REPLICATION OF A MAMMALIAN GENOME: THE ROLE OF *DE NOVO* PROTEIN BIOSYNTHESIS DURING G1 PHASE

by

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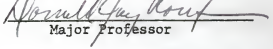

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ABSTRACT

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INTRODUCTION

The mammalian cell life cycle has been the subject of much study in recent years, yet relatively little is known about the control of particular events in the cycle. This is due largely to the complexity of the cell itself and to the multitude of factors that appear to affect the cell as it traverses its life cycle.

The cell's life cycle consists of four consecutive stages: Gap 1 (G1), synthesis phase (S), Gap 2 (G2), and mitosis (M) (1). Gap 1 marks the beginning of a cell cycle. It is a time of RNA and protein synthesis, and is considered to be a state of preparation for DNA synthesis. S phase is the period in which nuclear DNA synthesis occurs. Gap 2 marks the period of mitochondrial DNA synthesis (2) and in our assays the start of nuclear chromosome condensation. The last phase of the cycle, mitosis, involves the alignment of condensed chromosomes on a metaphase plate, their migration to opposite poles of the cell, and the subsequent division of the cell.

Since cells traverse the cell cycle in an ordered manner, there must be cellular controls inherent in the cycle which regulate the timing of various events. G1 has been considered a state of preparation for DNA synthesis, and as such should contain one or more control points for the initiation of S phase (2). It is known that a great deal of protein synthesis occurs throughout G1, and so several investigators have examined the relationship between G1 protein biosynthesis and DNA replication.

Previous experiments performed in our laboratory had shown that there was a requirement for protein biosynthesis during G1 phase which had to be fulfilled before initiation of S phase could occur in Chinese hamster cells (CHL cells). The first evidence for this was obtained with

ts 14, a CHL cell mutant which was temperature sensitive for DNA synthesis (3). Extensive characterization of this mutant showed it to be defective in protein synthesis. At 39°C, ts 14's 60S ribosomal subunit dissociates, inhibiting protein synthesis. ts 14 ceased DNA synthesis six hours after being shifted to 39°C (3).

A second experiment indicating the relationship between G1 phase protein biosynthesis and programmed DNA replication was performed by monitoring the effect of protein synthetic inhibitors and amino acid analogs on DNA synthesis in wild type Chinese hamster cells. When wild type HT-1 cells were treated with cycloheximide, puromycin, emetine or amino acid analogs, they exhibited a DNA^- phenotype with the same kinetics as did ts 14 (4). Cellular protein synthesis continues in the presence of amino acid analogs, but the proteins synthesized often are incapable of the functions for which they were intended due to incorporation of the analogs (5). These data led to the conclusion that synthesis of one or more G1 phase proteins is necessary for either the initiation or the maintenance of S phase in CHL cells.

Many investigators (6-8) correlated histone biosynthesis with DNA replication during S phase. Therefore, many believed that protein synthesis might be necessary for the maintenance of DNA replication.

Further experiments with S phase populations of both wild type and ts 14 CHL cells have shown that both cell lines are able to complete precisely one round of DNA replication in cases where protein synthesis is inhibited (9). In these experiments, wild type cells were treated with cycloheximide or emetine and ts 14 was transferred to nonpermissive temperature (39°C) at various times throughout S phase. Therefore, synthesis of protein is not necessary for the maintenance and completion of S phase once it has begun, but it is necessary for the initiation of S phase in CHL cells.

Schneiderman, Highfield and Dewey monitored the effect of cycloheximide mediated inhibition of G1 protein synthesis on the initiation of S phase in CHO cells. This work showed that G1 protein synthesis was required for the initiation of DNA synthesis in Chinese hamster ovary (CHO) cells (10).

Studies involving other mammalian cell lines also point to this conclusion. The activities of thymidine kinase (11), thymidylate kinase, deoxycytidine monophosphate deaminase and ribonucleotide reductase (12, 13) greatly increase at the beginning of S phase. These enzymes therefore must be synthesized prior to S phase. Synthesis of the DNA polymerases and ligases remains constant throughout G1 phase until after S phase has begun (14, 15). These data suggest that a signal for the synthesis of replicative enzymes occurs just prior to or at the beginning of S phase. Thus, some other signal must regulate the initiation of S phase itself.

Consequently, a number of investigators studying the initiation of DNA synthesis in drug treated wild type and mutant Chinese hamster cells, as well as in other cell lines, have all concluded that protein biosynthesis during G1 phase is required for cellular entrance into S phase. In contrast, once S phase has begun, no further protein biosynthesis appears to be required for the completion of that round of DNA replication (9).

Therefore, a number of questions about G1 phase protein biosynthesis necessary for DNA replication in Chinese hamster cells now can be asked. For example:

1) When during G1 phase are proteins necessary for DNA replication synthesized?

2) How many such proteins are synthesized?

3) Is it possible to assess synthesis of these life cycle-specific proteins independent of continued growth in tissue culture?

4) Where in the cell do these proteins reside (i.e., function)?

5) What regulates the synthesis of these G1 phase proteins (i.e., is regulation occurring at the level of mRNA synthesis or at the level of protein synthesis)?

The experiments described below were designed to investigate these questions.

In order to approach Question 1), an experiment using cytochalasin B was performed with ts 14 (unpublished data). Cytochalasin B inhibits cytokinesis, thus rendering mitotic cells binucleate (16). This creates a synchronous binucleated subpopulation of cells within an asynchronous cell culture. Using this technique, a nine hour G1 phase in wild type CHL cells at 33°C was observed. ts 14 then was treated with cytochalasin B at 33°C for one hour and subsequently placed at 33°C to grow. At various times replicate cultures of ts 14 were shifted to 39°C and grown until the ninth hour after cytochalasin B treatment. At the ninth hour, cultures were pulse-labelled with ^{3}H-thymidine for one hour. They then were analyzed by autoradiography, and only binucleate cells in which both nuclei were radiolabelled were scored. These results are shown in Figure 1. As can be seen in the figure, only when binucleate cells had remained at 33°C for three or more hours prior to being shifted to 39°C did they subsequently enter S phase. Thus, in ts 14, one or more proteins must be synthesized before the third hour of G1 phase in order for cells to enter S phase.

With regards to Question 2) and 3), Ley reported finding a CHO cell G1 phase-specific protein of 30,000 molecular weight, using SDS polyacrylamide gel electrophoresis (17). In his experiments, CHO cells were prelabelled with ^{14}C-isoleucine and then were arrested in G1 phase by

isoleucine deprivation (a state analogous to G_0). Subsequently, they were released from isoleucine deprivation and then were labelled with ^{3}H-isoleucine at hourly intervals. Nuclear and cytoplasmic subcellular fractions were prepared and analyzed by polyacrylamide disc gel electrophoresis. Ley observed a single peak of protein of 80,000 molecular weight enriched in ^{3}H in the cytoplasm of G1 phase CHO cells. He repeated the experiment using mitotically synchronized cells, and reported observing the same G1 phase-specific peak. Therefore, it appears that there is at least one protein synthesized specifically during mid-G1 phase in CHO cells, and this protein can be assayed by SDS polyacrylamide gel electrophoresis. Thus, polyacrylamide gel electrophoresis might also be used to examine Question 4), the subcellular localization of G1 phase-specific proteins.

Experiments described by Kit and Jorgensen indicated that inhibitors of transcription (Actinomycin D) and translation (cycloheximide) both restricted cellular entrance into S phase in a mutant of Chinese hamster cells (18). Based on these results, regulation of at least some of the G1 phase-specific proteins appeared to be at the level of transcription. In order to examine Question 5) in more detail, I have used the highly specific transcriptional inhibitor, alpha-amanitin.

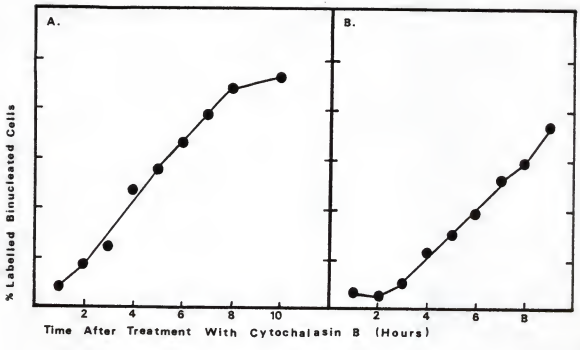

FIGURE 1. Effect of temperature on ts 14's entry into S phase. Wild type CHL cells (A) were treated with cytochalasin B for one hour at 33°C and then were pulse-labelled with ^{3}H-thymidine for 60 minutes at the times shown. They then were autoradiographed and only binucleate cells in which both nuclei were radiolabelled were scored. ts 14 (B) was treated for one hour at 33°C with cytochalasin B and then placed at 33°C to grow. At the times shown, ts 14 was shifted to 39°C and incubated until the ninth hour after cytochalasin B treatment. At the ninth hour, the cells were assayed for entrance into S phase by treatment with ^{3}H-thymidine for one hour at 39°C. They then were autoradiographed and scored.

MATERIALS AND METHODS

MATERIALS

Cell Culture. Powdered Dulbecco's modification of Eagle's minimal essential medium (DMEM), trypsin, sterile fetal calf serum (FCS) and colcemid were purchased from Grand Island Biological Company (GIBCO). Thymidine was from Sigma Chemical Company; cycloheximide from Calbiochem and alpha-amanitin from Boerhinger Mannheim. Plastic petri dishes and plastic tissue culture flasks were purchased from Corning and Falcon. Cells were incubated in New Brunswick CO-20 incubators with forced air circulation.

Radioisotopes. Radioisotopes were obtained as follows: Schwarz Mann: ^{3}H-arginine, ^{3}H-lysine, ^{3}H-alanine, ^{3}H-proline, ^{3}H-thymidine, ^{3}H-BrdUrd, ^{14}C-alanine and ^{14}C- thymidine. New England Nuclear: ^{14}C-proline.

Autoradiography. Nuclear Track Emulsion NT-B2, D-19 developer and paper fixer were purchased from Eastman Kodak Company.

