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Decrease in ADP-Ribosylation of HeLa Non-Histone Proteins from Interphase to Metaphase†

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ABSTRACT: Variations for non-histones in the ADP-ribosylating activities of interphase and metaphase cells were investigated. ^{32}P-Labeled nicotinamide adenine dinucleotide ([^{32}P]NAD), the specific precursor for the modification, was used to radioactively label proteins. Permeabilized interphase and mitotic cells, as well as isolated nuclei and chromosomes, were incubated with the label. One-dimensional and two-dimensional gels of the proteins of total nuclei and chromatin labeled with [^{32}P]NAD showed more than 100 modified species. Changing the labeling conditions resulted in generally similar patterns of modified proteins, though the overall levels of incorporation and the distributions of label among species were significantly affected. A less complex pattern was found for nuclear scaffolds. The major ADP-ribosylated proteins included the lamins and poly(ADP-ribose) polymerase. Inhibitors of ADP-ribosylation were effective in preventing the incorporation of label by most non-histones. Snake venom phosphodiesterase readily removed protein-bound ^{32}P radioactivity. A fundamentally different distribution of label from that of interphase nuclei and chromatin was found for metaphase chromosome non-histones. Instead of 100 or more species, the only major acceptor of label was poly(ADP-ribose) polymerase. This profound change during mitosis may indicate a structural role for ADP-ribosylation of non-histone proteins.

Posttranslational modification of nuclear proteins by ADP-ribosylation is being increasingly recognized as having a significant role in the regulation of nuclear function (Hilz & Stone, 1976; Hayaishi & Ueda, 1977; Purnell et al., 1980; Smulson & Sugimura, 1980). In particular, ADP-ribosylation has been implicated in the control of gene expression and cell differentiation (Farzaneh et al., 1982; Althaus et al., 1982). A related role is in the repair of DNA damage caused by X-rays, ultraviolet light, and chemical carcinogens (Borek et al., 1984). The enzyme responsible for the synthesis of protein-bound poly(ADP-ribose), poly(ADP-ribose) polymerase, is tightly associated with chromatin (Chambon et al., 1966; Hasegawa et al., 1967; Nishizuka et al., 1967), and the histones are themselves major acceptors of poly(ADP-ribose) (Adamietz et al., 1978; Braeuer et al., 1981; Riquelme et al., 1979; Ogata et al., 1980a,b; Smith & Stocken, 1975; Wong et al., 1983; Burzio et al., 1979). Because of such evidence that ADP-ribosylation is an important posttranslational modification of nuclear proteins, this investigation was undertaken to determine basic differences in the ADP-ribosylation of non-histone proteins from interphase nuclei and metaphase chromosomes. ADP-ribosylation of non-histones had not previously been well characterized, and so an assessment of the solution conditions to investigate the modification with radioactive isotopes was required. In addition to metaphase chromosomes, the experiments focused upon the two major components of the interphase nucleus: chromatin and the nuclear scaffold (the protein framework of the nucleus, similar to the nuclear matrix). The ultimate aim of these studies is to relate cell cycle changes in ADP-ribosylation of non-histones to changes in the structural organization of the HeLa genome.

Previous cell cycle studies of the ADP-ribosylation of nuclear proteins were concerned with the levels of ADP-ribosylation at different phases of the cell cycle and with the state of modification of poly(ADP-ribose) polymerase. These reports did not emphasize variations in the modification of non-histones. For example, an antibody specific for poly(ADP-ribose) revealed peaks at mid-S and S/G_2 phases (Kidwell & Mage, 1976), and immunofluorescence techniques demonstrated the in situ presence of poly(ADP-ribose) for interphase HeLa cells and metaphase chromosomes (Kanai et al., 1981). Metaphase proteins showed the greatest degree of modification with an in vitro system of HeLa nuclei (Tanuma et al., 1978), and measurements of the level of modification showed a 4-5 times higher level in metaphase (Holtlund et al., 1983). Furthermore, poly(ADP-ribose) polymerase was present in an active form on metaphase chromosomes (Holtlund et al., 1980).

No investigations of ADP-ribosylation have been concerned with the structural protein framework of the nucleus (the nuclear scaffold or nuclear matrix), but reports on the modification of chromatin-associated proteins have appeared. In particular, modifications of the histones and poly(ADP-ribose) polymerase, considered to be a non-histone protein, have received considerable research attention. For chromatin fibers,

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the activity of the polymerase was found to be greatest for extended chromatin (Jump et al., 1979), and condensation of polynucleosomes into 250-Å fibers at high ionic strengths was inhibited by poly(ADP-ribosylation) (Poirier et al., 1982). A dimer of modified H1 molecules has been proposed to link nonadjacent regions of the fibers, thereby forming chromatin loops (Wong et al., 1983). Poly(ADP-ribose) polymerase is itself the main acceptor of ADP-ribose in HeLa chromatin (Jump & Smulson, 1980). The chemistry of the modification of histone H1 was influenced by the labeling conditions (Adamietz et al., 1978), and multiple units of ADP-ribose were found to be added (Braeuer et al., 1981). Sites of ADP-ribosylation on histone H1 were reported to be either glutamic acid residues or COOH-terminal lysine residues (Riquelme et al., 1979; Ogata et al., 1980a,b) or phosphoserine residues (Smith & Stocken, 1975).

Because of the possible significance of ADP-ribosylation of nuclear proteins other than histones in regulating nuclear function, this study was carried out to determine differences in the modification of non-histone proteins from interphase nuclei and metaphase chromosomes. The results demonstrate fundamentally different patterns of modification. Over 100 proteins of interphase nuclei and chromatin were substantial acceptors of radioactivity from ^{32}P-labeled nicotinamide adenine dinucleotide ([^{32}P]NAD),[1] while the labeling pattern of metaphase chromosomes was much simpler. Labeling of poly(ADP-ribose) polymerase and the histones dominated the metaphase pattern.

