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

II. The Kinetics of Residual Protein Synthesis and Transport

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ABSTRACT

The kinetics of acidic residual chromosomal protein synthesis and transport were studied throughout the cell cycle in HeLa S-3 cells synchronized by 2 mM thymidine block and selective detachment of mitotic cells. Pulse labeling the cells with leucine-^{3}H for 2 min and then "chasing" the radioactive proteins for up to 3 hr showed that the amount of protein synthesized, transported, and retained in the acidic residual chromosomal protein fraction is greater immediately after mitosis and later in G_1 than in the S or G_2 phases of the cell cycle. During S, only 20–25% of the proteins synthesized and transported to the acidic residual chromosomal protein fraction are chased during the first 2 hr after pulse labeling, whereas up to 40% of the material entering the residual nuclear fraction in mitosis, G_1, and G_2 leaves during a 2 hr chase. Polyacrylamide gel electrophoretic profiles of these proteins, at various times after pulse labeling, reveal that the turnover of individual polypeptides within this fraction has kinetics of synthesis and turnover which are markedly different from one another and undergo stage-specific changes.

INTRODUCTION

A number of investigators have reported that a significant increase in the rate of incorporation of radioactive amino acids into tightly bound acidic nuclear proteins occurs before the initiation of DNA replication in cells stimulated to proliferate by hormones[1] (9, 10, 14), serum (7), or explantation (13). In the preceding paper (12), we demonstrated that the maximum rate of pulsed leucine-^{14}C-incorporation into acidic residual nuclear proteins and the maximum rate of accumulation of proteins in this fraction both occur in late G_1, just before the initiation of DNA replication in continuously dividing cells. To understand the significance of these observations in stimulated and continuously dividing cells, it is necessary to determine what an increased rate of incorporation of amino acids into tightly bound acidic nuclear proteins represents. Since the majority, if not all, of the acidic proteins remaining in the nucleus after exhaustive dilute salt and acid extractions are synthesized in the cytoplasm in HeLa S-3 cells (11), it is evident that these residual nuclear proteins are transported to the nucleus after synthesis. Further, it is probable that after some time in the nucleus a number of proteins are degraded or transported again back into the cytoplasm. It is, therefore, clear that an increased rate of pulsed leucine-^{14}C-incorporation into the acidic residual nuclear

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

fraction could theoretically represent an increased rate either of synthesis, transport, or retention of proteins in this fraction, or some combination of these processes.

In the present paper we attempted the resolution of this problem by pulse labeling the HeLa S-3 cells for a very short period of time and "chasing" the radioactively labeled proteins in various nuclear fractions for relatively long times at different stages of the cell cycle. The nuclear residual fraction was resolved on sodium dodecyl sulfate (SDS)–acrylamide gels in these experiments to determine if different proteins in this fraction had different kinetics of synthesis and turnover.

MATERIALS AND METHODS

Cell Culture and Synchronization

The culture (2) and synchronization of HeLa S-3 cells by selective detachment of mitotic cells (15, 6) and 2 mM thymidine block (1, 16) have been reported before and are detailed in the preceding paper (12).

Leucine-^{3}H Pulse-Chase Labeling at Different Stages of the HeLa S-3 Cell Cycle

To pulse-label cells in mitosis and G_1, 1×10^8 cells, just after selective detachment (mitosis) and then at 2 hr later (G_1), were harvested by centrifugation at 600 *g* for 3 min at 37°C and resuspended at the same temperature in 25 ml of Joklik-modified Eagle's Minimal Essential Spinner Medium (SM)[2] (2) minus leucine, plus 250 μCi leucine-^{3}H and 2% fetal calf serum for 2 min. The 4 ml "pulse" sample, containing 1.6×10^7 cells, was then withdrawn, diluted into 45 ml of ice-cold spinner salts solution, and harvested by centrifugation at 600 *g* for 3 min at 3°C. The remaining cells were diluted into 210 ml of SM at 37°C, harvested by centrifugation at 600 *g* for 3 min at 37°C, and were resuspended and incubated in 210 ml of SM at 37°C for up to 3 hr. During this chase period, 40-ml samples containing 1.6×10^7 cells were withdrawn at the indicated times and pelleted by centrifugation at 600 *g* for 3 min at 3°C. After washing three times in 50 ml of ice-cold spinner salts, all samples were fractionated into acid-soluble and residual nuclear protein fractions and were counted as described in the preceding paper (12). To obtain similar preparations of cells in early and late S as well as early G_2 phase of the HeLa S-3 cell cycle, samples of 1×10^8 cells, synchronized by two cycles of 2 mM thymidine block (1, 16), were harvested at 2 hr (early S), 5 hr (late S), and 7 hr (early G_2) after the reverse of the second thymidine treatment, and were labeled as described above.

