Phosphorylation of Nonhistone Proteins during the HeLa Cell Cycle

RELATIONSHIP TO DNA SYNTHESIS AND MITOTIC CHROMOSOME CONDENSATION* (Received for publication, November 15, 1982)

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Cell cycle variations in the phosphorylation of chromatin-associated nonhistones were determined. Cells were radiolabeled with $^{32}$Porthophosphate and chromatin was obtained by mild digestion of nuclei with micrococcal nuclease. The experiments were performed in the presence of a substrate inhibitor of alkaline phosphatase, $\beta$-glycerophosphate. The results show that, while similar molecular weight species of phosphorylated nonhistones are associated with interphase chromatin through the HeLa cell cycle, the incorporation of $^{32}$P cpm/µg of protein) profiles of selected major phosphononhistones show substantial changes. The most prominent peaks of specific radioactivity occur in the DNA synthesis phase (S phase). The phosphorylation states of the proteins of isolated metaphase chromosomes were also determined. Nonhistone proteins of isolated metaphase chromosomes are strikingly dephosphorylated, especially in comparison to histone H1. The phosphorylation of the major phosophonohistone of chromatin, which has a molecular weight of 55,000, was further characterized by techniques that included one-dimensional peptide mapping in sodium dodecyl sulfate-polyacrylamide gels and nonequilibrium pH gradient slab gel electrophoresis.

Phosphoproteins are also components of the nuclear scaffold, and cell cycle variations in these proteins were investigated. The primary phosphorylated species has a molecular weight of 119,000. As with chromatin-associated nonhistones, this nuclear scaffold protein shows substantial incorporation of $^{32}$P in S phase, and a high level of incorporation also occurs close to mitosis.

Covalent chemical modifications of chromosomal proteins have been implicated to change the structure of chromatin and thus alter various DNA activities such as gene expression, DNA replication, and chromosomal condensation during mitosis. Post-translational modifications of histones include acetylation, methylation, poly(ADP-ribo)sylation, and phosphorylation. Nonhistone modification, while recognized as being significant, has received less research attention. Nonhistone chromosomal proteins in animal tissues or organs are reported to be acetylated (1, 2) or methylated (3, 4). In addition to histones, several other chromosomal proteins and peptides are found to serve as acceptors for poly(ADP-ribose) (for review, see Refs. 5 and 6). Among the unique nonhistone proteins poly(ADP-ribo)sylated in mid-S phase HeLa nuclei, the major protein acceptor of ADP-ribose copurified with poly(ADP-ribose) polymerase from HeLa nuclei (7). Changes in the level of poly(ADP-ribo}sylation) during the cell cycle of HeLa S-3 cells have been examined with in vitro systems (8). The postsynthetic phosphorylation of nuclear nonhistone proteins has been studied by a number of investigators (9-13). More than 90% of the nuclear protein-bound phosphorus was found to be associated with nonhistone chromosomal proteins. The phosphorylation and dephosphorylation of chromosomal nonhistone proteins have been reported to be involved in the regulation of gene activity since phosphorylated nonhistone proteins are heterogeneous and tissue specific (14, 15), selectively bind to DNA (14, 16), and modulate the rate of RNA synthesis in vitro (14, 17, 18).

Changes in the phosphorylation of nuclear nonhistone proteins during the HeLa cell cycle have been investigated (19, 20). Platz and co-workers (19) observed the rate of phosphorylation of salt-extracted nuclear proteins to be maximal during G1 and G2 phases, somewhat decreased during S phase, and almost 90% depressed during mitosis. The phosphorylation of nuclear acidic proteins in HeLa S-3 cells during synchronous growth was examined by Karn et al. (20), who found that the rates of phosphate uptake into most major phosphoprotein species increased during early G1 phase and early S phase and were minimal during the late G2 to M period. Karn et al. (20) also found that the phosphate incorporated into nuclear acidic proteins turned over at rates which were different from one protein species to another.

