THE SYNTHESIS OF ACIDIC CHROMOSOMAL PROTEINS DURING THE CELL CYCLE OF HeLa S-3 CELLS

I. The Accelerated Accumulation of Acidic Residual Nuclear Protein before the Initiation of DNA Replication

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ABSTRACT

The synthesis and accumulation of acidic proteins in the tightly bound residual nuclear fraction goes on throughout the cell cycle of continuously dividing populations of HeLa S-3 cells; however, during late G_1 there is an increased rate of synthesis and accumulation of these proteins which precedes the onset of DNA synthesis. Unlike that of the histones, whose synthesis is tightly coupled to DNA replication, the synthesis of acidic residual nuclear proteins is insensitive to inhibitors of DNA synthesis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of acidic residual nuclear proteins shows different profiles during the G_1, S, and G_2 phases of the cell cycle. These results suggest that, in contrast to histones whose synthesis appears to be highly regulated, the acidic residual proteins may have a regulatory function in the control of cell proliferation in continuously dividing mammalian cells.

INTRODUCTION

Recent results from several laboratories suggest that an accelerated accumulation of tightly bound acidic nuclear proteins on chromosomes precedes the initiation of DNA replication in quiescent cells which are stimulated to proliferate. An increase in the specific activity of tightly bound acidic nuclear proteins occurs during the prereplicative period of the estrogen- (35) or progesterone- (26) stimulated rat uterus pulse labeled in vivo and in vitro with radioactive amino acids. An increased specific activity of the chromosomal proteins remaining in the nucleus after salt and dilute mineral acid extraction is evident before the beginning of DNA replication in isoproterenol-stimulated mouse salivary glands pulse labeled in vitro (30) or in vivo[1] with leucine-^{3}H. Similar findings have been reported in rat mammary gland stimulated to proliferate by explantation (34) and in contact-inhibited fibroblasts stimulated to divide by medium change (21). The role of acidic nuclear proteins in the control of RNA-transcription and cell proliferation has been the subject of several recent reviews (31, 33).

Stein and Baserga (32) have shown that acidic nuclear proteins, like histones (3, 28), are synthesized in the cytoplasm and transferred to the nucleus just after synthesis. Given this fact, it is not clear whether an increase in specific activity of tightly bound acidic nuclear proteins represents an increased rate of synthesis and/or transport of these proteins to the nucleus or a decreased rate of degradation and/or transport of these proteins out of the nucleus. Further, it is not clear whether an increase in the specific activity of these protein species before DNA replication occurs only in the

[1] Borun, T. W., G. S. Stein, and R. Baserga. Unpublished observations.

cells which were quiescent and have been stimulated to divide or occurs also in continuously dividing cells in G_1. The present investigation attempted the resolution of these questions by studying the synthesis and turnover of tightly bound acidic nuclear proteins throughout the life cycle of synchronized populations of continuously dividing HeLa S-3 cells.

MATERIALS AND METHODS

Cells and Synchronization

Logarithmically growing HeLa S-3 cells were maintained in suspension culture in Joklik-modified Eagle's Minimal Essential Spinner Medium (SM)[2] (10) supplemented with 3.5% each of calf serum and fetal calf serum. The cells were a gift from Dr. M. D. Scharff of the Department of Cell Biology, Albert Einstein College of Medicine, Bronx, N. Y. and were obtained free of mycoplasma contamination.

Synchronization of the cells was carried out at 37°C in a warm room, either by selective detachment of mitotic cells (20, 36) or by two cycles of 2 mM thymidine block (2, 37). Cells in the mitotic, G_1, S, and G_2 phases of cell cycle were obtained by blocking randomly growing cultures with 2 mM thymidine for 12 hr, releasing the cells into fresh, thymidine-free Eagle's Minimal Essential Medium (MEM) (10), and plating in 1 liter Blake bottles. 6 hr after plating, loosely adherent cells were removed by shaking the bottles vigorously, then washing and overlaying the monolayer with warm complete SM. At 9–10 hr after plating, mitotic cells were selectively detached in complete SM from the semiconfluent monolayers remaining after the previous treatments. 95% of the detached cells were in mitosis when examined by phase-contrast microscopy just after harvest. During the subsequent 16–18 hr in culture, the detached cells proceeded through G_1, S, and G_2 as indicated in Fig. 1 A. Cells in the S and G_2 phases of the cell cycle were obtained by blocking randomly growing suspension cells with thymidine (final concentration 2 mM) for 12 hr, releasing the cells into thymidine-free SM for 9 hr and blocking the cells with 2 mM thymidine for an additional 12 hr. After release from the second thymidine block, the cells progressed through S and into G_2 as shown in Fig. 1 B.

[2] *Abbreviations used in this paper*: ara C, cytosine arabinoside; EDTA, ethylenediaminetetraacetate; MEM Eagle's Minimal Essential Medium; POPOP, *p*-bis[2-(5-phenyloxazolyl)] benzene; PPO, 2,5-diphenyloxazole; SDS, sodium dodecyl sulfate; SM, Eagle's Spinner Medium; TCA, trichloroacetic acid.

Labeling with Radioisotopes

To determine the *cumulative incorporation* of isotope into total cellular and nuclear protein from G_1 through S into G_2, 400 ml of cells at a concentration of 4×10^5 cells/ml were continuously labeled with 15 μCi of leucine-^{14}C from 0.5 through 16–18 hr after mitosis. 50-ml samples were withdrawn from the culture at the indicated times and processed as described below.

To estimate the *rate* of protein synthesis, $1.25–2.5 \times 10^7$ cells at various stages of the cell cycle were harvested by centrifugation at 600 *g* at 37°C and pulse labeled for 30 min at that temperature in 5 ml of leucine-free SM containing 2% fetal calf serum and 1 μCi/ml of L-leucine-^{14}C or 5 μCi/ml of L-leucine-^{3}H, depending upon the experiment as indicated in the captions of the figures.

In one experiment, 2.5×10^7 selectively detached G_1 cells at 2 hr after mitosis and the same number of S cells at 8 hr after mitosis were pulse labeled for 30 min at 37°C in 5 ml of SM minus tryptophan and lysine plus 2% fetal calf serum, 1 μCi/ml L-lysine-^{14}C and 10 μCi/ml L-tryptophan-^{3}H.

The rate of DNA replication was estimated by pulse labeling 1×10^6 cells at various stages in the cell cycle in 2 ml of complete SM containing 0.2 μCi of thymidine-2-^{14}C for 30 min at 37°C.

In all experiments, isotope incorporation was terminated by the addition of at least 10 volumes of ice-cold spinner salts solution and pelleting the cells by centrifugation at 600 *g* for 3 min at 3°C.

There were a minimum of six determinations for each point, unless otherwise indicated, and the range of values for each point did not exceed 10% on either side of the mean.

Isolation of Nuclear Proteins

The entire procedure was carried out at 4°C. Labeled cell pellets were washed three times by suspension in 40 volumes of ice-cold spinner salts and centrifugation at 600 *g* for 3 min in an International PR-6 refrigerated centrifuge. On the last resuspension, 50-μl samples were removed, precipitated, and washed with cold 20% trichloroacetic acid (TCA). The precipitates were collected on 0.45 μ Millipore filters, and then dissolved in 10 ml of Bray's solution minus ethylene glycol and counted in an Intertechnique Liquid Scintillation Spectrometer (Intertechnique, Dover, N. J.). The isotope incorporation into this total cellular fraction, and all of the other nuclear protein fractions which were similarly prepared, was expressed in terms of disintegrations per minute per 5×10^7 cells. Cytoplasm was removed from the cells by three washes in 5 ml of 80 mM NaCl; 20 mM ethylenediaminetetraacetate (EDTA), pH 7.2; and 1%

