribose) polymerase activity. A sample of sonicated HeLa cell chromatin, prepared as described in Materials and Methods, was mixed with 0.13 ml of 75.1% glycerol and 1.4 ml of 10 mM Tris-HCl, pH 7.2 (final glycerol concentration = 4%), and layered onto a 13-ml 7–75% glycerol gradient in 10 mM Tris-HCl, pH 7.2. Centrifugation was for 16.5 h at 22 000 rpm and 4 °C in a Beckman SW 27 rotor. Rate measurements of poly(ADP-ribose) polymerase activity were performed as described in Materials and Methods on individual fractions, and on pooled regions, as indicated by the open bars.

gels according to the procedure of Sharp et al. (1973), except that ethidium bromide was eliminated from the gels. *Hin*d II and III digested SV40 DNA fragments were used as molecular weight markers, and the data of Danna and Nathans (1971) were used to construct a plot, by regression analysis, of log mol wt vs. mobility of these fragments. Following electrophoresis, the gels were stained with ethidium bromide and viewed under short wave UV light.

Nuclease-digested chromatin fractions, prepared from nuclei which had been previously incubated with [*adenine*-2,8-^{3}H]NAD, were treated with 250 μg/ml snake venom phosphodiesterase for 30 min at 37 °C in order to liberate 5′-AMP and Ado(P)-Rib-P from the endogenous poly(ADP-ribose). The digestion products were mixed with 0.1 μmol each of 5′-AMP and ADP-ribose as markers, spotted onto 20 × 20 cm cellulose thin-layer chromatography plates (Merck) and developed essentially according to the method of Yamada and Sugimura (1973).

The mixing experiment was performed as follows: chromatin was prepared from 3.5×10^8 HeLa cell nuclei according to the procedures of Noll et al. (1975) with the use of 80 u/ml of micrococcal nuclease for 2 min at 37 °C [0.2 ml (4.5 A_{260nm} units) was layered onto each of two 5–20% linear sucrose gradients and centrifuged for 16 h at 32 500 rpm (4 °C) in a Beckman SW 50.1 rotor as previously described]. Following centrifugation, the gradients were fractionated by upward displacement with 60% sucrose and monitored at 254 nm, also as described above. Fractions corresponding to 11S and >11S material were separately pooled, and the sample of >11S material was subsequently divided into two equal aliquots. One aliquot of this material was heat inactivated at 75 °C for 10 min to destroy the enzyme activity and then mixed with an equal volume of 11S material before assaying for poly(ADP-ribose) polymerase activity. The remaining samples of chromatin were also assayed for activity, as previously described.

Results

ECTHAM-Cellulose Chromatography. In order to determine whether poly(ADP-ribose) polymerase is physiologically associated with a unique species or subfraction of chromatin, solubilized chromatin was prepared from HeLa cell nuclei by the method of Marushige and Bonner (1966) and subjected to chromatography on ECTHAM-cellulose (Figure 1). The early fractions of rat liver chromatin elution patterns on ECTHAM-cellulose (corresponding to fractions 30–36 of Figure 1) have been previously characterized (Reeck et al., 1972; Simpson, 1974) and shown to consist primarily of more condensed chromatin containing a full complement of histones and devoid of any sequences which denature below 70 °C. The late-eluting fractions (37–45, Figure 1), on the other hand, are reported by Simpson (1974) to be enriched in the transcriptionally active regions of chromatin, as judged by relative template efficiency with RNA polymerase. In addition, these late fractions were shown to be depleted of histone H1, to have a higher content of nonhistone chromosomal proteins, and were enriched in low-melting sequences. Although poly(ADP-ribose) polymerase activity was found in all fractions assayed, the highest specific activity of the enzyme, as determined by rate measurements performed on individual pooled fractions (hatched bars), appeared to be associated with a unique species of chromatin which eluted considerably later than the bulk of the condensed chromatin. This result suggested that the transcriptionally active fractions of sheared chromatin might be enriched in poly(ADP-ribose) polymerase activity. In order to confirm this finding, an alternate method of fractionating chromatin into transcriptionally active and inactive regions was employed.