Experimental Procedures

Materials. Nicotinamide [*adenylate*-^{32}P]adenine dinucleotide was purchased from New England Nuclear. Phosphodiesterase (*Crotalus durissus terrificus*) was obtained from Boehringer Mannheim, and micrococcal nuclease was from Millipore. Ampholines were from LKB, and metrizamide (Nyegaard) was from Accurate Chemical.

Cell Culture. HeLa S-3 cells were maintained at 37 °C in suspension culture in minimum essential medium (Joklik-modified) supplemented with 5% fetal bovine serum.

Preparation of Interphase Samples. Nuclei were obtained by using the procedure described previously (Adolph, 1980a) and were further purified by sucrose density gradient centrifugation. Purification of nuclei on sucrose gradients and extensive washing of nuclei with isolation buffer containing 0.5% NP40 ensured that unincorporated [^{32}P]NAD was completely washed away after radioactive labeling of nuclear proteins. For this reason, the labeling patterns were not substantially altered during nuclease treatment, which could greatly stimulate poly(ADP-ribose) synthesis, in the preparation of nuclear scaffolds and chromatin.

Nuclear scaffolds were isolated by first treating purified nuclei with DNase I (100 μg/mL for 60 min on ice) or with micrococcal nuclease (15 units/mL for 5 min at 37 °C). An equal volume of high-salt buffer (4 M NaCl, 20 mM Tris-HCl, pH 7.4, 20 mM EDTA, and 0.2% Ammonyx LO) was then mixed with the nuclei. After 30 min on ice, nuclear scaffolds were centrifuged through 5% sucrose in half-diluted high-salt buffer.

Chromatin was prepared by treatment of nuclei with micrococcal nuclease (15 units/mL, 5 min, 37 °C). Nonchromosomal soluble nuclear proteins were removed by pelleting the treated nuclei and discarding the supernatants. The pellets were resuspended in 5 mM EDTA, pH 7.4, and 0.1% NP40, and after 30 min on ice, the supernatant of chromatin was collected after nuclear debris was pelleted. Further fractionation of chromatin fragments on sucrose density gradients was not found to be essential, since similar patterns of associated non-histones were detected whether or not the samples were additionally purified (Adolph & Phelps, 1982).

Preparation of Metaphase Chromosomes. Metaphase chromosomes were isolated in a buffer containing 50 mM NaCl, 5 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 0.1 mM PMSF (Adolph, 1980b). After twice washing colchicine-arrested cells with this buffer, chromosomes were released by mechanically disrupting the cells with the aid of 0.5% NP40 and 0.1% sodium deoxycholate. Density gradients of metrizamide (0.3–0.8 M) were used to further purify the chromosomes.

Labeling of Proteins with [^{32}P]NAD. To radioactively label non-histones with [^{32}P]NAD, either permeabilized interphase and mitotic cells or isolated nuclei and chromosomes were incubated with the isotope. To produce permeabilized interphase cells, the samples were resuspended in (a) nuclei isolation buffer (10 mM NaCl, 10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, and 0.1 mM PMSF) containing 0.5% NP40, (b) chromosome isolation buffer containing 0.5% NP40, and (c) chromosome isolation buffer without detergent. With isolated nuclei, the solutions used were (d) nuclei isolation buffer containing 0.5% NP40 and (e) 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 0.1 mM PMSF, and 0.5% NP40. In each case, cells (200 mL, 2×10^5 cells/mL) were washed twice in the appropriate buffer, and the cells were resuspended in 400 μL of buffer (4×10^7 cells). Portions of 100 or 200 μL were radioactively labeled. Incubation was with 20–25 μM [^{32}P]NAD (21–25 mCi/μmol) for 10–15 min at 23–25 °C.

The procedure for permeabilized mitotic cells utilized cells arrested in mitosis with colchicine (0.4 μg/mL, 12–20 h). In a typical experiment, cells (200 mL, 2×10^5 cells/mL) were concentrated 5-fold and treated with colchicine. The cells were washed in chromosome buffer and resuspended in 1.0 mL of buffer. Aliquots of 0.5 mL (containing 2×10^7 cells) were then incubated with [^{32}P]NAD. Labeling employed 20–25 μM [^{32}P]NAD (21–25 mCi/μmol, 10–15 min, 23–25 °C). The buffers used were (a) chromosome isolation buffer containing 0.5% NP40 and (b) chromosome isolation buffer without detergent. Chromosomes were then isolated as described above and purified on metrizamide gradients. In other experiments, purified chromosomes, in chromosome isolation buffer containing detergent, were labeled.

The level of total ADP-ribose incorporation for interphase cells permeabilized in isolation buffer containing NP40 had a typical value of 13 pmol/10^6 cells. Incorporation of ADP-ribose by isolated nuclei was considerably greater, typically 79 pmol/10^6 cells. Mitotic cells that were permeabilized in isolation buffer containing detergent showed a level of incorporation of 6.9 pmol/10^6 cells, or approximately 0.17 pmol/10^6 chromosomes. For isolated chromosomes, 1.0 pmol/10^6 chromosomes was incorporated.

