DETECTION OF G_1 PROTEINS IN CHINESE HAMSTER CELLS SYNCHRONIZED BY ISOLEUCINE DEPRIVATION OR MITOTIC SELECTION

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ABSTRACT

Examination of labeling patterns of proteins in Chinese hamster cells (line CHO) revealed the presence of a class of protein(s) that is synthesized during G_1 phase of the cell cycle. Cells arrested in G_1 by isoleucine (Ile) deprivation were prelabeled with [^{14}C]Ile, induced to traverse G_1 by addition of unlabeled Ile, and labeled with [^{3}H]Ile at hourly intervals. Cells were fractionated into nuclear and cytoplasmic portions, and proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Gel profiles of proteins in the 45,000–160,000 mol wt range from the cytoplasm of cells in G_1 were similar to those from cells arrested in G_1 except for the presence of a major peak of [^{3}H]Ile incorporated into a protein(s) of approximately 80,000 mol wt. Peaks of net [^{3}H]Ile incorporation were not detected in nuclear preparations. Cellular fractionation by differential centrifugation showed that peak I protein was located in the soluble supernatant fraction of the cytoplasm. Time-course studies showed that synthesis of this protein began 1–2 h after initiation of G_1 traverse; the protein reached maximum levels in 4–6 h and was reduced to undetectable levels by 9 h. A cytoplasmic protein with similar electrophoretic mobility was found in G_1 phase of cells synchronized by mitotic selection. This class of proteins is synthesized by cells before entry into S phase and may be involved in initiation of DNA synthesis.

Elucidation of biochemical events which occur during the $G_0 \rightarrow G_1$ transition and subsequent traverse of the G_1 phase of the cell cycle may contribute to our understanding of how initiation of DNA synthesis is regulated. The isoleucine (Ile)[1] deprivation technique for synchronizing Chinese hamster cells in G_1 phase (13, 24) has provided one means of determining some of the sequential biochemical events that occur during G_1 progression (22).

Chinese hamster cells (line CHO) deprived of Ile accumulate in G_1 until resupplied with adequate amounts of Ile, at which time the cells resume traverse of G_1, initiate nuclear and mitochondrial DNA synthesis (12), and divide in synchrony. The effect of Ile deprivation on DNA synthesis is rapid; synthesis is reduced to 44% within 1 h, whereas synthesis of RNA and protein is relatively unaffected (4). DNA synthesis in CHO cells is dependent upon a relatively sharp threshold concentration of Ile. Upon fourfold reduction of the minimum concentration of Ile required for all G_1 cells to initiate DNA synthesis, virtually all cells are

[1] *Abbreviations used in this paper:* Ile, isoleucine; SDS, sodium dodecyl sulfate; TdR, thymidine.

prevented from entering S phase (13). Because entry of G_1 cells into S phase is dependent upon the concentration of available Ile, it is logical to suspect that Ile is required for synthesis of a protein(s) involved in initiation of DNA synthesis.

The present study utilized double isotope labeling techniques and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis to detect proteins which are synthesized in CHO cells after reversal of G_1-arrest.

MATERIALS AND METHODS

Propagation of Cells

Chinese hamster cells (line CHO) were grown as suspension cultures in Ham's F-10 without $CaCl_2$ (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 5% fetal calf and 10% calf sera (Flow Laboratories, Rockville, Md.) and penicillin-streptomycin. In our hands the CHO grew with a doubling time of 17 h. Pleuropneumonia-like organism contamination was not detected when cells were routinely cultured on medium described by Chanock et al. (2) which contained yeast extract prepared by the method of House and Waddell (8).

Production of Synchronized G_1 Cells

Exponentially growing CHO cells at 3.4×10^5/ml were pelleted by centrifugation at 500 *g* and washed once with Ile$^-$ F-10 that contained twice the normal concentration of glutamine and 10% dialyzed calf-fetal calf sera (20). The cells were resuspended in Ile$^-$ F-10 medium at an initial concentration of 1.7×10^5 cells/ml medium. Cell concentration of these cultures after G_1 arrest was about 2.5×10^5 cells/ml.

Populations of G_1 cells were also produced by the mitotic selection technique as originally described by Terasima and Tolmach (19) and modified by Tobey et al. (21) Metaphase inhibitors were not used. The initial mitotic index of these populations was 0.90–0.95.