Electrophoresis. Molecular weight standards were purchased from Schwarz Mann. Bovine pancreatic RNase A (type I-A) was purchased from Sigma Chemical Company. DNase I was from Worthington Biochemical Corporation, and Pronase from Calbiochem. Acrylamide, N'-N'-Bis-methylene acrylamide and Photo Flo were obtained from Eastman Kodak Company. Ammonium persulfate and N,N,N'N'-tetramethylethylenediamine (TEMED) were purchased from Sigma Chemical Company. A Buchler Instruments Polyanalyst electrophoresis chamber and a Heathkit power supply were used. Tubular polyacrylamide gels were sliced with a Biorad gel slicer, Model 190 (Biorad Laboratories), incubated in Protosol (New England Nuclear) to extract the ^{3}H and ^{14}C CPM, and assayed in a Beckman LS-100C scintillation counter. Gels were destained electrophoretically using an Ames Model 190 Quick Gel Destainer.

CELL CULTURE

Cell Lines. The two cell lines used in these experiments were V79 Chinese hamster lung cells (CHL) and the K-1 strain of Chinese hamster ovary cells (CHO). The CHL cell wild type clone, HT-1, was isolated in this laboratory (4). At 37°C, HT-1 has a generation time of fifteen hours and forms colonies with 100% efficiency in DMEM.

A mutant CHL cell derivative used in these studies was ts 14. This clone was obtained from wild type CHL cells using BrdUrd - black light selection to yield clones defective in DNA replication at high temperature (4). ts 14 was shown to be temperature sensitive for protein synthesis, however (3). This lesion is manifest in the 60S ribosomal subunit, which is nonfunctional at 39°C (the nonpermissive culture temperature). At 33°C, ts 14 has a generation time of approximately 30 hours in DMEM.

The CHO cell line used in these experiments was obtained from Dr. Larry Thompson of Lawrence Livermore Laboratories, Livermore, California. These cells, derived from the K-1 strain, were selected to grow in monolayer culture and to exhibit the property of rounding up and detaching from the culture dish during mitosis. Thus, they are suited for synchronization by mitotic detachment. These cells contained outside marker mutations for proline and glycine auxotrophy.

Media. The media used in the routine culture of Chinese hamster lung cell derivatives was DMEM adjusted to 10% FCS, 4 mM glutamine and buffered with sodium bicarbonate at a concentration of 0.38%. Chinese hamster ovary cells were cultured in DMEM that also contained 3×10^{-4}M proline and 3×10^{-4}M glycine. This medium will be termed "DMEM*". All media were incubated in humidified CO_2 incubators for at least 24 hours before use so as to bring the media to the proper temperature and CO_2 tension. Special media used in various experiments will be described in relation to the technique in which they were employed.

Growth. All cultures were maintained in humidified CO_2 incubators. HT-1 and the CHO cell line were cultured at 37°C, while ts 14 was cultured at 33°C or 39°C, as required for particular experiments. All cells were grown as monolayers without antibiotics. No cells that had been passaged more than ten times from a cloning procedure were used in the experiments reported here. Each passage was performed by diluting the cells 1:50 in fresh medium, and was performed approximately once a week. Sterile phosphate buffered saline (PBS) was used to rinse cells (19) and 0.05% sterile trypsin without EDTA was used to detach cells from the surface of the plate or flask during passaging. Trypsinization was terminated by addition of DMEM* plus 10% FCS, which contains an endogenous trypsin inhibitor.

Cell Synchrony. Cell synchrony was achieved by using a double thymidine block (20) followed by mitotic selection (21) with or without the use of colcemid. A double thymidine block synchronized cells to the G1-S phase boundary. This technique, first developed by T. T. Puck (20), uses 2 mM thymidine to inhibit the cells' ribonucleotide reductase, and thus starves the cells for deoxyribonucleoside triphosphates. The procedure adopted for synchronizing CHL and CHO cells is as follows:

10 mls of medium adjusted to be 2 mM thymidine in DMEM* was fed to each 75 cm^2 flask of CHO cells. The cells then were incubated for 14 hours at 37°C. The 2 mM thymidine-DMEM* then was removed; the cells were rinsed twice with sterile PBS; refed DMEM*; and incubated at 37°C for nine hours. The DMEM* was again replaced with 2 mM thymidine-DMEM*, and the cells were incubated for 14 additional hours at 37°C to accumulate at the G1-S phase boundary.

In some experiments colcemid was added to the media at this time. Colcemid is a metaphase spindle inhibitor, and was used in some studies

to collect cells during mitosis. It is readily reversible and can be used to obtain large populations of synchronous cells (22). Colcemid diluted in DMEM* (1.0 µg/ml) was found to be the most effective concentration to accumulate metaphase cells. As shown in Figure 2, in a preliminary experiment, CHO cells were treated with colcemid at concentrations of 0.125, 0.5, 0.75, 1.0, and 1.5 µg/ml, and mitotic indices were determined on replicate cultures at several times. As can be seen, a 1.0 µg/ml colcemid concentration is effective in blocking cells at mitosis, and yields a rate of mitotic accumulation of 5-6% per hour. This is consistent with a CHO cell steady state generation time of approximately 18 hours at 37°C. When colcemid was used to accumulate mitotic cells, the cells were incubated at 37°C in DMEM* that contained 1.0 µg/ml colcemid for the first nine hours after reversal of the double thymidine block. Mitotic cells then were harvested by the technique described below.

Mitotic selection is based upon the fact that some mammalian cell lines "round up" during mitotic metaphase, and thus are easily detached from their growth surface. In this state they may be separated from the nonmitotic cells by shaking the flask. Thus, mitotic CHO cells may be removed from the culture and collected by centrifugation. They may then be resuspended in fresh medium and plated to a desired cell density as a synchronous population of M phase cells. This technique was described first by Terasima and Tolmach (21) (See Appendix 1 for details of my technique).

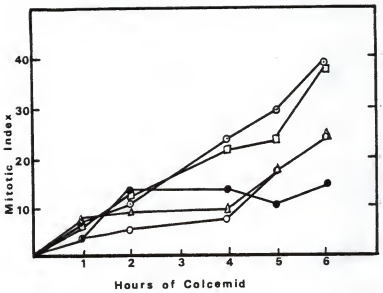

FIGURE 2. Effect of various concentrations of colcemid on mitotic indices. Asynchronous replicate cultures of CHO cells were treated with 0.125 (O—O), 0.5 (●—●), 0.75 (Δ—Δ), 1.0 (□—□) and 1.5 (⊖—⊖) μg/ml colcemid at 37°C and mitotic indices were determined at the times indicated in the figure.

METHODS

Methods of Assaying Cell Synchrony.

A.) Determination of mitotic indices. Mitotic indices are the ratio of mitotic cells to the total number of cells in a population. This ratio is useful in assaying the synchrony of a mitosis in culture and in assaying the purity of mitotic cell populations. The procedure employed to determine mitotic indices is a modification of that described by T. T. Puck (23), and is detailed in Appendix 2.

B.) Measurement of S phase by ^{3}H-thymidine incorporation. One method of monitoring the initiation and duration of S phase is to assay the rate of incorporation of radioactive thymidine into acid insoluble DNA. The procedure used in these experiments is one described by Roufa and Reed (4). Cells were pulse-labelled with ^{3}H-thymidine for one hour at 37°C, lysed with 1% SDS and precipitated with 10% TCA onto glass fiber filters (Whatman GF/C). The labelling medium consisted of DMEM* adjusted to be 10^{-6}M thymidine with a final radioactivity of 2 µC/ml ^{3}H-thymidine.

C.) Autoradiography. Autoradiography was used to assay the proportion of S phase cells in a population. The method used is an adaptation of one developed by Robert Mackay (unpublished), and is described in Appendix 3. The labelling medium used in this procedure also was DMEM* adjusted to be 10^{-6}M thymidine with a final radioactivity of 2.5 µC/ml ^{3}H-thymidine.

D.) Cesium chloride density gradients. These were used as a measure of both cell viability and culture synchrony. Cesium chloride density gradients were used to determine the ability of a cell population to replicate its DNA semiconservatively and therefore served as an index of cell viability. The protocol used to make and analyze the CsCl gradients as well as to label and purify CHO cell DNA have been described before (24).

Use of α-amanitin to Inhibit Transcription of mRNA in Cultures of CHO Cells. Alpha-amanitin was observed to inhibit Chinese hamster cell transcription in a reversible manner, and thus, was suitable for experiments described below (Moats, unpublished). Several studies have suggested that α-amanitin inhibits elongation of nascent RNA molecules (29, 30) by inhibiting eukaryotic RNA polymerases II and III specifically (25-28).

The concentration of α-amanitin necessary to inhibit mRNA transcription in isolated nuclei has been determined by a number of investigators (25-29), but there were no similar data for the use of α-amanitin in tissue cultures of CHO cells. Thus, it was necessary to determine the minimum concentration of α-amanitin that inhibits mRNA transcription in CHO cell cultures. Therefore, I decided to determine the minimum amount of α-amanitin that inhibits transcription of histone mRNA in synchronous S phase cultures of CHO cells. Since histone mRNA transcription is induced during S phase and histone proteins are synthesized only during S phase, I used histone protein synthesis to assess histone mRNA synthesis in vivo. This was done by treating synchronous S phase cultures with α-amanitin in DMEM*-^{3}H-arginine-^{3}H-lysine medium (final radioactivity = 5.0 μC/ml) at concentrations of 0, 1, 5, 10, and 50 μg/ml of α-amanitin. These media were fed to S phase CHO cells at 37°C for 6 hours (the length of S phase). At the end of S phase, nuclear extracts were prepared, and electrophoretic analysis of the ^{3}H-arginine and ^{3}H-lysine labelled proteins was carried out. These results are summarized in Figure 3. As shown in the figure, 10 μg/ml α-amanitin inhibited the synthesis of virtually all histones, and therefore appeared to inhibit transcription of histone mRNA. This concentration of α-amanitin was used in all subsequent experiments.

