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Nuclear Protein Modification and Chromatin Substructure. 1. Differential Poly(adenosine diphosphate) Ribosylation of Chromosomal Proteins in Nuclei versus Isolated Nucleosomes†

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ABSTRACT: Poly(adenosine diphosphate-ribose) polymerase has been shown to modify a number of histone and nonhistone proteins. The species of proteins which were modified varied according to the conditions under which the reaction was carried out. Development of a two-dimensional gel system has allowed the identification of modified proteins not only in the nucleus as a whole, but also, for the first time, in nucleosomal fragments of chromatin generated by digestion with micrococcal nuclease. When ADP ribosylation was carried out in intact nuclei, H1 and H2B were found to be major acceptors, with H2A, H3, HMG, and M1-M4 proteins being modified to a lesser extent. After digestion with micrococcal nuclease, modified histones were associated preferentially with mononucleosomes and dimers, whereas higher oligomers displayed a wide variety of heavily modified nonhistone proteins. The specific activity of the enzyme was shown to increase with increasing nucleosome repeat number, and considerable activity was found in subnucleosomes. No H4 ADP ribosylation was detected in this study. The evidence relating to the modification of basic proteins was corroborated when the proteins isolated from mononucleosomes, dimers, and trimers were analyzed in a single-dimension electrophoresis, where quantitation was more easily achieved. This pattern of histone modification was shown to be dependent on chromatin being in its "native" conformation within the nucleus and emphasized the importance of interaction between the ADP-ribosylating system and the core particles. If the reaction was carried out with isolated nucleosomes, very little histone modification, with the exception of H1 and H3.1, occurred, but the ADP ribosylation of the HMG proteins, M1, and M4 was greatly enhanced.

The interactions between proteins and DNA play a vital role in determining the organization and expression of the genome. A knowledge of the ways in which these proteins can be modified by chromatin-associated enzymes is consequently of great importance in understanding the functioning of the nucleus.

Poly(ADP-Rib)[1] polymerase, a tightly bound chromatin enzyme, catalyzes the successive transfer of ADP-Rib units from NAD to nuclear proteins (see reviews by Hayaishi and Ueda, 1977; Smulson and Shall, 1976; Hilz and Stone, 1976). This results in the generation of short poly(ADP-Rib) chains, covalently attached to both histone and nonhistone chromosomal proteins. The enzyme may also cross-link proteins (Lorimer et al., 1977). The purified enzyme shows an absolute requirement for DNA, and addition of histones further stimulates the activity (Okayama et al., 1977; Yoshihara et al., 1977).

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[1] Abbreviations used are: ADP-Rib, adenosine diphosphate-ribose; Ado(P)-Rib-P and ψ-ADP-Rib, 2′-(5′-phosphoribosyl)-5′-AMP; NAD, nicotinamide adenine dinucleotide; $NaDodSO_4$, sodium dodecyl sulfate; Cl_3AcOH, trichloroacetic acid; $PhCH_2SO_2F$, phenyl methanesulfonyl fluoride; HMG, high-mobility group proteins; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; NHP, nonhistone protein; NMN, nicotinamide mononucleotide.

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Complete Amino Acid Sequence of the Major Component Myoglobin from the Humpback Whale, *Megaptera novaeangliae*†

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ABSTRACT: The complete primary structure of the major component myoglobin from the humpback whale, *Megaptera novaeangliae,* was determined by specific cleavage of the protein to obtain large peptides which are readily degraded by the automatic sequencer. Over 80% of the amino acid sequence was established from the three peptides resulting from the cleavage of the apomyoglobin at the two methionine residues with cyanogen bromide along with the four peptides resulting from the cleavage of the acetimidated apomyoglobin at the three arginine residues with trypsin. The further digestion of the central cyanogen bromide peptide with trypsin and *S. aureus* strain V8 protease enabled the determination of the remainder of the covalent structure. This myoglobin differs from that of sperm whale, *Physeter catodon,* at 12 positions, and dwarf sperm whale, *Kogia simus,* at 14 positions, finback whale *Balaenoptera physalus* at 3 positions, minke whale, *Balaenoptera acutorostrata* at 2 positions, and California gray whale *Eschrichtius gibbosus,* at 1 position. All of the substitutions observed in this sequence fit readily into the three-dimensional structure of sperm whale myoglobin.