[2] *Abbreviations used in this paper*: MEM, Eagle's Minimum Essential Medium; SDS, sodium dodecyl sulfate; SM, Eagle's Spinner Medium.

Electrophoresis of the Residual Nuclear Fraction

To obtain sufficient leucine-^{3}H–labeled material for electrophoresis of the residual nuclear fraction, 1×10^8 cells were harvested at 37°C as described above, at 2 hr after selective detachment of mitotic cells (G_1), at 2 hr after reverse of the second of two cycles of thymidine treatment (S), and at 7 hr after reverse of thymidine (G_2). Each preparation was resuspended in 20 ml of SM (2) minus leucine plus 400 μCi leucine-^{3}H and 2% fetal calf serum and incubated at 37°C for 2 min. The 6 ml pulse sample containing 3×10^7 cells was then withdrawn, diluted into 40 ml of ice-cold spinner salts, and harvested at 3°C as described above. The remaining cells were diluted with SM to a total volume of 230 ml and were harvested at 37°C as described above. After resuspension and incubation in 140 ml of SM at 37°C, chase samples of 60 ml containing 3×10^7 cells were withdrawn at 30 and 120 min after the end of the pulse period and were harvested by centrifugation at 600 *g* at 3°C for 3 min. After washing three times with 50 ml of ice-cold spinner salts, all of the samples were fractionated into acid-soluble and residual nuclear fractions as described in the preceding paper. The residual nuclear fraction was resuspended in 4 ml of 1% SDS and 0.01 M sodium phosphate buffer, pH 6.8, and was dialyzed against 0.1% SDS, 0.01 M sodium phosphate, pH 6.8 overnight. Samples of 0.25 ml of the preparation were mixed with 50 μl of 60% sucrose and were electrophoresed on 7.5% acrylamide, 0.2% bis acrylamide gels, 20 cm long and 6 mm wide, made up in 0.1 M sodium phosphate pH 6.8, 0.1% SDS tray buffer. Electrophoresis was carried out at 90 v (constant) for 11 hr, after which each gel was mechanically fractionated (4) into 70 scintillation vials and was counted in an Intertechnique Scintillation Spectrometer (Intertechnique, Dover, N. J.) as described in the preceding paper (12).

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 and amino acids were supplied by Sigma Chemical Co., St. Louis,