This paper describes detailed studies on cell cycle-dependent changes in the phosphorylation of nonhistone proteins associated with chromatin in synchronously growing HeLa S-3 cells. In these experiments, unshaved chromatin was prepared by mild micrococcal nuclease treatment of nuclei and all the preparations were made in the presence of an effective substrate inhibitor of alkaline phosphatase, $\beta$-glycerophosphate. The phosphorylation pattern of nonhistones present in metaphase chromosomes was also obtained to compare with that of interphase chromatin. Experimental procedures that included one-dimensional peptide mapping in SDS-polyacrylamide gels and nonequilibrium pH gradient slab gel electrophoresis were used to examine the nature of the phosphorylation. Another major emphasis in this paper is to analyze the phosphorylation pattern of the HeLa nuclear scaffold. Changes in the phosphorylation of these residual nuclear proteins during the cell cycle were determined.

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1 The abbreviations used are: SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; TLCK, N-a-p-tosyl-L-lysine chloromethyl ketone HCl; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NP40, Nonidet P-40; BSA, bovine serum albumin protein-55K, protein-138K, etc., protein with molecular weight of 55,000, 138,000, etc.; HMG, high mobility group.
EXPERIMENTAL PROCEDURES

RESULTS

Phosphatase Inhibitor—For a meaningful comparison of protein phosphorylation at different phases of the cell cycle, it was necessary to find a proper inhibitor for phosphoprotein phosphatase to prevent dephosphorylation during the isolation of nuclei, chromatin, metaphase chromosomes, or nuclear scaffolds from HeLa cells. Previously reported effective inhibitors of phosphohistone phosphatases such as p-chloromercuri phenyl sulfonate (0.01 to 1 mM) (21), 5,5'-dithiobis-(2-nitrobenzoate) (1 to 5 mM) (21), and sodium bisulfite (50 mM) (22) were first employed during chromatin isolation. However, they interfered with the isolation procedures and gave rise to different sets of nonhistone protein species. Another phosphatase inhibitor, NaF (10 to 50 mM) (23), was also included during the preparation of chromatin without efficient inhibition of phosphononhistone phosphatase.

Since β-glycerophosphate is reported to be one of the best substrates for mammalian alkaline phosphatase (24, 25), β-glycerophosphate was tested as a substrate inhibitor of phosphononhistone phosphatase. In Fig. 1 an autoradiogram of 32P-labeled chromatin-associated nonhistone proteins prepared in the presence of varying concentrations of β-glycerophosphate is shown. β-Glycerophosphate, at concentrations higher than 10 mM, was found to be strikingly efficient in preventing dephosphorylation of phosphononhistone nonhistone proteins during the isolation of chromatin. Therefore, all the isolation buffers included 10 mM β-glycerophosphate as an effective substrate inhibitor of phosphononhistone phosphatase.

Phosphorylation States of Nonhistone Proteins during the HeLa Cell Cycle—To investigate the differential phosphorylation of chromatin-associated nonhistone proteins during the cell cycle, synchronized populations of HeLa S-3 cells in phosphate-free medium containing 2.5% dialyzed fetal calf serum were pulse-labeled for 1.0 h with [32P]orthophosphate at 2- or 4-h intervals, and chromatin was prepared by digestion with micrococcal nuclease. The nonhistone proteins present in chromatin were analyzed by SDS-polyacrylamide slab gel electrophoresis, and the Coomassie staining patterns of these proteins are presented in Fig. 2A. In this experiment, the same relative concentration of nonhistone proteins associated with each chromatin preparation was applied. Although there were some variations in the amount of each nonhistone protein at different times, the protein species present in chromatin were the same for all preparations.

Fig. 2B shows an autoradiogram of this gel. A large number of nonhistone proteins were found to be phosphorylated and to be more strongly phosphorylated than histones H1B and H1A. The autoradiograms indicated that nonhistone proteins associated with chromatin from HeLa cells in mid-S phase were the most strongly phosphorylated. Among these phosphononhistone proteins, 8 protein species (indicated with arrows) with molecular weights of 138,000 (protein-138K), 133,000 (protein-133K), 122,000 (protein-122K), 107,000 (protein-107K), 101,000 (protein-101K), 55,000 (protein-55K), 40,000 (protein-40K), and 36,000 (protein-36K) were selected to be examined more precisely.

