Differential Phosphorylation and Turnover of Nuclear Acidic Proteins during the Cell Cycle of Synchronized HeLa Cells*

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SUMMARY

The phosphorylation of non-histone nuclear proteins has been studied in HeLa S-3 cells during synchronous growth. The uptake of [32P]phosphate into individual protein bands separated on sodium dodecyl sulfate polyacrylamide gels varies at different stages in the cell cycle while the banding patterns themselves are remarkably constant. Rates of phosphate uptake into most major phosphoprotein species are increased during the early G1 and early S phases and are minimal during the late G2 to M period.

The turnover of previously incorporated phosphoamides during a cold chase following a 23-hour exposure to [32P]orthophosphate shows that some proteins lose their phosphoamides much more rapidly than do others. The half-lives vary from 5 to 12 hours, with an overall half-life of 6.7 hours. In contrast, the [32P]phosphate is a more sensitive index of differential phosphoryl group turnover in different nuclear acidic proteins than is the uptake of [32P]phosphate in short term labeling experiments.

If the nuclear phosphoproteins play a role in differential transcription during the cell cycle, changes in phosphorylation would appear to be more significant than changes in relative concentrations of individual protein species.

The experiments to be described deal with changes in the metabolic activities of nuclear phosphoproteins during the replication cycle of synchronously growing mammalian cells. Interest in this class of proteins stems from observations that they show strong indications of involvement in the positive control of gene activity (e.g., 1–13).

The question arises as to whether the nuclear phosphoproteins are altered at times of gene activation and repression during the cell cycle. It is known, for example, that the synthesis of RNA in synchronously dividing cells is suppressed during mitosis (14–16), while the synthesis of particular messenger RNAs (e.g., histone mRNAs) is restricted to the late G1 and early S phases of the cycle (17–19).

A number of observations have been made of synthesis and phosphorylation of nuclear acidic proteins in HeLa cells (20–23). The synthesis and accumulation of acidic nuclear proteins goes on throughout the cell cycle of continuously dividing populations of HeLa S-3 cells (20). In synchronously dividing cell populations there is an increased rate of synthesis and accumulation of these proteins which precedes the onset of DNA synthesis (21). The amount of nuclear protein synthesized, transported, and retained in the acidic chromosomal protein fraction is greater immediately after mitosis and later in G1 than in the S or G2 phases of the cell cycle (22). Preliminary studies of phosphorylation of HeLa nuclear proteins during the cell cycle indicate that the rate of phosphorylation is maximal in the early S phase and decreases in the late S and G2 phases when RNA synthesis is also reduced (23). The suppression of nuclear acidic protein phosphorylation during M has recently been reported by Platz et al. (24).

The present study is a detailed analysis of the uptake and turnover of phosphate in the phenol-soluble nuclear acidic proteins of synchronously and nonsynchronously growing HeLa S-3 cells.

MATERIALS AND METHODS

Cell Culture and Synchronization—HeLa S-3 cells1 were maintained in suspension culture at 2 to 6 x 10⁴ cells per ml by daily dilution with fresh Joklik-modified minimal essential medium (Grand Island Biological Co., Grand Island, N. Y.) containing 10% fetal calf serum and supplemented with 2.5 units per ml of penicillin G, 2.5 μg per ml of streptomycin, and 20 units per ml of mycostatin.

Synchronization was obtained by the double thymidine block method (25, 26), exposing the cells to 2 mM thymidine for 14 hours, to normal medium for the following 9 hours, and to 2 mM thymidine for an additional 14 hours. Cells were harvested by centrifugation at 1,500 x g for 4 min and resuspended in one-fifth of the original volume of thymidine-free medium. The time of release from the thymidine block is taken as starting at this resuspension. The cells were centrifuged and resuspended in the original volume of thymidine-free medium. The cell cycle was monitored by measurements of cell concentration, 