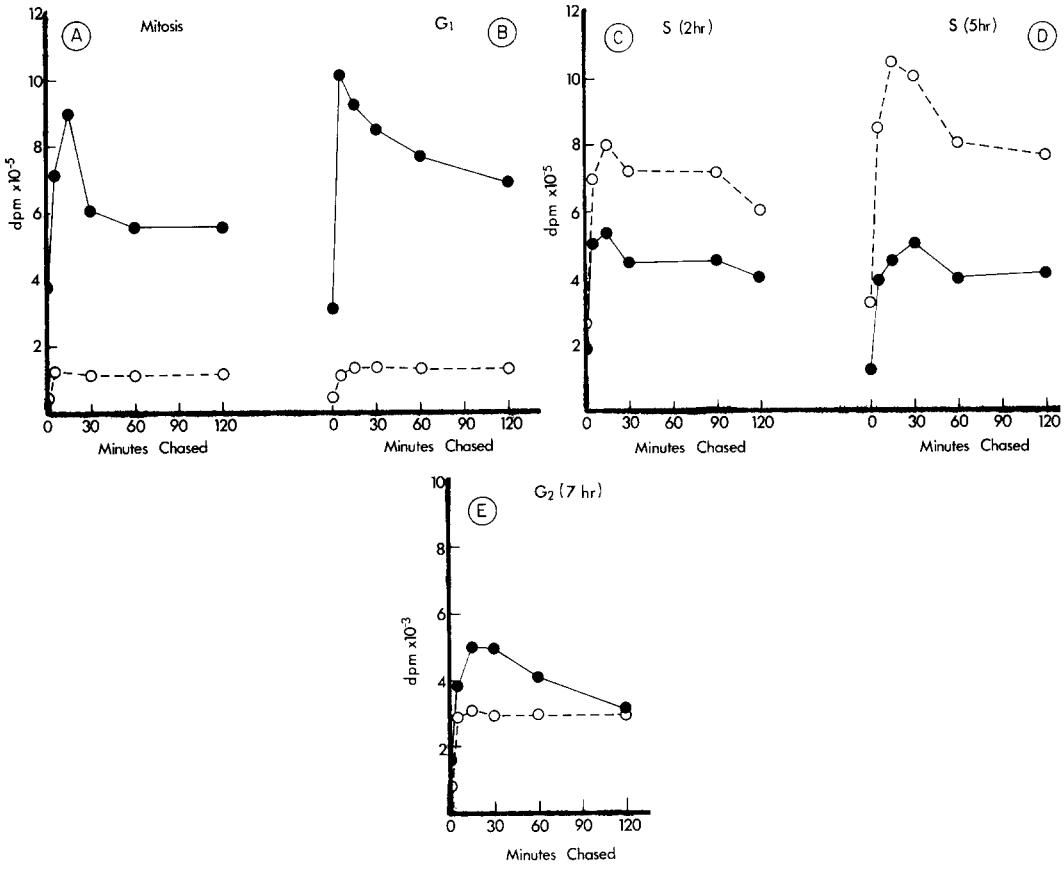


FIGURE 1 (A) Pulse-chase labeling of mitotic HeLa S-3 cells. Immediately after selective detachment, 1×10^8 mitotic cells were harvested and labeled at a concentration of 4×10^6 for 2 min in leucine-free SM containing 250 μCi *l*-leucine–^{3}H and 2% fetal calf serum. 1.6×10^7 cells (pulse) were then withdrawn, diluted into 45 ml of ice-cold spinner salts, and pelleted by centrifugation at 600 *g* for 3 min. The remaining cells were diluted into 210 ml of SM at 37°C, harvested by centrifugation at 600 *g* for 3 min at 37°C, and resuspended in 210 ml of SM. 1.6×10^7 of these cells were withdrawn at the chase times indicated on the abscissa, and all samples were processed as indicated in Materials and Methods. There was a minimum of six determinations for each point, and the range of values for each point did not exceed 10% on either side of the mean. 0.25 N H_2SO_4–soluble fractions (○--○--○); residual acidic nuclear proteins (●——●——●).

(B) Pulse-chase labeling of G_1 HeLa S-3 cells. 2 hr after selective detachment, 1×10^8 cells were harvested, pulse labeled, and chased as described in Fig. 1 A.

(C) Pulse-chase labeling of early S-phase HeLa S-3 cells. 2 hr after release from a double 2 mM thymidine block, 1×10^8 cells were harvested, pulse labeled, and chased as described in Fig. 1 A.