Glycerol Gradient Centrifugation. Sheared chromatin isolated from HeLa cell nuclei was subjected to centrifugation in a steep glycerol gradient essentially according to the procedure of Lapeyre and Bekhor (1974). The optical density profile of the solubilized chromatin on the gradient (Figure 2) is seen to consist of a broad distribution of three peaks, generally consistent with the findings of Howk et al. (1975) using sheared 3T3 mouse cell chromatin. On the basis of circular dichroism spectra, electron microscopy, and template restriction studies, the slower sedimenting form of chromatin on such gradients has recently been shown to possess properties characteristic of extended, template-active chromatin (Murphy et al., 1973). As can be seen from the data in Figure 2, it is this region (fractions 6–14) of the HeLa cell chromatin which exhibits the highest specific activity of associated poly(ADP-ribose) polymerase. Individual fractions (15–18) assayed from

TABLE I: Approximate Molecular Weights and Sizes of DNA Fragments Isolated from Nucleosome Monomers, Dimers, and Trimers and Separated by Agarose Gel Electrophoresis.[a]

Chromatin Fraction	No. of Bands	Interpolated mol wt ($\times 10^{-4}$)	No. of Base Pairs (mol wt/327)
11S (ν bodies)	1	5.1–5.6	156–170
16S (dimers)	2	5.1–5.6	156–170
		8.1–10.7	247–328
21S (trimers)	3	5.1–5.6	156–170
		8.1–10.7	247–328
		12.3–14.7	375–450

[a] DNA, isolated from pooled nucleosome monomer, dimer, and trimer sucrose gradient fractions by the method of Noll et al. (1975), was subjected to electrophoresis in 1.5% agarose gels according to the procedure of Sharp et al. (1973). SV40 DNA fragments, produced by digestion with *Hin*d II and III restriction endonucleases, were used as molecular weight markers, and the data of Danna and Nathans (1971) were used to construct a least-squares plot of log mol wt vs. mobility for these fragments. The mol wt values of the nuclease-digested chromatin DNA fragments were then estimated by interpolation, and the approximate number of base pairs in each fragment was obtained by dividing this mol wt value by 327 (the average mol wt of the deoxyribonucleoside 5′-monophosphates).

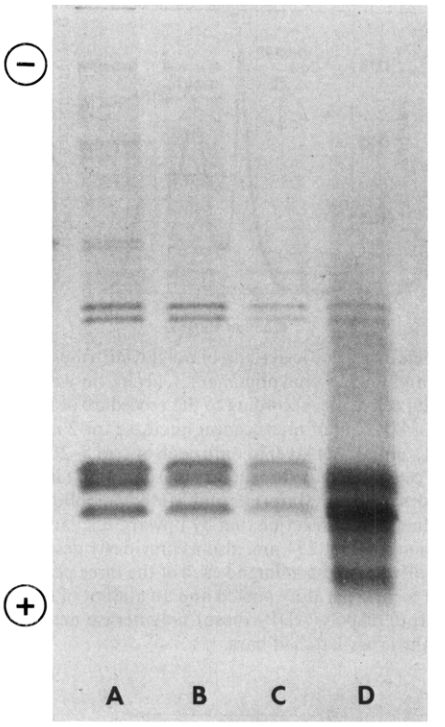

FIGURE 3: Polyacrylamide–sodium dodecyl sulfate gel electrophoresis of commercially prepared calf-thymus histones (General Biochemicals) and proteins extracted from nuclease-digested HeLa cell chromatin subunits. Electrophoresis was carried out in a 15% acrylamide slab gel having an acrylamide:bisacrylamide ratio of 45:0.4, and a 3% stacking gel, as described by Laemmli (1970) (see Materials and Methods): (A) 11S monomer (ν-body) proteins; (B) 16S dimer proteins; (C) 21S trimer proteins; (D) commercial calf-thymus histones.

the central region of the gradient showed intermediate and decreasing levels of enzyme activity. Thus, in confirmation of the data of Figure 1, only minimal poly(ADP-ribose) polymerase activity was detected in the fast sedimenting, condensed chromatin fractions (heterochromatin).