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mitotic index, and [\text{H}]thymidine incorporation rate. The cell number was measured at different times in the cell cycle using a Coulter counter. For determination of the mitotic index, the cells were fixed in 3:1 ethanol-acetic acid and stained with 1% (w/v) crystal violet in water. The number of mitotic figures was scored in at least 500 cells at each time point. Rates of thymidine incorporation into DNA were determined by incubating 1-ml aliquots of the cell suspension in the presence of 5 \mu Ci of [\text{H}]thymidine of specific activity 20 Ci per mmole (New England Nuclear, Inc., Boston, Mass.) for 30 min at 37°. Cold medium, 3.0 ml, was added at the end of the incubation period and the cells were collected by centrifugation at 2,000 \times g for 3 min. The cells were resuspended in 3 ml of 5% trichloroacetic acid and transferred to a Millipore filter (pore size 0.45 \mu). The filter was finally washed with 10 ml of 70% ethanol (v/v) and dried at 60° for 30 min. Bray’s scintillation liquid (27), 20 ml, was added to the dried filters and \text{H} activity was measured in a Packard Tri-Carb model 3375 scintillation spectrometer.

Isotopic Labeling of Nuclear Proteins—At different times after removal of the thymidine block, 4 to 8 \times 10^6 cells were harvested by centrifugation at 1,500 \times g for 4 min and gently resuspended in 5 ml of culture medium containing 2 \muCi of carrier-free [\text{P}]orthophosphate (New England Nuclear, Inc., Boston, Mass.). After 15 min incubation at 37° the cells were centrifuged as before and the cell pellet was frozen by immersing the tube in acetone at −70°. The cells were stored at −80°. Nuclei were isolated and the cell pellet was frozen by immersing the tube in acetone at −70°. The frozen cells were then homogenized in 4 ml of ice-cold 0.6 \text{M} \text{HClO}_4, centrifuged and re-extracted with 2:1 \text{M} \text{HClO}_4. The distribution of individual protein bands were based on mobility versus molecular weight.
supernatant was filtered through Whatman No. 3 paper. Aliquots of the clear supernatant were subjected to chromatography on Dowex 1 (formate) columns (0.6 × 5.0 cm) as described by Hurlbert (30). After application of the sample, the columns were washed with distilled water and with 1.0 m ammonium formate. The ATP fraction was eluted with 2 m ammonium formate-0.75 m formic acid, and the ATP was further purified by chromatography on Whatman No. 3 paper in ethanol-1 m ammonium acetate, pH 7.3-water, 66:5:30:3.5 (v/v/v). Spots corresponding in Rf to authentic ATP standards were cut out and the ATP eluted and analyzed by measuring absorbance at 260 nm and 32P activity.

Measurement of Cyclic AMP-dependent Protein Kinase Activity—The levels of cyclic AMP-dependent protein kinase activity were measured in high speed supernatant fractions obtained after homogenization of HeLa cells in 0.32 m sucrose containing 0.1% Triton X-100, using a Potter-Elvehjem homogenizer (0.15-mm clearance) at 2,000 rpm with 20 up-and-down strokes. The homogenate was centrifuged for 60 min at 100,000 × g, and aliquots of the supernatant containing 20 μg of protein were incubated at 31° with 100 μg of histone Fl as substrate and [γ-32P]ATP in the presence or absence of 10-6 m cyclic AMP as described previously (31, 32). The reaction was stopped by the addition of 5% trichloroacetic acid containing 0.25% sodium tungstate. The protein precipitate was washed and assayed for 32P activity as described (32).

Chemical Analyses—Protein was determined by the method of Lowry et al. (33) using bovine serum albumin as a standard for nuclear acidic protein determinations, and calf thymus histone as a standard for histone analyses. DNA was determined by the diphenylamine reaction as modified by Burton (34), using highly polymerized calf thymus DNA as a standard.

The amino acid compositions of the phosphoprotein fractions were determined by ion exchange chromatography after the method of Spackman et al. (35). The Beckman amino acid analyzer 120B was modified for a 7-fold increase in sensitivity by addition of a Honeywell expanded range card. For determination of alkali-labile phosphate, the proteins were first dialyzed exhaustively against distilled water and hydrolyzed in 1.0 N NaOH at 100° for 5 min. Inorganic phosphate released was analyzed as described previously (31, 32). The reaction was stopped by the addition of 5% trichloroacetic acid containing 0.25% sodium tungstate. The protein precipitate was washed and assayed for 32P activity as described (32).