The complete sequence reported here for the myoglobin from the humpback whale, *Megaptera novaeangliae,* is in total agreement with that found for the first 60 residues by Edman & Begg (1967) in their classical introduction of automated sequencing methodology. The complete amino acid sequence of the myoglobin from Amazon River dolphin (Dwulet et al., 1975), California gray whale (Bogardt et al., 1976), Atlantic bottlenosed dolphin (Jones et al., 1976), arctic minke whale (Lehman et al., 1977), dwarf sperm whale (Dwulet et al., 1977), Pacific common dolphin (Wang et al., 1977), finback whale (DiMarchi et al., 1978a), pilot whale (Jones et al., 1978), and Dall porpoise (Meuth et al., 1978) have been reported. All of these sequences of cetacean myoglobin were determined by automated Edman degradation. This paper reports the application of the peptide fragmentation and analytical procedures that were used in these papers in determining the complete amino acid sequence of the major component myoglobin from the humpback whale. Completion of this sequence extends the number of known cetacean myoglobin sequences to 14. In addition to the above-mentioned proteins, the primary structures of the myoglobins from the Black Sea dolphin (Kluh & Bakardjieva, 1971), common porpoise (Bradshaw & Gurd, 1969; Meuth et al., 1978), sperm whale (Edmundson, 1965; Romero-Herrera & Lehmann, 1974), and killer whale (Castillo et al., 1977) have also been reported.

Experimental Section

Materials

The principal component of humpback whale myoglobin was isolated from muscle tissue as described by Hapner et al. (1968). Phosphate buffer (pH 6.4, 0.1 ionic strength) was used to effect the purification of the crude homogenate on Sephadex CM-50. The homogeneity of the purified myoglobin was shown

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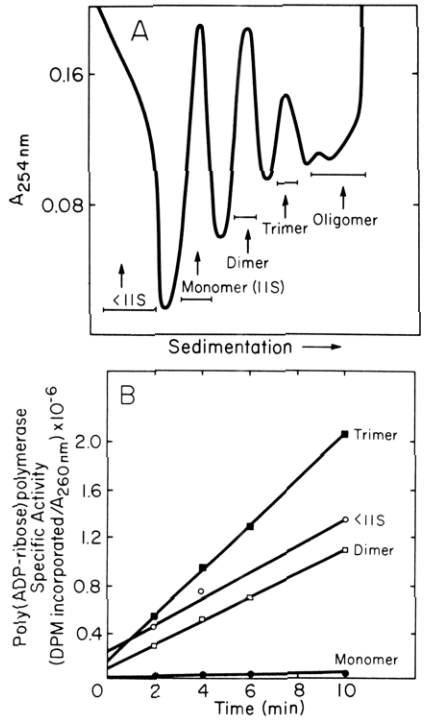

FIGURE 1: Relative specific activity of poly(ADP-Rib) polymerase in nucleosome monomer, dimer, trimer, and higher oligomers. Micrococcal nuclease digested chromatin was prepared from isolated HeLa cell nuclei by the method of Noll et al. (1975) with slight modifications as described in the following paper of this issue (Giri et al., 1978). The resultant chromatin fragments were fractionated into nucleosome monomer, dimer, trimer, and oligomer by sucrose gradient centrifugation. As shown in Figure 1A, appropriate fractions were pooled as indicated by the horizontal bars, and poly(ADP-Rib) polymerase activity was assayed in an aliquot of each. The results are expressed as a function of time of incubation (B).