Sucrose Gradient Centrifugation of Nuclease-Treated Chromatin. Having obtained evidence to suggest that poly(ADP-ribose) polymerase activity is not homogeneously distributed among the various fractions of solubilized HeLa cell chromatin, an effort was next made to determine the distribution of this chromosomal enzyme, as well as its homopolymeric product, among the various chromatin structural subfractions (i.e., nucleosome monomers and oligomers) produced by endonucleolytic digestion. Isolated HeLa cell nuclei were subjected to a limited digestion, in situ, with micrococcal nuclease, essentially according to the method of Noll and his co-workers (1975). The limited conditions employed for digestion were designed such that several differently sized chromatin fragments could be isolated, thus enabling us to assay enzyme activity and the presence of product specifically in nucleosome monomers, dimers, and trimers. Following digestion, the nuclei were hypotonically lysed and the chromatin was separated into ν-body monomers and oligomers by sucrose gradient centrifugation. Catalase and human IgG were used as markers on identical gradients to establish approximate S values for the various chromatin structural subunits.

In order to further characterize these various chromatin fractions, DNA was isolated by the method of Noll et al. (1975) from each of the three peaks routinely observed on sucrose gradients (11, 16, and 21 S) and subjected to electrophoresis in 1.5% agarose gels, essentially according to the procedure of Sharp et al. (1973). SV40 DNA fragments, produced by digestion with a mixture of the restriction endonucleases *Hin*d II and III, were used as molecular weight markers, and the sizes of the DNA fragments associated with the nucleosome monomer, dimer, and trimer bands were estimated by regression analysis and interpolation (Table I). These base-pair values, while approximate, are generally consistent with the findings of others (Sollner-Webb and Felsenfeld, 1975; Oosterhof et al., 1975; Lacy and Axel, 1975), although the DNA fragments associated with the 11S material appeared to contain somewhat less than the expected 200 base pairs.

In addition, polyacrylamide–sodium dodecyl sulfate gel electrophoresis was carried out by the method of Laemmli (1970) on proteins extracted from each of the three peaks obtained by sucrose gradient centrifugation of nuclease-digested chromatin (Figure 3). The results of this study showed that all three major chromatin subunit fractions (11, 16, and 21 S) possess a full complement of the histones, including histone H1, as well as a quite complex array of nonhistone chromosomal proteins. Densitometry scans of the gel patterns indicated that the ratio of histone H1 to the small molecular weight histones (H2A, H2B, H3, and H4) in the 21S material was approximately twice that observed for the 11S and 16S chromatin subunits. Since histone H1 is thought not to be a structural component of the ν-bodies themselves, but rather associated with the internucleosomal "bridge" regions of chromatin (Gorovsky and Keevert, 1975; Honda et al., 1975; Whitlock and Simpson, 1976), these data imply that at least some of the 11S material isolated by sucrose gradient centrifugation consists of nucleosome particles which possess attached DNA fragments, or tails, containing histone H1 and, possibly, many of the nonhistone chromosomal proteins seen on the gels.

Fractions representing each of the three peaks routinely observed on sucrose gradients (11, 16, and 21 S) were separately pooled and assayed for poly(ADP-ribose) polymerase activity as described in the experimental procedures. The data of Figure 4 (and independently in Figure 8) show that the ac-