Treatment of Samples with Inhibitors of ADP-Ribosylation. Nuclei were prepared, purified on sucrose density gradients, and resuspended in nuclei isolation buffer containing 0.5% NP40 and 1 mM PMSF. The preparation was divided into

[1] Abbreviations: ADP, adenosine 5′-diphosphate; NAD, nicotinamide adenine dinucleotide; DTT, DL-dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; NP40, Nonidet P-40; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; 1-D, one dimensional; 2-D, two dimensional.

three equal portions; 3-aminobenzamide was added to one portion to a concentration of 10 mM, and diadenosine tetraphosphate (10 mM final concentration) was added to another. The nuclei were incubated with 2.0–2.5 μM [^{32}P]NAD (21–25 mCi/μmol) for 10 min at 23–25 °C before being washed with nuclei isolation buffer (4 °C). Samples were then resuspended in the SDS-containing buffers used for 1-D gel electrophoresis, or the urea-containing buffer used for nonequilibrium pH gradient electrophoresis.

Treatment of Samples with Snake Venom Phosphodiesterase. HeLa nuclei suspended in 10 mM NaCl, 10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM PMSF, and 1 mM TLCK were warmed at 37 °C for 5 min. Nuclei were further incubated at 37 °C in the presence or absence of snake venom phosphodiesterase (0.15–5.0 units/mL depending on the nuclei concentration). The treatment was terminated by immediately pelleting the nuclei at 4 °C and washing with the buffer described above.

Polyacrylamide Gel Electrophoresis. Two systems of SDS–polyacrylamide gel electrophoresis were used to separate proteins. The phosphate-buffered gel system of Weber & Osborn (1969) provided a low-pH gel system, while the Tris-buffered, discontinuous pH gels of Laemmli (1970) gave sharper protein bands and thus higher resolution. The phosphate-buffered gels (normally 5% and 8% w/v acrylamide) contained 50 mM sodium phosphate, pH 6.8, and 0.1% SDS. The electrophoresis running buffer was also 50 mM sodium phosphate, pH 6.8, and 0.1% SDS. Protein samples were in 10 mM sodium phosphate, pH 6.8, 1% SDS, 10% glycerol, and 10% β-mercaptoethanol. Prior to electrophoresis, the samples were heated at 37 °C for 10 min. The running buffer was changed during electrophoresis to prevent the pH rising above pH 7.

The gel system of Laemmli (1970), with a pH 6.8 sample buffer and spacer gel and a pH 8.8 running buffer and separating gel, was also used. Although the linkage of ADP-ribose to acceptors is known to be labile at alkaline pH, in practice the results with Laemmli gels were similar to the results with phosphate-buffered gels at pH 6.8. The sample buffer contained 0.0625 M Tris-HCl, pH 6.8, 1% SDS, 10% glycerol, and 10% β-mercaptoethanol. The preparations were heated at either 37 °C for 10 min or 100 °C for 90 s. Little difference was detected in the lability of the poly(ADP-ribose) linkage between the two temperatures.

The procedures of O'Farrell (1975) were used for two-dimensional separation of proteins. The first-dimension separation was by nonequilibrium pH gradient electrophoresis (O'Farrell et al., 1977).

Results

ADP-Ribosylation of Non-Histone Proteins from Interphase Nuclei Incubated with [^{32}P]NAD. Isolated nuclei were incubated for 10 min with [^{32}P]NAD, and nuclear proteins which had incorporated the radioactive label as ADP-ribose were separated on one-dimensional SDS–polyacrylamide gels. The gel system of Laemmli (1970) was used because of the increased resolution compared to phosphate-buffered gels, while the higher pH of Tris-buffered gels did not appear to change the pattern of modified species. Autoradiograms of 12.5% and 8% gels are included in Figure 1. Besides total nuclear proteins, the autoradiograms also show the ADP-ribosylated proteins of two major nuclear components, chromatin and nuclear scaffolds.

The most highly labeled non-histone of total nuclei has a molecular weight slightly above the marker of β-galactosidase (M_r 116 000). The same species is also the major ADP-ribosylated protein of nuclear scaffolds and chromatin. This protein appears to be poly(ADP-ribose) polymerase (Jump & Smulson, 1980). Similar patterns of modified species are seen for total nuclei and chromatin, but a different and much simpler pattern is found for nuclear scaffolds. Several dozen bands can be detected on autoradiograms of nuclei and chromatin, and these cover the entire molecular weight range of the gels (from 10 000 to 200 000). But for nuclear scaffolds, a group of modified proteins with molecular weights of 65 000–70 000 and poly(ADP-ribose) polymerase are the only major species. Two-dimensional gels, which are discussed in a section below, allow a more accurate estimate of the number of ADP-ribosylated non-histones and show that, while more than 100 species can be distinguished for total nuclei and chromatin, only these few major species are found for nuclear scaffolds.

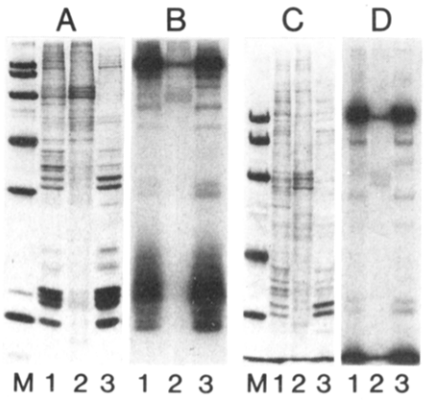

FIGURE 1: ADP-ribosylated total proteins of interphase HeLa nuclei. Nuclei were labeled with [^{32}P]NAD, and nuclear scaffolds and chromatin were prepared for electrophoresis on one-dimensional SDS–polyacrylamide gels (Tris buffered). (A) 12.5% gel, stained with Coomassie brilliant blue, of proteins from (1) total nuclei, (2) nuclear scaffolds, and (3) chromatin. The molecular weight markers in lane M include β-galactosidase (M_r 116 000), phosphorylase *b* (M_r 97 000), bovine albumin (M_r 66 000), chicken egg albumin (M_r 45 000), carbonic anhydrase (M_r 30 000), and lysozyme (M_r 14 000). (B) Autoradiogram of the nuclear proteins shown in (A). (C) Stained 8% gel of proteins from (1) total nuclei, (2) nuclear scaffolds, and (3) chromatin. (D) Autoradiogram of the samples in (C). For both the 12.5% and 8% gels, the amounts of nuclei from which the samples applied to the gels were derived are as follows: lane 1 (nuclei), 1.3×10^5; lane 2 (nuclear scaffolds), 5.7×10^5; lane 3 (chromatin), 2.6×10^5.