TABLE I: Average Chain Length of Poly(ADP-Rib) Associated with Micrococcal Nuclease Digested Chromatin Fractions.[a]

fraction	snake venom phosphodiesterase digest. prod[b] (^{3}H cpm) ψ-ADP-rib (A)	5′-AMP (B)	av chain length (1 + A/B)
top (<11 S)	494	79	7.25
monomer (11 S)	686	236	3.90
oligomer (>11 S)	1602	452	4.54

[a] The conditions of poly(ADP) ribosylation of an isolated HeLa nuclear preparation with [^{3}H]NAD, followed by nuclease digestion, chromatin isolation, and fractionation, were performed as described before (Mullins et al., 1977). [b] The methods for snake venom phosphodiesterase digestion of ^{3}H-labeled poly(ADP) ribosylated chromatin fractions and the separation of digestion products by thin-layer cellulose chromatography were described elsewhere (Mullins et al., 1977).

ternucleosomal regions or from regions where the typical nucleosome structure was altered.

By digestion with snake venom phosphodiesterase, the polymer was shown to be poly(ADP-Rib). The average chain length (Table I) of the polymer synthesized by the enzyme in this "top" (<11S) fraction was about twofold greater than that synthesized by the enzyme in the monomers and oligomers. We are currently investigating the significance of this observation.

It was found that the specific activity of the enzyme in oligomer particles (Figure 1A) was slightly greater than that of

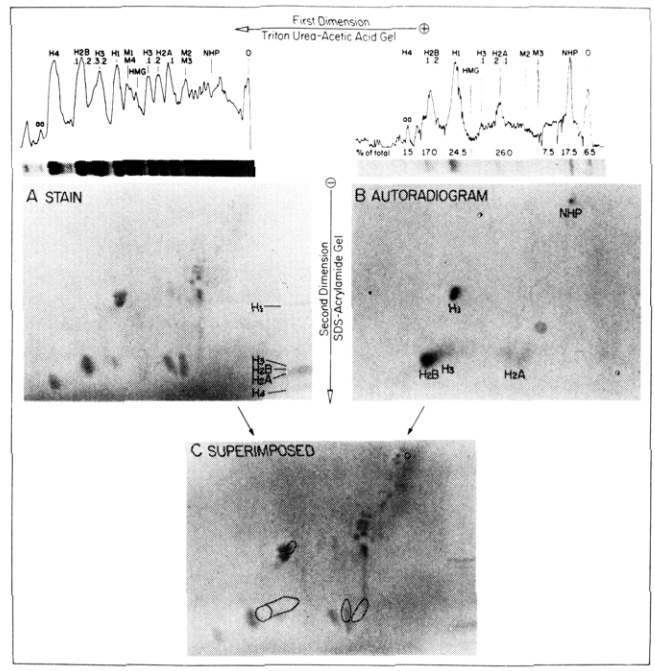

FIGURE 2: Two-dimensional gel electrophoretic analysis of acid-soluble proteins from [α-^{32}P]NAD-incubated nuclei. 4×10^7 HeLa nuclei were incubated with [α-^{32}P]NAD (2 μCi, 0.1 nmol) in a total volume of 1.5 mL for 30 min at room temperature (Mullins et al., 1977). As described under Materials and Methods, an aliquot of 0.4 N H_2SO_4-extracted proteins from the washed nuclei was subjected to Triton–urea–acetic acid tube gel electrophoresis in the first dimension, followed by second-dimensional electrophoresis in an $NaDodSO_4$–acrylamide slab gel, with purified calf thymus histones as standards. After staining (A) with Coomassie blue, the gel was subjected to autoradiography (B) as indicated under Materials and Methods. The photographs and densitometer scans of the negatives refer to the one-dimensional electrophoretic run of an identical sample in a Triton–urea–acetic acid–acrylamide slab gel. The nomenclature of histones and their variant species is described by Franklin and Zweidler (1977). Percent total refers to radioactivity in the gel computed by integrating the peaks from densitometer scans. The radioactive spots are outlined in the diagrammatic representation (C) of the autoradiogram superimposed on the stained gel.

trimers (data not shown). However, recently published results from our laboratory indicate that an increase in specific activity occurs until the fragment reaches six to nine nucleosomes in length (Batt et al., 1978). This trend may be related either to the increasing numbers of intact linker regions (where we believe the enzyme to be located) or to the arrangement of these nucleosomes in a higher order of structure. It is possible that one of the functions of poly(ADP-Rib) polymerase is to pack the chromatin in a more condensed form.