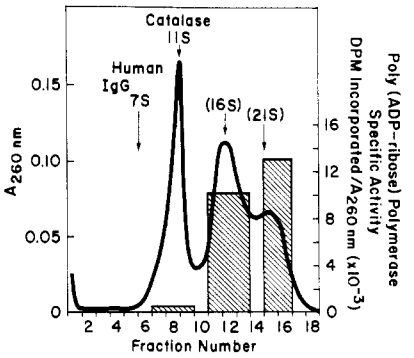

FIGURE 4: Relative specific activity of poly(ADP-ribose) polymerase in nucleosomes and nucleosome oligomers. Chromatin was prepared from 3.5×10^8 HeLa cell nuclei according to the procedure of Noll et al. (1975) with the use of 80 u/ml of micrococcal nuclease for 2 min at 37 °C; 0.2 ml (4.5 A_{260nm} units) was layered onto each of two 5–20% linear sucrose gradients and centrifuged for 16 h at 32 500 rpm (4 °C) in a Beckman SW 50.1 rotor as described in Materials and Methods. Following centrifugation, the gradients were fractionated by upward displacement with 60% sucrose and monitored at 254 nm, also as previously described. Fractions from both gradients corresponding to each of the three peaks observed (11, 16, and 21 S) were separately pooled and an aliquot of each was used to measure the rate of poly(ADP-ribose) polymerase activity/A_{260nm}, as indicated by the cross-hatched bars.

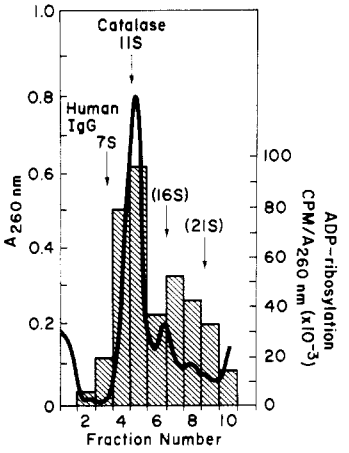

FIGURE 5: Generation of ADP-ribosylated chromosomal proteins in intact HeLa cell nuclei and their distribution among various subchromatin fractions. Chromatin from 5.8×10^8 HeLa cell nuclei which had been previously incubated with [*adenine*-2,8-^{3}H]NAD was prepared according to the method of Noll et al. (1975) with the use of 70 u/ml micrococcal nuclease for 2 min at 37 °C; 0.5 ml (8.5 A_{260nm} units) was layered onto each of two 12-ml 5–20% linear sucrose gradients and centrifuged for 10 h at 40 000 rpm (4 °C) in a Beckman SW 40Ti rotor as described in Materials and Methods. Fractions from both gradients corresponding to each of the three peaks observed (11, 16, and 21 S) were separately pooled and used to assay for the presence of Cl_3AcOH-insoluble radioactivity as indicated by the hatched bars.

tivity of this enzyme could not be observed in the ν-body monomer fraction, high activity being found only in those chromatin fractions sedimenting faster than 11 S. This observation suggests either that the individual ν bodies lack poly(ADP-ribose) polymerase, or a suitable acceptor, or that the activity of the enzyme is dependent upon the physical integrity of the oligomeric structures themselves.

In a similar type experiment, isolated HeLa cell nuclei were first incubated with [^{3}H]NAD, the substrate for poly(ADP-ribose) polymerase, prior to the preparation of nuclease-treated chromatin. The micrococcal nuclease digestion step resulted in an approximate 30% loss of Cl_3AcOH-insoluble radioactivity. Following the separation of the resultant chromatin

TABLE II: Effect of Alkali, Neutral Hydroxylamine, and Various Enzymes on the Poly(ADP-ribose) Associated with 11S Nucleosome Monomers (ν Bodies).[a]

Treatment	% Cl_3AcOH-Insoluble cpm Remaining
Control	100
0.1 N NaOH, pH 12.5[b]	54
1 M NH_2OH, pH 7[b]	71
DNase I[c]	117
Pancreatic RNase[c]	118
Micrococcal nuclease[c]	110
Pronase[c]	7
Snake venom phosphodiesterase[d]	29