Conditions for Labeling with Radioactive Precursors

24 h after cells were deprived of Ile, 0.25 μCi of uniformly labeled [^{14}C]Ile (312 μCi/mmol; Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) was added per ml of cell suspension. 32 h after deprivation, the cells were spun out of the medium containing [^{14}C]Ile and resuspended in "conditioned" Ile$^-$ F-10 which had been used to grow CHO cells to a state of G_1 arrest. After a wait of 4 h for incorporation of residual [^{14}C]Ile, cells were released from G_1 by the addition of 2×10^{-5} M Ile, and 25-ml aliquots containing 2.4×10^5 cells/ml were labeled with 3 μCi or 10 μCi L-[4,5-^{3}H]isoleucine (73.5 Ci/mmol, New England Nuclear, Boston, Mass.) per ml of medium at hourly intervals.

Cell Fractionation

Cells were fractionated into crude cytoplasmic and nuclear portions as described by Becker and Stanners (1). This procedure involved treating cells with a mixture of Tween 40 and sodium deoxycholate (16), rupturing the cells by twice forcing the suspension through a 26-gauge needle, and pelleting the nuclei by centrifugation at 800 *g* for 10 min. The nuclei were washed twice and dissolved in 1% SDS buffer at a concentration of 5×10^6 nuclei/0.5 ml. The cytoplasmic portion was treated with an equal volume of 2% SDS buffer. Preparations were made 4% in mercaptoethanol, boiled for 2–3 min, and chilled on ice. Protein was determined by the method of Lowry et al. (14) with bovine serum albumin as the standard.

An alternate method of cell fractionation was used to determine the subcellular component with which the peak I protein was associated. Cells were washed three times with phosphate-buffered saline and allowed to swell in a hypotonic buffer (0.01 M NaCl, 0.0015 M $MgCl_2$, 0.01 M Tris, pH 7.4) for 10 min, and cells were then lysed with a Dounce homogenizer until about 90% of the cells were broken, as judged by phase-contrast microscopy. Nuclei were pelleted by centrifugation at 800 *g* for 10 min at 4°C, and the cytoplasmic fraction was spun at 100,000 *g* for 1 h at 4°C to yield the microsomal pellet and soluble supernatant. Each of these fractions was dissolved in SDS buffer, and the polypeptides were separated by gel electrophoresis.

Gel Electrophoresis

Electrophoresis was carried out on 7% SDS-polyacrylamide gels as described by Becker and Stanners (1) after the method of Laemmli (10). Approximately 100 μg of protein contained in 50 μl of reservoir buffer plus 0.25 M sucrose were layered on 5 × 58-mm gels and electrophoresis was run at 1.7 mA/gel for 2 h. Bromphenol blue was used to mark the buffer front. Gels were then fixed overnight in 7% acetic acid and sliced into 0.75-mm slices with an automatic slicing devise (11). The slices were treated with 0.5 ml of NCS (Amersham/Searle, Arlington Heights, Ill.) which contained 10% H_2O and heated at 50°C for 3 h. After the samples were chilled, 10 ml of Omnifluor (New England Nuclear) liquid scintillation cocktail were added per scintillation vial.

Radioactivity was measured with a Packard Tri-Carb 3330 liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Discriminators were set such that counts attributable to tritium were not detected in the ^{14}C channel and about 25% of counts from ^{14}C spilled over into the tritium channel. Tritium counts were corrected for ^{14}C spillover, ^{3}H or ^{14}C was summed

for the gel slices, and the percentage of total ^3H or ^{14}C was determined for each slice.

Appropriate mol wt markers (Worthington Biochemical Corp., Freehold, N. J.) were dissolved in 1% SDS buffer and separated by electrophoresis. Gels were fixed in 5% TCA for 30 min at 56°C, stained for 30 min at 56°C with a solution of Coomassie brilliant blue R (Sigma Chemical Co., St. Louis, Mo.), and destained by frequently changing the destaining solution. These solutions were prepared according to the method of Weber and Osborn (25).