Poly(ADP) Ribosylation of Proteins in Nuclei. The patterns of modification of chromatin-associated proteins (both histones and nonhistone proteins), which were labeled with [^{32}P]NAD in the nucleus, were analyzed (Figure 2). Separation of ^{32}P-labeled histones in the first dimension was carried out in the Triton–urea–acetic acid gel system described earlier. This allowed a very detailed analysis of histone variants (Alfageme et al., 1974; Cohen et al., 1975; Franklin and Zweidler, 1977) and posttranscriptionally modified histones (Riggs et al., 1977) to be made. Since the separation is partially dependent on molecular charge, addition of the negatively charged polymer may reduce the mobility of the modified species. This did occur to a limited extent (Figure 2), since the chain lengths were small (Table I) and, as a result, questioned the positive identification of the modified proteins. For example, no modification of H4 was noted and this may have resulted from an altered mobility of the modified molecules. To eliminate

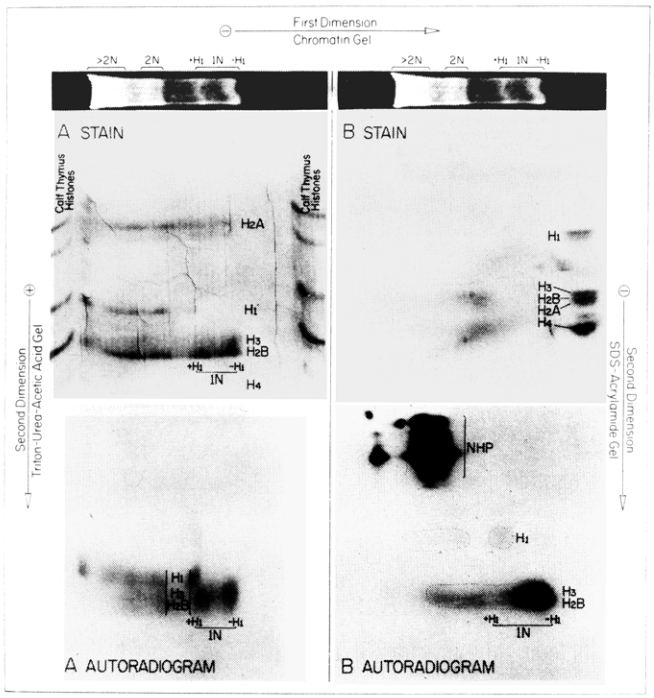

FIGURE 3: The modification of nucleosomal proteins by ^{32}P-labeled NAD. $\sim 2.6 \times 10^7$ HeLa nuclei were incubated with [α-^{32}P]NAD (0.7 μCi) in a total volume of 1 mL for 30 min at room temperature (Mullins et al., 1977). Micrococcal nuclease digested chromatin prepared from the washed nuclei (Giri et al., 1978) was resolved into nucleosome monomer (1N) with and without H1-containing linker regions, dimer (2N), and higher oligomers, by electrophoresis in first-dimension 5% acrylamide tube gels (in duplicates) as described by Varshavsky et al. (1976). The proteins associated with the nucleosomes are displayed in second dimension, as indicated under Materials and Methods, by electrophoresis either in Triton–urea–acetic acid (A) or in $NaDodSO_4$–acrylamide (B) slab gels, along with purified calf thymus histones as standards. After staining with Coomassie blue, the gels were subjected to autoradiography.

this ambiguity, the histones were then separated on the basis of their mobility in $NaDodSO_4$–acrylamide gel system and were precisely identified (Figure 2C).