[a] Micrococcal nuclease digested chromatin was isolated from HeLa cell nuclei previously incubated with [*adenine*-2,8-^{3}H]NAD, as described in Materials and Methods, and fractionated into nucleosome monomers and oligomers by sucrose gradient centrifugation. Fractions corresponding to 11S nucleosome monomer material were subsequently pooled and assayed for Cl_3AcOH-insoluble radioactivity, also as described previously (control). Aliquots of this pooled preparation were then treated as shown above, and the Cl_3AcOH-precipitable counts again assayed. [b] Treatment was for 60 min at 37 °C. [c] Assay mixture contained, in a volume of 1 ml, 50 μmol of Tris-HCl, pH 7.4, 5 μmol of $MgCl_2$, 100 μg of enzyme. Incubation was for 30 min at 37 °C. The micrococcal nuclease assay contained, in addition, 1 mM $CaCl_2$. [d] Same as *c*, except 250 μg of enzyme/ml.

fractions by sucrose gradient centrifugation (Figure 5), aliquots of each fraction were removed and assayed for the presence of Cl_3AcOH-insoluble radioactivity. In contrast to the data of Figure 4, which showed negligible enzymatic activity in the 11S nucleosome peak, the results of this experiment indicated that those chromatin fractions which possess significant poly(ADP-ribose) polymerase activity (nucleosome oligomers) are relatively deficient in the accumulation of the labeled product of this enzyme, and that a considerable amount of this homopolymeric material is ultimately found associated with the 11S nucleosome monomers.

Control experiments were performed to verify that the acid-insoluble material derived from labeled NAD was, in fact, covalently bound poly(ADP-ribose). Nicotinamide, an inhibitor of the covalent attachment of ADP-ribose to nuclear proteins, significantly reduced the number of Cl_3AcOH-precipitable counts associated with 11S material, as did treatment of labeled nucleosomes with 0.1 N NaOH to cleave the alkali-labile linkage of ADP-ribose to protein. Similarly, treatment with 1 M neutral hydroxylamine resulted in a 29% decrease in the number of Cl_3AcOH-insoluble counts sedimenting with the ν-body fraction. Furthermore, the acid-insoluble radioactivity associated with 11S material was shown to be sensitive to treatment with both pronase (possibly indicating that many of the modified sites contained single ADP-ribose units, or at least very short chains) and snake venom phosphodiesterase, but insensitive to digestion with DNase I, pancreatic RNase, or micrococcal nuclease. These data are summarized in Table II. Finally, the material released from labeled 11S nucleosomes by treatment with phosphodiesterase was identified as 5′-AMP and ψ-ADP-ribose, or Ado(P)-Rib-P, by thin-layer chromatography on cellulose plates (Figure 6), thus firmly establishing that the labeled material is poly(ADP-ribose).

One explanation for the lack of detectable in vitro poly(ADP-ribose) polymerase activity in 11S nucleosome monomers might be a high level of poly(ADP-ribose) breakdown in these structures, relative to other chromatin sub-

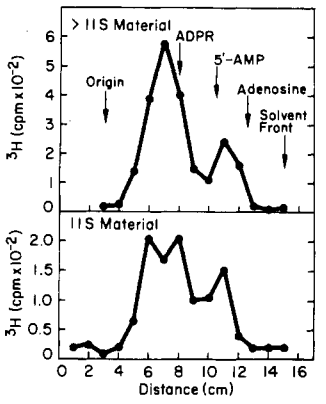

FIGURE 6: Cellulose thin-layer chromatography of material released from labeled nucleosome monomeric (11 S) and oligomeric (>11 S) material by treatment with snake venom phosphodiesterase (250 μg/ml, 30 min, 37 °C). Chromatographic development was carried out essentially according to the procedure of Yamada and Sugimura (1973). ADP-ribose and 5'-AMP were employed as markers, as described in Materials and Methods, and the labeled products were identified as Ado(P)-Rib-P, or ψ-ADP-ribose (major peak), and 5'-AMP (minor peak).