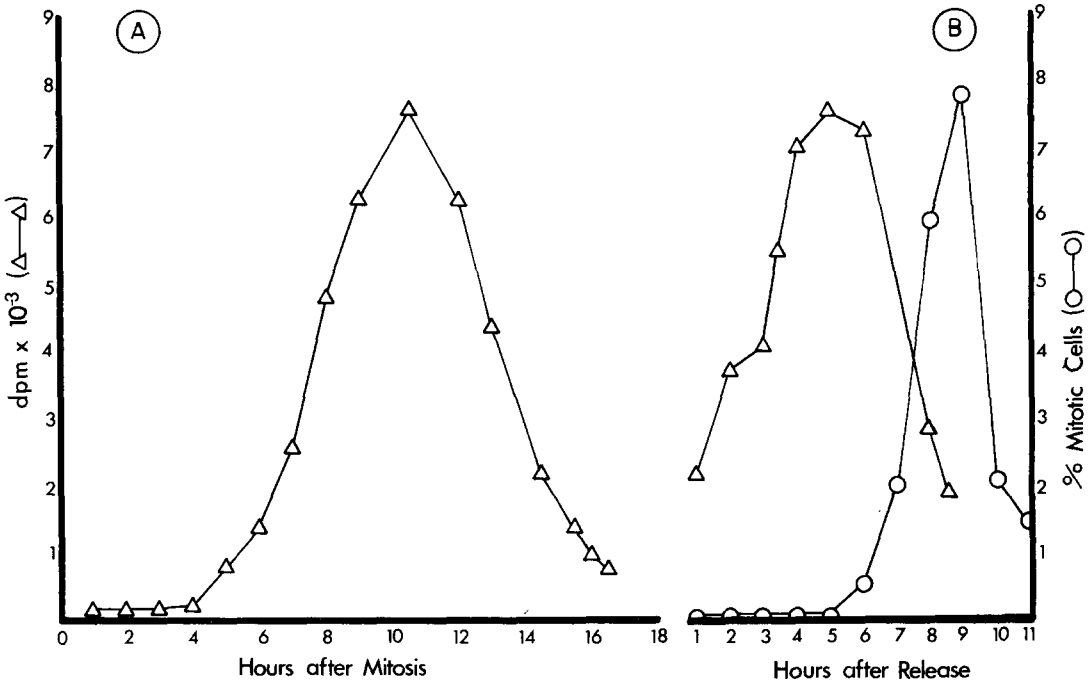


FIGURE 1 Incorporation of thymidine-2-^{14}C into DNA (A) at various times after selective detachment of mitotic cells and (B) after release from a double 2 mM thymidine block. 10^6 cells were labeled for 30 min with 0.2 μCi of thymidine-2-^{14}C, and the amount of radioactivity incorporated into cold 20% TCA-precipitable material was determined. It can be seen that G_1 lasts for 4–5 hr in these cells and that S lasts for 8–10 hr. The values are reported as disintegrations per minute per 10^6 cells, and there was a minimum of six determinations for each point.

Triton X-100 (13), and nuclei were pelleted by centrifugation for 3 min at 600 *g*. Nuclei prepared in this fashion were free of visible cytoplasmic contamination when examined by phase-contrast microscopy, and had a protein: DNA ratio of 3.1 and an RNA:DNA ratio of 0.32. The nuclei were then extracted twice with 2 ml of 0.15 M NaCl and centrifuged at 600 *g* for 3 min. The pellet was extracted twice with 2 ml of 0.35 M NaCl and centrifuged at 600 *g* for 3 min. The pellet was then extracted three times with 1 ml of 0.25 N H_2SO_4 and centrifuged at 600 *g* for 3 min. Control experiments have shown that over 95% of the radioactivity and protein extractable after a total of six similar extractions with 0.25 N H_2SO_4 are removed in the first three extractions. The combined 0.25 N H_2SO_4 supernatants constituted the crude histone extract while the pellet remaining after these salt and dilute mineral acid washes contained the tightly bound acidic residual nuclear proteins which were solubilized by homogenization in 4 ml of 1% sodium dodecyl sulfate (SDS), 0.01 M sodium phosphate buffer pH 6.8. This fractionation procedure is outlined in Fig. 2; the amino acid analysis of the acidic residual nuclear proteins has been shown previously (29).

The amounts of DNA and RNA were determined by the method of Scott, Fraccastoro, and Taft (24) and the method of Burton (4). The amount of protein was determined by the method of Lowry et al. (16).

Electrophoresis of Nuclear Protein Fractions

To separate proteins according to their molecular weights by means of SDS acrylamide gel electrophoresis (17, 25), nuclear protein fractions were brought to a final SDS concentration of 1% and dialyzed for 12 hr against 0.01 M sodium phosphate, pH 6.8 containing 0.1% SDS at room temperature. Electrophoresis of 0.25 ml samples, mixed with 50 μl of 60% sucrose, was carried out at room temperature for 11 hr at 90 V (constant) on 7.5% acrylamide, 0.8% bisacrylamide gels, 20 cm long and 6 mm in diameter, made up in 0.1 M sodium phosphate, 0.1% SDS, pH 6.8 tray buffer. To separate histones according to charge, the acetic acid–urea electrophoretic method of Panyim and Chalkley was used (18). Gels were frac-

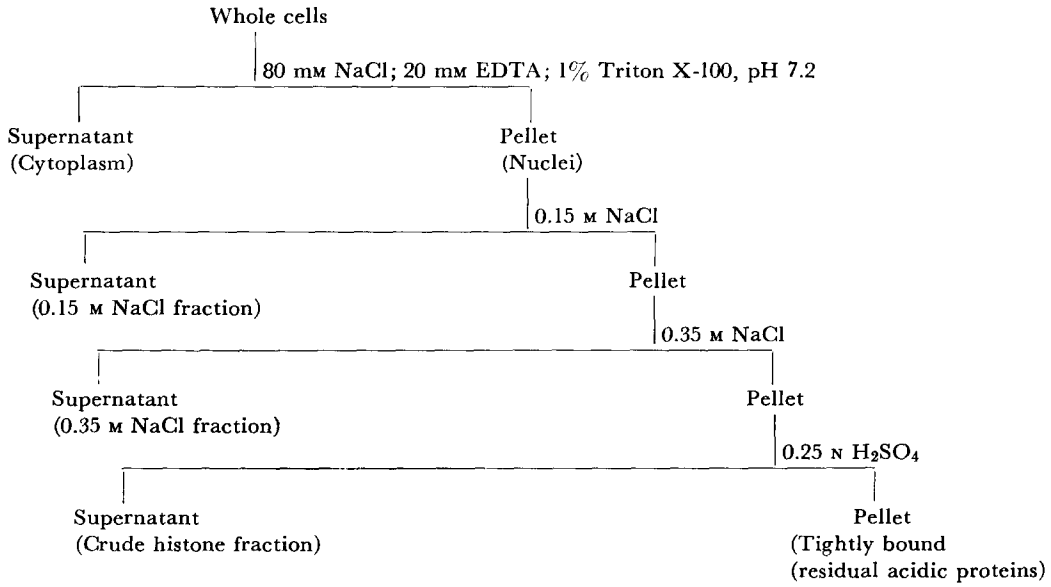


FIGURE 2 Scheme for isolation of nuclei and fractionation of nuclear proteins from HeLa S-3 cells. The procedure is described in the text (see Materials and Methods).

tionated mechanically (17) and collected in 70 or 100 liquid scintillation vials. 10 ml of 1:3 Triton X-100: toluene containing 4 g of 2,5-diphenyloxazole (PPO) and 50 mg of dimethyl *p*-bis[2-(5-phenyloxazolyl)]-benzene (POPOP)/liter were added to each vial, and radioactivity was determined in an Intertechnique Liquid Scintillation Counter.