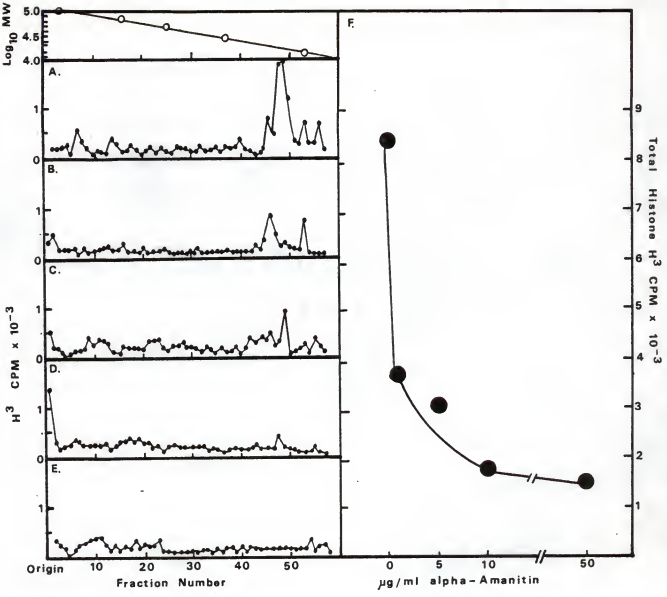

FIGURE 3. Effect of various concentrations of α-amanitin on histone synthesis during S phase in synchronous CHO cells. S phase CHO cells were treated with 0 (A), 1 (B), 5 (C), 10 (D) and 50 (E) µg/ml α-amanitin-^{3}H-arginine-^{3}H-lysine medium at 37°C for an entire S phase. Histone synthesis was monitored by incorporation of ^{3}H-arginine-^{3}H-lysine into nuclear protein and was assayed by SDS polyacrylamide gel electrophoresis. At the end of S phase, nuclear extracts were prepared and electrophoresis carried out as described in the text. Gels were then sliced and assayed in scintillation counters to detect ^{3}H. The migration of molecular weight standards also is shown. In panel F the total histone radioactivity observed in Panels A-E is plotted vs. the concentration of α-amanitin used in each experiment.

Analysis of Cell Cycle-Specific Proteins in Extracts of Synchronous CHO Cells by SDS Polyacrylamide Gel Electrophoresis.

Labelling cellular proteins for electrophoresis. Cells were treated with radioactive amino acids at various times during G1 and S phases, so that newly synthesized proteins could be monitored on electrophoretic gels. The proteins synthesized during the short labelling periods were so numerous that staining gels with Coomassie blue yielded a continuum of blue dye. Distinct peaks could be visualized from a gel only as radioactively labelled proteins. By using a single amino acid labelled either with ^{3}H or with ^{14}C to detect these proteins, the amount of each protein species of a particular molecular weight could be calculated from the radioactivity on the gel. Alanine was chosen for the majority of the studies because it is a prevalent amino acid in many proteins and because it is nonessential for growth, i.e., it is not contained in DMEM. Therefore, it is possible to add high specific activity alanine at low molarity without reducing the medium's ability to support cell growth. ^{3}H- and ^{14}C-proline also were used to confirm the alanine labelling studies and to make certain that proteins deficient in alanine might be seen in the gel profiles. Proline was selected because it also is not in DMEM and because the CHO cell line is a proline auxotroph. Therefore, CHO cells utilize only the radioactive proline supplied in the medium to synthesize protein.

Below are the various procedures used to label cells. In all cases, 1×10^{6} mitotic CHO cells were radiolabelled, and each sample analyzed was derived from $1 - 5 \times 10^{5}$ radioactive cells.

^{14}C-alanine or ^{14}C-proline labelled proteins:

A.) Asynchronous cells. Asynchronous cells were innoculated into culture at one third confluency, and labelled for 48 hours in 5 mls DMEM*

with 1.25 μC/ml ^{14}C-alanine (8 x 10^{-6}M) at 37°C. This amount of ^{14}C-alanine was established by treating cultures with various concentrations of ^{14}C-alanine and monitoring the amount incorporated into TCA insoluble protein over a 24 hour period. These data are summarized in Figure 4.

B.) Synchronous cells. (1) 10^6 mitotic CHO cells were plated into DMEM* and incubated at 37°C. Sixty minutes later floating cells were decanted. The monolayer then was fed with 2 mls (DMEM* without FCS) plus 3.2 x 10^{-5}M ^{14}C-alanine (5 μC/ml) or 10^{-4}M ^{14}C-proline (10 μC/ml) for the labelling period described in each experiment. (2) ^{3}H-alanine or ^{3}H-proline labelled proteins: Only synchronous cells were labelled with ^{3}H amino acids. Mitotic CHO cells were cultured in DMEM* at 37°C. After 60 minutes floating cells were removed. When a particular phase of the cycle was labelled, the monolayer was fed with 2 mls of media that contained 10^{-6}M radioactive ^{3}H-alanine at a specific activity of 30,000 μC/μmole or 10^{-4}M ^{3}H-proline at 300 μC/μmole for the length of time indicated for each experiment. (3) ^{3}H-arginine plus ^{3}H-lysine labelled proteins: ^{3}H-arginine and ^{3}H-lysine medium was used during S phase to label histones. As described above, cells were treated with 5 μC/ml each of these amino acids at a concentration of 10,000 μC/μmole for ^{3}H-arginine and 43,000 μC/μmole for ^{3}H-lysine.

Protein extracts for electrophoreses. The protocols utilized for making radiolabelled whole cell, nuclear and cytoplasmic protein preparations were adapted from the methods of Penman (31), and are detailed in Appendix 4. All extracts were adjusted to contain 100,000 CPM per 40 μl sample, and each electrophoretic sample was derived from 1 - 5 x 10^5 cells.

Mixed samples for electrophoreses. The mixed samples for electrophoreses were composed of 40 μl ^{3}H-alanine or ^{3}H-proline labelled extracts and 40 μl ^{14}C-alanine or ^{14}C-proline labelled extracts. 8 μl of BME was added to these mixtures and the samples were boiled for three minutes and then applied to a gel.

Procedure for SDS polyacrylamide gel electrophoresis.

Gel preparation. SDS polyacrylamide gel electrophoresis was used to assay the proteins synthesized by CHO cells during their life cycle. A modification of the Laemmli system (32) was developed using a 6 cm, 15% acrylamide resolving gel and a 1 cm, 4% acrylamide stacking gel. Appendix 5 describes the exact composition of the gels.

Electrophoresis. Gels were electrophoresed for 3 hours at 100 V, constant voltage (unless otherwise stated). When samples contained radioactive amino acid labels, gels were sliced into 1 mm segments and incubated in Protosol for 8 hours at 37°C. Then they were assayed in a scintillation counter to detect ^{3}H and ^{14}C CPM. The analysis of these double label gels appears below.

When the samples were not radioactive, as in the case of the molecular weight standards, the gels were stained with 0.2% Coomassie blue in 7% acetic acid for 2 hours. These gels then were destained in 7% acetic acid and 10% methanol electrophoretically. The molecular weight standards used in these studies were human gamma globulin (160,000 daltons), bovine serum albumin (67,000 daltons), ovalbumin (45,000 daltons), chymotrypsinogen (25,000 daltons), and cytochrome C (12,000 daltons), each at a concentration of 1 µg/gel. A standard gel was included in each experiment.

Analysis of ^{3}H and ^{14}C labelled proteins on electrophoretic gels. Samples containing a mixture of 10^{5} ^{3}H CPM and 10^{5} ^{14}C CPM of radioactive proteins were electrophoresed and the gels sliced and assayed in a scintillation counter. The amounts of each isotope per gel slice were determined and the ratios of % total ^{3}H: % total ^{14}C were calculated. These ratios reflect differences between the ^{3}H and ^{14}C proteins synthesized by a cell. That is, ratio values larger than 1.00 might be due to

an increase in the ^{3}H CPM or to a decrease in the ^{14}C CPM, and so indicate either the increased synthesis of particular ^{3}H proteins or the decreased synthesis of specific ^{14}C proteins. Ratios close to 1.00 indicate that ^{14}C and ^{3}H samples contain equivalent amounts of particular molecular weight classes of proteins.

In order to establish whether ratios which differed from 1.00 were significant, it was necessary to determine the confidence limits of the above assay. This was done by analyzing a series of gels which contained mixtures of identical ^{3}H- and ^{14}C-labelled protein extracts. The % ^{14}C CPM and % ^{3}H CPM were calculated for each gel slice, and the means of the ratios and their standard deviations ( ) were determined. The 99% confidence limits were computed as the Mean $\pm(2.58)$ ( ), and were $0.4649 \leq x \leq 1.7101$. Ratio peaks falling outside of these limits then were considered significant. Figure 5 summarizes such a control gel. The dashed lines indicate the 99% confidence limits. These confidence limits subsequently were applied to all similar gels.

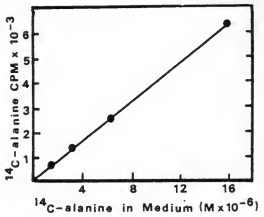

FIGURE 4. Incorporation of ^{14}C-alanine into TCA precipitable protein in asynchronous CHO cells at 37°C in 24 hours in culture. Cultures of 10^6 CHO cells were allowed to incorporate various concentrations of ^{14}C-alanine (156 μC/μmole) as indicated in the figure at 37°C for 24 hours. At this time, the cells were lysed and the radioactivity in the acid precipitable protein assayed as described in the text.

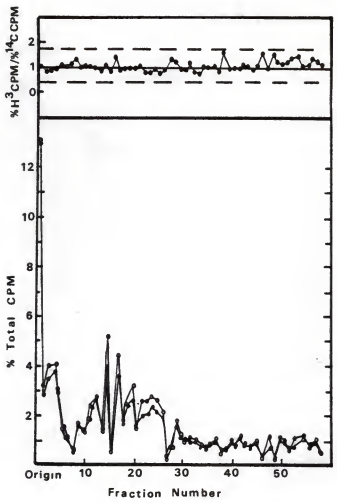

FIGURE 5. Electrophoretic analysis of a mixture of identical G1 phase proteins labelled with ^{3}H-alanine and with ^{14}C-alanine. Two identical synchronous cultures of CHO cells were labelled during hours 3-7 of G1 phase either with 10^{-6}M ^{3}H-alanine (30 μC/ml, ●——●) or with ^{14}C-alanine (5 μC/ml, O——O). Cytoplasmic protein extracts were prepared and mixed in equal amounts on a radioactivity basis (10^{5} CPM each per sample). The mixed sample was analyzed by electrophoresis as described in the text, and the % ^{3}H/% ^{14}C ratios for each slice were calculated. The dashed lines indicate the 99% confidence limits calculated from the data as described in the text.