RESULTS

Labeling Patterns of Proteins in G_1-Arrested Cells

Since our objective was to prelabel with [^{14}C]Ile those proteins that are synthesized in cells arrested in G_1 by Ile deprivation, it was first necessary to determine the maximum amount of [^{14}C]Ile that could be added to the G_1 cells without inducing traverse of G_1. This was accomplished by adding varying amounts of Ile to arrested cells and measuring the relative amount of DNA synthesis by incorporation of ^3H-labeled thymidine ([^3H]TdR). An increase in DNA synthesis would indicate that cells had escaped the G_1 block and entered S phase. Data presented in Fig. 1 illustrate the dose-response curve for incorporation of [^3H]TdR as a function of Ile concentration. DNA synthesis did not occur at concentrations below 1.25×10^{-6} M, whereas a fourfold increase to 5×10^{-6} M stimulated about 90% of maximum [^3H]TdR incorporation. The amount of [^{14}C]Ile added for prelabeling in the following experiments did not exceed a total Ile concentration of 8×10^{-7} M. At this concentration of [^{14}C]Ile, proteins in the mol wt range of interest were labeled such that about $4-6 \times 10^4$ cpm were separated on each gel.

Labeling Patterns of Proteins in Cells Traversing G_1

Cells prelabeled with [^{14}C]Ile were induced to traverse G_1 by addition of Ile, labeled with [^3H]Ile after 4–5 h, and fractionated into crude cytoplasmic and nuclear portions. Polypeptides were subjected to electrophoresis on SDS-polyacrylamide gels as described in Materials and Methods. A typical separation profile of the distribution of radioactively labeled cytoplasmic proteins is presented in Fig. 2. Distribution of polypeptides less than 45,000 mol wt were not included because they

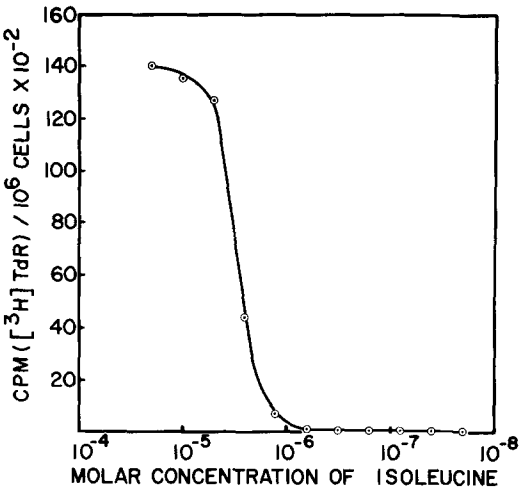

FIGURE 1 Effect of varying concentrations of isoleucine on initiation of DNA synthesis in cells synchronized by ile deprivation. Suspension cultures containing 2.5×10^5 G_1 cells/ml were exposed to [^3H]TdR (0.1 μCi/ml; sp act = 24 Ci/mmol) and twofold dilutions of 2×10^{-5} M Ile. Cells were counted after 16 h, concentrated to 1/25th of the original volume, and 100 μl of the suspension were placed on Whatman 3MM filter paper disks which had been pretreated with 0.1 N NaOH. Disks were then washed three times with 5% TCA, three times with ethanol, once with ether, and dried. Disks were placed in vials, scintillation fluor was added, and radioactivity was quantitated by liquid scintillation spectrometry.

were too small to be properly resolved at this concentration of acrylamide. Gel profiles of polypeptides in the 45,000–160,000 mol wt range from cells traversing G_1 were similar to those from cells arrested in G_1 except for the presence of a major peak of [^3H]Ile in gel slices that corresponds to a mol wt of approximately 80,000. A lesser and perhaps insignificant amount of [^3H]Ile was present in peak II. Peaks of net [^3H]Ile incorporation were not detected in nuclear preparations. Further cell fractionation by Dounce homogenization and differential centrifugation revealed that peak I protein is located in the soluble supernatant fraction of the cytoplasm.

Time-Course Pattern of Peak I Synthesis

The rate of appearance of peak I protein during G_1 traverse was measured by labeling cells during early, middle, or late G_1 phase (Fig. 3). As seen from these profiles, net incorporation of [^3H]Ile into peak I was greatest during mid-G_1. This

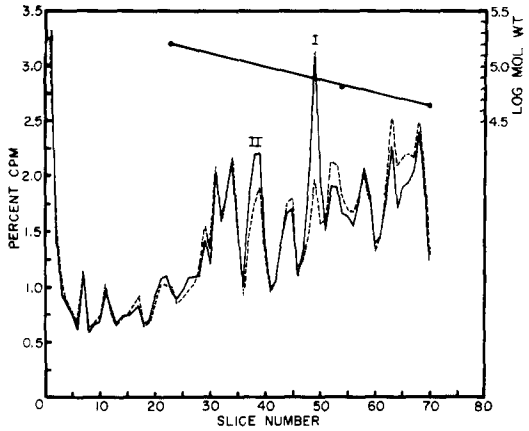

FIGURE 2 SDS-polyacrylamide gel electrophoretic patterns of proteins prelabeled with [^{14}C]Ile (---) before release of cells in G_1 arrest and proteins labeled with [^{3}H]Ile (—) 4–5 h after reversal of G_1 arrest. Total radioisotope associated with proteins separated on each gel was 6.5×10^4 cpm [^{14}C]Ile and 2.5×10^5 cpm [^{3}H]Ile. Mol wt markers used were: human gamma globulin, 160,000 daltons; bovine serum albumin, 67,000 daltons; and ovalbumin, 45,000 daltons. Electrophoresis was from left to right.