The autoradiograms of 12.5% gels in Figure 1 reveal a ladder of highly labeled bands in the molecular weight region which stained gels show is occupied by the nucleosome core histones. The ladder of bands extends from the molecular weight position of histone H4 to a molecular weight approaching 25 000. The labeled bands may include core histones with poly(ADP-ribose) chains of varying lengths. Free poly(ADP-ribose) could also occur with these molecular weights (Holtlund et al., 1983). Comparing the autoradiograms and stained gels shows, in addition, bands at the positions of histones H1A and H1B that have incorporated label from [^{32}P]NAD.

The major ADP-ribosylated proteins of nuclear scaffolds that have molecular weights of around 65 000–70 000 evidently include the lamins, structural proteins of the nuclear envelope lamina (Gerace & Blobel, 1980). The peripheral nuclear envelope lamina proteins, along with residual nucleoli and other internal material, are the predominant structural components of nuclear scaffolds. The identification of the species seen on the autoradiograms in Figure 1 with the lamins is strengthened by two-dimensional gel electrophoresis (described below).

Influence of Labeling Conditions on the Patterns of Mod-

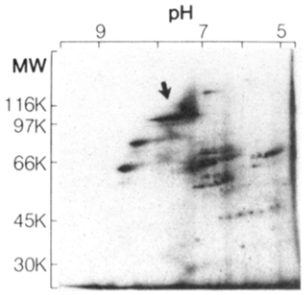

FIGURE 2: Autoradiogram of a two-dimensional polyacrylamide gel showing the ADP-ribosylated proteins of HeLa nuclei labeled with [^{32}P]NAD. Permeabilized cells, resuspended in chromosome isolation buffer containing 0.5% NP40 (see Experimental Procedures), were incubated with [^{32}P]NAD before nuclei were isolated for electrophoresis. Nonequilibrium pH gradient electrophoresis was used in the first dimension and an 8% SDS–polyacrylamide slab gel (Tris buffered) in the second. The number of labeled nuclei added to the first-dimension tube gel was 2.8×10^6 in 70 μL of urea lysis buffer. The arrow indicates the position of poly(ADP-ribose) polymerase.

ification: *Two-Dimensional Gel Electrophoresis*. The high resolution provided by two-dimensional gel electrophoresis was used to investigate the effect of the labeling conditions on the incorporation of radioisotope from [^{32}P]NAD into proteins. Both permeabilized cells and isolated nuclei were incubated with label. The composition of the buffer was also adjusted. The presence or absence of detergent and the ionic strength of the buffer were the most important variables.

Figure 2 shows an autoradiogram of a two-dimensional gel of total nuclear proteins labeled by incubating permeabilized cells with [^{32}P]NAD. The cells were in metaphase chromosome isolation buffer (see Experimental Procedures) containing 0.5% NP40 detergent. This was to allow the most direct comparison with the results for metaphase chromosome non-histones (Figure 8). Following incubation of the interphase cells with [^{32}P]NAD, nuclei were isolated, and the proteins were separated on two-dimensional gels.

The autoradiogram demonstrates that a large number of nuclear non-histones have incorporated the radioactive label and are therefore posttranslationally modified by ADP-ribosylation. On these two-dimensional gels, over 100 ^{32}P-labeled species can be counted. In counting this number, we considered a single species to be either an individual spot or a series of related spots with differing isoelectric points. Fifteen or more spots can be counted in some of these series, which presumably arise from single polypeptides with ADP-ribose chains of variable lengths.

The modified nuclear proteins that can be identified on autoradiograms of 2-D gels include structural proteins of the nuclear protein framework, evidently the lamins and poly(ADP-ribose) polymerase. The identification of the proteins as the lamins (Kaufmann et al., 1983) and poly(ADP-ribose) polymerase is based on the molecular weights and isoelectric points of these species and on the modification pattern for nuclear scaffold proteins. The definition of nuclear scaffold structural proteins is operational and includes all the species which copurify with the residual nuclear material during digestion with nuclease and high-salt extraction.

Variations in the patterns of ADP-ribosylation that result from changing the labeling conditions are revealed in Figure 3. In panels A and B, permeabilized cells were incubated with [^{32}P]NAD, while in panels C and D isolated nuclei were incubated with radioactive isotope. A basic difference between the labeling conditions was the low incorporation of [^{32}P]NAD with permeabilized cells. This was especially evident in the absence of detergent, as shown in panel B, where the major