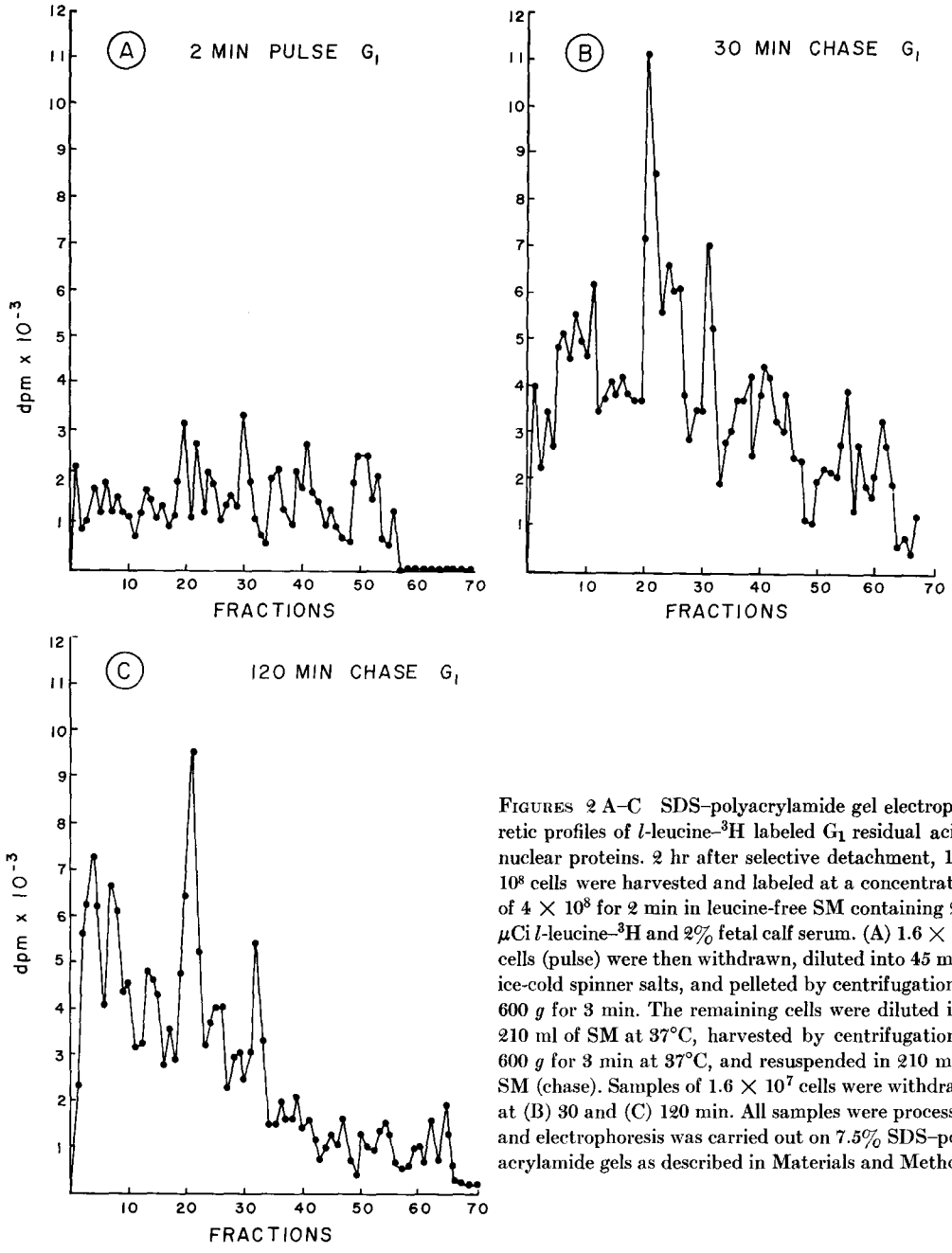
(D) Pulse-chase labeling of late S-phase HeLa S-3 cells. 5 hr after release from a double 2 mM thymidine block, 1×10^8 cells were harvested, pulse labeled, and chased as described in Fig. 1 A.

(E) Pulse-chase labeling of G_2 HeLa S-3 cells. 7 hr after release from a double 2 mM thymidine block, 1×10^8 cells were harvested, pulse labeled, and chased as described in Fig. 1 A.

Mo. L-leucine–^{3}H (58 Ci/mmole) was obtained from New England Nuclear Corporation, Boston, Mass.