RESULTS AND DISCUSSION

Composition of HeLa Nuclei (or Chromatin) at Different Stages in Cell Cycle—Nuclear protein and DNA contents have been examined in synchronously growing HeLa S-3 cells at different times after release from a double thymidine block (25, 26). The degree of synchrony obtained by this procedure is illustrated in Fig. 1, which plots three parameters of growth: cell number, and rate of [3H]thymidine incorporation into DNA. The timing of these events is highly reproducible, and was determined for each of the isotope-labeling experiments described in this communication. The degree of synchrony, as calculated by the method of Engelberg (37, 38) is 63% for the cells shown in Fig. 1. On the basis of this data, we consider the G2 period of the cell cycle to extend from 5 1/4 to 7 1/2 hours, while the G1 phase of the following cycle is taken as the period from 8 1/4 to 12 hours.

In order to monitor changes in nuclear proteins during the cell cycle, we have counted the number of cells in the population and analyzed for DNA content and protein distribution in different nuclear extracts. The results are summarized in Table I. A comparison of the figures for DNA content of the original cell suspension (Column 3) and the DNA recovered in the nuclear (or chromatin) fractions (Column 4) shows that recovery is very high at all stages in the cell cycle, with an average recovery of 86.1 ± 1.7%. This minimizes the risks of artifact due to the selection of a small or variable fraction of the nuclei for analysis. There are significant variations in the protein to DNA ratio of the nuclei during synchronous cell growth, ranging from 2.19 to 3.27 to 1 (Column 5). This ratio drops in the late S phase and increases during the G1 period of the following cycle. The changes are largely due to varying proportions of the non-histone nuclear proteins, because the histone to DNA ratios are not appreciably altered during the cell cycle but remain constant at about 1.1 to 1. This is consistent with observations that histone and DNA synthesis proceed concomitantly throughout the S phase of HeLa cells (19, 28, 39).

In the fractionation procedure employed to separate nuclear proteins about 40% of the total nuclear protein is removed when the nuclei are washed twice in 0.14 m NaCl (Table I, Column 8). The acid-soluble proteins, mainly histones, comprise another 40% of the total nuclear proteins. Little less of protein occurs in the chloroform-methanol-HCl washes; the bulk of the extracted material comprises lipids and phospholipids.

The residual proteins, which are then extracted in phenol, comprise about 13% of the total protein in the isolated nuclei. This fraction includes many of the nuclear phosphoproteins.
The amount of phenol-soluble protein per nucleus varies during the cell cycle, as judged by the phosphoprotein to DNA ratio at different times after release from the thymidine block. This ratio falls from 0.42 to 1 in the early S phase to 0.28 to 1 in G2 (Table I, Column 12). It follows that a substantial increase in the acidic protein complement of the nucleus must occur in the prereplicative phase of the cycle.

It should be noted that the high recoveries of protein obtained (approximately 96%) minimize the possibilities of artifact due to differential extraction of proteins depending upon stages in the cell cycle. As a further check, we have compared the recovery of phenol-soluble proteins from metaphase chromosomes (isolated from cells blocked in mitosis by exposure to vinblastine sulfate for 16 hours) and from nuclei obtained from an unsynchronized cell population. No indications of differential extractability were obtained.

The distribution of alkali-labile phosphorus in the different nuclear subfractions is shown in Table I. It can be seen that the phenol-soluble proteins, representing only 13 to 14% of the total nuclear protein, contain about one-third of the total phosphorus and also account for about one-third of the total radioactivity incorporated in a long term (23-hour) labeling period.

The proteins extractable in 0.14 M NaCl contain only 7% of the total radioactivity incorporated. The HCl-soluble protein fraction, mainly histones, contains about half of the total counts. It should be noted that the proteins of the phenol-soluble fraction have both the highest specific activity and the highest phosphorus content of the nuclear fractions analyzed.

Characterization of Nuclear Phosphoprotein Fraction—The nuclear phosphoprotein fraction comprises a heterogeneous mixture of proteins differing in molecular weight, amino acid composition, and degree of phosphorylation. The molecular size heterogeneity is indicated by differences in electrophoretic mobility in SDS polyacrylamide gels. A complex banding pattern is obtained which shows the presence of multiple polypeptide chains ranging in molecular weight from 18,000 to 170,000 (Fig. 2). At least 21 major bands are detectable; each of these, in turn, may include different protein species of similar or identical molecular weights.