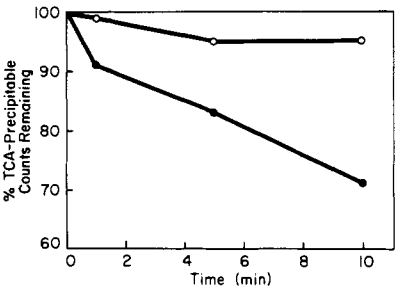

FIGURE 7: Relative rates of poly(ADP-ribose) breakdown in monomeric (11 S) and oligomeric (>11 S) HeLa cell chromatin subunits. Nuclease-digested chromatin was isolated from HeLa cell nuclei previously incubated with [*adenine*-2,8-^{3}H]NAD and fractionated on a 5-20% linear sucrose gradient as described in Materials and Methods. Fractions corresponding to 11S and >11S material were separately pooled and assayed at various times for Cl_3AcOH-insoluble radioactivity under incubation conditions optimal for poly(ADP-ribose) glycohydrolase activity: 11S material (○); >11S material (●).

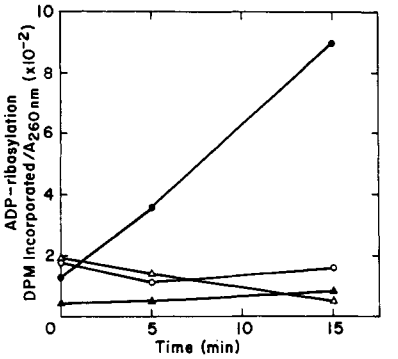

FIGURE 8: Mixing experiment. The details are given in Materials and Methods: (▲) 11S material; (●) >11S material; (△) heat-inactivated >11S material; (○) heat-inactivated >11S material + 11S material.

structural elements (nucleosome dimers and trimers). To test this, fractions corresponding to labeled 11S and oligomeric material from a gradient similar to that shown in Figure 5 were separately pooled and incubated for 10 min, at 37 °C, under conditions optimal for poly(ADP-ribose) glycohydrolase activity (25 mM KH_2PO_4, pH 7, 10 mM β-mercaptoethanol). At various times during the incubation, duplicate aliquots were withdrawn from each sample and assayed for Cl_3AcOH-insoluble radioactivity. As can be seen from the data of Figure 7, very little radioactivity (<5%) was lost from the 11S monomer material during this 10 min incubation, although the endogenous glycohydrolase (or some other polymer degradation activity) associated with the oligomeric fractions resulted in a 30% decrease in the Cl_3AcOH-insoluble counts associated with these structures. Thus, the pattern of glycohydrolase activity in the chromatin substructure may parallel that of poly(ADP-ribose) polymerase, and the lack of detectable polymerase activity in the ν bodies themselves cannot be due simply to more rapid polymer degradation.

It is well established that poly(ADP-ribose) polymerase requires both DNA and nuclear proteins for optimal activity (Yamada et al., 1971; Yoshihara, 1972). The lack of detectable poly(ADP-ribose) polymerase activity in the 11S nucleosome monomers (Figure 4), therefore, is most probably due to a deficiency of the enzyme itself, rather than the absence of a suitable acceptor, since the data in Figures 3 and 5 clearly show that these structures do contain a great variety of known acceptors, some of which are certainly ADP-ribosylated. However, chromatin-bound poly(ADP-ribose) polymerase may require DNA of a particular configuration not found in the 11S monomers alone for maximal activity. For example, the enzyme may require the presence of adjacent ν bodies, or even adjacent bridge regions, if chromatin coiling is invoked, for binding or activity. In order to distinguish between these two possibilities, a mixing experiment was performed (Figure 8) in which pooled fractions exhibiting poly(ADP-ribose) polymerase activity (nucleosome oligomers) from the gradient shown in Figure 4 were heat inactivated at 75 °C for 10 min, mixed with pooled fractions containing the 11S ν-body material, and incubated in the presence of [^{3}H]NAD as described. As before, high activity was found associated with the chromatin fractions containing both ν bodies and intact internucleosomal bridges, whereas no activity was found in the monomer material alone. As can be seen, poly(ADP-ribose) polymerase activity was not restored to the 11S monomers by adding DNA and acceptors having a more natural configuration, thus suggesting that the enzyme itself is missing from these structures. This interpretation is strengthened by our recent observation that the tops of sucrose gradients, like that shown in Figure 4, possess remarkably high levels of poly(ADP-ribose) polymerase activity (manuscript in preparation), presumably released during endonucleolytic digestion, and suggesting that the enzyme may require intact nucleosome oligomers, not so much for activity, as for binding to the chromatin.