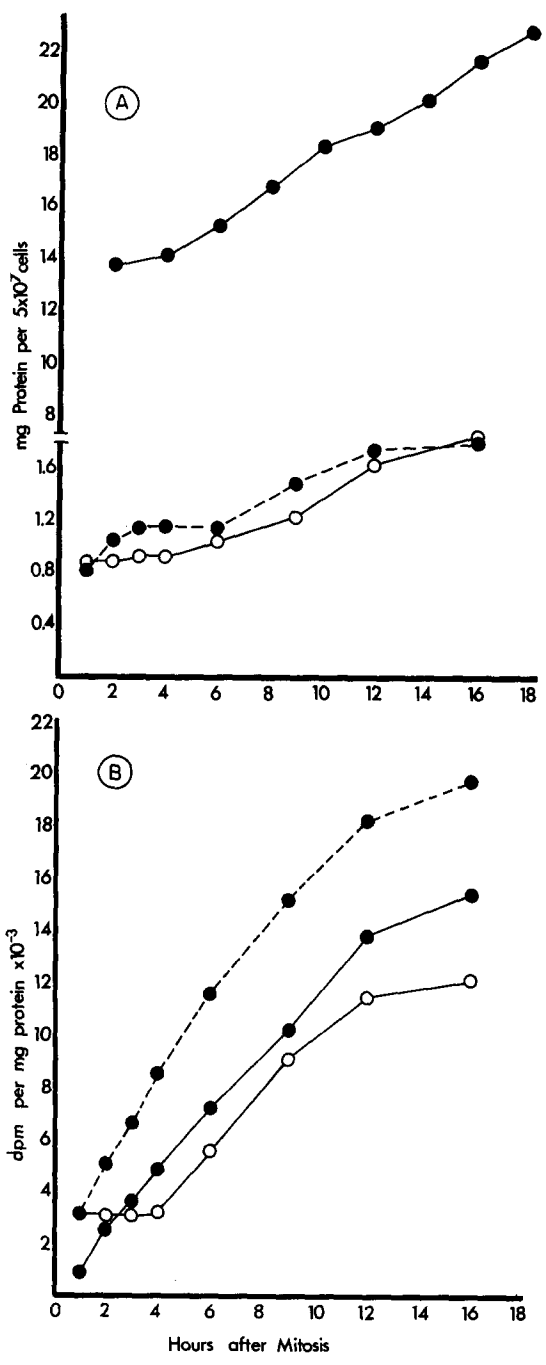
Materials

Eagle's Minimum Essential Medium (MEM), Joklik modified Eagle's Spinner Medium (SM), and Earle's balanced spinner salt solution were obtained from Grand Island Biological Co., Grand Island, N. Y., and serum was purchased from Flow Laboratories, Rockville, Md. Thymidine, cytosine arabinoside, and amino acids were supplied by Sigma Chemical Co., St. Louis, Mo. L-leucine-^{3}H (58 Ci/mmole), L-leucine-^{14}C (30 mCi/mmole), L-lysine-^{14}C (250 mCi/mmole), thymidine-2-^{14}C (25 mCi/mmole), and L-tryptophane-^{3}H (4 Ci/mmole) were obtained from New England Nuclear Corporation, Boston, Mass.

RESULTS

The Accumulation of Leucine-^{14}C-Labeled Protein through the HeLa S-3 Cell Cycle

The net result of the synthesis, transport, and turnover of nuclear protein fractions will be reflected in the way these fractions accumulate through the cell cycle. The accumulation of leucine-^{14}C in total cellular and nuclear protein was measured by continuously labeling a culture of selectively detached mitotic cells from 0.5 through 16–18 hr after selective detachment. Samples of 2×10^7 cells were withdrawn at the indicated times and fractionated as described in the Materials and Methods. Fig. 3 A and 3 B show that both the amount and the specific activity of the total cellular and the tightly bound residual nuclear protein fraction increase continuously throughout the HeLa S-3 cell cycle, in contrast to the H_2SO_4-soluble nuclear protein (crude histone) fraction which does not begin an appreciable increase in amount or specific activity until the beginning of S phase, about 4–5 hr after mitosis. Thus, while both nuclear protein fractions double in about 18–19 hr, and each comprises about 5–8% of the total cellular protein, depending upon the stage of the cell cycle, the residual nuclear protein fraction begins increasing immediately after mitosis and has a higher specific activity than either the total cellular or the crude histone fraction, throughout the cell cycle. The rates at which specific activities of these protein fractions change with time was calculated from the data in Fig. 3 B and is shown in Fig. 3 C. It is clear that the maximum rate of accumulation



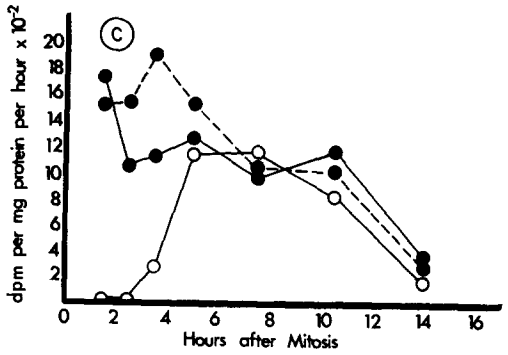


FIGURE 3 (A) Amount of total cellular and nuclear proteins in cells at various times after mitotic selective detachment. Nuclei were isolated as described in Methods, and the proteins were fractionated according to the procedure shown in Fig. 2. The quantity of protein present was determined by the method of Lowry et al. (16), and the values are reported as milligram of protein per 5×10^7 cells. There was a minimum of six determinations for each point. Total cellular protein (●——●——●), histone (○——○——○), and acidic residual nuclear protein (●--●--●).

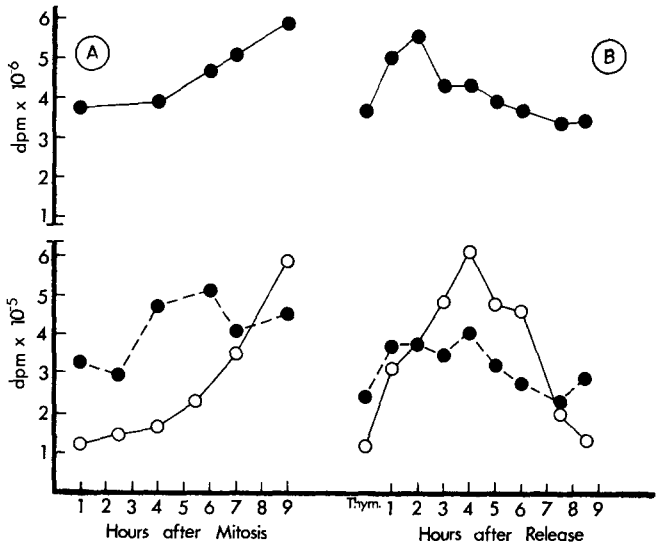
(B) Specific activities of total cellular and nuclear proteins at various times after selective detachment of mitotic cells. 400 ml of cells at 5×10^5/ml were labeled with 15 μCi of L-leucine-^{14}C from 0.5 to 16 hr after selective detachment. At the indicated times, 50-ml portions were harvested and processed as described in Materials and Methods. The amounts of proteins were determined as previously described (Fig. 3 A). The values are reported as disintegrations per minute per milligram of protein, and there was a minimum of six determinations for each point. Total cellular protein (●——●——●), histone (○——○——○), and residual acidic nuclear protein (●--●--●).

(C) The rates at which the specific activities of total cellular, crude histone, and residual acidic proteins change during the cell cycle. These values were calculated from the data in Fig. 3 B, and are expressed as disintegrations per minute per milligram of protein per hour; there was a minimum of three determinations for each point. Total cellular protein (●——●——●), histone (○——○——○), and acidic residual nuclear protein (●--●--●).

of leucine-^{14}C in the residual nuclear fraction occurs in late G_1, just before the initiation of DNA replication, while the crude histone fraction accumulates label at peak rates when the rate of DNA replication is maximal.

The Rate of Leucine-^{14}C Pulse-Label Incorporation into Protein through the Cell Cycle

In the following paper, it will be shown that the pulse-labeling kinetics of both the dilute acid-soluble and the residual nuclear protein fractions are complex, with material entering and leaving



FIGURES 4 A and 4 B The rate of incorporation of L-leucine-^{14}C into total cellular and nuclear proteins at various times (A) after selective detachment of mitotic cells and (B) after release from a double 2 mM thymidine block. Cells were pulse labeled with L-leucine-^{14}C for 30 min (see Methods). The nuclei were isolated as described in Methods, and the proteins were fractionated according to the procedure shown in Fig. 2. The values are reported as disintegrations per minute per 5×10^7 cells, and there was a minimum of six determinations for each point. Total cellular protein (●——●——●), histone (○——○——○), and acidic residual nuclear protein (●--●--●).