A comparison of the banding patterns of the phenol-soluble nuclear proteins at different stages in the cell cycle is presented in Fig. 2. The results show a remarkable uniformity in the relative concentrations of the different protein bands. Some minor differences are detectable by densitometry, in agreement with the data presented in Table I.
Hours after release from thymidine block

| Hours after release from thymidine block |
|----------------------------------------|
| 0 | 1.5 | 3 | 4.5 | 6 | 7.5 | 9 | 10.5 | 12 |

FIG. 2. Electrophoretic patterns in SDS-polyacrylamide gels of HeLa nuclear phenol-soluble proteins prepared at different stages of the cell cycle. The proteins were extracted from nuclei isolated at the indicated times after release from the thymidine block. All gels in this and in succeeding figures contained 125 µg of total protein. Note the uniformity in protein-banding patterns throughout the cycle.

with the findings of Bhorjee and Pederson (40), but, on the whole, it is the uniformity of the protein pattern, rather than its variability, which attracts attention. This result is in contrast with findings that the nuclear phosphoprotein complement changes appreciably in cells during the course of differentiation (41-45). It has been noted previously that the synthesis of nuclear acidic proteins proceeds throughout the HeLa cell cycle (20-22), with increasing rates of synthesis in late G1 (21). The reproducibility of the gel patterns at different times, shown here, suggests that the synthesis and accumulation of many of the major nuclear acidic proteins are under close coordinate control.

The amino acid analyses presented in Table III confirm the impression of constant proportionality of the phenol-soluble proteins throughout the HeLa cell cycle, although one would only expect to detect gross differences by this technique. The average amino acid composition of the nuclear phenol-soluble protein fraction indicates a clear predominance of the acidic amino acids, aspartic and glutamic acid (21 mole %) over the basic amino acids, lysine, arginine, and histidine (15 mole %). This is in accord with findings in other cell types (2).

Nuclear Protein Phosphorylation during Cell Cycle—Rates of [32P]orthophosphate incorporation into the nuclear phosphorylation fraction vary at different stages in the cell cycle. Comparisons were made by selecting aliquots of the cell suspension at different times after release from the thymidine block and pulse-labeling for 15 min in the presence of [32P]orthophosphate. The

| Amino acid     | Hrs after release of cells from thymidine block |
|----------------|-------------------------------------------------|
| Lysine         | 6.3 6.8 6.4 6.6 6.3                             |
| Histidine      | 2.2 2.4 2.5 2.4 2.3                             |
| Arginine       | 6.0 5.7 5.8 6.4 5.9                             |
| Aspartic acid  | 9.7 9.5 9.7 9.8 9.6                             |
| Threonine      | 5.4 5.6 5.1 4.7 5.5                             |
| Serine         | 7.3 7.3 7.3 7.2 6.7                             |
| Glutamic acid  | 12.5 12.5 12.7 11.9 11.5                        |
| Proline        | 4.6 4.6 5.1 4.6 4.9                             |
| Glycine        | 9.0 9.5 9.3 9.2 9.0                             |
| Alanine        | 7.2 7.0 7.2 7.3 7.0                             |
| Valine         | 6.2 6.3 6.4 6.2 5.9                             |
| Methionine     | 1.1 1.5 1.7 2.0 1.5                             |
| Isoleucine     | 5.8 4.8 4.7 4.9 5.8                             |
| Leucine        | 9.7 9.4 8.9 9.3 10.4                            |
| Tyrosine       | 2.0 3.3 3.1 3.4 3.6                             |
| Phenylalanine  | 3.8 3.9 3.7 4.0 3.9                             |

* Values not corrected for hydrolytic losses.

FIG. 3. Changes in rate of nuclear phenol-soluble protein phosphorylation at different times in the cell cycle. Aliquots of the HeLa cell suspension were taken at the indicated times and incubated for 15 min with [32P]orthophosphate. The nuclear phosphoprotein fraction was isolated and its [32P] activity measured. The specific activity is plotted against time after release from the thymidine block. This is maximal in S and in G1, but minimal in the G2 to M period.