In a final study designed to determine whether the poly(ADP-ribose) polymerase activity associated with the nucleosome oligomers (16S and 21S material) was, in fact, dependent upon a higher order of chromatin structure than is found within the monomers, micrococcal nuclease digested chromatin was prepared and fractionated by centrifugation on isokinetic sucrose gradients according to the procedures of Noll et al. (1975). Fractions representing the various chromatin subunits (11, 16, and 21 S) were then separately pooled, assayed for poly(ADP-ribose) polymerase activity, and the oligomer fractions again treated with micrococcal nuclease, this time for 10 min, in order to convert most of this material to nucleosome monomers. As can be seen from the data of Table III, further digestion with micrococcal nuclease decreased the activity of the associated poly(ADP-ribose) polymerase by about 37% in both the 16S and 21S oligomeric structures, and sucrose gradient centrifugation of the redi-

TABLE III: Effect of Further Nuclease Digestion on the Poly(ADP-ribose) Polymerase Activity of Various HeLa Cell Chromatin Subunits (Nucleosome Monomers, Dimers, and Trimers).[a]

Region of Gradient	Poly(ADP-ribose) Polymerase Act. (dpm incorp/A_{260nm}) Before Digestion (A)	After Digestion (B)	% Decrease
11 S	364	350	
16 S	9421	5760	39
21 S	6787	4348	36

[a] Micrococcal nuclease digested (110 u/ml, 2 min, 37 °C) chromatin was prepared from 5.2×10^8 HeLa cell nuclei and fractionated into nucleosome monomers and oligomers by isokinetic sucrose gradient centrifugation (22 h at 27 000 rpm, 4 °C, in a Beckman SW 27 rotor), as described in Materials and Methods. Following centrifugation, the regions of the gradients corresponding to nucleosome monomers (11S), dimers (16S), and trimers (21S) were separately pooled and assayed for poly(ADP-ribose) polymerase activity, also as previously detailed. These initial levels of enzyme activity are shown in column A. The remaining volumes of the three samples were then dialyzed overnight against the nuclease digestion buffer (50 mM Tris-HCl, pH 7.4, 25 mM KCl, 1 mM $MgCl_2$, 0.25 M sucrose, 15 mM β-mercaptoethanol, and 1 mM $CaCl_2$) at 3 °C. After dialysis, the samples were again treated with 110 u/ml micrococcal nuclease at 37 °C, this time for 10 min. After assaying for enzyme activity (column B), the oligomer samples were pooled, dialyzed overnight against water, and then lyophilized to dryness. After redissolving in water to an appropriate dilution, the redigested samples were again subjected to isokinetic sucrose gradient centrifugation in order to determine the extent of oligomer breakdown (see text).

gested fractions, following dialysis and concentration by lyophilization, revealed that about 40% of the oligomeric material had been converted to 11S monomers. These fractions could not be assayed for poly(ADP-ribose) polymerase activity, however, as the lyophilization step apparently destroyed the activity of the enzyme.

Discussion

The present study was undertaken in order to gain information about the distribution of a chromatin-bound protein modifying enzyme, poly(ADP-ribose) polymerase, and its product, poly(ADP-ribose), within the newly emerging chromatin substructure. Poly(ADP-ribose) polymerase is ideally suited to this task since it is one of the most tightly bound chromosomal proteins known, requiring 1 M salt for dissociation (Sugimura, 1973).