these fractions at different rates at different times of the HeLa S-3 cell cycle. In spite of these variations, it was found that a 30 min leucine-^{14}C pulse is an accurate means of estimating the maximum amount of protein being synthesized and transported to the nucleus at a given point in the cell cycle, even though some of the labeled material present after a 30 min pulse will subsequently be chased out of the nuclear protein fractions. The incorporation of leucine-^{14}C into total cellular and nuclear proteins was measured by pulse labeling 2.5×10^7 cells for 30 min at various times after selective detachment in G_1 and early S. To achieve maximum synchrony in late S and G_2, the same number of cells were pulsed at various times after release from the second of two cycles of thymidine block (see Materials and Methods). Figs. 4 A and 4 B show that the rate of leucine-^{14}C incorporation into the tightly bound, residual nuclear protein fraction increases immediately after mitosis and reaches a maximum in late G_1, just before the initiation of DNA replication. The rate then declines by about 40% through the remainder of the cell cycle and reaches its initial, early G_1 value just before mitosis. This is in sharp contrast to the incorporation of leucine-^{14}C into 0.25 N H_2SO_4–soluble nuclear proteins which reaches a maximum rate at mid–S phase, which is six times the rate observed in G_1 or G_2 and closely parallels the variation in the rate of thymidine-^{14}C incorporation into DNA (Figs. 1 A and 1 B). The change in the rate of leucine-^{14}C incorporation into total cellular protein follows a pattern which is intermediate between that observed for the acid-soluble nuclear protein fraction and that for the residual nuclear protein fraction, that is, it reaches a peak in S but does so before the peak of DNA replication. Even though thymidine block has been reported to produce unbalanced growth (6, 7, 15), the data on the pulse-label rate of incorporation shown in Figs. 4 A and 4 B, which reflect the synthesis and transport of labeled proteins into the nucleus at different times in selectively detached and in thymidine-synchronized cells, are in good agreement with each other at overlapping portions of the cell cycle. These data are also in substantial agreement with the data on the rate of accumulation shown in Fig. 3 C, which reflect a combination of synthesis, transport, and retention of proteins in the nucleus. Thus, both of these kinds of evidence indicate that, in contrast to that of histones (19, 28), the synthesis and trans-

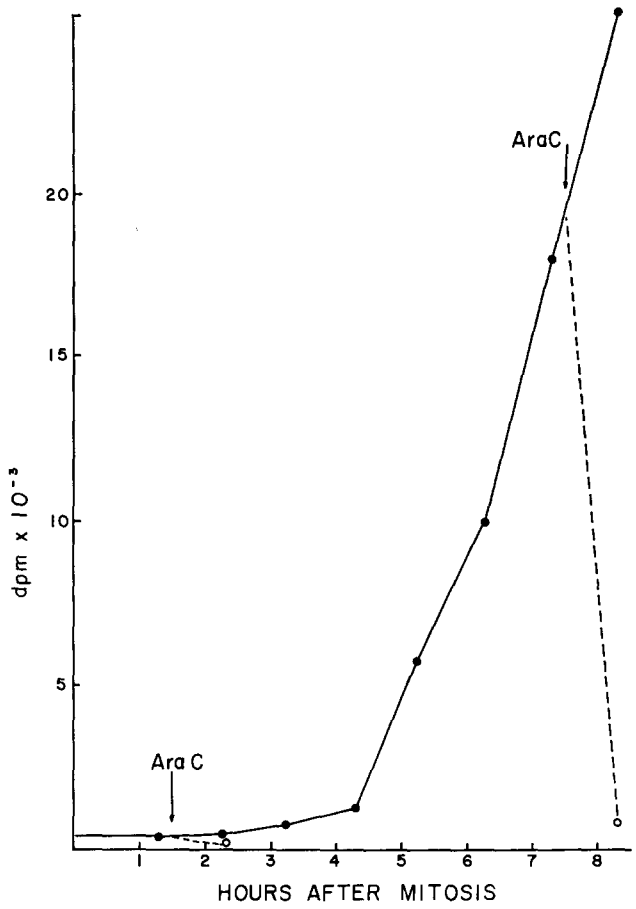


FIGURE 5 Effect of cytosine arabinoside on the incorporation of thymidine-^{14}C into DNA. At 1.5 (G_1) and 7.5 (S) hr after mitotic selective detachment, samples of cells were treated with cytosine arabinoside (final concentration 40 μg/ml). 30 min after addition of the drug, the rate of DNA synthesis was determined by labeling 2 ml of cells at 5 $\times$ 10^5/ml for 30 min with 0.2 μCi of thymidine-^{14}C and determining the amount of radioactivity incorporated into 20% TCA-precipitable material (○--○--○). The rate of DNA synthesis was also determined in untreated G_1 and S cells (●——●——●) at various times after mitotic selective detachment. There was a minimum of six determinations for each point.

port of proteins to the tightly bound residual nuclear fraction is continuous through the cell cycle and is not tightly coupled to DNA replication. It should be pointed out that these results are representative of many similar experiments carried out in this laboratory over a period of 18 months in which a variety of amino acids-^{14}C and ^{3}H were used to label proteins.

Effect of the Inhibition of DNA Replication on Nuclear Protein Synthesis

Since it has been previously demonstrated that HeLa S-3 histone polypeptide synthesis is restricted almost entirely to the S phase of the cell cycle and ceases if DNA replication is inhibited (19, 28), it is of interest to determine whether the synthesis of residual nuclear proteins occurring in S is affected by the inhibition of DNA replication. Cytosine arabinoside (ara C) is a potent inhibitor of HeLa cell DNA replication (5), and at a concentration of 40 μg/5 $\times$ 10^5 cells per ml it has no significant effect on the bulk rate of RNA or protein synthesis. At 1.5 (G_1) and 7.5 (early S) hr after selective detachment, 2.5 $\times$ 10^7 cells in 50 ml of SM were treated with 40 μg/ml of ara C for 30 min, pulse labeled with lysine-^{14}C and tryptophan-^{3}H for 30 min, and then fractionated as described

TABLE I

Effect of Cytosine Arabinoside (AraC) on HeLa S-3 Cell Protein Synthesis

Fraction	dpm/10^6 cells			
	G_1 (2 hr)		S (8 hr)	
	Tryptophan-^{3}H	Lysine ^{14}C	Tryptophan-^{3}H	Lysine-^{14}C
Total cell control	557,000	74,000	574,000	88,000
Total cell Ara C	534,000	69,000	577,000	83,000
0.25 N H_2SO_4 control	11,000	5,000	11,000	15,000
0.25 N H_2SO_4 Ara C	16,000	5,000	14,000	4,000
Residual control	60,000	13,000	49,000	11,000
Residual Ara C	66,000	14,000	51,000	8,000

The rates of tryptophan and lysine incorporation into total cellular and nuclear proteins were determined during the G_1 and S phases of the cell cycle (2 and 8 hr after mitotic selective detachment), by labeling 2.5 × 10^7 cells with L-tryptophan-^{3}H (10 μCi/ml) and L-lysine-^{14}C (1 μCi/ml) for 30 min after 30 min of treatment with cytosine arabinoside (final concentration 40 μg/ml). The rates of L-tryptophan-^{3}H and L-lysine-^{14}C incorporation were also determined in untreated controls. The nuclei were isolated and the proteins were fractionated as described in Methods. There was a minimum of three determinations for each point.

in the Materials and Methods. Fig. 5 shows that a 30 min pretreatment of the cells with ara C has no effect on the minimal G_1 rate of thymidine-^{14}C incorporation but reduces the rate of S phase thymidine incorporation by 95% to the G_1 background level. Table I summarizes the effect of ara C treatment on the incorporation of lysine-^{14}C and tryptophan-^{3}H in G_1 and S cells. Neither drug treatment nor phase of the cell cycle has much affect on the rate of tryptophan-^{3}H incorporation into total cellular or 0.25 N H_2SO_4–soluble nuclear proteins. Tryptophan incorporation into nuclear residual proteins is about 16% higher in G_1 than in S, but it is not significantly affected by ara C in either phase of the cell cycle. While lysine-^{14}C incorporation into total cellular protein increases only about 10% in the transition from G_1 to S and is not affected by ara C treatment, lysine incorporation into the 0.25 N H_2SO_4–soluble nuclear fraction increases threefold at this point in S over its G_1 rate, and this increased rate of incorporation is sensitive to the inhibition of DNA replication. The rate of lysine-^{14}C incorporation into the residual nuclear protein fraction is somewhat higher in G_1 than in S but, while not sensitive to ara C in G_1, it is reduced by about 27% in S when DNA replication is inhibited.