From densitometer scans of Coomassie stained gels (Fig. 2A) and autoradiograms (Fig. 2B) and the direct measurement of radioactivities in protein bands, the specific radioactivity (32P cpm/μg of protein) in each protein was calculated and these values are plotted in Fig. 3. Even though each protein showed some differences in the degree of phosphorylation during the cell cycle, all 8 proteins displayed similar profiles, i.e. the maximum rate of phosphorylation was observed during mid-S phase (about 6 h after the G1/S boundary). A slightly increased phosphorylation was also seen during early G1 phase (11 h). The chromatin-associated nonhistone proteins...
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FIG. 3. Specific radioactivity ($^{32}$P cpm incorporated/µg of protein) variations of eight phosphorylated nonhistones during the cell cycle. The values were calculated from densitometer scans of stained gels and autoradiograms and from direct measurement of radioactivities. The highest level of phosphorylation occurs in mid-S phase, though the selected proteins differ in the degree of this increase. Protein-55K shows the greatest increase in specific radioactivity.

To study the phosphorylation states of nonhistone proteins associated with HeLa metaphase chromosomes, randomly growing HeLa S-3 cells in phosphate-free medium were arrested in metaphase by colchicine block and labeled with $[^{32}$P]orthophosphate. Metaphase chromosomes were isolated and purified by sedimentation into 0.3 to 0.8 M Metrizamide density gradients. Fig. 4 presents the Coomassie staining pattern of $^{32}$P-labeled metaphase chromosomal proteins analyzed in a SDS-polyacrylamide gel (lane A) and the autoradiogram of this gel (lane B). Even though the nonhistone proteins associated with metaphase chromosomes and interphase chromatin were not identical (Figs. 2A and 4A), all the nonhistone proteins were greatly dephosphorylated in mitosis while histones H1B and H1A were highly phosphorylated. Densitometer scans of autoradiograms of S phase chromatin and metaphase chromosomal proteins (Fig. 4, C and D) clearly illustrate the relative differences in the degree of phosphorylation of the proteins. These relative differences are quantitated in Table I. Among other histones, H3, H2A, and H4 were found to be phosphorylated at M phase (data not shown).

Stability of the Phosphate Label Incorporated into Nonhistones—Experiments were undertaken to examine whether the incorporated $^{32}$P was covalently bound to nonhistone proteins via phosphomonoester linkages, i.e. via phosphorylation of amino acids in proteins rather than poly(ADP-ribosylation), tightly bound nucleic acids, etc. Nuclei were isolated from $^{32}$P-labeled HeLa S-3 cells and incubated at 25 °C in the presence of alkaline phosphatase. After 1, 2, or 4 h of incubation, the nonhistone proteins present in each aliquot of nuclei were separated on SDS-polyacrylamide gels as shown in Fig. 5A (inset). Proteolysis was minimized during the 4-h incubation by including two protease inhibitors, PMSF (1 mM) and TLCK (1 mM), in the incubation buffer. Fig. 5A (inset) also shows an autoradiogram of the gel which demonstrates that most nonhistone proteins lost the $^{32}$P label in the 4-h incubation with alkaline phosphatase.

Densitometric scanning of autoradiograms made it possible to estimate the percentage of $^{32}$P radioactivity that remained in nonhistone proteins after incubation with alkaline phosphatase. Fig. 5A shows the loss of $^{32}$P label from total nuclear nonhistone proteins when the $^{32}$P-labeled nuclei were treated with alkaline phosphatase for various lengths of time at 25 °C. After 4 h of treatment, 13% of the untreated control $^{32}$P radioactivity remained in total nuclear nonhistone proteins. The phosphatase-resistant radioactivity (13%) was probably due to phosphate groups attached to proteins which were highly folded or embedded in the nuclear structure. When the stability of the phosphate label associated with protein-55K

were found to be less phosphorylated during G2 (9 h) and G1 (13-24 h) phases. Among these major phosphorylated nonhistones, the protein with a molecular weight of 55,000 (protein-55K, Fig. 3F) showed the greatest changes in the incorporation of $^{32}$P label through the cell cycle. The ratio of the specific radioactivities of protein-55K of S phase (4-5 h) chromatin to that of G2 phase (8-9 h) chromatin was 3.3:1, while the ratio for S phase (4-5 h):G1 phase (20-21 h) chromatin was 4.3:1.