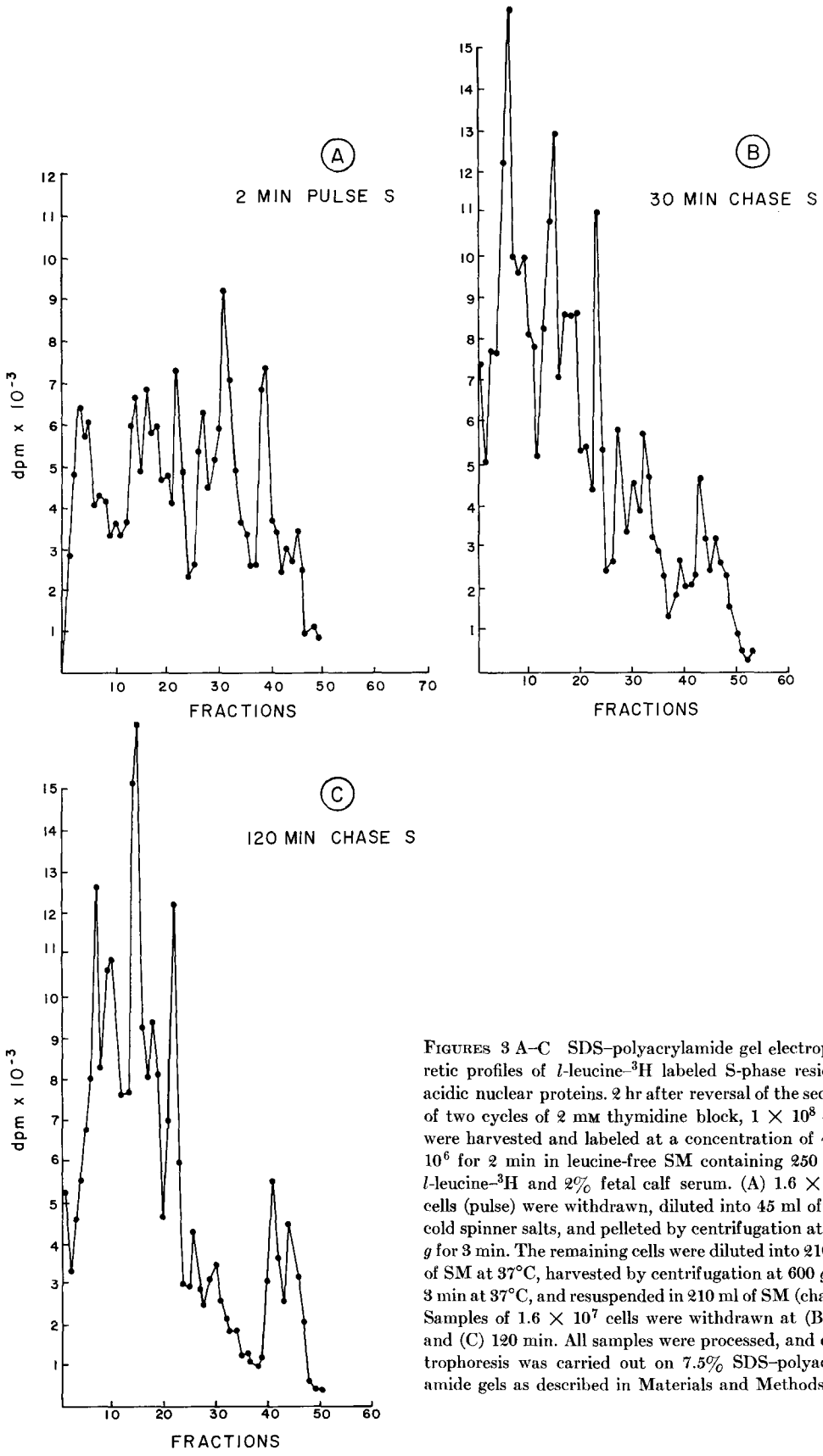
RESULTS

By pulse labeling cells with leucine-^{3}H for a short time, such as 2 min, and then chasing radioactive proteins for a relatively long time, such as 2 or 3 hr, one can observe the arrival of labeled protein in a given nuclear fraction and follow the redistributions of these proteins which occur during the chase period. Figs. 1 A–E show that the synthesis and transport of proteins into and out of the