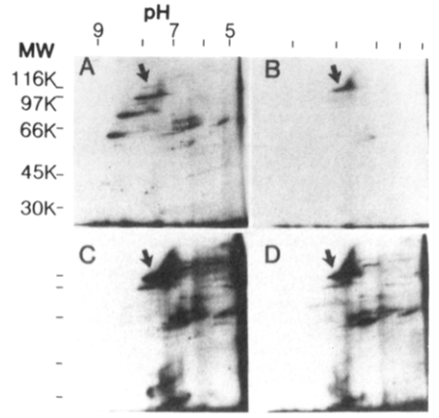

FIGURE 3: Effect of labeling conditions on the incorporation of label from [^{32}P]NAD into nuclear proteins. Autoradiograms of two-dimensional gels of total nuclear proteins are shown. Permeabilized cells were incubated with [^{32}P]NAD in (A) nuclei isolation buffer containing 0.5% NP40 and in (B) chromosome isolation buffer without detergent. Isolated nuclei were incubated with label in (C) nuclei isolation buffer containing 0.5% NP40 and in (D) 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 0.1 mM PMSF, and 0.5% NP40. With permeabilized cells, nuclei were isolated and purified before electrophoresis was performed. For each tube gel, 2.1×10^6 nuclei in 70 μL of urea buffer were added. The location of poly(ADP-ribose) polymerase is shown by an arrow in each panel.

protein to have incorporated label is poly(ADP-ribose) polymerase. The addition of 0.5% NP40 substantially increased the permeability of the cells to [^{32}P]NAD so that at least 100, and perhaps several hundred, of the non-histones incorporated ^{32}P label (Figure 2 and Figure 3A). The patterns of modification were not extremely sensitive to the composition of the NP40-containing buffer. In Figure 2, the cells were incubated with the isotope in metaphase chromosome isolation buffer (50 mM NaCl, HEPES buffered) while in Figure 3A nuclei isolation buffer (10 mM NaCl, Tris buffered) was used, yet the autoradiograms are quite similar.

Stained, two-dimensional gels of nuclei labeled under these various conditions showed identical patterns. The differences that are seen in the autoradiograms of Figure 3 are therefore not due to differences in protein extractability but do reflect differences in the labeling protocols.

The use of isolated nuclei greatly increased the incorporation of label from [^{32}P]NAD. Incorporation into nuclear non-histones was at least 40 times greater for isolated nuclei in nuclei isolation buffer containing NP40 compared to permeabilized cells in the same solution, and at least 100 times greater compared to cells in hypotonic buffer without detergent. But in spite of this increased incorporation for isolated nuclei, the number of ^{32}P-labeled proteins did not increase. The biggest changes were in the distribution of label. The lamins and, especially, poly(ADP-ribose) polymerase showed relatively greater incorporation of label with isolated nuclei than with permeabilized cells. This is perhaps understandable for the lamins since, with isolated nuclei, these proteins are directly exposed to the [^{32}P]NAD-containing solution. The largest variation in the level of incorporation was found for poly(ADP-ribose) polymerase.

Proteolytic Analysis of the 116 000-Dalton Protein. To investigate whether some of the ^{32}P-labeled bands in Figures 1–3 result from partial proteolysis of the major 116 000-dalton acceptor, one-dimensional peptide mapping was performed according to the procedure of Cleveland et al. (1977). Permeabilized cells were incubated with [^{32}P]NAD, labeled proteins were resolved by SDS–polyacrylamide gel electrophoresis, and gel segments containing the 116 000-dalton band

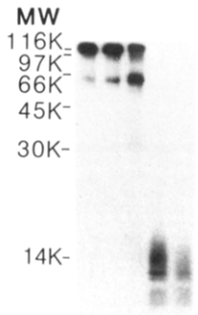

FIGURE 4: Partial proteolysis of the 116 000-dalton protein [poly(ADP-ribose)polymerase] labeled with [^{32}P]NAD. One-dimensional peptide mapping in SDS–polyacrylamide gels was applied to characterize the ^{32}P-labeled peptides generated by protease digestion. Gel segments corresponding to the 116 000-dalton species were excised from a SDS–polyacrylamide gel, and the protein was electrophoresed into a second gel (15% acrylamide) after the gel segments were overlayed with chymotrypsin (0, 1, 5, 25, and 100 μg in 30 μL of sample buffer for lanes 1, 2, 3, 4, and 5, respectively). The figure includes an autoradiogram which reveals the radioactively labeled peptides.

were directly excised. Electrophoresis in a second gel (15% acrylamide) followed overlaying the segments in the sample wells with chymotrypsin (0, 1, 5, 25, and 100 μg in 30 μL of sample buffer).

An autoradiogram which shows the ^{32}P-labeled peptides that are generated by partial proteolysis is included in Figure 4. The primary fragment of intermediate size that results from chymotryptic digestion has a molecular weight of 63 000, while another large peptide that is produced has a molecular weight of 33 000. These fragments are most prominent in lane 3. A limit digestion series of small fragments results from more extensive proteolytic digestion, and these ^{32}P-labeled peptides are seen in lanes 4 and 5. The molecular weights of the bands cover the range of 7000–18 000.

Comparing these results with the autoradiograms of nuclear proteins in Figures 1–3 suggests that most of the ^{32}P-labeled nuclear species are not created by digestion of the 116 000-dalton band by a protease with the specificity of chymotrypsin. However, the molecular weight of the 63 000-dalton chymotryptic band is close to the molecular weight of a major nuclear protein (61 000), and the series of limit digestion peptides overlaps with the region of the histones. Therefore, this molecular weight region could contain proteolytic peptides in addition to ADP-ribosylated histones and free poly(ADP-ribose).

Effect of Inhibitors of ADP-Ribosylation. The results of including inhibitors of ADP-ribosylation on the protein species that incorporate radioactivity from [^{32}P]NAD were investigated. Nuclei were isolated and incubated for 10 min at 24 °C with [^{32}P]NAD in the presence of 3-aminobenzamide and diadenosine tetraphosphate (Ap_4A), both at concentrations of 10 mM. The incorporation of label from [^{32}P]NAD was drastically inhibited with 3-aminobenzamide to 0.6% of controls, while the incorporation of label was reduced to 6.4% of controls with diadenosine tetraphosphate.