To study the phosphorylation states of nonhistone proteins associated with metaphase chromosomes, randomly growing HeLa S-3 cells in phosphate-free medium were arrested in metaphase by colchicine block and labeled with $[^{32}$P]orthophosphate. Metaphase chromosomes were isolated and purified by sedimentation into 0.3 to 0.8 M Metrizamide density gradients. Fig. 4 presents the Coomassie staining pattern of $^{32}$P-labeled metaphase chromosomal proteins analyzed in a SDS-polyacrylamide gel (lane A) and the autoradiogram of this gel (lane B). Even though the nonhistone proteins associated with metaphase chromosomes and interphase chromatin were not identical (Figs. 2A and 4A), all the nonhistone proteins were greatly dephosphorylated in mitosis while histones H1B and H1A were highly phosphorylated. Densitometer scans of autoradiograms of S phase chromatin and metaphase chromosomal proteins (Fig. 4, C and D) clearly illustrate the relative differences in the degree of phosphorylation of the proteins. These relative differences are quantitated in Table I. Among other histones, H3, H2A, and H4 were found to be phosphorylated at M phase (data not shown).
were used to estimate the fraction with total nonhistone proteins and histone H1B and H1A present in the weights in total nonhistones and in H1B plus H1A was calculated for the two preparations. The positions of histones H1B and H1A in the densitometer scans are indicated.

**TABLE I**

Percentage of $^{32}$P radioactivity associated with proteins in S phase (4-5 h) chromatin and metaphase chromosomes

| S phase (4-5 h) chromatin | Metaphase chromosomes |
|---------------------------|-----------------------|
| Nonhistones               | 93% 28%               |
| H1B + H1A                 | 7% 72%                |
| Nonhistones/H1B + H1A     | 13 0.4                |

during incubation with alkaline phosphatase was examined (Fig. 5B), only 1% of the control $^{32}$P radioactivity was found to be resistant to phosphatase treatment. Therefore, the phosphate label incorporated into nuclear nonhistone proteins was predominantly due to the phosphorylation of proteins via phosphomonoester linkages.

**Characterization of Protein-55K Phosphorylation**—Among 8 major phosphorylated chromatin-associated nonhistones (Fig. 3), protein-55K showed the greatest changes in the degree of phosphorylation during the HeLa cell cycle and so a series of experiments were performed to further characterize this phosphorylation.

One-dimensional peptide mapping in SDS-polyacrylamide slab gels (see under "Experimental Procedures") was employed to characterize the nature of the sites that incorporate $^{32}$P label. Pronase (Streptomyces griseus protease) was used and Fig. 4A shows an autoradiogram of a peptide map produced with increasing amounts of pronase. This demonstrates that the $^{32}$P label was incorporated in protein since almost complete digestion was observed with 10 µg of pronase. Two specific proteases, chymotrypsin and Staphylococcus aureus V8 protease, were employed to examine the nature of the phosphorylation sites in the protein molecule. In a chymotryptic peptide map (Fig. 6B), protein-55K was digested in the presence of 200 µg (8.8 units) of chymotrypsin, such that most of the $^{32}$P label ended up in a single peptide with a molecular weight of 25,000. An autoradiogram of a peptide map generated by increasing amounts of S. aureus V8 protease is presented in Fig. 6C. Protein digestion was complete with...
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Phosphorylated nonhistones in proteins can be readily released by treatment with mild alkali, but not with acid (26). N-Phosphorylated amino acids such as phosphohistidine and phospholysine, however, are known to be labile in acidic pH and relatively stable under basic conditions (27). Therefore, the experiments for the determination of phosphohistidine residues in protein-55K included studies on the pH stability of the chemical bonds between protein and phosphates.

Alkali treatment of chromatin-associated nonhistone proteins was performed in SDS-polyacrylamide slab gels. Both the regularly stained and destained gel (control) and the same gel incubated in alkaline solution were exposed to x-ray film for an identical period of time, and these autoradiograms were compared. Even though the amount of each protein recovered after alkali treatment was the same as that in the control gel when the Coomassie staining patterns were compared to each other, most of the $^{32}$P label was released from nonhistone proteins by incubating the gel in 1 M NaOH for an hour at 40°C. Protein-55K bands were excited from both the control and alkali-treated gels, and the $^{32}$P radioactivity present in each protein band was measured. The results in Table II revealed that 9% of the control radioactivity remained in protein-55K after this treatment.