indicated that the rate of synthesis of peak I protein was highly variable during G_1. The kinetics of peak I synthesis during G_1 progression were established by labeling traversing cells at hourly intervals for 9 h after release. Percentage net [^{3}H]Ile in peak I was calculated by determining the distribution of label as described in Materials and Methods, except that, instead of whole gels, only 10–15 gel slices in the area of peak I were included in the calculations. Net percentage of Ile in peak I was equal to Σ %[^{3}H]Ile – %[^{14}C]Ile for each slice under peak I curve, and this value was plotted as a function of time after release from G_1 arrest. Net synthesis began during the 1–2-h labeling interval, was maximal in the 5- and 6-h samples, and was greatly reduced by 9 h (Fig. 4). Maximum synthesis of peak I protein occurred at the time when the fraction of cells initiating DNA synthesis was about 0.15.

Synthesis of Peak I Protein in G_1 Cells Synchronized by Mitotic Selection

Since the above data indicated that a class of proteins could be detected with isotopically labeled Ile in cells synchronized by Ile deprivation, we then sought a similar protein in G_1 cells synchronized by an alternate method. Mitotic selection was used to produce G_1 cells that are relatively unperturbed biochemically and possess time-course patterns of DNA synthesis and cell division that are similar if not identical to those of cells synchronized by Ile

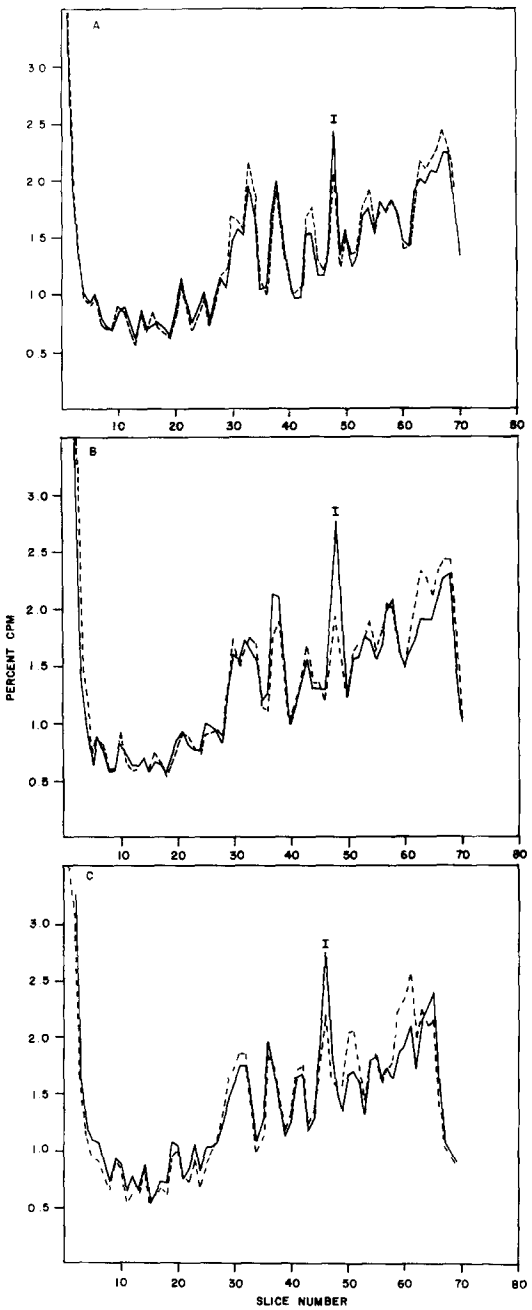

FIGURE 3 SDS-polyacrylamide electrophoretic profiles of cytoplasmic proteins from cells in (A) early G_1 (t = 2 h), (B) mid-G_1 (t = 5 h), and (C) late G_1 (t = 8 h). Proteins prelabeled with [^{14}C]Ile (---) and postlabeled with [^{3}H]Ile (—).