RESULTS

Previous experiments performed in our laboratory had shown that protein biosynthesis is required during G1 phase before initiation of S phase can occur in CHL cells. Experiments with ts 14 had established that one or more proteins must be synthesized before the third hour of G1 phase in order for cells to initiate S phase (see above).

In order to study the synthesis of individual proteins during G1 phase and their subcellular distribution, it was necessary to obtain homogeneous populations of CHL cells. I attempted unsuccessfully to synchronize HT-1 to the G1-M phase boundary by mitotic selection. If these attempts had proved successful, the method would have been applied to cultures of ts 14, and all further experiments would have been performed with that clone. HT-1 cells were synchronized to S phase with a double thymidine block. Then they were released and allowed to traverse the cycle to mitosis, at which time I tried to collect the mitotic cells by variations of the mitotic detachment technique. However, under none of the conditions attempted did mitotic CHL cells detach from the monolayer. The methods used to selectively detach mitotic CHL cells included lowering the Ca^{++} and Mg^{++} ion concentrations, adding EDTA, using suspension culture media, and trying differential trypsinization with and without EDTA. All of these methods failed to yield a synchronous population of CHL cells which detached from the monolayer during mitosis.

Thus, I extended my study of G1 phase proteins to use the CHO K-1 cell line, a wild type sister cell line of the CHL cells originally used. This cell line readily yields populations which are 96-99% mitotic by a straight forward application of the procedure described by Terasima and Tolmach (21).

The CHO Cell Life Cycle

In order to document the CHO cell replicative requirement for G1 phase protein synthesis originally observed in CHL cells, the CHO cell life cycle was first determined. CHO cells were synchronized by mitotic detachment and innoculated into DMEM* at 37°C. The cultures were treated with 1.0 µg/ml colcemid in DMEM* and mitotic indices were determined at hourly intervals. Also, replicate cultures were pulse labelled for one hour with ^{3}H-thymidine and the radioactivity incorporated into acid insoluble DNA was assayed to measure cellular entrance into S phase. These data are illustrated in Figure 6. As can be seen, G1 and S phases each were 7 hours in length. G2 phase was one to two hours long. Note that after one generation the cultures have lost much of their synchrony, since M phase, which appears here to last approximately 5 hours, is known to require less than one hour in individual cells (23). Approximately 78% of the synchronous mitotic cells innoculated into culture were viable, as can be seen by the percent of the cells which complete one round of growth to the next mitosis. The viability of mitotic cells also was assessed by their ability to replicate their genome within 14 hours after innoculation into synchronous culture (G1 + S phase). Again the cells exhibited 80% viability. The latter study, illustrated in Figure 7, involved the use of a density transfer protocol into medium that contained 5-bromodeoxyuridine (24). Approximately 80% of the ^{14}C-thymidine prelabelled DNA within a synchronous culture of CHO cells replicated in DMEM* medium that contained ^{3}H-BrdUrd. Thus, 80% of the ^{14}C-DNA was converted from a light fraction (ρ = 1.70) to unifilar BrdUrd-DNA (ρ = 1.75), and the latter peak of DNA contained both ^{3}H-BrdUrd and ^{14}C-thymidine labels.

A Replicational Requirement for G1 Protein Synthesis in Wild Type CHO Cells

Having established the length of G1 phase in synchronous cultures of mitotically selected CHO cells to be 7 hours, it was next determined whether a G1 phase protein biosynthetic restriction point for DNA replication occurred in CHO cells as it did in CHL cells. To perform this experiment, a series of identical cultures of synchronous G1 phase CHO cells were innoculated into DMEM*. Commencing one hour after innoculation, duplicate cultures were adjusted to 2 μM cycloheximide each hour and were maintained in the presence of the drug at 37°C until the end of the seventh hour (the end of G1 phase, see Figure 6). Thus, some cells were cultured in medium that contained cycloheximide for 7 hours (a complete G1) while others were in medium that contained the drug for as little as one hour (the final hour of G1 phase). Two cultures that were not exposed to cycloheximide served as controls. Seven hours after innoculation into culture, all monolayers were rinsed with DMEM* and the cells' entry into S phase was assessed by measuring their ability to synthesize DNA in culture. DNA synthesis was assayed by monitoring the incorporation of ^{3}H-thymidine into acid precipitable DNA during a one hour period (hours 8-9 post mitosis). The results of an experiment in which S phase was assayed in the absence of cycloheximide are shown in Figure 8. Similar results also were obtained when DNA synthesis was measured in the presence of cycloheximide. As indicated by the data, CHO cells become resistant to cycloheximide-inhibition of entry into S phase after the fifth hour of G1 phase. An identical experiment was performed using emetine, another inhibitor of mammalian protein synthesis, and again similar results were obtained. Therefore, as had been observed in asynchronous cultures of ts 14 (Figure 1), synchronous cultures of a wild type CHO

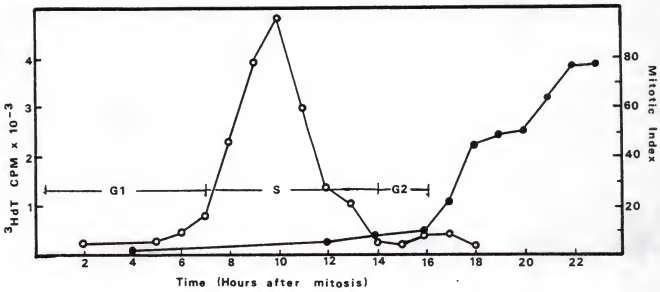

FIGURE 6. CHO cell life cycle. Synchronous mitotic metaphase populations of CHO cells were innoculated into culture at 37°C and grown through an entire cell cycle. Commencing at the times indicated, ^{3}H-thymidine incorporation into acid insoluble DNA was measured in replicate cultures at 37°C (0——0). Mitotic indices also were determined (●——●) at the times shown in the figure.

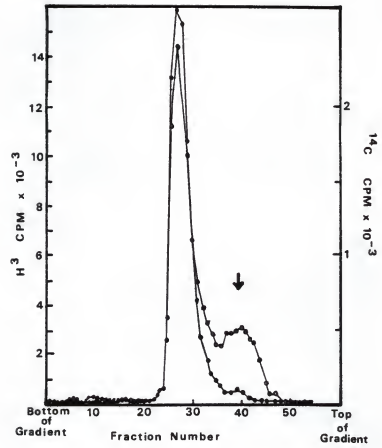

FIGURE 7. CsCl density gradients from S phase CHO cells. CHO cells were labelled with ^{14}C-thymidine (0——0) for 24 hours, then synchronized with a double thymidine block and collected by mitotic detachment. 10^6 metaphase cells were innoculated into culture at 37°C and allowed to progress through G1 phase. These cultures then were labelled with 10^{-5}M ^3H-BrdUrd (0.5 μC/ml, ●——●) for the length of S phase (hours 7-14). At this time, the DNA was extracted and analyzed by isopycnic CsCl gradient centrifugation (24). The arrow indicates the position at which E. coli DNA banded (ρ = 1.70).

cell line also exhibited a strict replicational requirement for de novo protein biosynthesis specifically during mid-G1 phase. Furthermore, the last protein required for initiation of S phase appeared to be synthesized by the end of the fifth hour of G1 phase in a sufficient amount to enable some CHO cells to initiate S phase at hour 7. The precise time of replicative protein synthesis differed between ts 14 cultured at 33°C and CHO cells grown at 37°C, but these discrepancies appear due to the difference in the cell lines and culture temperature. As shown by others, the length of G1 phase varies greatly among cell lines and appears to vary markedly with culture conditions (33). We conclude that during mid-G1 phase CHO cells synthesize one or more proteins that are required for subsequent initiation of S phase.

Transcription of mRNA(s) Also Is Required During G1 Phase for Initiation of S Phase Functions.

Were the mRNA's that specified the replicative proteins synthesized each G1 phase or did these mRNA's preexist in the cell as it entered G1 phase? If the former were the case, synthesis of these mRNA's could be located temporally during G1 phase by cellular sensitivity to transcriptional inhibitors. I attempted to use two transcriptional drug inhibitors, Actinomycin D and α-amanitin, to ask these questions of synchronous G1 phase CHO cultures by experiments similar to those described above. However, Actinomycin D treatment during any hour of G1 or S phase precludes the assay of DNA synthesis, presumably by irreversibly binding to DNA thus inhibiting the movement of the DNA polymerases across the chromatin template (34). Thus, my studies relied exclusively upon the use of α-amanitin. I have observed that this drug does not inhibit DNA replication during S phase, and that it reversibly inhibits G1 phase transcription without subsequently effecting the rate of S phase DNA replication (Moats, unpublished observations).

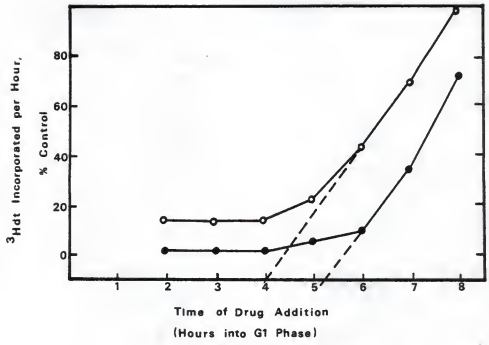

FIGURE 8. Effects of antibiotic drug inhibitors administered during G1 phase on the initiation of DNA replication in CHO cells. Each hour of G1 duplicate cultures of synchronous CHO cells were treated with 2 μM cycloheximide and were maintained in the presence of the drug at 37°C until the end of the seventh hour (●——●). Two dishes not exposed to the drug served as controls. At the end of the seventh hour, the cycloheximide was removed and the cells were pulse labelled for one hour at 37°C with ^{3}H-thymidine (10^{-6}M, 2 μC/ml). S phase entry was assayed by monitoring the ^{3}H-thymidine incorporated into acid insoluble DNA. An identical experiment was performed using 10 μg/ml α-amanitin (O——O).