The acid stability of the phosphate in amino acids was examined with electrophoretically eluted protein-55K. This protein eluate was subjected to hot acid treatment, and the cold trichloroacetic acid-precipitable radioactivity was measured. Table II also summarizes the results of this experiment and indicates that 90% of the $^{32}$P label in protein-55K was stable in hot 10% trichloroacetic acid.

If the bonding of phosphate with protein was acyl linkage to carboxyl groups, such acyl phosphates will be rapidly hydrolyzed at both extremes of pH or by hydroxylamine treatment at pH 5.5 (28). And if the phosphoryl groups were joined to tyrosine residues, the phosphoprotein should be stable in 1 N acid and in 1 N alkali (29). All these experiments, therefore, suggested that the phosphates were monoesterified to hydroxyl groups of serine or threonine residues of protein-55K.

Hydrolysis of the protein-55K eluate in 6 N HCl for 2 h at 110°C and high voltage paper electrophoresis of the hydrolysate (Fig. 7F) demonstrated that the phosphate label was associated with serine residues. Although phosphotyrosine has been reported to be stable in this hydrolysis condition (30), no tyrosine was found to be phosphorylated in protein-55K.

55K. In addition to protein-55K, other phosphohistidine protein eluates were subjected to mild acid hydrolysis, and the phosphoamino acids were analyzed by paper electrophoresis (Fig. 7). All eight proteins contained phosphorylated serine residues, but neither phosphothreonine nor phosphotyrosine.

Since protein-55K was one of the phosphorylated nonhistone proteins associated with metaphase chromosomes and was greatly dephosphorylated at mitosis, it was of interest to find the differences in the isoelectric points of protein-55K present in interphase chromatin or metaphase chromosomes.

Before performing nonequilibrium pH gradient slab gel electrophoresis, it was important to prove the identity of protein-55K associated with chromatin and that present in metaphase chromosomes. For this purpose, the one-dimensional peptide mapping technique was again employed and the results are presented in Fig. 8. Protein-55K bands in SDS-polyacrylamide gels of chromatin isolated from synchronized HeLa S-3 cells at either early S phase (Fig. 8A) or mid-S phase (Fig. 8B) and protein-55K of metaphase chromosomes (Fig. 8C) were overlayed with three concentrations of S. aureus V8 protease. Essentially identical species of phosphopeptides were generated by treating each protein with the same amount of enzyme, indicating that protein-55K present in interphase chromatin and metaphase chromosomes are the same.

To separate species of protein-55K with varying states of phosphorylation at different stages of the cell cycle, nonequilibrium pH gradient slab gel electrophoresis was performed with protein bands cut from preparative SDS-polyacrylamide gels. Since the protein in the gel retained residual bound SDS, the effect of SDS in a nonequilibrium pH gradient slab gel was studied with BSA. A band of BSA cut from a SDS slab gel and a control sample of BSA that was directly dissolved in O'Farrell's urea lysis buffer displayed identical isoelectric points (5.6 to 5.7) in this gel electrophoresis. Even though the recovery of the protein from the band of polyacrylamide was not complete (32% recovery), this method was considered valuable in working with highly radiolabeled proteins.

Fig. 9 shows an autoradiogram of a nonequilibrium pH

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**Table II**

$^{32}$P radioactivity in protein-55K resistant to alkali or hot acid treatment

| Treatment          | Control | Resistant* |
|--------------------|---------|------------|
|                    | cpm     | cpm        |
| NaOH (1 M)         | 3,470   | 330 (9)    |
| Hot trichloroacetic acid (10%) | 11,200  | 10,100 (90) |

* Percentage of control is shown in parentheses.
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The phosphorylated protein species of the HeLa nuclear scaffold were analyzed on SDS-polyacrylamide gels, and cell cycle changes in the degree of incorporation of $^{32}$P into these proteins were investigated. The procedures to synchronize cells and to label proteins with $^{32}$P were the same as for the experiments with chromatin. Fig. 10A presents the Coomassie blue staining pattern of the gel; B, autoradiogram of the phosphorylated nuclear scaffold proteins. The cells were labeled for the following times after release from the G1/S boundary: 1, 0–1 h; 2, 5–6 h; 3, 8–9 h; 4, 10–11 h; 5, 15–16 h; 6, 20–21 h. The most strongly phosphorylated nuclear scaffold protein has a molecular weight of 119,000.