The lack of an ara C effect on the rate of tryptophan-^{3}H incorporation into S phase residual nuclear protein at the same time that a 27% inhibition of lysine-^{14}C incorporation into this fraction is observed, suggests that the residual nuclear fraction might contain a histone-like component which is sensitive to the inhibition of DNA replication. Conversely, the lack of an ara C effect on tryptophan incorporation into the acid-soluble (crude histone) nuclear fraction, together with the absence of tryptophan in histones (8, 9), strongly suggests that the 0.25 N H_2SO_4–soluble nuclear protein fraction is contaminated with nonhistone polypeptides. To more clearly demonstrate the latter two points, the acid-soluble and residual nuclear proteins labeled with tryptophan-^{3}H and lysine-^{14}C in this experiment were resolved according to molecular weight on SDS acrylamide gels (17, 25) as described in the Materials and Methods. Figs. 6 A–D show the distribution of tryptophan-^{3}H and lysine-^{14}C radioactivity in different proteins of the 0.25 N H_2SO_4–soluble nuclear fraction from control and ara C–treated G_1 and S phase cells. It is clear that there is tryptophan-^{3}H–containing protein distributed throughout each of the gels, that incorporation of tryptophan into these proteins is not dependent upon DNA replication, and that the rate of this incorporation is similar in G_1 and S. Lysine-^{14}C incorporation, on the other hand, while occurring at a low level during G_1 which is not sensitive to ara C treatment, increases dramatically in S and is found to largely occur in one of two principal

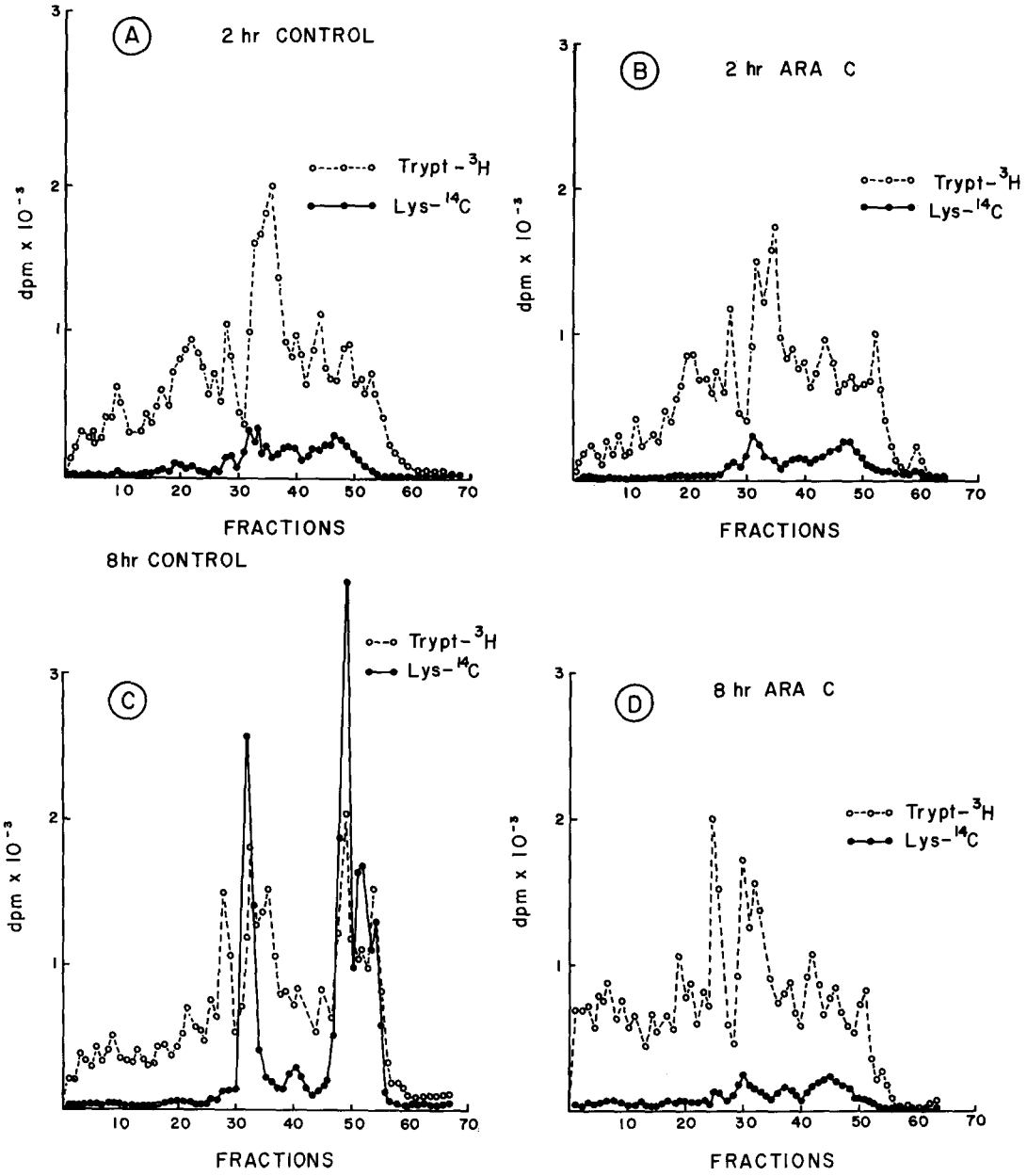


FIGURE 6 (A) SDS–polyacrylamide gel electrophoretic profiles of L-tryptophan-^{3}H– and L-lysine-^{14}C–labeled G_1 phase 0.25 N H_2SO_4–soluble nuclear proteins. 2.5×10^7 cells were labeled for 30 min with L-tryptophan–^{3}H (10 μCi/ml) and lysine-^{14}C (1 μCi/ml) at 2 hr after mitotic selective detachment (G_1). The nuclei were isolated, the nuclear proteins were extracted, and electrophoresis was carried out as described in Methods.

(B) Effect of cytosine arabinoside on the SDS–polyacrylamide gel electrophoretic profiles of L-tryptophan-^{3}H– and L-lysine-^{14}C–labeled G_1 0.25 N H_2SO_4–soluble nuclear proteins. The same procedures described in Fig. 6 A were used, except that the cells were treated with cytosine arabinoside (final concentration 40 μg/ml) 30 min before labeling.

(C) SDS–polyacrylamide gel electrophoretic profiles of L-tryptophan-^{3}H– and L-lysine-^{14}C–labeled S phase (8 hr after mitotic selective detachment) 0.25 N H_2SO_4–soluble nuclear proteins. The same procedures described in Fig. 6 A were used.

(D) Effect of cytosine arabinoside on the SDS–polyacrylamide gel electrophoretic profiles of L-tryptophan-^{3}H– and L-lysine-^{14}C–labeled S phase (8 hr after mitotic selective detachment) 0.25 N H_2SO_4–soluble nuclear proteins. The same procedures described in Fig. 6 A were used, except that the cells were treated with cytosine arabinoside (final concentration 40 μg/ml) 30 min before labeling.