As described in Methods, 10 µg/ml α-amanitin inhibited synthesis of histone mRNA completely. Consequently, G1 cells were treated with DMEM* containing either 10 µg/ml or 50 µg/ml α-amanitin at hourly intervals throughout G1 phase. Thus, some cells were exposed to the drug for the entire seven hours of G1, while others were exposed to α-amanitin only during the last hour of G1. At the end of the seventh hour, the monolayers were rinsed twice with DMEM* and pulse labelled for one hour at 37°C with ^{3}H-thymidine. The amount of ^{3}H-thymidine incorporated into DNA was determined as described above. These data involving 10 µg/ml α-amanitin also are shown in Figure 8, and demonstrate that RNA, presumably mRNA, necessary for entrance into S phase were synthesized by the end of the fourth hour of G1, one hour before the synthesis of the required G1 protein(s).

These experiments suggest that RNA synthesized during hour 4 of G1 could be an mRNA which codes for replicative proteins synthesized during the fifth hour of G1 phase. In animal cells the synthesis of several RNA classes is sensitive to inhibition by α-amanitin. mRNA, transcribed by RNA polymerase II in vitro, is inhibited by low levels of α-amanitin (0.1-1.0 µg/ml). 5S and transfer RNA's, transcribed by RNA polymerase III, are sensitive only to high levels of α-amanitin in vitro (50-300 µg/ml) (29). On the other hand, several investigators have shown that RNA primers are involved in replication of eukaryote DNA (35, 36). Synthesis of these primers might also have given rise to α-amanitin sensitivity during G1 phase. One attractive interpretation of the data in Figure 8, is that mRNA made during hour 4 of G1 codes for the protein(s) synthesized during the fifth hour. In order to distinguish between an interpretation of Figure 8 involving synthesis of mRNA and one involving primer RNA synthesis, I performed the following experiment.

Four identical cultures of G1 phase CHO cells were treated with 10 μg/ml α-amanitin for six hours after mitosis. α-amanitin then was removed, and two dishes were assayed for their ability to synthesize DNA with ^{3}H-thymidine. The other two dishes were assayed with ^{3}H-thymidine in the presence of 2 μM cycloheximide. If the α-amanitin sensitive step preceded the cycloheximide sensitive step and was a necessary requirement for the hour 5 protein's synthesis, dishes incubated in medium that contained cycloheximide after removal of α-amanitin should not be able to translate newly synthesized mRNA's. On the other hand, if the required protein's biosynthesis had occurred in the presence of α-amanitin, then cultures released from α-amanitin into cycloheximide medium should be able to enter S phase and incorporate ^{3}H-thymidine. As seen in the data summarized in Table 1, the cells treated with cycloheximide after release from the α-amanitin blockade were precluded from entering S phase, while the cells which were not treated with cycloheximide after release from α-amanitin did commence S phase normally. Therefore, the α-amanitin sensitive step precedes the cycloheximide sensitive step during G1 phase and appears to be required for the protein biosynthesis that is necessary for cells to enter S phase. The simplest interpretation of these results is that the RNA which is transcribed by the end of the fourth hour of G1 phase codes for protein(s) (i.e., is mRNA) whose translation is required for the initiation of S phase.

Analysis of Proteins Synthesized by CHO Cells Specifically During G1 Phase

Inasmuch as my data indicated that particular mRNA's and their protein products are synthesized in the mid-G1 phase of each cellular generation and that these proteins are required by the cell for subsequent entry into S phase, I next wanted to determine how many such proteins are synthesized before the fifth hour of the CHO cell seven hour G1 phase.

TABLE 1

Amount of ^{3}H-thymidine incorporated into DNA during the six hours after treatment with alpha-amanitin and its sensitivity to cycloheximide

Treatment during G1, hours 1-6	Treatment during hours 6-12	^{3}H-thymidine incorporated into DNA CPM ± SEM
α-amanitin (10 µg/ml)	NONE	35359 ± 2059
	2 µM cycloheximide	879 ± 52

In order to visualize specific proteins synthesized during the cell's life cycle and to attempt to correlate one or more proteins with the G1 phase replicative protein biosynthetic requirement, I employed the mixed sample SDS polyacrylamide gel electrophoresis procedure described in Methods. In each experiment, synchronous CHO cells were treated with media that contained high specific activity, tritiated amino acids (^{3}H-alanine or ^{3}H-proline) during the fifth through seventh hours of G1 phase. Tritium labelled proteins then were compared to reference CHO cell proteins extracted from cultures treated with the same amino acids labelled with ^{14}C. Since the only assay of these proteins which I possessed was the bioassay shown in Figure 4, I was compelled to use unfractionated, whole cell, nuclear and cytoplasmic extracts for the electrophoretic analyses. Crude extract from approximately 10^{6} cells was the maximum sample size accomodated by the electrophoretic procedure. For this reason and reasons relating to the maximum specific activity of the radioactive proteins obtainable in culture, my analysis was limited to cellular proteins present in each cell in a very large number of copies. Several investigators have pointed out that cell cycle specific proteins are difficult to visualize by electrophoresis (37, 38), but that it is possible if the proteins are made in many copies per cell. Thus, if I were to observe a specific G1 phase protein, it would have been synthesized in many copies per cell each generation (see below). However, if such a peak were observed, as it was by K. D. Ley in his studies of G1 phase CHO cells (17), and if the protein's synthesis correlated with the fifth to seventh hours of G1 phase, the electrophoretic procedure would provide me with a chemical assay for these replicative proteins. Based upon such an assay, I might then determine the number of G1 phase protein species required for the initiation of DNA replication and the subcellular distribution of these proteins during G1 and subsequent S phases.

To verify that cell cycle-specific protein biosynthesis could be observed by my analysis, I again took advantage of the S phase localization of histone protein biosynthesis. S phase cells were radiolabelled for five hours with ^{3}H-arginine and ^{3}H-lysine. Nuclear fractions then were prepared, and the extracts were examined by SDS disc gel electrophoresis. In addition, ^{3}H-alanine-labelled S phase nuclear and cytoplasmic protein extracts also were examined by electrophoresis in mixed samples with ^{14}C-alanine-labelled G1 phase proteins. The mixed sample gels then were analyzed by the procedures described in Methods. These data are summarized in Figures 9 and 10. As can be seen from the data and Figure 9A, fractions 34-39 contain the newly synthesized arginine and lysine rich, S phase nuclear proteins with molecular weights between 12,000 and 18,000. Based upon these proteins' content of basic amino acids, their molecular weights, and their subcellular distribution, they correspond to the cells' histones. In Figure 9B, the same proteins, in this case labelled with ^{3}H-alanine, are contained in S phase nuclear proteins, but not in G1 phase nuclear (^{14}C-alanine label) proteins. Neither the cytoplasmic protein extracts from S phase cells (^{3}H label) or from G1 phase cells (^{14}C label) contained radioactive proteins whose molecular weights corresponded to the histones (Figure 9C).

These same data also are represented as normalized % ^{3}H/% ^{14}C ratios in Figure 10. Note that in the nuclear extracts only (Figure 10A) are there significant G1 vs S phase differences. These differences involve primarily proteins which migrate with the mobility of histones. No significant deviations in the % ^{3}H/% ^{14}C ratios in samples that contained cytoplasmic protein fractions were noted (Figure 10B). Since a wealth of evidence indicates that many enzymes are induced in particular stages of the cell's life cycle, particularly during the G1 and S phases (11-15),

it is clear that double label electrophoretic analyses such as those illustrated in Figures 9 and 10 detect only the prevalent cellular proteins, i.e., proteins which occur in a large number of copies per cell.

How sensitive then is the electrophoretic assay? As noted in the data contained in Figures 9 and 10, histone proteins synthesized by 5 x 10^5 cells during one S phase yield a % ^3H/% ^{14}C ratio of 6.5 (Figure 10A). Other investigators have estimated that every S phase mammalian cell must synthesize approximately 10^8 molecules of histone protein as it replicates its chromatin (39). Thus, in the 5 hours of S phase labelling, each CHO cell that contributed to the extracts examined in the experiment summarized by Figure 9, synthesized approximately 7 x 10^7 molecules of histone. This resulted in a peak of histone protein containing 28,000 CPM of ^3H-alanine (Figure 9B, fractions 32-40), and corresponded to a radioactivity of 32,000 CPM per µg of histone protein. Assuming that a peak of radioactive protein that contains 500 CPM can be detected by electrophoresis, this assay should detect all proteins synthesized in amounts greater than 30 fg per cell during the labelling period. 30 fg of protein is equivalent to approximately 10^6 copies of a protein of 15,000 daltons molecular weight or to approximately 10^5 copies of a protein of 150,000 daltons. For fewer copies of a protein species to be visualized, the proteins must contain more radioactivity.

In order to increase the specific activity with which CHO cells incorporated radioactive amino acids into protein, I also used ^3H- and ^{14}C-proline, an amino acid which the cell line cannot synthesize de novo. Because it was necessary to add 10^{-4}M proline to growth medium in order to maintain the cells' progression through their life cycle, it was not feasible to employ specific proline radioactivities higher than 300 µC/µmole, i.e., 30 µC/ml. Thus, I was not surprised that fewer

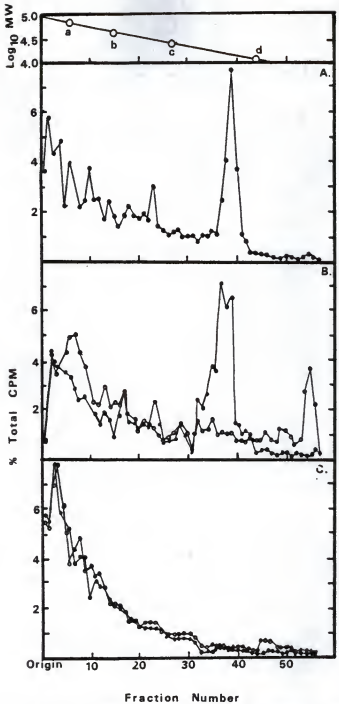

FIGURE 9. Electrophoretic analysis of S phase proteins labelled with ^{3}H-arginine and ^{3}H-lysine or with ^{3}H-alanine and G1 phase proteins labelled with ^{14}C-alanine. Two identical synchronous cultures of CHO cells were labelled during an entire S phase either with ^{3}H-arginine + lysine (0.5 μC/ml, A), ^{3}H-alanine (30 μC/ml, panels B and C, ●——●). A culture was labelled during early G1 phase (hours 0-3) with ^{14}C-alanine (5 μC/ml, panels B and C, O——O). Nuclear and cytoplasmic protein extracts were prepared and mixed in equal amounts on a radioactivity basis for the ^{3}H- and ^{14}C-alanine labelled proteins. The ^{3}H-arginine + lysine labelled nuclear proteins and the mixed samples were analyzed by electrophoresis as described in the text. The nuclear extracts are in Panel B and the cytoplasmic in Panel C. The migration of the molecular weight standards is indicated at the top of the figure. a = bovine serum albumin, MW 67,000 daltons; b = ovalbumin, MW 45,000 daltons; c = chymotrypsinogen, MW 25,000 daltons; d = cytochrome C, MW 12,000 daltons.