Variations in the Phosphorylation of Nuclear Scaffold Proteins during the Cell Cycle—The phosphorylated protein species were investigated. The experiments indicate that the protein-55K molecule is di- or triphosphorylated in mid-S phase, primarily diphosphorylated in interphase, and mono- or diphosphorylated in mitosis.

Fig. 8. Identification of protein-55K in isolated metaphase chromosomes by one-dimensional peptide mapping. Protein bands with molecular weights of 55,000 were excised from SDS-polyacrylamide gels of A, early S phase chromatin; B, mid-S phase chromatin; and C, isolated metaphase chromosomes. The protein bands were then digested with the same increasing amounts of S. aureus V8 proteinase: 1, 0.05 μg; 2, 5 μg; 3, 200 μg. The position of protein-55K in these maps is indicated with an arrow.

Fig. 9. Nonequilibrium pH gradient slab gel electrophoresis of protein-55K associated with chromatin and metaphase chromosomes. The figure displays an autoradiogram of protein-55K samples which have been radioactively labeled with $^{32}$P. Gel segments containing the protein were cut from SDS-polyacrylamide slab gels and applied to the nonequilibrium pH gradient slab gel. 1, protein-55K from chromatin from synchronized cells in mid-S phase; 2, protein-55K from unsynchronized cells; 3, protein-55K of isolated metaphase chromosomes.

Fig. 11. Changes during the cell cycle in the specific radioactivity ($^{32}$P cpm/μg protein) of the nuclear scaffold protein with a molecular weight of 119,000. The values were obtained from densitometer scans of stained gels and autoradiograms and from direct measurement of radioactivities. A high degree of phosphorylation occurs from S phase through mitosis and the level decreases markedly in G1 phase.
in the molecular weight range of 65,000–70,000 (2190 cpm versus 110 cpm) with the M-G1 nuclear scaffold sample. The specific radioactivity (32P cpm/µg of protein) of the 119,000-dalton protein was calculated for the selected times during the cell cycle and Fig. 11 contains this profile. A substantial incorporation of radiolabel is found for the mid-S phase sample (5–6 h). This is similar to the situation with chromatin. In addition, the 119,000-dalton protein in samples close to mitosis (8–9 h and 10–11 h) shows a high level of phosphorylation. The incorporation of 32P label declines markedly, however, in G1 phase.

**DISCUSSION**

This investigation of nonhistone phosphorylation is part of a larger study that has the goal of understanding the role of nonhistones in the structural changes of the HeLa genome through the cell cycle. For interphase cells, the present study has been concerned with the nonhistone proteins that are associated with the two primary nuclear components, chromatin fragments produced by mild micrococcal nuclease digestion and the nuclear scaffold. The nuclear scaffold is the residual protein framework of the nucleus that is produced by extensive nuclease digestion of isolated nuclei and extraction of histones plus other proteins with high salt concentrations or polyanions. The results of this investigation have demonstrated that the phosphorylation of nonhistone proteins is a major modification of both chromatin and nuclear scaffolds. With chromatin, emphasis was placed on characterizing the phosphorylation of the species that showed the greatest incorporation of 32P, a protein with a molecular weight of 55,000 (protein-55K). The peak of specific radioactivity of incorporated 32P was found in S phase and the incorporation was minimal prior to mitosis. This general result was shared by the seven other chromatin-associated phosphoproteins that were examined, although the peaks of incorporation were less pronounced. Experiments with alkaline phosphatase and one-dimensional peptide mapping in polyacrylamide gels demonstrated that the 32P label was indeed incorporated in protein, and high voltage paper electrophoresis showed the modified residues to be serine for all eight proteins. One-dimensional peptide mapping also indicated that the same limit peptides to be serine for all eight proteins. One-dimensional peptide mapping in polyacrylamide gels demonstrated that the 32P label was indeed incorporated in protein, and high voltage paper electrophoresis showed the modified residues to be serine for all eight proteins. One-dimensional peptide mapping also indicated that the same limit peptides for protein-55K are phosphorylated in mid-S phase cells and in metaphase chromosomes. Nonequilibrium pH gradient slab gels revealed differences in the isoelectric points of protein-55K from mid-S phase cells and metaphase chromosomes which are compatible with the existence of multiple phosphorylation sites.