FIGURES 2 A–C SDS–polyacrylamide gel electrophoretic profiles of *l*-leucine-^{3}H labeled G_1 residual acidic nuclear proteins. 2 hr after selective detachment, 1×10^8 cells were harvested and labeled at a concentration of 4×10^8 for 2 min in leucine-free SM containing 250 μCi *l*-leucine-^{3}H and 2% fetal calf serum. (A) 1.6×10^7 cells (pulse) were then withdrawn, diluted into 45 ml of ice-cold spinner salts, and pelleted by centrifugation at 600 *g* for 3 min. The remaining cells were diluted into 210 ml of SM at 37°C, harvested by centrifugation at 600 *g* for 3 min at 37°C, and resuspended in 210 ml of SM (chase). Samples of 1.6×10^7 cells were withdrawn at (B) 30 and (C) 120 min. All samples were processed, and electrophoresis was carried out on 7.5% SDS–polyacrylamide gels as described in Materials and Methods.

acid-soluble and residual nuclear fractions vary in a complex manner throughout the HeLa S-3 cell cycle. About twice as much protein, pulse labeled for 2 min with leucine-^{3}H, is synthesized, transported to, and retained in the residual nuclear fraction just after mitosis and in later G_1 than in either the S or G_2 phases of the cell cycle. These results are consistent with those reported in the preceding paper (12), even through the amount of protein chased out of the nuclear residual fraction by the end of 2 hr is twice as large in G_1 as in S or G_2. When DNA is being replicated in S, only about



FIGURES 3 A–C SDS–polyacrylamide gel electrophoretic profiles of *l*-leucine-^{3}H labeled S-phase residual acidic nuclear proteins. 2 hr after reversal of the second of two cycles of 2 mM thymidine block, 1×10^8 cells were harvested and labeled at a concentration of 4×10^6 for 2 min in leucine-free SM containing 250 μCi *l*-leucine-^{3}H and 2% fetal calf serum. (A) 1.6×10^7 cells (pulse) were withdrawn, diluted into 45 ml of ice-cold spinner salts, and pelleted by centrifugation at 600 *g* for 3 min. The remaining cells were diluted into 210 ml of SM at 37°C, harvested by centrifugation at 600 *g* for 3 min at 37°C, and resuspended in 210 ml of SM (chase). Samples of 1.6×10^7 cells were withdrawn at (B) 30 and (C) 120 min. All samples were processed, and electrophoresis was carried out on 7.5% SDS–polyacrylamide gels as described in Materials and Methods.

20–25% of the proteins synthesized and transported to the nuclear residual fraction leave again during the chase period, whereas up to 40% of the material entering in G_1 or G_2 leaves this fraction during the chase.