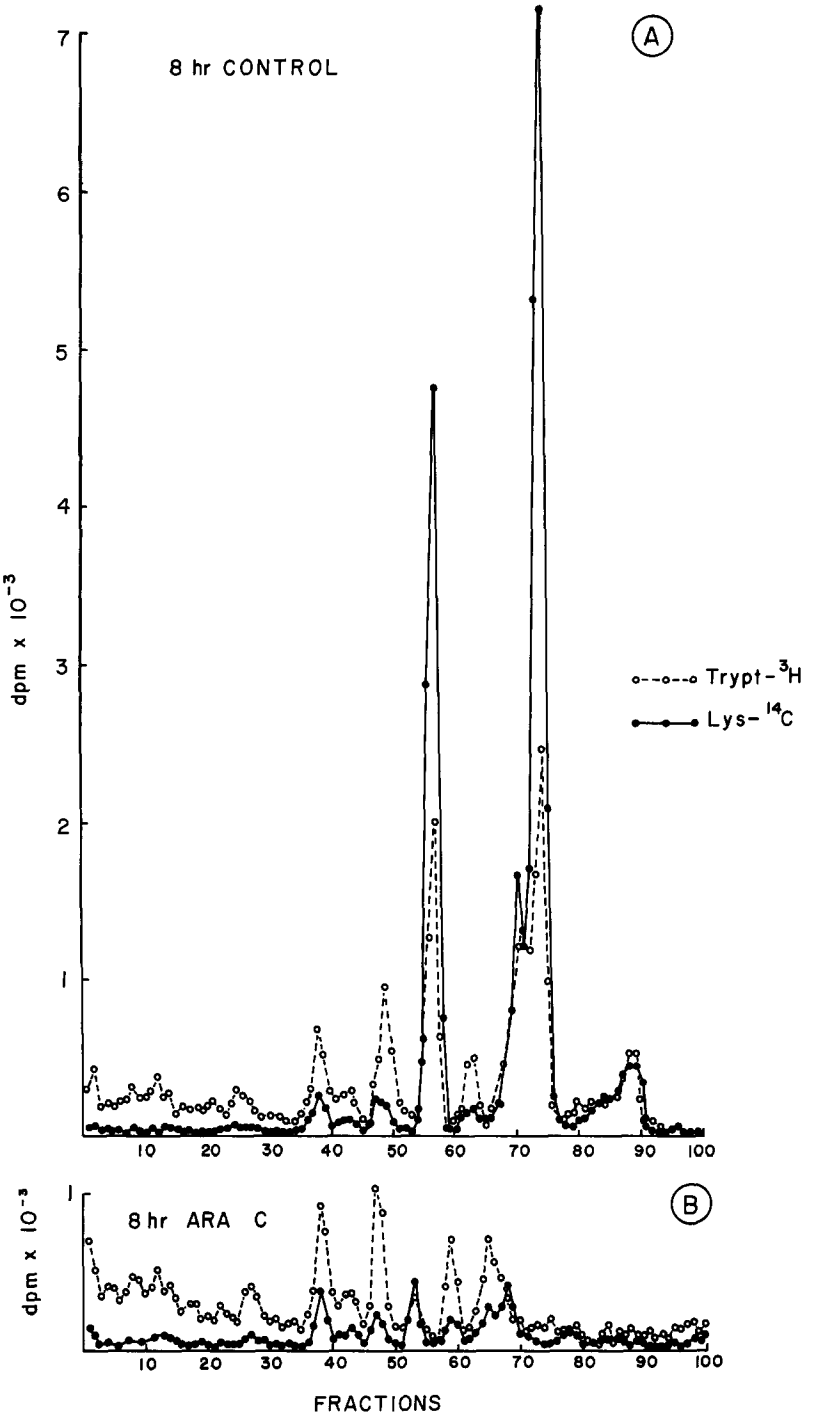


FIGURE 7 (A) Polyacrylamide gel electrophoretic profiles of L-tryptophan-^{3}H– and L-lysine-^{14}C–labeled S phase (8 hr after mitotic selective detachment) 0.25 N H_2SO_4–soluble nuclear proteins, dialyzed overnight against 0.9 N acetic acid. Electrophoresis was carried out on acetic acid–urea gels, 20 cm long, 5 mm in diameter, according to the method of Panyim and Chalkley (18), at 2 mA per gel for 11 hr. Gels were mechanically fractionated and processed as described in the Methods.

(B) Effect of cytosine arabinoside on the polyacrylamide gel electrophoretic profiles of L-tryptophan-^{3}H– and L-lysine-^{14}C–labeled S phase (8 hr after mitotic selective detachment) 0.25 N H_2SO_4–soluble nuclear proteins. The same procedures described in Fig. 7 A were used, except that the cells were treated with cytosine arabinoside (final concentration 40 μg/ml) 30 min before labeling.

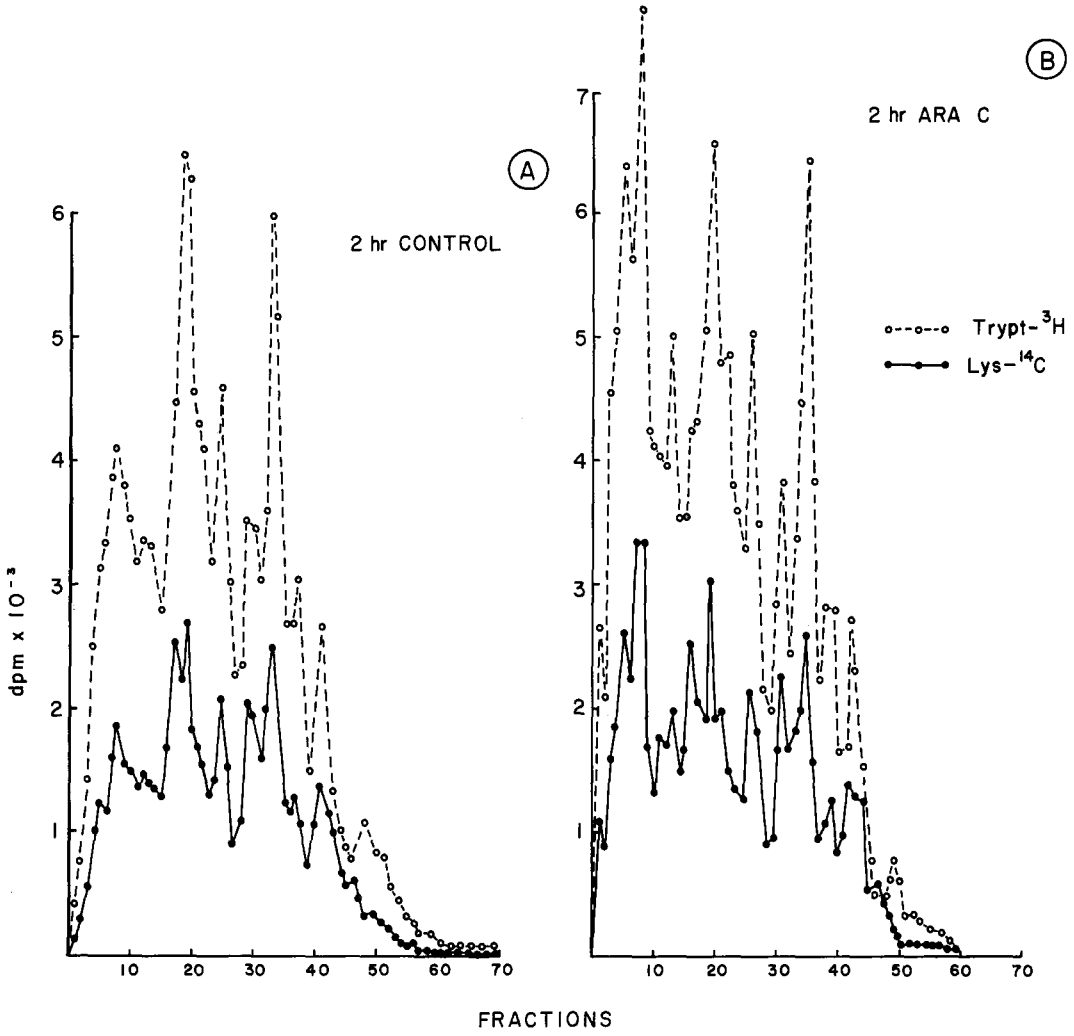


FIGURE 8 (A) SDS–polyacrylamide gel electrophoretic profiles of L-tryptophan-^{3}H– and L-lysine-^{14}C–labeled G_1 phase (2 hr after mitotic selective detachment) residual acidic nuclear proteins. The same procedures as described in Fig. 6 A were used.

(B) Effect of cytosine arabinoside on the SDS–polyacrylamide gel electrophoretic profiles of L-tryptophan-^{3}H– and L-lysine-^{14}C–labeled G_1 phase (2 hr after mitotic selective detachment) residual acidic nuclear proteins. The same procedures described in Fig. 6 A were used, except that the cells were treated with cytosine arabinoside (final concentration 40 μg/ml) 30 min before labeling.

groups of polypeptides. The more rapidly migrating group has a molecular weight of about 12,000 when compared with marker protein standards[3] and contains the f_{2a1}, f_{2a2}, f_{2b}, and f_3 monomer fractions of HeLa S-3 histones (11). The more slowly migrating species, which run to about the middle of the gel under these conditions, have a molecular weight of about 24,000 and contain the f_1 and possibly the f_3 dimer histone fractions.[3] As has been previously shown (3), lysine-^{14}C incorporation into these polypeptides decreases to background G_1 levels within 30 min of the inhibition of DNA replication with ara C (cf. Figs. 6 A, B, and D with Fig. 6 C). While the nature of this background incorporation is unknown, it probably accounts for reports of net histone synthesis in G_1 (12, 22, 23). Since SDS acrylamide

[3] Borun, T. W. Unpublished results.