Two peaks of phosphate incorporation are evident. The first occurs early in the S phase (between 1/2 and 3 hours) and the second peak occurs in early G1 (at about 10 hours). The rate of phosphate uptake appears to be somewhat greater in S than
TABLE IV
Tests for contamination of nuclear phosphoproteins by radioactive cytoplasmic proteins, and for possible effects of 2 mM thymidine on protein phosphorylation by HeLa cells

| Conditions of experiment                                      | Specific activity of phenol-soluble proteins (cpm/mg protein) |
|---------------------------------------------------------------|---------------------------------------------------------------|
| Nonradioactive nuclei + [*P*-labeled cytoplasm]                | 992                                                          |
| Cells incubated without thymidine                             | 20,590                                                       |
| Cells exposed to 2 mM thymidine for 2 hrs.                    | 19,030                                                       |
| Cells exposed to 2 mM thymidine for 4 hrs.                    | 20,050                                                       |

* Nuclei from $4 \times 10^7$ nonradioactive cells were mixed with the postnuclear supernatant fraction obtained from $4 \times 10^7$ cells which had been labeled for 15 min in the presence of 2 mCi of [*P*-orthophosphate. The nuclei were then reisolated and the phenol-soluble proteins were prepared and counted.

* Cells ($4 \times 10^7$) were pulse labeled for 15 min in the presence of 2 mCi of [*P*-orthophosphate in the presence or absence of 2 mM thymidine as described under "Materials and Methods.""

in G1; an uptake ratio of about 1.2:1.0 was observed consistently. The phosphorylation of the phenol-soluble nuclear acidic protein is markedly reduced in the late S and G2 phases of the cell cycle and remains low in the M period. Thus, the rate of [*P*-phosphate incorporation into the nuclear phosphoproteins is high at periods of intense RNA synthesis and low when transcription is suppressed (14–16).

The possibility that estimates of the rate of nuclear protein phosphorylation might be in error due to injurious effects of the thymidine double block was tested by comparing [*P*-uptakes in control (unsynchronized) cell cultures and in cultures exposed to 2 mM thymidine for 2 or 4 hours. As can be seen from the data in Table IV, the phosphate uptake into the nuclear proteins of control and thymidine-treated cells is virtually identical. Moreover, no differences in gel electrophoretic patterns could be discerned. Other control experiments have established that cytoplasmic protein fractions do not contribute significantly to the radioactivity of the nuclear fractions we have analyzed. This possibility was tested by preparing the nuclear phosphoproteins from unlabeled cells which were homogenized in the presence of a [*P*-labeled postnuclear supernatant fraction from homogenates of cells incubated in the usual way with 2 mCi of [*P*-orthophosphate. Less than 4.8% contamination was observed (Table IV).

The distribution of [*P*-phosphate in different size classes of nuclear phosphoproteins after pulse-labeling for 15 min was determined by radiodensy of the multiple bands separated by SDS-polyacrylamide gel electrophoresis (Fig. 4). The staining pattern shown at the bottom of the figure is aligned with the corresponding densitometer tracing in the top panel. The other panels compare the distribution and specific activities of the nuclear phosphoproteins at the indicated stages in the cell cycle.

It is evident that there is a marked heterogeneity in [*P*-phosphate incorporation into the proteins at different regions of the gel. In some cases the peaks of [*P* activity do not coincide exactly with the positions of the major protein bands. This strongly suggests that minor bands, not visible because of their low concentration, may be contributing disproportionately to the [*P*-uptake measurements.

The [*P*-activity of the individual protein bands follows quite
closely the cell cycle-dependent changes described for the total phosphol soluble protein fraction. Labeling is greatest in the S and Gt phases and depressed in the G2 to M phase. Some minor differences in the rate of labeling of different bands can be observed, but, on the whole, the pulse labeling experiments indicate a parallel metabolic response of many diverse nuclear proteins to events occurring at different stages of the cell cycle.