The results of experiments involving the fractionation of sheared HeLa cell chromatin by ECTHAM-cellulose chromatography and glycerol gradient centrifugation indicated that poly(ADP-ribose) polymerase activity is associated primarily with the transcriptionally active regions of the chromatin. Similar results were obtained by Keller et al. (1975) with respect to the chromatin-bound kinase responsible for the phosphorylation of nonhistone chromosomal proteins. And while this latter finding is not particularly surprising in view of the considerable body of evidence which has accumulated in recent years implicating nonhistone phosphoproteins in gene regulation (Allfrey et al., 1973), the apparent association of poly(ADP-ribose) polymerase with this chromatin subfraction is somewhat puzzling. Thus far, no firm evidence has been obtained to indicate that ADP-ribosylated chromosomal proteins are similarly involved in the regulation of gene transcription, although a recent report has suggested a role in differentiation (Caplan and Rosenberg, 1975). On the contrary, most evidence to date seems to indicate that poly(ADP-ribose) polymerase activity is correlated with some aspect of replication or repair, rather than gene transcription (Preiss et al., 1971; Smulson and Rideau, 1972; Roberts et al., 1973, 1975; Yoshihara et al., 1975). Furthermore, while the concentration of histone H1, a known acceptor of poly(ADP-ribosylation), is low in transcriptionally active chromatin as compared with the other major histone species, it is still premature to assume that this protein is the only acceptor of poly(ADP-ribose) polymerase. There is, in fact, good evidence to indicate that several other chromosomal proteins, including histone H3 (Ord and Stocken, unpublished observations) and a heterogeneous population of nonhistone proteins (Nishizuka et al., 1969; Otake et al., 1969; Burzio and Koide, 1972; Miwa and Sugimura, unpublished observations), are also ADP-ribosylated.

The nuclease digestion studies reported above have presented evidence to suggest that poly(ADP-ribose) polymerase requires, for its association with chromatin and concomitant activity, regions of chromosomal DNA between adjacent and intact ν bodies since enzyme activity was found associated only with nucleosome oligomers (Figure 4). The presence of appreciable poly(ADP-ribose) polymerase activity in the nonsedimenting regions of the sucrose gradients (manuscript in preparation) implies that the enzyme is released from the chromatin upon nuclease digestion, and that very little, if any, remains bound to the 11S ν-body "tail" regions during centrifugation (as does, for example, histone H1). Thus, while poly(ADP-ribose) polymerase is a strongly bound chromosomal enzyme, this association is apparently dependent upon a higher order of chromatin structure than is found in just the ν bodies themselves. In addition, since both heterochromatin and euchromatin appear to possess similar substructural elements (nucleosomes), we must tentatively conclude that poly(ADP-ribose) polymerase resides primarily within the internucleosomal regions of euchromatin, and that it is present only minimally, if at all, in transcriptionally inert chromatin.

Perhaps the most interesting aspect of the present investigation is the finding that the eventual acceptors of ADP-ribosylation appear to be the nuclear proteins of the 11S nucleosome itself (Figure 5), probably including histones H3 and H1, as well as several nonhistone chromosomal proteins (Ord and Stocken, unpublished observations; Nishizuka et al., 1969; Otake et al., 1969; Burzio and Koide, 1972; Miwa and Sugimura, unpublished observations). Since many of the nonhistone chromosomal proteins, as well as histone H1, are probably associated mainly with the internucleosomal "bridge" regions of chromatin, this finding implies that the 11S material in our sucrose gradients consists largely of ν bodies and attached internucleosomal material, or "tails", and this conclusion is supported by the histone gel electrophoresis patterns (Figure 3).

In conclusion, although the exact functions of poly(ADP-ribose) polymerase and its product, poly(ADP-ribose), are not known, a variety of evidence suggests possible roles in the modulation of gene replication, repair or expression, as well as in the maintenance of the chromatin architecture. Our results indicate that the association of this enzyme with chromatin is not random but appears to be localized primarily within the internucleosome regions of transcriptionally active chromatin and is strongly dependent upon the physical integrity of this structural organization, both for its association and

activity. It is hoped that this finding will aid in the future elucidation of the precise relationship of poly(ADP-ribose) polymerase to chromatin physiology, as well as in an understanding of the chromosome itself.

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