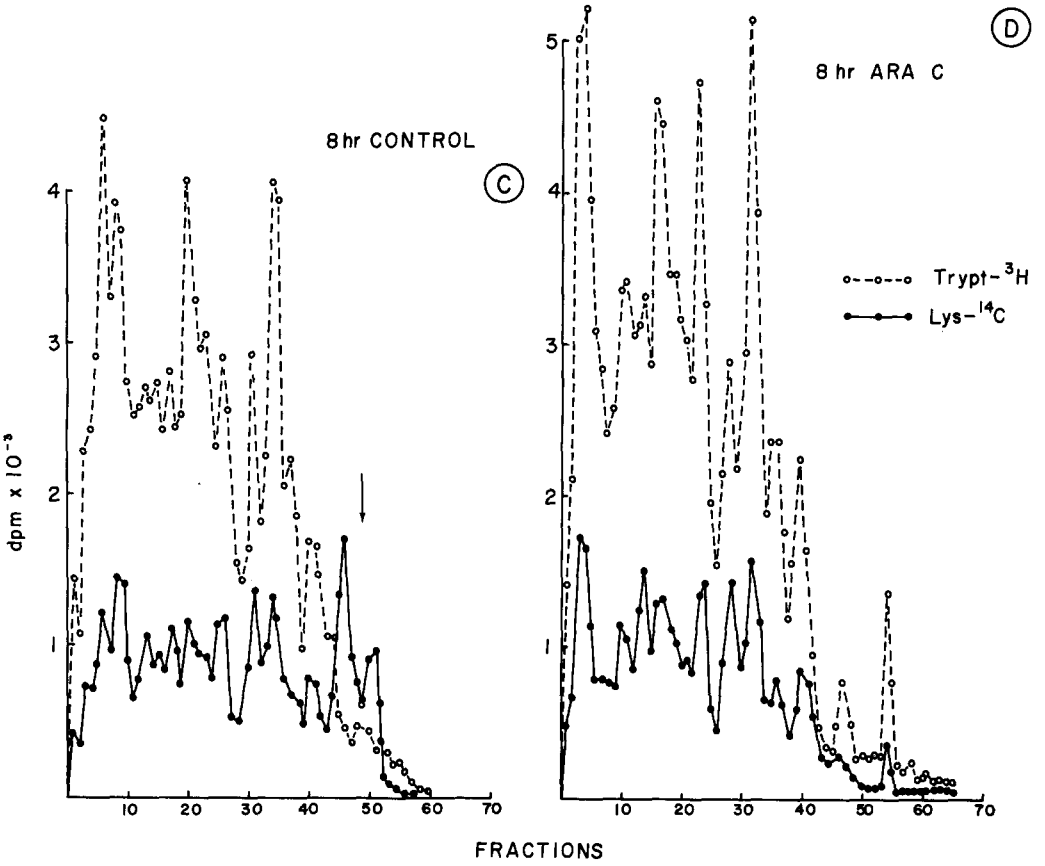


FIGURE 8 (C) SDS-polyacrylamide gel electrophoretic profiles of L-tryptophan-^{3}H– and L-lysine-^{14}C–labeled S phase (8 hr after mitotic selective detachment) residual acidic nuclear proteins. The same procedures described in Fig. 6 A were used.

(D) Effect of cytosine arabinoside on the SDS–polyacrylamide gel electrophoretic profiles of L-tryptophan-^{3}H– and L-lysine-^{14}C–labeled S phase (8 hr after mitotic selective detachment) residual acidic nuclear proteins. The same procedures described in Fig. 6 A were used, except that the cells were treated with cytosine arabinoside (final concentration 40 μg/ml) 30 min before labeling.

gel electrophoresis resolves proteins according to their molecular weight, irrespective of their charge, it was thought useful to try to resolve out the negatively charged components of the tryptophan-^{3}H–, lysine-^{14}C–labeled acid-soluble nuclear fraction on 15% acrylamide gels, using the high resolution acetic acid–urea method of Panyim and Chalkley (18). A comparison of Figs. 6 A–D and Figs. 7 A–B shows that a large amount of the tryptophan-^{3}H–containing proteins which enter the SDS gels do not migrate into these gels. However, some tryptophan radioactivity, not sensitive to ara C treatment, does co-electrophorese with the histone polypeptides resolvable by this method. The nature of these histone-associated, tryptophan-containing proteins, which occur in trace amounts, is at present unknown.

Electrophoresis of the residual nuclear fraction of control and ara C–treated G_1 and S phase cells labeled with tryptophan-^{3}H and lysine-^{14}C shows that this fraction is quite heterogeneous and confirms the hypothesis that the residual fraction contains histone-like polypeptides whose synthesis is sensitive to the inhibition of DNA replication. Figs. 8 A–D also show that, except for a group of rapidly migrating polypeptides which have the electrophoretic mobility in these SDS acrylamide gels of histone fractions f_{2a1}, f_{2a2}, f_{2b}, and f_3 (indicated by the arrow in Fig. 8 C), neither the

tryptophan-^{3}H nor the lysine-^{14}C incorporation into the many other polypeptides found in the nuclear residual fraction in G_1 or S seems to be sensitive to ara C treatment. The histone-like polypeptides are found only in the S phase residual nucleus (Fig. 8 C); they account for about 25% of the total lysine incorporation into this fraction at this time, and this incorporation stops when DNA replication is inhibited (Fig. 8 D and Table I). Since these nuclei have been exhaustively extracted with 0.25 N H_2SO_4 (see Materials and Methods), the retention of histone-like material in the residual fraction may reflect some special state of this material in the nucleus. In any event, these data clearly demonstrate that the majority of nonhistone residual nuclear proteins can be synthesized in S phase at normal rates when DNA replication is inhibited.

Co-Electrophoresis of Residual Nuclear Proteins fro G_1, S, and G_2 Cells

To determine if stage-specific differences in the rate of synthesis of different proteins could be demonstrated, 1.25×10^7 S phase HeLa S-3 cells, pulse labeled for 30 min with leucine-^{14}C, were mixed with the same numbers of G_1 or G_2 cells labeled for 30 min with leucine-^{3}H and were fractionated as described in the Materials and Methods. The residual nuclear fractions of these cell mixtures were then electrophoresed on SDS acrylamide gels. Figs. 9 A and B show that there are some stage-specific differences demonstrable by this technique. Peak *C* is present in both G_1 and S but relatively more of it seems to be synthesized in G_2. Peak *E* is present to a significant extent in S and G_2 but appears to be absent in G_1. As has been shown in the preceding section, peak *J*, here labeled with leucine-^{14}C, is found only in S and probably represents some nonacid extractable form of histone polypeptide.