The incorporation of 32P into proteins is strikingly different with metaphase chromosomes. Densitometer scans of autoradiograms are dominated by the incorporation into histones H1B and H1A, whereas for chromatin the degree of H1 phosphorylation is comparable to nonhistone phosphorylation. This net result is due both to a decrease in nonhistone phosphorylation and an increase in H1 phosphorylation in mitosis. Other detailed studies of the phosphorylation of histone H1 (31, 32) have shown, for example, that the number of moles of phosphate per mol of protein increases by 3–4 in the transition from S phase to mitosis. Thus, while the correlation of H1 phosphorylation with the onset of mitosis may indicate a role in mitotic chromosome condensation, a general increase in the degree of phosphorylation of nonhistones is clearly not a prerequisite for chromosome condensation.

A significant result of the experiments concerned with the phosphorylation of nuclear scaffold proteins was the identification of a 119,000-dalton protein as the major phosphorylated species. Coomassie blue staining of SDS-polyacrylamide gels showed proteins with molecular weights of 65,000–70,000 to be the predominant species. These are probably identical with the "laminas," structural proteins of the peripheral nuclear lamina that are more highly phosphorylated during mitosis than during interphase (33). But while autoradiograms of gels of nuclear scaffold proteins show that the 65,000–70,000-dalton species are phosphorylated, the degree of phosphorylation of these proteins is substantially less (20-fold) than the 119,000-dalton protein. Indeed, the degree of phosphorylation of the 65,000–70,000-dalton group is similar to that of a number of the minor proteins of the nuclear scaffold. A high level of incorporation (32P cpm/µg of protein) for the 119,000-dalton protein was found from S phase to mitosis, though the level declined markedly in G1 phase.

Other investigations have been concerned with HeLa cell cycle changes in the phosphorylation of histones and HMG proteins. As mentioned earlier, histone H1 has been shown to undergo a greatly increased phosphorylation which immediately precedes mitosis. Histones H3, H2A, and H4 are also phosphorylated (34–36); the phosphorylation of H2A is constant through the cell cycle but that of H3 varies with the stage (37). Among HMG proteins, HMG14 and HMG17 are phosphorylated (38); the incorporation of 32P into HMG14 and HMG17 in G2 phase is increased 7-fold compared to that in G1 phase, and there is a 2-fold increase of 32P incorporation into HMG17 in early S phase relative to that in the G1 and G2 phases.

A related study from this laboratory on changes in HeLa cell nonhistones was concerned with cell cycle variations in the synthesis of chromatin nonhistones and nuclear scaffold proteins (39). One-dimensional and two-dimensional polyacrylamide gel electrophoresis were used to resolve the patterns of incorporation of [35S]methionine label using synchronized cells. The results of this study demonstrated that substantial variations in [35S]methionine incorporation occurred during the cell cycle, but these changes were not dramatic nor was there a strong correlation with the onset of mitosis. The same result was found with the nonhistones associated with chromatin fragments and with nuclear scaffolds. Thus, both the synthesis and the phosphorylation of nonhistones are not obviously related to the morphological events that lead to the formation of metaphase chromosomes. The possibility remains open that the synthesis or modification of certain minor proteins may have a crucial role. These species may not be readily recognized among the 250 or so chromatin-associated nonhistones or the dozens of minor nuclear scaffold proteins. Also the dephosphorylation, and not the phosphorylation, of nonhistones may be a factor in triggering mitosis. However, the positive correlation of nonhistone phosphorylation with DNA synthesis would suggest that this modification is connected more with the organization and activity of chromatin during interphase than with chromosome condensation.