Two-dimensional polyacrylamide gels were employed to characterize the effect of the inhibitors of ADP-ribosylation (Figure 5). Panels A and B show 2-D gels in which the second dimension is a pH 6.8, phosphate-buffered slab gel. Nonequilibrium pH gradient electrophoresis was used for the separation in the first dimension. The results using the discontinuous, Tris–glycine-buffered gel system of Laemmli are in panels C and D. Both of these gel systems show that incorporation of ^{32}P is eliminated with 3-aminobenzamide.

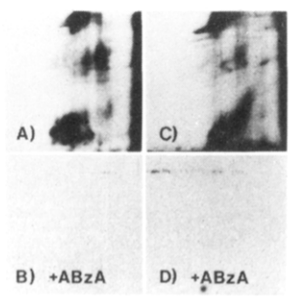

FIGURE 5: Effect of 3-aminobenzamide in inhibiting ADP-ribosylation of nuclear non-histones shown by autoradiograms of two-dimensional gels. (A and B) pH 6.8, phosphate-buffered gels (5% acrylamide): (A) control; (B) 3-aminobenzamide treated. (C and D) Tris- buffered gels (8% acrylamide): (C) control; (D) 3-aminobenzamide treated.

With Ap_4A, labeling of most species was dramatically reduced, but the species identified as automodified poly(ADP-ribose) polymerase continued to be a major acceptor of label from [^{32}P]NAD. It has been reported that Ap_4A specifically inhibits the modification of chromatin proteins rather than the auto-ADP-ribosylation of the polymerase (Tanaka et al., 1981).

Removal of Incorporated Radioactive Label with Snake Venom Phosphodiesterase. Experiments were undertaken to determine whether the incorporated ^{32}P radioactivity could be removed by treating nuclei with snake venom phosphodiesterase. Nuclei isolated from randomly growing HeLa cells were labeled with [^{32}P]NAD, and the nuclei were then incubated at 37 °C in the presence or absence of snake venom phosphodiesterase. After 0.5, 1, 2, 4, and 22 h of incubation at 37 °C, ^{32}P radioactivity remaining in nuclei was measured for both control and phosphodiesterase-treated samples. Figure 6A shows that 70% of the control ^{32}P radioactivity could be removed by 0.5-h treatment of nuclei with snake venom phosphodiesterase. After a 22-h incubation, only 8% of the control ^{32}P cpm was resistant to phosphodiesterase.

No significant proteolysis was observed in nuclei during incubation with snake venom phosphodiesterase in the presence of two protease inhibitors, PMSF (1.0 mM) and TLCK (1.0 mM). The inset in Figure 6A shows the staining pattern, using Coomassie brilliant blue, of a one-dimensional SDS–polyacrylamide gel in which nuclear proteins treated at 37 °C with venom phosphodiesterase for 0, 1, 2, and 4 h are resolved.

Figure 6B contains an autoradiogram of a two-dimensional gel of non-histone proteins associated with nuclei incubated for 22 h at 37 °C in the absence of enzyme. The majority of the proteins still retain their label in ADP-ribose residues, although an increased background of ^{32}P label is observed. Figure 6C is an autoradiogram of a gel of nuclei incubated for 22 h at 37 °C with snake venom phosphodiesterase. This autoradiogram demonstrates that most radioactivity is removed.

ADP-Ribosylation of Metaphase Chromosome Non-Histones. The modification of metaphase non-histone proteins was studied by arresting cells in mitosis with colchicine and then incubating permeabilized mitotic cells or isolated chromosomes with [^{32}P]NAD. In Figure 7, one-dimensional gels of the ADP-ribosylated proteins of metaphase chromosomes are shown. For these experiments, metaphase-arrested cells were permeabilized by being resuspended in chromosome isolation buffer containing 0.5% NP40, and the cells were then incubated for 10 min with [^{32}P]NAD. Chromosomes were isolated and purified, and the labeled proteins were separated on both Tris–glycine gels (Figure 7, lane 2) and phosphate-

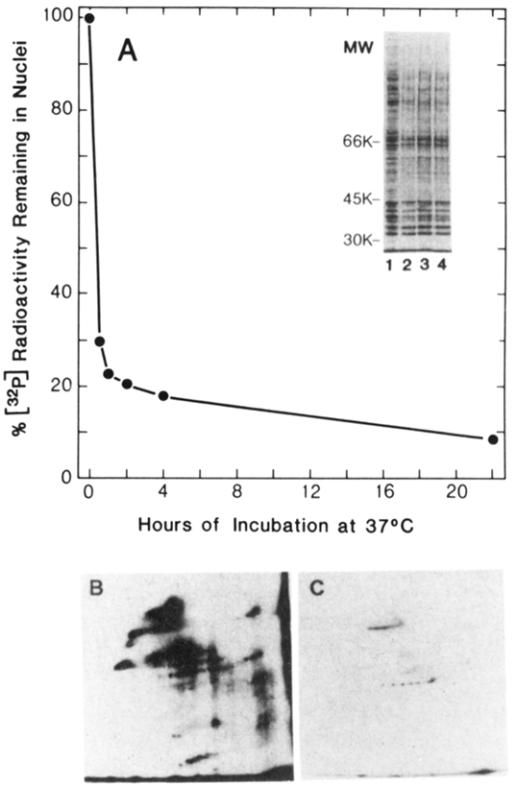

FIGURE 6: Removal of ^{32}P label from ADP-ribosylated nuclear proteins with snake venom phosphodiesterase. Phosphodiesterase treatment was as described under Experimental Procedures. (A) Percentage of ^{32}P radioactivity remaining in nuclei. Only 8% of incorporated ^{32}P was resistant to enzyme after 22-h incubation. The inset contains a stained gel (8%) of proteins from nuclei incubated with phosphodiesterase for (1) 0, (2) 1, (3) 2, and (4) 4 h at 37 °C and demonstrates that proteolysis is not significant under these conditions. (B) Autoradiogram of a two-dimensional gel of proteins from HeLa nuclei incubated for 22 h at 37 °C *without* added enzyme. (C) Autoradiogram of a two-dimensional gel of proteins from nuclei incubated for 22 h at 37 °C *with* snake venom phosphodiesterase.