The fluctuation observed in the rates of [3P] incorporation into the non-histone proteins at different stages in synchronous cell growth does not appear to depend on fluctuations in the specific activity of the cellular ATP pools. The incorporation of [3P]orthophosphate into ATP pools was measured in cells at different stages of the cycle. As the results in Table V show, the specific activity of the cellular ATP pools remains relatively uniform in the course of synchronous growth. The minor variations listed in Table V do not seem significant and are not likely to account for the large differences in the specific activity of the phospho protein fraction at different stages. The fluctuations in [3P]-phosphate incorporation into the nuclear proteins are more likely to represent alterations in protein kinase activities in nuclei at different stages of the cycle. It is known that the phosphorylation of some acidic nuclear proteins is CAMP-dependent (32, 46). Cyclic AMP levels are known to fluctuate throughout the cell cycle of synchronously growing HeLa cells (47) with minimal concentrations in the late G2 to M period. This suggests that the major changes in nuclear protein phosphorylation in the HeLa cell cycle reflect the changing activities of the CAMP-dependent protein kinase.

Histone Phosphorylation in Cell Cycle—The rate of [3P]orthophosphate incorporation into the histones of synchronized HeLa cells varies throughout the cell cycle as shown in Fig. 5. The peak of phosphorylation is observed at 3 hours, which coincides with the peak of DNA synthesis. Correlations between histone phosphorylation and DNA synthesis have been noted before (49-52); the phosphorylation of histone fraction F1 is known to be dependent on cell cycle position and to be active in the S phase (50-52).

Some differences exist between the timing of histone phosphorylation and phosphate uptake into the non-histone proteins of the nucleus. The peak of histone phosphorylation occurs somewhat later in the cell cycle than the corresponding peak for the nuclear phosphol soluble proteins (Fig. 3). Moreover, no major peak in histone phosphorylation appears in G1 as it does for the more acidic protein fraction. These differences argue strongly against the view that the variable rates of phosphorylation of the nuclear acidic proteins simply reflect differences in the specific activities of nuclear ATP pools at different phases of the cycle.

| Table V |
|---|
| [3P]Phosphate incorporation into ATP pools of HeLa cells at different times of cell cycle |

| Time after release from thymidine block | Specific activity of [3P] orthophosphate incorporation into total histones (percent O:time) |
|---|---|
| hrs | % |
| 0 | 60 |
| 6 | 80 |
| 12 | 100 |
| 24 | 120 |

Some preliminary experiments have been carried out to determine whether corresponding changes in kinase activity toward histone F1 can be detected at different stages of synchronous growth. The soluble protein kinase activity of HeLa cell homogenates was compared at different times after release from the thymidine block, both in the presence and absence of cyclic AMP. The activation of histone F1 phosphorylation by cyclic AMP varies from 3- to 6-fold during the cycle, being highest at early S and lowest in early M (Table VI).

### Table VI

| Time after release from thymidine block | Specific activity of soluble protein kinase during HeLa cell cycle |
|---|---|
| hrs | units |
| 0 | 182 |
| 1 | 125 |
| 2 | 139 |
| 3 | 166 |
| 4 | 94 |

^a Assay performed as described under "Materials and Methods" with histone fraction F1 as substrate in the presence of 10^-6 M cyclic AMP.

^b One unit of enzyme activity = 1 pmole of phosphate incorporated per mg of enzyme protein in 5 min at 31°.
the cells were incubated in a nonradioactive medium. Aliquots of the suspension were withdrawn at the indicated times for preparation and analysis of the nuclear phosphoprotein fraction. As in the synchronously growing cell cultures, the rate of 32P loss from the phenol-soluble proteins exceeds the rate of decline of the 14C specific activity. The ratio of 32P:14C activities consequently decreases rapidly with time during the cold chase (Fig. 8). The kinetics of 32P turnover appear to be characteristic for the different nuclear subfractions.

In agreement with the results obtained in synchronously growing cells, the rate of 32P turnover varies for different proteins in the phenol-soluble fraction. The distribution of [32P]phosphate in different protein bands separated by SDS-polyacrylamide gel electrophoresis is shown in Fig. 9 for 3 and 9 hours after commencement of the cold chase. Three major phosphorylated bands (of molecular weights 28,000, 47,000 and 55,000) are evident. The rapid 32P turnover in proteins of molecular weight 55,000 and the relatively slow turnover in proteins of molecular weight 28,000 are in agreement with results obtained in the synchronously growing cell populations (see Fig. 7). Fig. 9 also shows the distribution of the [14C]leucine-labeled proteins in the electrophoretic pattern. It is clear that in some cases, the peaks of 32P activity do not coincide exactly with the positions of the major [14C]leucine-labeled bands.