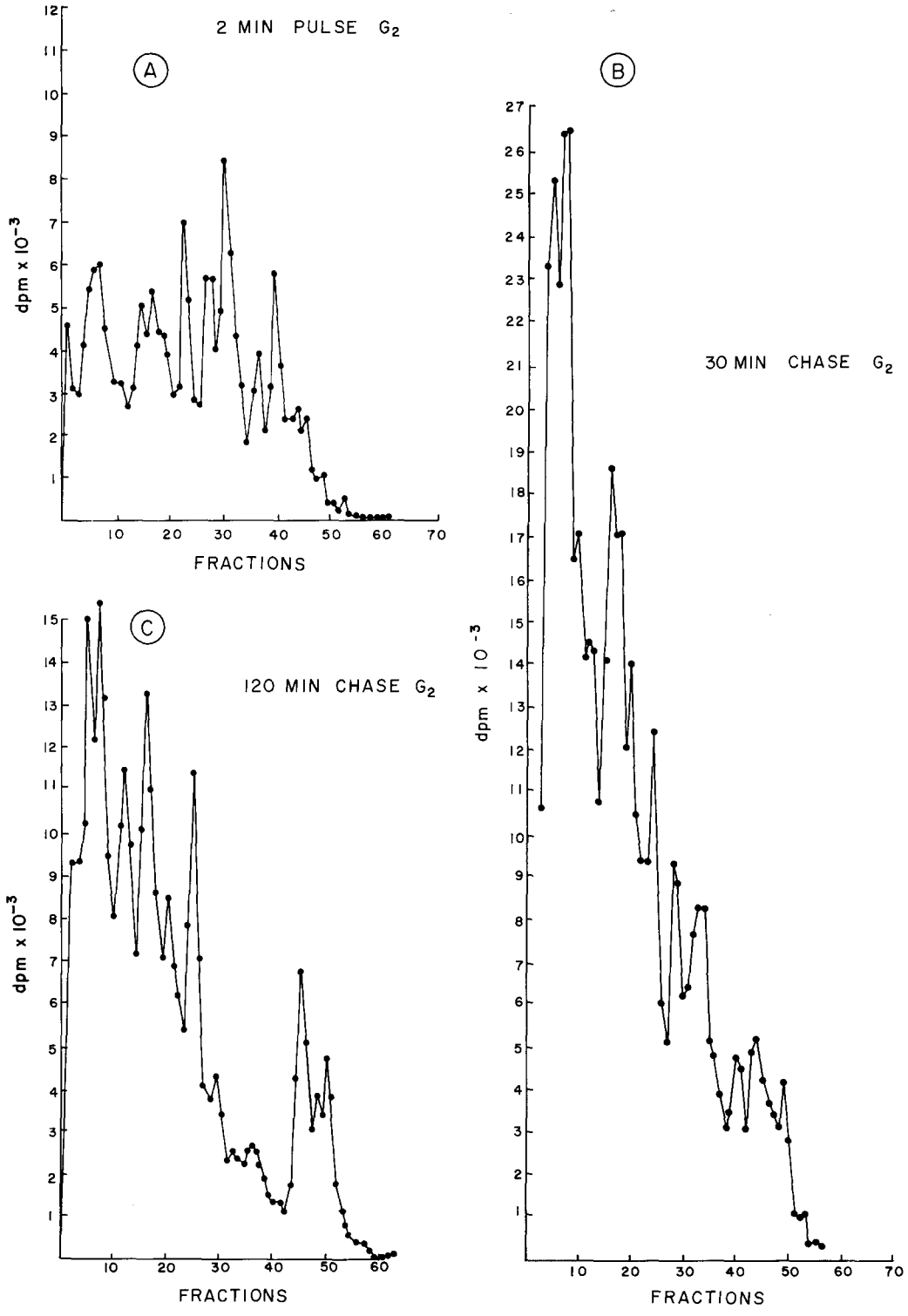
Nearly the opposite is true of the acid-soluble fraction. Although there is a low level of incorporation of leucine-^{3}H into the nonhistone proteins of the 0.25 N H_2SO_4-soluble nuclear fraction during G_1 and G_2, less than 10% of the material entering this fraction at these times leaves during the chase periods. When DNA replication commences in S, the incorporation of leucine-^{3}H into the acid-soluble nuclear fraction also begins to increase and it reaches levels at mid-S which are 10–12 times those observed in G_1 or G_2. During S, however, up to 25% of the total amount of pulse-labeled acid-soluble protein entering the nucleus leaves again during the subsequent chase period. Since it has been previously shown that HeLa S-3 histone polypeptides do not turn over at a rate of more than 1–2% per hour (3, 5), it is evident that the acid-soluble nuclear proteins chased out of this fraction during S phase are probably not histone polypeptides.