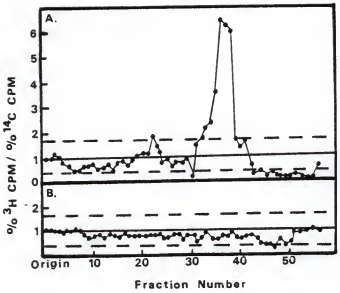

FIGURE 10. % ^{3}H-alanine/% ^{14}C-alanine ratios were determined for each slice of the nuclear and cytoplasmic extracts shown in Figure 9B, C. 10A shows the ratios of the nuclear extracts, 10B the cytoplasmic extracts, and the dashed lines indicate the 99% confidence limits on the assay (see the text).

^{3}H-proline CPM were incorporated on a per cell basis than were ^{3}H-alanine CPM. In summary, the sensitivity of the electrophoretic assay depends directly upon the specific activity of radioactive proteins labelled in synchronous tissue cultures. So far I have not been able to achieve specific activities greater than 32,000 CPM per µg protein, and this limits the sensitivity of the electrophoretic assay to prevalent cellular proteins.

Seven independent double-label experimental approaches were attempted to visualize G1 phase-specific CHO cell proteins by SDS polyacrylamide gel electrophoresis. The first approach involved electrophoresis of a mixture of extracts that contained ^{14}C-alanine labelled reference cell proteins and ^{3}H-alanine labelled synchronous cell proteins radiolabelled for 1 hour periods from the second through the eighth hours of G1 phase. The ^{14}C-alanine labelled extracts were obtained from asynchronous CHO cell cultures grown for 48 hours (3 generations) in ^{14}C-alanine. ^{3}H-protein extracts from cultures radiolabelled each hour of G1 thus were electrophoresed with the ^{14}C protein reference extract, and I analyzed the % ^{3}H/% ^{14}C ratios in each gel slice. The % ^{3}H/% ^{14}C ratios calculated from the data are summarized in Figure 11. As can be seen in the figure, none of the gels contained fractions with % ^{3}H/% ^{14}C ratios that differed significantly from 1.00.

Since it was possible that some of the proteins synthesized during G1 phase were not synthesized in sufficient quantity in one hour to incorporate enough ^{3}H-alanine to be visualized on the gel (i.e., such a protein would necessarily be synthesized at 10^{6} copies/hour, see above), the next method that I attempted to visualize G1-specific proteins involved electrophoresis of extract obtained from cells labelled for 4 hours during G1. The results of these experiments are illustrated in

Figure 12. Again, no significant peaks appeared in either the nuclear (12A) or in the cytoplasmic (12B) extracts.

A third approach that I attempted to visualize G1 phase-specific CHO cell proteins involved ts 14, the temperature sensitive mutant. In this experiment, ts 14 was labelled with ^{14}C-alanine at 33°C for 48 hours (1.5 generations) to label all of its proteins, and a cell free protein extract was prepared. A second culture of ts 14 was transferred to 39°C for 14 hours so that all cells would come to rest at or near the G1-S phase boundary (4), and then were returned to 33°C. Since ts 14 cells commence DNA replication rapidly when returned to permissive temperature (4), they must immediately synthesize all G1 proteins necessary for entrance into S phase. Therefore, the cultures were treated with ^{3}H-alanine for the 8 hours following return to permissive temperature. These data appear in Figure 13. Again, no significant peaks in the % ^{3}H/% ^{14}C ratios were observed.

The asynchronous cultures used to generate reference ^{14}C protein extracts might have accumulated large amounts of stable proteins synthesized each cell generation. If this were the case, and if the cycle-specific proteins which I sought were not degraded rapidly, newly synthesized ^{3}H-alanine labelled proteins might comigrate with the stable ^{14}C labelled proteins and yield % ^{3}H/% ^{14}C ratios not significantly greater than 1.00. Therefore, in order to visualize stable cell cycle-specific proteins, three additional protocols were attempted. Cultures were labelled during early G1 phase (hours 0-3) with ^{14}C-alanine and these nuclear and cytoplasmic reference extracts were electrophoresed in combination with extracts from cells radiolabelled with ^{3}H-alanine during late G1 phase (hours 3-7). These data are represented in Figure 14A. Figure 14B summarizes the data obtained when ^{3}H-alanine labelled

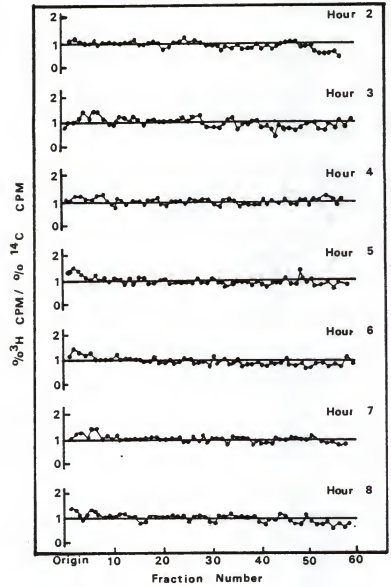

FIGURE 11. Electrophoretic analyses of whole cell protein extracts obtained from each hour of G1 phase. Synchronous G1 cells were radiolabelled with ^{3}H-alanine (30 µC/ml) for one hour periods from the second through the eighth hours of G1 phase and whole cell protein extracts were prepared. The ^{14}C-alanine whole cell reference protein extracts were obtained from asynchronous cells which had been radiolabelled for 48 hours. The ^{3}H-alanine labelled proteins were mixed with equal amounts of ^{14}C-alanine labelled proteins (on a radioactivity basis), and the mixed samples analyzed by SDS polyacrylamide gel electrophoresis. % ^{3}H/% ^{14}C ratios were determined for each gel slice. The 99% confidence limits are $0.4649 \leq x \leq 1.7101$.

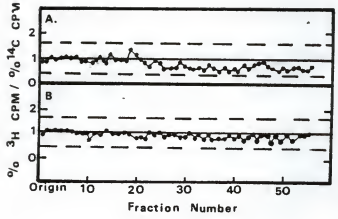

FIGURE 12. Electrophoretic analysis of ^{3}H-alanine and ^{14}C-alanine labelled proteins. Synchronous CHO cells were labelled during hours 3-7 of G1 phase with ^{3}H-alanine (30 μC/ml) and nuclear and cytoplasmic extracts were prepared. Mixed samples composed of these late G1 phase ^{3}H-alanine labelled proteins and ^{14}C-alanine labelled proteins from asynchronous cells were analyzed by SDS polyacrylamide gel electrophoresis. The % ^{3}H/% ^{14}C ratios for nuclear (panel A) and cytoplasmic (panel B) extracts were calculated. The 99% confidence limits of the assay are indicated by the dashed lines.

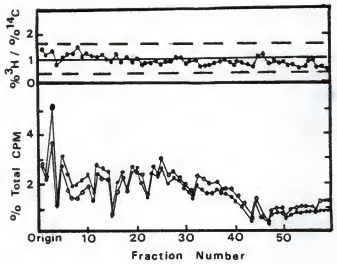

FIGURE 13. Electrophoretic analysis of ^{3}H- and ^{14}C-alanine labelled proteins from ts 14. One culture of ts 14 was transferred to 39°C for 14 hours, returned to 33°C, and subsequently radiolabelled for 8 hours after return to 33°C with ^{3}H-alanine (30 µC/ml, ●——●). Whole cell protein extract was prepared and mixed with a whole cell extract obtained from a culture of ts 14 which had been radiolabelled with ^{14}C-alanine (1.25 µC/ml, O——O) for 48 hours at 33°C.

nuclear and cytoplasmic proteins synthesized during late G1 phase (hours 3-7) were analyzed with nuclear and cytoplasmic proteins synthesized during late G1 in ^{14}C-alanine medium that contained 10 µg/ml α-amanitin. This dosage of α-amanitin inhibits cellular entry into S phase, presumably by inhibiting particular G1 phase-specific mRNA's (see above). A sixth set of experiments also was performed using ^{14}C-proline labelled late G1 phase proteins synthesized in the presence of α-amanitin and ^{3}H-proline labelled late G1 phase proteins. These data are represented in Figure 14C. As in the previous attempts to visualize G1 phase-specific proteins, no significant electrophoretic ratio peaks were observed.

The seventh attempt to visualize specific G1 phase proteins involved a combination of two previous approaches. As before, ts 14 preincubated at nonpermissive temperature was radiolabelled with ^{3}H-alanine during late G1 to 33°C and was electrophoresed with ^{14}C-alanine labelled proteins from CHO cells labelled in the presence of α-amanitin after return to (hours 3-7). These data are shown in Figure 15. Again, no significant ratio peaks appeared.

Thus, seven independent experimental approaches all provided no evidence for the synthesis of cell cycle-specific prevalent proteins in synchronous cultures of G1 phase CHO cells. Apparently, all cell cycle-specific proteins synthesized during mid-G1 phase are synthesized in amounts less than 30 fg per cell, and therefore cannot be visualized in total cell extracts radiolabelled at a specific activity of 32,000 CPM/µg protein.