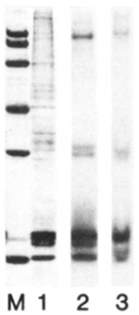

FIGURE 7: ADP-ribosylated proteins of metaphase chromosomes. Metaphase-arrested cells were resuspended in chromosome isolation buffer containing 0.5% NP40. The permeabilized cells were labeled with [^{32}P]NAD for 10 min at 24 °C before chromosomes were isolated and purified on metrizamide density gradients. (1) Staining pattern, using Coomassie brilliant blue, of the proteins of isolated metaphase chromosomes. The molecular weight standards of Figure 1 are in lane M. (2) Autoradiogram of a Tris-buffered gel (12.5%) of chromosomal proteins. (3) Autoradiogram of a pH 6.8, phosphate-buffered gel (8% acrylamide) of chromosomal proteins. Lanes 2 and 3 each contained 1.2×10^7 labeled chromosomes in SDS final sample buffer.

buffered gels at pH 6.8 (lane 3). The pattern of modified species is seen to be similar with the two gel systems and is impressive in its lack of complexity. The pattern is dominated by the histones and poly(ADP-ribose) polymerase.

Two-dimensional gel electrophoresis was also employed. Figure 8A includes an autoradiogram of a 2-D gel of the

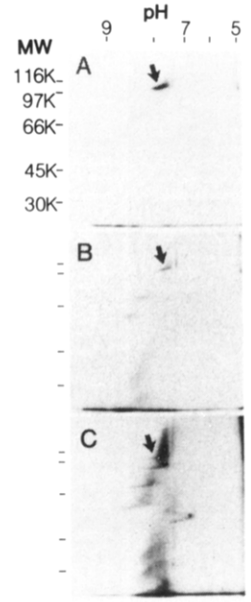

FIGURE 8: Effect of labeling conditions on ADP-ribosylated non-histones of metaphase chromosomes. Proteins were labeled with [^{32}P]NAD, and autoradiograms of two-dimensional gels are shown. (A) Permeabilized mitotic cells were radioactively labeled in chromosome isolation buffer containing 0.5% NP40. (B) Permeabilized mitotic cells were labeled in chromosome isolation buffer without detergent. (C) Isolated metaphase chromosomes were labeled in chromosome isolation buffer containing 0.5% NP40. Following incubation with [^{32}P]NAD, metaphase chromosomes were prepared from the permeabilized cells and purified on metrizamide density gradients. The amount of chromosomes (in 70 μL of urea lysis buffer) electrophoresed in each tube gel was 4×10^7. An arrow in each panel indicates poly(ADP-ribose) polymerase.

non-histones of metaphase chromosomes from permeabilized mitotic cells labeled with [^{32}P]NAD in chromosome isolation buffer containing 0.5% NP40. The appearance of the autoradiogram is strikingly different from Figure 2 where interphase nuclear non-histones were labeled under the same conditions. Instead of a pattern consisting of over 100 ^{32}P-labeled species, the autoradiogram of metaphase non-histones shows only a single major acceptor of label. This is poly(ADP-ribose) polymerase.

Because of this significant change during mitosis in the modification of non-histones, the effect of the labeling conditions on the incorporation of ^{32}P was examined. Besides incubation of metaphase-arrested cells in chromosome isolation buffer containing NP40 (Figure 8A), mitotic cells were labeled in this buffer without detergent. Isolated metaphase chromosomes were also labeled in chromosome buffer containing NP40. Figure 8B includes an autoradiogram of the results with permeabilized cells without detergent and shows a simple pattern that is similar to the one in panel A. Without detergent, relatively less ^{32}P label is found in poly(ADP-ribose) polymerase compared to other labeled species. The greatest change between the two conditions is a 25-fold reduction in total incorporation of label from [^{32}P]NAD that occurs when detergent is omitted. Thus, the apparent increase in the modification of proteins other than poly(ADP-ribose) polymerase in Figure 8B compared to Figure 8A is actually due to the longer exposure for Figure 8B (24 times longer, 14 days vs. 14 h). Figure 8C shows the labeled species when isolated chromosomes are incubated with the isotope. The differences with the results when permeabilized cells are used are similar to the situation for interphase nuclei (Figure 3). An overall increase in the incorporation of label is found (4-fold compared to permeabilized cells in chromosome buffer containing NP40),

and incorporation into species other than poly(ADP-ribose) polymerase is the major feature. But even for isolated metaphase chromosomes, the pattern of modified proteins is less complex than that for interphase nuclei.

Discussion

The profound difference between the ADP-ribosylation of the non-histones of interphase nuclei and metaphase chromosomes is perhaps the most important finding of this investigation. Although both structures have complex non-histone compositions, 100 or more interphase non-histones were major acceptors of label, while the principal non-histone acceptor in metaphase was poly(ADP-ribose) polymerase. What is the significance of this difference? ADP-ribosylation of non-histone proteins could have both structural and functional consequences. The structural significance of differences during the cell cycle might relate to changes in the state of the genome. Another posttranslational modification, phosphorylation of histone H1, has been proposed to be involved in the condensation of metaphase chromosomes. Similarly, ADP-ribosylation of non-histones, as well as histones, could regulate aspects of the short-range and long-range structure of chromatin. Such roles for non-histone ADP-ribosylation will require experimental verification, but as was mentioned in the introduction, some results are becoming available which support a structural role for ADP-ribosylation of histones.