The major ADP-ribosylated histones (Figure 2) were H1 and H2B (0.1 and 0.2), with minor modification of H2A and H3 (see also Figure 4A). Extensive modification was also found in a nonhistone chromosomal protein. The data suggested selective modification of nonallelic histone variants (Cohen et al., 1975), but this must remain a tentative conclusion. No modification of histone H4 was detected in these studies.

We were concerned that conversion of the labeled NAD into another metabolite might allow ^{32}P-labeling by an alternative modification, such as histone phosphorylation. The experiment was therefore repeated using [*adenine*-^{14}C]NAD (results not shown), and the qualitative labeling patterns noted above were confirmed.

ADP Ribosylation of Proteins in Nucleosomes of Increasing Size. The novel two-dimensional gel system of Todd and Garrard (1977) was modified to enable assessment to be made of the patterns of ADP-ribosylated proteins in differing classes of mononucleosomes, as well as in nucleosomes of increasing number.

After labeling with [^{32}P]NAD in the nuclei, as described under Materials and Methods, nucleosomes were generated and separated in a nondenaturing gel system (Varshavsky et al., 1976). Labeled proteins from these nucleosomes were analyzed in the second dimension, as described under Materials and Methods, either in a Triton–urea–acetic acid gel (Figure 3A) or in a $NaDodSO_4$–acrylamide gel (Figure 3B). Some loss was experienced in the protein content as a result of the extraction technique but this was not a selective loss. When only the histones were examined (Figure 3A), the most extensively modified types were H1 and H2B, with H3 also showing some modification. However, modification of these histones in particles larger than dimer seemed considerably reduced. Histone H1 and, consequently, its modified species were completely absent from the "core" nucleosomes. In this experiment we show definitively that poly(ADP-Rib) polymerase (located between the nucleosomes; part 2) can modify histones within core particles. This emphasizes the importance of the native chromatin conformation in allowing a "linker" enzyme to interact with core particles.

When all the proteins were analyzed (Figure 3B), those particles larger than dimer were found to contain a great number of heavily modified proteins which migrated to the regions of the gel where nonhistone proteins are found. These results were confirmed in a one-dimension $NaDodSO_4$–acrylamide gel (results not shown), where 70% of the radioactivity was in nonhistone proteins. It is possible that these represented nonhistone proteins or histones cross-linked by poly(ADP-Rib), which were not acid extractable. It is hoped that the two-dimensional gel technique will enable a positive identification of these modified proteins to be made in the future.

Modification in Nuclei and in Isolated Nucleosomes. To evaluate the influence of chromatin conformation on ADP ribosylation, a comparison was made between histones modified in isolated chromatin subunits (in vitro) and in particles modified in nuclei (in situ). A Triton–urea–acetic acid gel system was run in one dimension only, since previous results (Figure 2) had shown that radioactive bands could be assigned with certainty to stained bands in the gel.

(a) When modification was carried out in the nucleus (Figure 4A), three regions of radioactivity, corresponding to labeled histones or histone-like proteins, were noted (II, III, and IV). These represented modified M3/M2 and H2A; H1; and H3/H2B, respectively. Slight differences in the labeling patterns obtained in this experiment and that shown in Figure 2 were probably a result of selecting chromatin and chromatin fragments for analysis in the latter experiment. These modified histones were seen, irrespective of their source (i.e., monomer, dimer, etc.); however, the modified nonhistone protein (region I) previously described (Figure 2A) was only detected here in chromatin. When nucleosomes were prepared, this protein was presumably lost. By integrating the area under each region in the scans, the relative amounts of each modified species of histone could be compared. There were no significant differences in the relative amounts between histones isolated from chromatin and histones isolated from either monomer, dimer, or particles larger than dimer (containing three to six nucleosomes).

These results were in sharp contrast to results obtained after modification was carried out in isolated dimer, trimer, or particles larger than trimer.