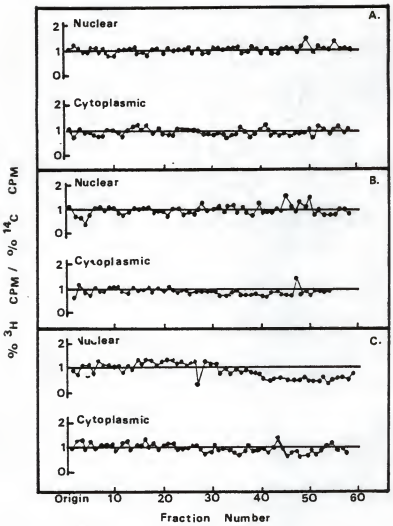

FIGURE 14. Use of ^{14}C-alanine labelled proteins synthesized early in G1 phase, as well as in lage G1 phase in the presence of α-amanitin, as electrophoretic standards. Cultures were labelled during early G1 phase (hours 0-3) with ^{14}C-alanine and nuclear and cytoplasmic reference extracts prepared. These were mixed with nuclear and cytoplasmic proteins which had been radiolabelled with ^{3}H-alanine during late G1 phase (hours 3-7) and electrophoresis carried out as described in the text. % ^{3}H/% ^{14}C ratios were determined (panel A). In panel B, ^{3}H-alanine labelled nuclear and cytoplasmic proteins synthesized during late G1 phase (hours 3-7) were analyzed with nuclear and cytoplasmic proteins synthesized during late G1 in ^{14}C-alanine medium that contained 10 μg/ml α-amanitin. % ^{3}H/% ^{14}C ratios from the electrophoreses of ^{14}C-proline radiolabelled late G1 proteins synthesized in the presence of 10 μg/ml α-amanitin with ^{3}H-proline radiolabelled late G1 proteins are shown in panel C. As before, the 99% confidence limits of the assay are $0.4649 \leq x \leq 1.7101$.

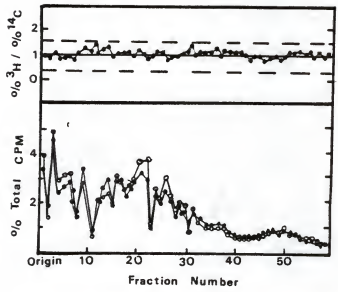

FIGURE 15. Electrophoretic analysis of ^{3}H-alanine (●——●) radiolabelled proteins from ts 14 and ^{14}C-alanine proteins (O——O) from CHO cells labelled in the presence of 10 µg/ml α-amanitin. % ^{3}H/% ^{14}C ratios were calculated for each gel slice. The 99% confidence limits of the assay are indicated by the dashed lines.

DISCUSSION

The CHO cell life cycle at 37°C consists of a seven hour G1 phase, a seven hour S phase, a G2 phase lasting approximately two hours, and mitosis, half an hour. The experiments summarized by Figures 1 and 8 establish that there is a requirement for protein biosynthesis during mid-G1 phase which must be satisfied before the initiation of S phase can occur in Chinese hamster cells. Synthesis of these proteins in synchronous cultures of CHO cells appears to be complete by the end of the fifth hour of G1 phase. The experiments illustrated in Figure 8 also indicate that mRNA's coding for these proteins are synthesized by the end of the fourth hour of G1 phase. Also the data in Figures 3 and 9-14 indicate that the protein synthesis required for entry into S phase is not histone (H2A, H2B, H3 or H4) synthesis. Histone synthesis, as well as synthesis of functional histone mRNA, is restricted to S phase in my synchronous CHO cultures (note data in Figure 3). The experiment in which α-amanitin treatment during G1 was followed by cycloheximide treatment (Table 1) demonstrated that the α-amanitin sensitive step preceded the cycloheximide sensitive step and appeared to be prerequisite for it. Thus, mRNA synthesized during the fourth hour of G1 phase appears to code for protein which is synthesized by hour 5 and which is required for cellular entry into S phase at hour 7.

The several electrophoretic analyses attempted in my studies were unable to detect this or any other G1 phase-specific protein synthesized by synchronous CHO cells, in agreement with the work of others (37, 38). My studies examined both cytoplasmic and nuclear proteins in the molecular weight range of 12,000 to 150,000 daltons, and, as discussed above, required that 30 fg of radioactive protein be synthesized per cell for electrophoretic detection. For proteins of 15,000 daltons,

this implies a minimum of 1.2×10^6 copies per cell; for proteins of 50,000 daltons, 3.6×10^5 copies per cell; and for proteins of 150,000 daltons, 1.2×10^5 copies per cell. Kolodny and Gross have observed G2 phase-specific proteins in synchronous cultures of HeLa cells (38). They employed labelling conditions very similar to those I used, but failed to comment on the sensitivity of their assay. I presume that the differences observed by these investigators, as well as by K. D. Ley (17), who reported a G1 phase-specific CHO cell protein, reflected approximately 10^6 molecules of the protein per cell.

Proteins synthesized during mid-G1 phase appear to control initiation of S phase in CHO cells either directly or indirectly (see Figures 1 and 8). Synthesis of these proteins therefore provides a biochemical marker which can be used to characterize the G1 phase. Since the proteins themselves could not be visualized by SDS polyacrylamide gel electrophoresis when labelled with a variety of radioactive amino acids, these proteins are probably not predominant cellular proteins (i.e., present at a level of 30 fg/cell). Inasmuch as one would not expect regulatory proteins to be present in the cell in large numbers, it is not surprising that proteins responsible for replicative control cannot be visualized by electrophoresis of unfractionated protein extracts.

Ley's electrophoretic data (17) directly conflict with the findings reported here. I have used the same cell line, similar labelling conditions and one of the two synchronization procedures that he used, and repeatedly attempted to confirm his observations. The time of synthesis of Ley's protein I (17) appeared to correlate closely with the time at which my cultures become insensitive to cycloheximide treatment (Figure 8). One possible explanation for the difference between my data and Ley's, is that Ley reported only his data from cell cultures synchronized

to G_0 by amino acid starvation. I also attempted to examine synthesis of cycle-specific CHO cell proteins after presynchronization by proline starvation. After reversal of the G_0 blockade, CHO cells appeared to enter S phase asynchronously over a 14 hour period (data not shown). In order to distinguish repair DNA synthesis from semiconservative, replicative DNA synthesis over this long time course, cellular DNA was prelabelled with ^{14}C-thymidine and then the cells were synchronized to G_0 by proline deprivation. Cells were assayed for DNA replication from hours 0-5 and hours 6-12 after release from the G_0 blockade by incubating them in medium that contained BrdUrd and $^{32}PO_4$. As shown in Figure 16A and B, after release from G_0 fewer than 5% of the cells entered S phase and replicated their DNA, as indicated by the lack of conversion of ^{14}C DNA to hybrid density DNA. Thus, in my hands, CHO cells did not synchronously reverse from G_0 quantitatively, and I was unable to confirm the precise experiments that Ley reported. (Perhaps the same was true of the "G_0" culture in Ley's experiments, and might account for differences between our results).

These data, as well as the data of others (4, 9, 10) suggest that initiation of DNA synthesis in animal cells is regulated by de novo protein synthesis. Whether this is a direct or an indirect control mechanism is not clear at present. Some investigators have examined the enzymatic requirements for DNA synthesis, which include induction of the DNA polymerases, ligases and enzymes necessary for the synthesis of the deoxyribonucleotides (11-15). The intracellular pools of deoxyribonucleotides might affect the control of DNA synthesis, but it is difficult to envision that substrate biosynthesis alone controls initiation of DNA replication. However, the activities of thymidine kinase (11), thymidylate kinase, deoxycytidine monophosphate deaminase and ribonucleotide reductase

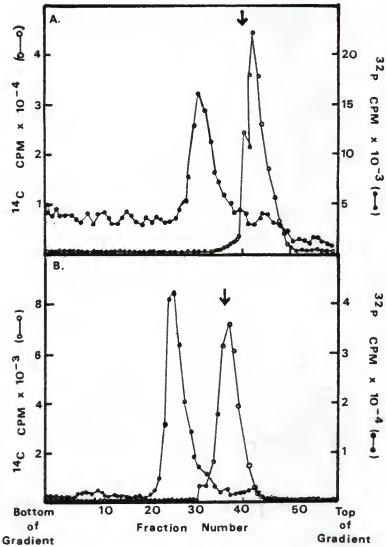

FIGURE 16. CsCl density gradients from G_0 cells. CHO cell DNA was prelabelled with ^{14}C-thymidine (O——O) and then the cells were synchronized to G_0 by proline deprivation. Cells were assayed for DNA replication from hours 0-5 (panel A) and hours 6-12 (panel B) after release from the G_0 blockade by allowing them to incorporate BrdUrd in the presence of $^{32}PO_4$ (●——●). The DNA was extracted and analyzed by isopycnic CsCl gradient centrifugation (24). The arrows indicate the position at which E. coli DNA banded (ρ = 1.70).

(12, 13) all increase greatly at or near the beginning of S phase. In cultured mouse embryo cells, Reichard and his colleagues found that the intracellular pools of deoxyribonucleotides do not increase until after DNA synthesis has begun, and that increases in the pool sizes were concomitant with increases in the cells' DNA synthetic activity (12).

The data presented in this thesis establish that histone synthesis per se is not the signal for initiation of S phase, since synthesis of histones and their mRNA appears to be restricted to the S phase. The possibility exists, however, that a modification of histones, such as phosphorylation (40, 41), signals the initiation of S phase. In addition, it is also possible that changes in the association of chromatin DNA with nonhistone chromosomal proteins (42-45) or with membrane components (40) might be involved in signalling the initiation of S phase. For example, Pederson and Robbins have shown that the affinity of chromatin for ^{3}H-Actinomycin D increases markedly during S phase in synchronous HeLa cell cultures (42). From this they inferred that the availability of ^{3}H-Actinomycin D binding sites is altered at the beginning of S phase. My data suggests that these types of alterations are not accompanied by synthesis of large amounts of cell cycle-specific proteins at or near the G1-S phase boundary.