Phosphopeptide Content of Isolated Nuclear Phosphoproteins—The average phosphorus content of the total nuclear phenol-soluble protein fraction was determined at different times after release from the thymidine block. There are no major differences in alkali-labile phosphorus content of the proteins prepared at different stages of the cycle (Table VI). The steady state level

release of the cells from the thymidine block. In these experiments, the acidic nuclear proteins were prelabeled for 23 hours in the presence of [3H]leucine and [32P]orthophosphate as described under "Materials and Methods." After washing, the cells were incubated under cold chase conditions, and aliquots of the suspension were withdrawn at the indicated times for preparation and analysis of the nuclear phosphoprotein fraction. A, the per cent of the maximal specific activity (counts per min per mg of protein) is plotted against time for [3H]leucine (O—O) and for [32P]phosphate (O—O). B, the ratio of 32P activity to 14C activity is plotted against time. The decreasing ratio indicates that phosphate groups in the proteins are subject to replacement without a corresponding degradation of the polypeptide chain.

Fig. 6. Relative rates of turnover of [32P]phosphate and [14C]leucine in nuclear phosphoproteins of synchronously growing HeLa S-3 cells. The acidic nuclear proteins were prelabeled for 23 hours in the presence of [3H]leucine and [32P]orthophosphate as described under "Materials and Methods." After washing, the cells were incubated under cold chase conditions, and aliquots of the suspension were withdrawn at the indicated times for preparation and analysis of the nuclear phosphoprotein fraction. A, the per cent of the maximal specific activity (counts per min per mg of protein) is plotted against time for [3H]leucine (O—O) and for [32P]phosphate (O—O). B, the ratio of 32P activity to 14C activity is plotted against time. The decreasing ratio indicates that phosphate groups in the proteins are subject to replacement without a corresponding degradation of the polypeptide chain.
Densitometry trace immediately after release

0 0 0 0 0

20 40 60 80 100
Slice number

FIG. 7

determined by direct analysis represents a balance between the turnover and replacement of phosphate groups on "old" protein molecules, as well as an incorporation of phosphate into newly synthesized acidic protein of the nucleus. It is clear from the

NaCl-extracted proteins

HCl-extracted proteins

Phenol-extracted proteins

Percent maximum specific activity

Hours in nonradioactive medium

Fig. 8. Relative rates of turnover of \(^{32}\text{P}\) phosphate and \(^{14}\text{C}\)-leucine in protein fractions of logarithmically growing HeLa S-3 cells. The proteins were prelabeled for 24 hours in continuously growing but asynchronous cultures. After washing, the cells were incubated under cold chase conditions, and aliquots of the suspension were withdrawn at the indicated times for preparation and analysis of the nuclear protein fractions. The per cent of the maximal specific activity (counts per min per mg of protein) is plotted against time for \(^{14}\text{C}\)-leucine (○—○) and for \(^{32}\text{P}\) phosphate (□—□) for proteins soluble in 0.14 M NaCl (top panel), 0.25 N HCl (center panel), and phenol (lower panel).

Fig. 7. Differential rates of \(^{32}\text{P}\) phosphate turnover in HeLa nuclear phosphoproteins during synchronous growth. The cell suspension was prelabeled with \(^{32}\text{P}\) orthophosphate for 24 hours as described under "Materials and Methods." After washing, the cells were incubated under cold chase conditions, and aliquots were withdrawn at the indicated times for preparation and electrophoretic analysis of the nuclear phenol-soluble phosphoprotein fraction. The proteins were separated by electrophoresis in 0.1% SDS-10% polyacrylamide gels. The protein-banding pattern is shown at the bottom of the figure and its densitometer tracing is shown in the top panel. Molecular weights are indicated above the major protein peaks. The intermediate panels show the patterns of \(^{32}\text{P}\) distribution in the nuclear proteins at different times after release from the thymidine block. Note the rapid loss of \(^{32}\text{P}\) activity from the band at molecular weight 65,000, and the relatively slow decreases in \(^{32}\text{P}\) activity of bands at molecular weights 28,000 and 47,000.
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fraction. The proteins were separated by electrophoresis in 0.1% suspension were withdrawn at the indicated times for preparation were incubated under cold chase conditions and aliquots of the phosphate and [Wleucine for 23 hours. After washing, the cells growing cell populations. The cells were prelabeled with [a2P]- in the nonradioactive medium. Note the rapid rate of loss of 32P nuclear phosphoproteins in unsynchronized, logarithmically 47,000. relatively slower loss from bands at molecular weights 28,000 and 1°C (C- - -0) activity is shown after 3 hours and after 9 hours from the protein band at molecular weight 55,000 and the Phosphorus content of nuclear phosphoprotein Tie after rel~~c$om thymidine