(b) When modification was carried out in isolated nucleosomes (Figure 4B), there was very little modification of proteins in regions II and IV. Histone H1 continued to be modified to a considerable extent, especially in particles greater than trimer (containing four to six nucleosomes), and histone H31 was modified to a much greater extent than in the nucleus. Additional modified proteins were also detected. These were the histone-like "high mobility group" proteins (HMG) and M1 and M4. This pattern was not due to an enrichment for nucleosomes containing these proteins, since their amounts

relative to the histones are slightly reduced in isolated particles (Figure 4B stain) when compared with the proteins isolated from nuclei (Figure 4A stain). In this particular gel (Figure 4B), H4 was not adequately stained, but similar gels of histones from these "core" particles showed the normal histone complement.

Discussion

The primary objectives of this investigation were to identify ADP-ribosylated proteins and to relate the types of modification to different orders of chromatin structure and conformation. By comparing ADP ribosylation in intact chromatin subfractions, the present studies emphasized the importance of the location of the modifying enzyme in relation to its acceptors. A number of studies have indicated that several chromatin proteins can act as acceptors for poly(ADP-Rib), although these studies have not been carried out at the nucleosome level. Thus, poly(ADP) ribosylation of H1 in vivo has been reported (Dixon, 1976; Smith and Stocken, 1973; Ueda et al., 1975; Riquelme et al., 1977; Roberts et al., 1975). Ueda et al. (1975) also showed modification of H2A, H2B, and H3, although Wong et al. (1977) were unable to show significant modification of these proteins in trout testis. In addition to simple modification, Lorimer et al. (1977) and Stone et al. (1977) have reported the formation of H1 dimers, linked by a chain of poly(ADP-Rib).

Isolation and identification of modified proteins have been hindered by the lability of the linkage between the polymer and the protein (Nishizuka et al., 1969; Adamietz and Hilz, 1976; Wong et al., 1977) by the high turnover of NAD (Rechsteiner et al., 1976), and by the presence of the poly(ADP-Rib) glycohydrolase (Lorimer et al., 1977).

The confusion in the literature regarding the extent of modification amongst proteins prompted this detailed investigation. By extracting proteins under conditions which preserved the linkage to the polymer and by separating histones into their variant types, we were able to catalogue a variety of ADP-ribosylated histone and nonhistone proteins. Use of the Triton–urea–acetic acid gel, which separates proteins mainly on the basis of their hydrophobicity, provided a unique advantage, since modification of proteins in their hydrophilic regions would not affect their binding to Triton. Their mobilities would therefore be affected only to the extent that ionic charge and molecular weight were altered (L. Cohen, personal communication). The importance of H1 in its role as an acceptor for the polymer was confirmed. In addition, it was shown that "core" histones, with the exception of H4, were modified in the nucleus. This contrasts with data regarding the acetylation of histones, where Davie and Candido (1977) showed H4 to be extensively modified. Modified proteins in this study were identified on the basis of their mobilities in a two-dimensional separation which utilized a different gel system in each dimension. This prevented identification errors (caused by altered mobilities), occurring as a consequence of adding the negatively charged polymer.

When modification was carried out in the nucleus, and chromatin fragments isolated, the pattern of modification depended on the size of particle which was analyzed. In monomers and dimers modified histones were predominant, whereas in larger particles nonhistone proteins were the modified species. There are three explanations for this: (a) Modification of histones by poly(ADP-Rib) polymerase either occurs in nucleosomes which are more susceptible to micrococcal nuclease or renders them more susceptible. (b) Digestion is random, and labeled nonhistone proteins are selectively lost from monomers and dimers. (c) Cross-linked proteins are selectively generated in larger particles as a result of folding and these have altered mobilities.