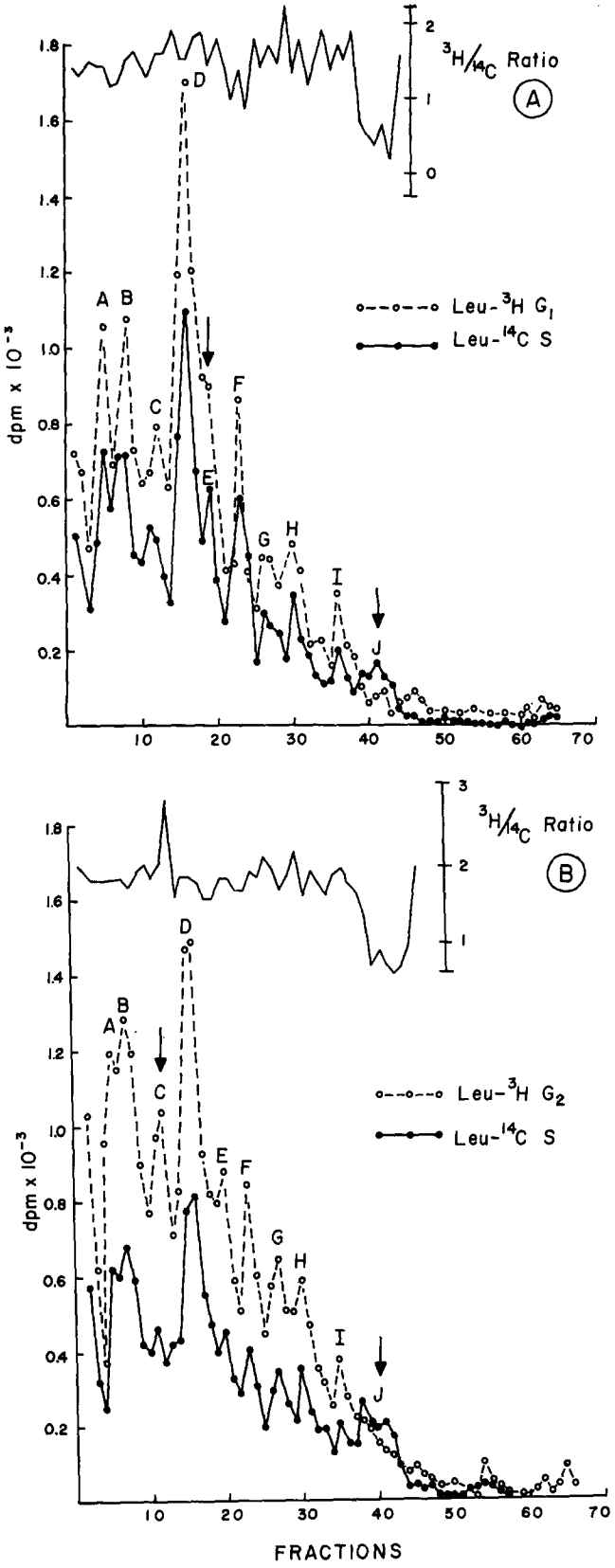
DISCUSSION

The leucine-^{14}C incorporation data presented here demonstrate that synthesis and accumulation of acidic proteins in the tightly bound residual nuclear fraction goes on throughout the cell cycle of continuously dividing populations of synchronized HeLa S-3 cells. After mitosis, this synthesis and accumulation increases by about 40% to its maximum rate in late G_1, just before the initiation of DNA replication. It then continues at a slowly declining rate through S and G_2 until it reaches its initial early G_1 rate, just before mitosis. It has been previously shown that the synthesis of HeLa histone polypeptides occurs almost exclusively in S phase and rapidly ceases if DNA replication is inhibited by treatment with cytosine arabinoside (3). In contrast to HeLa histone synthesis, the synthesis of most of the polypeptides found in the tightly bound residual nuclear fraction is not coupled to nor dependent upon DNA replication. The lack of coordination of DNA replication and residual nuclear protein synthesis was suggested by Zampetti-Bosseler et al. (38) who showed that the specific activity of these proteins was not affected by hydroxyurea; however, their experiments were done on randomly growing HeLa cells, and these nonhistone nuclear proteins were not separated electrophoretically.

Electrophoresis of the residual nuclear proteins from G_1, S, and G_2 cells labeled with various radioactive amino acids reveals several stage-specific peaks in the radio-electropherograms of the SDS acrylamide gels. These peaks include what appear to be lysine-rich, tryptophan-deficient, histone-like polypeptides which have the electro-

FIGURE 9 (A) SDS–polyacrylamide gel electrophoretic profiles of L-leucine-^{14}C–labeled S phase and L-leucine-^{3}H–labeled G_1 residual acidic nuclear proteins. 1.25×10^7 S phase cells at 3 hr after reversal of the second of 2 cycles of 2 mM thymidine block were labeled for 30 min with L-leucine-^{14}C (2 μCi/ml). 1.25×10^7 G_1 cells at 2 hr after selective detachment were labeled for 30 min with L-leucine-^{3}H (5 μCi/ml). The G_1 and S samples were combined, the nuclei were isolated, the nuclear proteins were extracted, and electrophoresis was carried out as described in Methods. The upper curve represents the ^{3}H to ^{14}C ratios throughout the gel.

(B) SDS–polyacrylamide gel electrophoretic profiles of L-leucine-^{14}C–labeled S phase and L-leucine-^{3}H G_2 residual acidic nuclear proteins. The same procedures described in Fig. 9 A were used, except that the G_2 sample was obtained using 1.25×10^7 cells at 8 hr after the reversal of the second of 2 cycles of 2 mM thymidine block.


$^3H/^{14}C$ Ratio
A
B
Leu-^3H G_1
Leu-^{14}C S
dpm x 10^{-3}
A B C D E F G H I J
$^3H/^{14}C$ Ratio
B
Leu-^3H G_2
Leu-^{14}C S
dpm x 10^{-3}
A B C D E F G H I J
FRACTIONS

phoretic mobility of several authentic histones but are not extractable from the nucleus with dilute mineral acid. Like the synthesis of acid-soluble histones, the synthesis of these polypeptides occurs only in S phase and is tightly coupled to DNA replication.

Electrophoresis of the 0.25 N H_2SO_4–soluble nuclear fraction of G_1 and S phase cells labeled with tryptophan-^3H and lysine-^{14}C shows that these extracts contain at least trace amounts of tryptophan-containing nonhistone proteins which actively incorporate other amino acids and co-electrophorese with histone polypeptides, even when the latter are resolved by their net positive charge on acetic acid-urea–containing acrylamide gels. As is the case with the residual nuclear protein fraction, these acid-soluble, nonhistone polypeptides are synthesized throughout the cell cycle, and their synthesis is not dependent upon DNA replication in S phase.

The present data establish that an increased rate of synthesis and accumulation of acidic residual nuclear proteins in late G_1 precedes the initiation of DNA replication in a cell that divides continuously, without apparent external stimulation. While similar to correlations previously observed in a wide variety of stimulated cell systems, such as hormone-induced uterine proliferation (26, 35), isoproterenol-stimulated rodent salivary gland proliferation[1] (30), serum-induced proliferation of contact-inhibited fibroblasts (21), and the lactating mammary gland (34), the present observations complicate speculations as to the role of acidic residual nuclear proteins in cellular regulation of mammalian DNA replication. Since the increased synthesis and accumulation of these proteins occur both in stimulated cells that are about to begin DNA replication before a round of induced cell division and, as a matter of course, in continuously dividing cells in late G_1, it cannot be maintained that this phenomenon is a *unique* characteristic of stimulated proliferating systems. Indeed, the converse may be true; that is, the phenomenon may be characteristic of a certain state in all cells that are about to begin DNA replication, and stimulation may merely transform the quiescent cell into this state. Thus, in spite of the strong correlation between the increased synthesis and accumulation of acidic residual nuclear proteins and the initiation of DNA replication, it is not yet possible to determine if the former is a fairly direct cause of the latter or whether both are merely effects of some other unknown cause.

The presence of nonhistone proteins in dilute mineral acid extracts of HeLa cell nuclei is consistent with some of the observations of Sadgopal and Bonner (22, 23). Since these nonhistone proteins are synthesized throughout the cell cycle and can contaminate histones prepared by chromatography on Amberlite CG-50 (22, 23) differential precipitation (14), as well as those resolved by acrylamide gel electrophoresis, they may be responsible for reports of significant histone synthesis in G_1 (12, 22, 23) which the present HeLa cell data clearly argue against (see Fig. 3 B). It is possible that the tryptophan-containing polypeptides co-electrophoresing with histones reported here are similar to the nonhistone polypeptides apparently covalently linked to histones in estrogen-stimulated uterus which have been reported by Barker (1).

It is not clear whether the labeled histone-like polypeptides found in the residual nuclear fraction during S phase are an artifact of acid extraction, as suggested by the work of Sonnenbichler and Norbis (27), or perhaps represent histone-regulator complexes of some sort which are insoluble in dilute mineral acids but become dissociated by treatment with the strong detergent SDS. The latter hypothesis would thus imply that a given histone can have different states of association with the chromosome, perhaps depending upon or being influenced by subtle modifications of the polypeptide backbone such as phosphorylation, methylation, or acetylation of the histone.

While still other speculations are possible, it is probably best to conclude here by emphasizing that all the data presented here, however diverse, are consistent with the hypothesis that the acidic residual nuclear proteins, unlike histones whose synthesis appears to be highly regulated, have *some* regulatory function, the exact nature of which is not yet clearly seen.

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