To determine if various proteins having different kinetics of synthesis and turnover could be resolved within the residual nuclear fraction, cells were pulse labeled for 2 min with leucine-^{3}H and then chased for 30 and 120 min in G_1, S, and G_2 as described in Materials and Methods. After isolation of the residual nuclear fraction from these cells, the proteins within the fractions were separated, according to molecular weight, on SDS–acrylamide gels (8) as described in the preceding paper (12). A comparison of the leucine-^{3}H incorporation profiles in Figs. 2 A–C, 3 A–C, and 4 A–C shows that heterogeneous polypeptides of many different molecular weights are synthesized at all times in the cell cycle shown here. Consistent with the pulse-chase data in Figs. 1 A–E, there are over-all stage-specific differences in the kinetic behavior of the nuclear fractions. For example, it is clear that S-phase cells retain more of the different kinds of polypeptides synthesized and transported to the residual nuclear fraction than do G_1 and G_2 cells. The gel electropherograms also demonstrate that individual polypeptide peaks within the residual nuclear fraction can have kinetics of synthesis and turnover which are very different from the kinetics of the bulk fraction of which they are a part. For example, in G_1 cells, relatively high molecular weight polypeptides running from the origin to about fraction 30 of the gels shown in Figs. 2 A–C tend to be retained in the residual nuclear fraction, whereas the smaller proteins running into gel fractions 31–60 are largely chased out of the nucleus by 120 min in these experiments. While most (75%) of the S-phase polypeptides made and transported to the residual nuclear fraction are retained during the chase period, the proteins migrating into fractions 25–35 of the gels shown in Figs. 3 A–C are largely chased out of the nucleus. Finally, G_2 residual nuclear proteins of high molecular weight have an extremely high turnover rate, whereas those proteins of a lower molecular weight which migrate into fractions 40–60 of the gels shown in Figs. 4 A–C are retained during the chase period.

DISCUSSION

The results presented here demonstrate that the maximal rate of leucine-^{14}C incorporation into the residual nuclear fraction of HeLa S-3 cells and the maximal rate of accumulation of proteins in this fraction in late G_1, which were reported in the preceding paper, most probably represent a definite acceleration of synthesis and transport of acidic proteins into the residual nuclear fraction of continuously dividing cells, just before the initiation of DNA replication. This accelerated incorporation and accumulation occur in spite of the fact that the most rapid transport of proteins out of the residual nuclear fraction also occurs in G_1 in the HeLa cell. As the cell proceeds into S phase and DNA replication commences, this rapid turnover of proteins declines somewhat and most of the polypeptides synthesized and transported to the residual fraction tend to remain there. A very rapid turnover of high molecular weight proteins begins again as the cells proceed from S phase to G_2.

It should be emphasized that the pulse chase and electrophoresis data presented here demonstrate that: (*a*) the residual nuclear fraction of HeLa S-3 cells is physically heterogeneous, containing polypeptides of many different molecular weights; (*b*) individual polypeptides within the residual nuclear fraction have kinetics of synthesis and turnover which are markedly different from one another and those of the fraction as a whole; and (*c*) the synthesis and turnover of proteins within the residual nuclear fraction undergo stage-specific changes, some of which may be related to



the initiation of DNA replication in continuously dividing cells in G_1, and perhaps in quiescent cells which have been stimulated to proliferate.

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Figures 4 A–C SDS–polyacrylamide gel electrophoretic profiles of *l*-leucine–^{3}H labeled G_2 residual acidic nuclear proteins. 7 hr after reversal of the second of two cycles of 2 mM thymidine block, 1×10^8 cells were harvested and labeled at a concentration of 4×10^6 for 2 min in leucine-free SM containing 250 μCi *l*-leucine–^{3}H and 2% fetal calf serum. (A) 1.6×10^7 cells (pulse) were then withdrawn, diluted into 45 ml of ice-cold spinner salts, and pelleted by centrifugation at 600 *g* for 3 min. The remaining cells were diluted into 210 ml of SM at 37°C, harvested by centrifugation at 600 *g* for 3 min at 37°C, and resuspended in 210 ml of SM (chase). Samples of 1.6×10^7 cells were withdrawn at (B) 30 and (C) 120 min. All samples were processed, and electrophoresis was carried out on 7.5% SDS–polyacrylamide gels as described in Materials and Methods.