FIG. 9. Differential rates of [32P]phosphate turnover in HeLa nuclear phosphoproteins in unsynchronized, logarithmically growing cell populations. The cells were pretreated with [32P]-phosphate and [14C]leucine for 23 hours. After washing, the cells were incubated under cold chase conditions and aliquots of the suspension were withdrawn at the indicated times for preparation and electrophoretic analysis of the nuclear phenol-soluble protein fraction. The proteins were separated by electrophoresis in 0.1% SDS-10% polyacrylamide gels. The distribution of 32P (O-----O) and 14C (C- - -C) activity is shown after 3 hours and after 9 hours in the nonradioactive medium. Note the rapid rate of loss of 32P from the protein band at molecular weight 55,000 and the relatively slower loss from bands at molecular weights 28,000 and 47,000.

TABLE VII
Phosphorus content of nuclear phosphoprotein fraction at different stages of cell cycle of synchronized HeLa cells

| Time after release from thymidine block | Phosphorus content of protein* |
|----------------------------------------|-------------------------------|
| hrs                                    | %                             |
| 0                                     | 0.099 ± 0.003                 |
| 1.5                                    | 0.144 ± 0.024                 |
| 3.0                                    | 0.115 ± 0.018                 |
| 4.5                                    | 0.091 ± 0.014                 |
| 6.0                                    | 0.100 ± 0.008                 |
| 7.5                                    | 0.083 ± 0.001                 |
| 9.0                                    | 0.101 ± 0.007                 |
| 10.5                                   | 0.079 ± 0.004                 |
| 12.0                                   | 0.100 ± 0.009                 |

* Phosphorus determined as alkali-labile phosphate as described under “Materials and Methods.” Data presented as average of three values ±S.E.

[32P] incorporation data that rates of phosphorylation vary in different proteins and change at different periods of the cell cycle. The studies of isotope retention in both synchronous and non-synchronous cell cultures show that individual nuclear proteins exchange their phosphate groups at differing rates. The complexity and diversity of these structural modifications are not evident in the over-all phosphate level of the phenol-soluble fraction.

Based on an average phosphorus content of about 0.1% (by weight), it can be estimated that an average protein of molecular weight 120,000 containing about 4 of these in the phosphorylated form. (Since not all proteins in the phenol extract are equally phosphorylated, some will be more and others less phosphorylated than this average figure indicates.)

CONCLUSION

Although the relative concentrations of the major nuclear phenol-soluble phosphoproteins appear to be stable, there are clear differences in rates of phosphorylation of the nuclear proteins at different stages in the cell cycle. Protein phosphorylation is most active at periods when RNA synthesis is high (G1 and S) and it is minimal in the G2 to M period when RNA synthesis is suppressed. These changes are not due to changes in the specific activity of the cellular ATP pools which remain relatively constant. They do parallel changes in cellular cyclic AMP concentrations and probably reflect alterations in nuclear protein kinase activities at different phases of the cycle.

Phosphate incorporated into the nuclear acidic proteins is not stable, but turns over at rates which differ from one protein species to another. The phosphate groups are subject to exchange without degradation of the polypeptide chain.

If the nuclear phosphoproteins play a role in the control of transcription during the cell cycle, changes in their phosphorylation would offer a mechanism for modifying their structure and influencing their interactions with other components of the chromosome. In a nondifferentiating cell population, this may be more important than changes in the relative concentrations of the individual protein species.

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