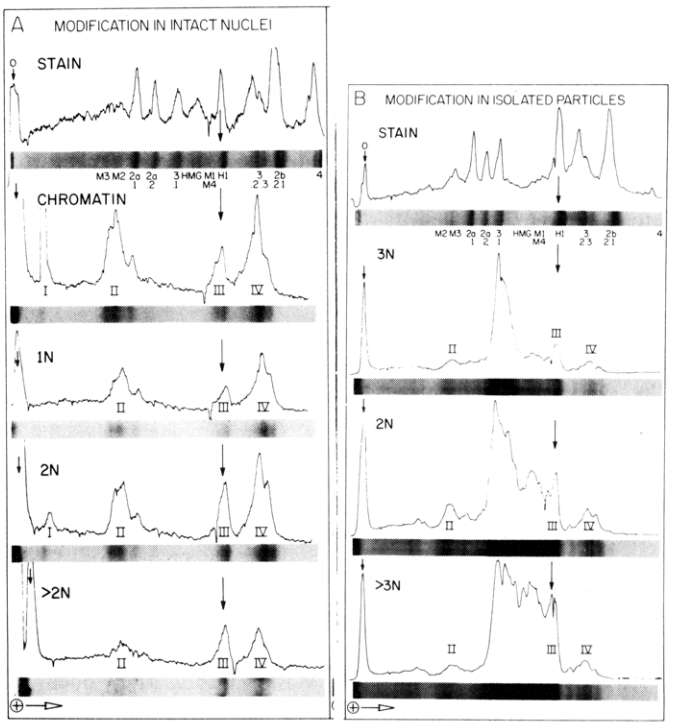

FIGURE 4: Differential pattern of ADP ribosylation in chromatin subunits from intact nuclei vs. isolated nucleosome particles. (A) Modification in intact nuclei: 6×10^7 HeLa nuclei were incubated with [^{32}P]NAD (4 μCi) in a total volume of 2 mL for 30 min at room temperature (Mullins et al., 1977). Preparation and fractionation of micrococcal nuclease digested chromatin fragments from these modified nuclei were carried out by the method of Noll et al. (1975) with slight modifications as described (Giri et al., 1978). (B) Modification in isolated particles: Micrococcal nuclease digested chromatin fragments were prepared from HeLa nuclei and then fractionated into monomer (1N), dimer (2N), trimer (3N), and oligomer as described (Giri et al., 1978). The appropriate fractions were pooled and concentrated by vacuum dialysis. These fractions (about 1.6 $A_{260\ nm}$ units) were subsequently incubated in a total volume of 0.5 mL with [^{32}P]NAD (0.12 μCi) for 30 min at room temperature as above. Extraction of histones and their separation in the two-dimensional gel system are described under Materials and Methods. Photographic negatives of the stained gels and of the autoradiograms were scanned in a densitometer.

The second alternative implies that modified histones should also occur and be detected in particles larger than dimer; this was not the case, even though there was a predominance of these particles in the gel. We have no evidence in favor of cross-linking and, for these reasons, we favor the first explanation.

Poly(ADP-Rib) polymerase, which is located between the nucleosomes (see part 2), is able to modify not only the nonhistone proteins and H1 which occur in that region but also some of the "core" histones. It is not known whether the nucleosomes are in their condensed form when this modification takes place, but it is important to note that when particles were isolated and then incubated under conditions where the polymer was synthesized, the enzyme was not able to modify those "core" histones to a significant extent, the sole exception being H3.1. It was just as efficient, however, at modifying H1, and additional modification of histone-like proteins was seen.

If artifactual modification occurs in the isolated nucleus, it would be expected to play a more important role in particles which were isolated and then modified. Similar studies concerning the phosphorylation of chromatin proteins (Bohm et al., 1977) have supported this idea. Fragmentation of the chromatin by nuclease digestion resulted in a wider spectrum

of proteins being made available for phosphorylation. This nonspecific modification with poly(ADP-Rib) was indeed observed in the case of some histone-like proteins (HMG proteins). However, it seems probable that modification of histones by poly(ADP-Rib) polymerase plays a significant role in the nucleus and may contribute to nucleosome structure. Although an exact function or functions cannot yet be ascribed to the enzyme, the results reported here and in part 2 provide some powerful techniques to investigate, in a very detailed way, the modification of chromatin proteins.

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