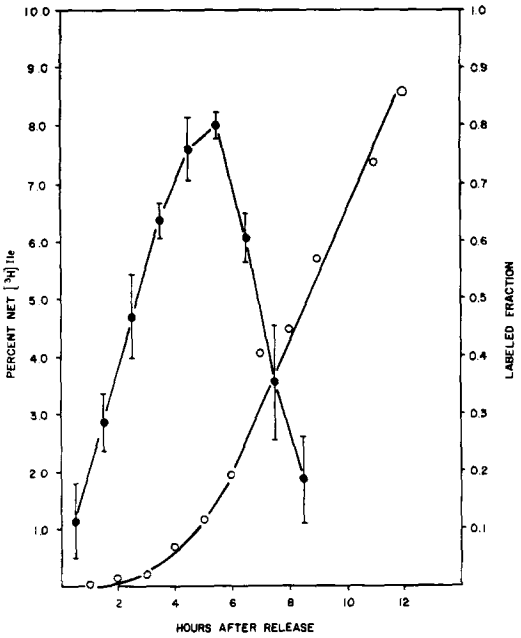

FIGURE 4 Time-course pattern of synthesis of peak I protein during G_1 traverse. Net incorporation of [^{3}H]Ile in peak I as described in text (●—●) after release from G_1 arrest by addition of Ile. Entry of cells into S phase was determined by continuously labeling a separate culture after release with 0.05 μCi [^{3}H]TdR/ml, sampling at hourly intervals, and determining the fraction of cells (N/N_0 − 1) labeled (○—○) by autoradiography as previously described (23).

deprivation (20). The profiles in Fig. 5 represent separation of cytoplasmic proteins from cells labeled in late interphase and collected at mitosis (Fig. 5 A), and G_1 cells synchronized by mitotic selection and then labeled 4 h after cells entered G_1 (Fig. 5 B). These data show a peak of radioisotope incorporation in G_1 cells produced by mitotic selection that corresponds to peak I protein in G_1 cells synchronized by Ile deprivation. Peak I in the separation patterns of proteins from cells in G_2 is very greatly reduced, which suggests that this protein may be specific for G_1 phase of the cell cycle.

DISCUSSION

The results presented here show that cells synchronized by Ile deprivation or mitotic selection synthesize a class of soluble cytoplasmic proteins of approximately 80,000 mol wt during the G_1 phase of the cell cycle.

Peak I protein was detected as a class of labeled polypeptides that migrated in SDS-polyacrylamide gels with an apparent mol wt of 80,000. Although a more definite characterization should be forthcoming, unpublished results by Darby and Ley indicate that the protein is composed of a single polypeptide.

The protocol used for prelabeling with [^{14}C]Ile those cytoplasmic proteins which are synthesized during G_1 arrest was based on the following characteristics of our system: By 24 h after CHO cells were initially deprived of Ile, cells in S, G_2, and M at the time of deprivation continued to traverse and are now arrested in G_1 (13, 5). Proteins being synthesized during G_1 arrest were labeled with [^{14}C]Ile. The fact that there is a threshold concentration of Ile above which cells begin to escape the Ile block (as determined by initiation of DNA synthesis) allowed us to prelabel