The changes in ADP-ribosylation that were found could also have functional importance connected with the activity of poly(ADP-ribose) polymerase. The increased automodification of the enzyme during mitosis may reflect a decreased requirement for the modification of other proteins. For example, gene transcription is minimal during mitosis, and the involvement of ADP-ribosylation in this and other processes may be correspondingly minimal. Differences in the localization of the polymerase on metaphase chromosomes compared to interphase may also be a factor contributing to the dominance of the labeling of the polymerase. This different localization could effectively decrease the capacity of the non-histone substrates for modification in metaphase. Additional factors which modulate the activity of enzymes could be involved. All these influences upon the activity of the enzyme associated with metaphase chromosomes could reflect a packaging phenomenon that is connected not with mitosis but with the next cell cycle. A requirement may exist for the availability of the enzyme in the early stages of the next cell cycle.

Permeabilized cells were produced for the in vitro labeling experiments by resuspending the cells in hypotonic buffers containing nonionic detergent. Examination of the treated cells in a phase-contrast microscope and counts of the numbers that remained after the treatments demonstrated that the cells were not lysed by these buffers. The condensed nuclear morphology was recognizable, though considerable cytoplasmic material had been depleted. Labeling interphase cells in the absence of detergent drastically reduced (more than 100-fold) the amount of label incorporated from [^{32}P]NAD by nuclear proteins (Figure 3B). Such a solution condition, which resulted in only a relatively small number of proteins accepting label, cannot therefore be considered representative of the pattern of ADP-ribosylation of nuclear proteins.

It could be argued that the fundamental differences between the ADP-ribosylation patterns for interphase nuclei and metaphase chromosomes, and for samples labeled under different protocols, result from performing the labeling in vitro. In particular, the relationship between poly(ADP-ribose) polymerase activity and the degree of DNA fragmentation could produce substantial variations in the labeling patterns because of different levels of experimentally induced DNA damage. The best argument for the veracity of these observations comes from experiments with in vivo labeling. When interphase and metaphase cells were labeled in normal growth conditions with [^{3}H]adenosine, the same basic decrease in the number of modified non-histones was found in comparing nuclei and chromosomes (K. W. Adolph and M.-K. Song, unpublished results). For metaphase chromosomes, the 116 000-dalton species was the only major acceptor of [^{3}H]adenosine label. The striking similarity of the results with [^{32}P]NAD and [^{3}H]adenosine lends confidence that the factors which influence in vitro labeling, such as the extent of DNA damage, are not leading to faulty conclusions.

The principal variation during the cell cycle in ADP-ribosylation of non-histones is therefore the change that is associated with mitosis. Although this difference is convincingly demonstrated, the implications of the modification for chromatin structure and metaphase chromosome condensation await further investigation.

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Hydrogen Exchange of Lysozyme Powders. Hydration Dependence of Internal Motions†

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ABSTRACT: The rate of exchange of the labile hydrogens of lysozyme was measured by out-exchange of tritium from the protein in solution and from powder samples of varied hydration level, for pH 2, 3, 5, 7, and 10 at 25 °C. The dependence of exchange of powder samples on the level of hydration was the same for all pHs. Exchange increased strongly with increased hydration until reaching a rate of exchange that is constant above 0.15 g of H_2O/g of protein (120 mol of H_2O/mol of protein). This hydration level corresponds to coverage of less than half the protein surface with a monolayer of water. No additional hydrogen exchange was observed for protein powders with higher water content. Considered in conjunction with other lysozyme hydration data [Rupley, J. A., Gratton, E., & Careri, G. (1983) *Trends Biochem. Sci. (Pers. Ed.) 8*, 18-22], this observation indicates that internal protein dynamics are not strongly coupled to surface properties. The use of powder samples offers control of water activity through regulation of water vapor pressure. The dependence of the exchange rate on water activity was about fourth order. The order was pH independent and was constant from 114 to 8 mol of hydrogen remaining unexchanged/mol of lysozyme. These results indicate that the rate-determining step for protein hydrogen exchange is similar for all backbone amides and involves few water molecules. Powder samples were hydrated either by isopiestic equilibration, with a half-time for hydration of about 1 h, or by addition of solvent to rapidly reach final hydration. Samples hydrated slowly by isopiestic equilibration exhibited more exchange than was observed for samples of the same water content that had been hydrated rapidly by solvent addition. This difference can be explained by salt and pH effects on the nearly dry protein. Such effects would be expected to contribute more strongly during the isopiestic equilibration process. Solution hydrogen exchange measurements made for comparison with the powder measurements are in good agreement with published data. Rank order was proven the same for all pHs by solution pH jump experiments. The effect of ionic strength on hydrogen exchange was examined at pH 2 and pH 5 for protein solutions containing up to 1.0 M added salt. The influence of ionic strength was similar for both pHs and was complex in that the rate increased, but not monotonically, with increased ionic strength.

An understanding of the properties and function of proteins requires knowledge of the protein-water interactions that contribute to these phenomena. Solvent modulates the thermodynamics and dynamics of protein folding, of protein-ligand reactions, and of protein interactions with membranes and other cell organelles. Study of the process of hydrating dry protein to the solution state, because it allows the water concentration to be varied over a wide range, has provided insight into relationships between protein-water interactions and special properties of proteins (Careri et al., 1980; Rupley et al., 1983).

Three stages have been observed for the hydration process for lysozyme: (1) interaction at strong water binding sites, principally charged groups, complete at 0.07 h^1 (grams of

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