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Additional references will be found on p. 3318.
Phosphorylation of Nonhistone Proteins during the HeLa Cell Cycle

EXPERIMENTAL PROCEDURES

Cell Culture and Synchronization

HeLa S3 cells (ATCC 3587) were maintained in suspension culture at a concentration of 3 to 6 x 10^6 cells per ml with fresh, dibutyryl-cyclic AMP (5 x 10^-4 M) containing either 1% newborn calf serum or 5% fetal calf serum.

Synchronization was obtained by the double thymidine block method (1). Cells were exposed to 2.5 or 5 mM thymidine for an additional 15-16 hr. At the end of this treatment, cells were washed and suspended in fresh medium with a doubling time of 0.5 hr before measuring the incorporation of [3H]thymidine into DNA. The number of viable cells, and thymidine incorporation, was determined at various times after release. The percentage of cells in the different stages of the cell cycle in the presence of 5% sodium dodecyl sulfate for 30 min at 37° C.

Radiolabeling and Preparation of Chromosomes

To determine the rate of phosphorylation of chromatin-associated nonhistone proteins at different phases in the HeLa S3 cell-cycle, aliquots of suspension cultures of cells synchronized by double thymidine blocks or with mitomycin C for 2 hr were pelleted after each intact cycle and reconstituted at a concentration of 2 x 10^6 cells per ml with the appropriate medium and 100 units/ml mitomycin C. The isolated chromosomes were then fixed in methanol and stored as described previously. The isolated chromosomes were denatured in 50 mM sodium hydroxide for 5 min at 37° C. 50% ethanol was used as the sodium hydroxide minimum essential medium with the exception that HEPES (10 mM sodium phosphate) for 1.0 hr at 37° C in a gasser water bath. After the end of the incubation period, cells were cooled on ice and nuclei were isolated as previously reported (2), with the exception of the addition of 10 mM polypropylene glycol to the isolation buffer.

Chromatin was prepared by mild micrococcal nuclease digestion of nuclei (3). The nuclei were treated with micrococcal nuclease (10 units/ml) in the presence of 2 ml/5 ml at 37° C. Digestion was terminated by shifting to ice and adding EDTA, pH 7.4, to a final concentration of 2 mM. The nuclei were pelleted and then swollen in a solution of 5 mM EDTA, pH 7.4, 0.15 M NaCl for 30 min on ice.

Radiolabeling and Preparation of Nuclei

HeLa S3 cells were isolated as previously described (4). For experiment A, nuclei were extracted with 2.5% sodium dodecyl sulfate and 300 mM phosphate-free medium and labeled with [3H]thymidine (250 pmol/ml) for 16 hr at 37° C.

After labeling, nuclei were washed and resuspended in 50 mM sodium hydroxide for 5 min at 37° C in the absence of sodium hydroxide. The isolation buffer consisted of 10 mM NaCl, 5 mM HEPES, pH 7.4, 0.5 M NaCl, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate. After pelleting, chromosomes in the supernatant were purified by centrifugation into density gradients of metrizamide (0.5-2.1 g/cm3) in a buffer containing 0.1 M NaF and 0.1 mM PEG 8000. For experiment B, the isolated chromosomes were denatured in 50 mM sodium hydroxide for 5 min at 37° C. 50% ethanol was used as the sodium hydroxide minimum essential medium with the exception that HEPES (10 mM sodium phosphate) for 1.0 hr at 37° C in a gasser water bath. After the end of the incubation period, cells were cooled on ice and nuclei were isolated as previously reported (2), with the exception of the addition of 10 mM polypropylene glycol to the isolation buffer.

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Phosphorylation of Nonhistones during the HeLa Cell Cycle

Figure 31. Stability of [32P] incorporated into chromatin-associated nonhistone proteins in HeLa cells. Cells cultured at the G1/S boundary were pulse-labeled with [32P]orthophosphate for 1 h at 37°C. The cells were washed, reseeded in [32P]-free medium, and further incubated at 37°C for (I) 0 h, (II) 2 h, (III) 2 h, (IV) 4 h, and (V) 8 h. Proteins in each isolated chromatin sample were separated by SDS-polyacrylamide gel electrophoresis and an autoradiogram of an 8% gel is shown in this figure.

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