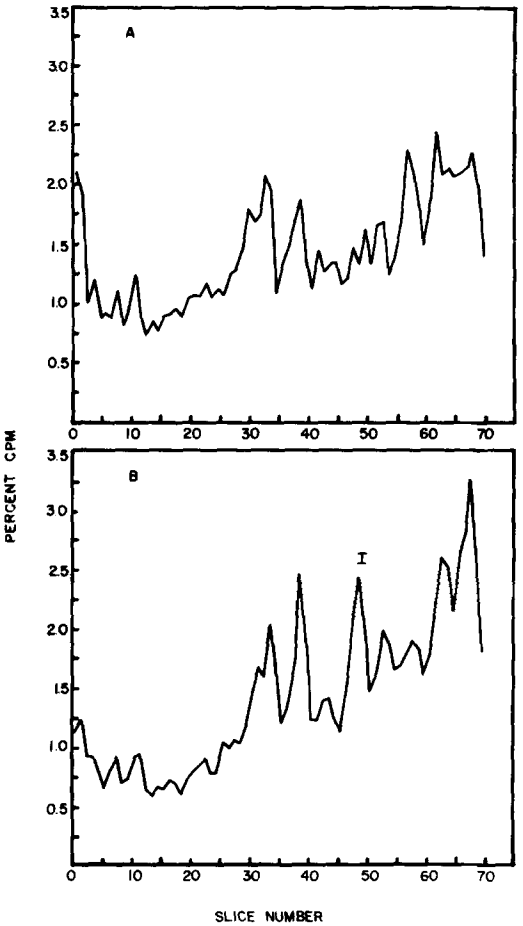

FIGURE 5 SDS-polyacrylamide electrophoretic profiles of cytoplasmic proteins from G_1 cells synchronized by mitotic selection. (A) Proteins from cells in G_2 labeled in monolayer cultures with [^{3}H]Ile 1–2 h before harvesting mitotic cells. (B) Proteins from cells labeled with [^{3}H]Ile 4–5 h into G_1.

with concentrations of [^{14}C]Ile that did not exceed 10^{-6} M (see Fig. 1) without inadvertently triggering G_1 traverse. This minimum concentration of Ile compares well with the minimum concentrations previously reported (13) which allowed a few mitotically selected cells to traverse G_1 and enter S phase (6.0×10^{-7} M) or some cells of an Ile-deprived culture to complete mitosis (1×10^{-6} M).

Evidence that depriving CHO cells of Ile did not cause abnormal labeling patterns is presented in Fig. 6 which illustrates the similarity between polyacrylamide gel profiles of G_1 proteins synthesized in cells which were synchronized by Ile deprivation or by mitotic selection. Cells synchronized by mitotic selection are *selected* out of a random population of cells and thus are less likely to be biochemically perturbed than cells synchronized by induction techniques (for discussion on methods of cell synchronization, see Mitchison [15]).

Other investigators have compared the patterns of proteins synthesized in resting and proliferating cells. Becker and Stanners (1) examined newly synthesized proteins of 30,000–150,000 mol wt in hamster cells growing in synchrony and cells in stationary phase by double-labeling techniques and SDS-polyacrylamide gel electrophoresis. These workers did not detect significant differences between gel profiles of cytoplasmic proteins synthesized in different phases of the cell cycle; however, they did detect differences when profiles from stationary phase cells were compared with any phase of proliferating cells. This is in contrast to our findings where we detected differences in the pattern of cytoplasmic proteins from different phases of the cell cycle, notably the peak I protein, but the patterns between traversing and G_1-arrested cells are similar. One explanation for the similarity of patterns for noncycling vs. cycling cells in our system may be the possibility that Ile-deficient G_1 arrest is not analogous to stationary phase. Indeed, the results of Enger and Tobey (4) indicate that the rates of macromolecular synthesis in high density, stationary phase cells are much lower than in cells arrested in G_1 by Ile deprivation. Kolodny and Gross (9) also reported differences in patterns of proteins synthesized during G_2 phase and patterns obtained in other phases of the cell cycle.

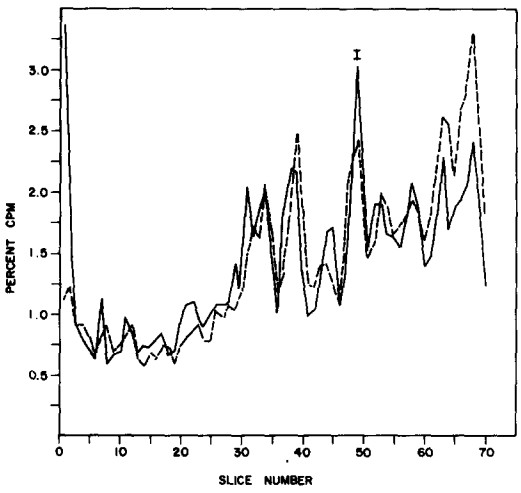

FIGURE 6 SDS-polyacrylamide patterns of cytoplasmic proteins from cells traversing G_1 after synchronization by Ile deprivation (—) or mitotic selection (---).

Numerous studies suggest that factors required for initiation of DNA synthesis are made in the cytoplasm (3, 6, 7, 17, 18, and others). Our results showed that the peak I protein was synthesized in the cytoplasm of cells traversing G_1 and that synthesis declined as the cells entered S phase. Because synthesis of peak I protein precedes initiation of DNA synthesis, we propose that this protein is required for initiation of DNA synthesis. Studies in our laboratory are currently directed at testing this hypothesis by attempting to clarify the role of this protein in G_1 progression and its relationship to DNA synthesis.

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