Nuclear transplantation data and cell fusion studies have demonstrated the presence of a factor or factors in the cytoplasm of S phase cells which induces the initiation of DNA replication in nuclei of G1 cells but not in G2 nuclei (46-49). Therefore, initiation of DNA replication in animal cells appears to be under positive control (2). Inherent in the many positive control models suggested for the initiation of DNA synthesis is the notion that the DNA itself possesses a sequence of nucleotides which specifies the initiation site or sites (2, 50).

My data indicate that a class of G1-specific proteins is translated from a class of newly synthesized mRNA during G1, and that these proteins are prerequisite for the initiation of DNA replication at the beginning of S phase.

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APPENDICES

APPENDIX 1. Technique for Mitotic Selection.

1) Nine hours after reversal of the second thymidine block, the cultures were examined under a phase microscope to estimate the percent rounded cells in the population. The flask then was incubated for ten or fifteen minutes at 37°C in the CO_2 incubator. After this time the flasks again were examined to see whether more cells had rounded up. When it seemed that the percent rounded cells was a maximum, the flasks were shaken. The time of harvesting varied from 9-10 hours after reversal of the second thymidine block.

2) To shake cells off -- NO media was removed prior to shaking. All operations were performed under sterile conditions. The flask was held horizontally, parallel to the floor and was shaken sharply two times by pulling it toward the chest and then pushing it away. Then the side of the flask was slapped sharply two times.

3) The media was swirled over the monolayer to collect all the cells and then the flask was tilted on end to collect the media and cells, and the media with the mitotic cells was transferred to a 50 ml conical centrifuge tube.

4) The cell suspensions were centrifuged at 2000 rpm at 4°C in the International centrifuge for 10 minutes. The media was decanted off and the cells were resuspended in fresh media and plated at the cell density desired. Cells that had been treated with colcemid prior to mitotic detachment, were resuspended in sterile PBS and centrifuged again at 2000 rpm for 10 minutes at 4°C. The PBS was removed and the cells were resuspended in fresh media and plated at the cell density desired.

APPENDIX 2. Procedures for Determination of Mitotic Indices.

Steps 1)-18) were followed when the percent mitotic cells in a population of cells was desired, i.e., as in cell cycle experiments. Steps 5)-18) were followed when a mitotically selected population of cells was analyzed.

1) The DMEM*-colcemid medium was removed, placed in a conical centrifuge tube and kept on ice.

2) The monolayer was trypsinized and the trypsin quenched with DMEM* with 10% FCS. This was pooled with the media in Step 1).

3) The pooled medium and cell suspension was centrifuged at 1500 rpm for 15 minutes in the International table top centrifuge to pellet the cells.

4) The supernatant was decanted and discarded.

5) The pellet was resuspended GENTLY in 1.0 ml half strength PBS (1 part PBS: 1 part water) and incubated for 3 minutes at room temperature. This swelled the cells, rendering mitotic figures easier to visualize.

6) The swollen cells were pelleted by centrifugation at 1500 rpm for 5 minutes in the International table top centrifuge.

7) The supernatant was aspirated off with a Pasteur pipette and discarded.

8) The cell pellet was resuspended in 0.5 ml MeOH:HOAc (3:1) by dripping the solution down the side of the tube and letting it flow onto the pellet. This then was incubated at room temperature for 5 minutes without shaking.

9) 1.0 ml of 67% HOAc was added to the pellet in MeOH:HOAc solution, again by dripping it down the side of the tube. The tube again was NOT disturbed.

10) The tube of cells was incubated at 4°C for at least 2 hours.

APPENDIX 2 (cont.)

11) After incubation in the cold, the cells were triturated to disperse cell aggregates.

12) To prepare slides, 3-5 drops of the cell suspension were placed on a slide with a Pasteur pipette and air dried.

13) After the slide was dry it was immersed in 1 M HCl for 3 minutes at 60°C.

14) After the HCl treatment, the slide was rinsed in water.

15) Then the slide was covered with 0.5% crystal violet and stained for 3 minutes.

16) The slide was rinsed by gently running water over it and then it was air dried.

17) Slides were covered using 3-4 drops of Permount (Fisher Scientific Company) to permanently seal the coverslip.

18) The slides were examined with a microscope, scoring the number mitotic cells in the total number of cells viewed. 300-500 cells were scored for each point taken in an experiment.

APPENDIX 3. Procedures Used for Autoradiography.

A) Darkroom Materials:

The following items were used in dipping and developing slides.

1) Kodak Nuclear Track Emulsion, NTB2 (Eastman Kodak Company) -- This was stored at 4°C in the dark and had to warm up to room temperature before use.

2) D-19 Developer (Eastman Kodak Company)

3) Paper Fixer (Eastman Kodak Company)

4) 2% Acetic acid

5) Safelight

6) Microscope Slides

7) Microscope Slide Box

B) Labelling cells:

^{3}H-thymidine media was used to label cells. This was prepared by mixing 0.5 ml ^{3}H-thymidine (Schwarz Mann, S. A. 0.5 mC/ml), 0.1 ml 10^{-3} M thymidine and 4.4 ml DMEM*. This gave a final concentration of 50 µC/ml. 100 µl of this solution was added to each dish of cells assayed. There were 2.0 ml DMEM* per dish of cells, thus 100 µl of ^{3}H-thymidine would give a final concentration of 2.5 µC/ml radioactive thymidine per dish. Cells were labelled for one hour at 37°C, in the CO_2 incubator.

C) Harvesting and Fixing cells:

After labelling for one hour with ^{3}H-thymidine, cells were fixed as follows:

If using flaskettes --

1) Media was aspirated off and discarded as radioactive waste.

2) The monolayer was treated with 2.0 ml MeOH:HOAc (3:1) and incubated for five minutes at room temperature.

3) The MeOH:HOAc was removed and the monolayer rinsed three times with PBS.

4) After the last rinse was removed, the monolayer was treated with 1.0 ml MeOH:HOAc (3:1) and 1.0 ml 67% HOAc and incubated five minutes at room temperature.

5) The top of the flaskette was removed from the slide bottom and the slide bottom was air dried overnight.

APPENDIX 3 (cont.)

If using flasks --

1) The monolayer was trypsinized and the trypsin quenched with DMEM* with 10% fetal calf serum.

2) The cells were pelleted by centrifuging at 1000 rpm for seven minutes.

3) The supernatant was discarded and the cell pellet was resuspended in 1.0 ml PBS, GENTLY. The cells were again pelleted by centrifuging at 1000 rpm for five minutes and the PBS decanted.

4) Step 3) was repeated twice more, GENTLY.

5) The pellet was resuspended after the final spin, in 0.5 ml MeOH:HOAc (3:1) and 1.0 ml 67% HOAc and incubated at 4°C for at least two hours.

6) This preparation was stored in the cold box until ready to use.

7) To use, it was triturated to distribute the cells evenly.

8) 2-5 drops of this cell suspension were placed on a slide and air dried.

9) The dry slide was then immersed in 60°C 1 M HCl for three minutes (This fixes the cells to the slide).

10) The slide was air dried.

D) Procedure for dipping the slides in emulsion:

1) The emulsion, double wrapped to protect it from light, distilled water, a 50 ml graduated cylinder, 2 wooden applicator sticks and a Copeland jar were incubated at 45°C for at least one and one half hours prior to use.

All of the following were performed in a darkroom using a safelight.

2) The emulsion was mixed with water, one volume emulsion: one volume water. To dip twenty slides, usually 20 mls emulsion plus 20 mls water was used. This mixed in a graduated cylinder and stirred very gently and SPARINGLY so as not to make bubbles.

3) The emulsion-water solution was then transferred to the Copeland jar and the slides were immersed one by one SLOWLY into the emulsion and then were SLOWLY withdrawn from the emulsion and leaned on the side of the box to dry in the dark (about 15 minutes). At all times the slides and the emulsion were kept at least three feet away from the safe lights.

4) The slides were placed in the slide box. The box was sealed with tape, wrapped twice in aluminum foil and taped shut again so that NO light could get into the box and expose the emulsion.

APPENDIX 3 (cont.)

6) The slides were incubated for one week at -90°C in the Revco freezer.

E) Developing the slides:

1) The slides were immersed in D-19 for five minutes and then rinsed quickly in distilled water.

2) They were then dipped in 2% HOAc for ten seconds and again rinsed quickly in distilled water.

3) Then the slides were soaked in paper fixer for two minutes and were subsequently rinsed in running distilled water for thirty minutes.

4) The slides were stained with 0.5% crystal violet, air dried, coverslipped with Permount (See Methods, "Mitotic Collection Functions") and % radiolabelled nuclei scored by examining them under

APPENDIX 4. Procedures used to make protein extracts for electrophoresis.

The three buffers used to make extracts are given below.

1) Lysis Buffer A:

0.05 M NaCl
1% SDS
0.03 M Tris HCl, pH 7.5
Stored at room temperature.

2) Lysis Buffer B:

0.03 M Tris HCl, pH 8.0
0.1 M NaCl
0.001 M $MgCl_2$
0.005 M $CaCl_2$
0.5% NP-40
Stored at 4°C.

3) Lysis Buffer B without NP-40:

0.03 M Tris HCl, pH 8.0
0.1 M NaCl
0.001 M $MgCl_2$
0.005 M $CaCl_2$
Stored at 4°C.

Whole Cell Extracts:

The media was aspirated off and the monolayer gently rinsed twice with 4°C PBS. 1.0 ml Lysis Buffer A was added and the dish incubated at room temperature for fifteen minutes. The plate was then tilted to collect the lysate and the lysate was transferred to a 12 ml conical tube. The lysate was adjusted to 0.01 M $MgCl_2$ and 5 µg deoxyribonuclease I (Worthington Biochemical Corporation) and 1 µg ribonuclease A added. This solution was vortexed and incubated at 37°C for thirty minutes. After thirty minutes, the lysate was adjusted to 0.01 M Na_2EDTA, and dialyzed for at least eight hours against 0.1% SDS at room temperature. After dialyzing, 10 µl of the extract were spotted on a glass fiber filter and counted in the scintillation counter. The remainder of the lysate was lyophilized. The lyophilized powder was dissolved in a quantity of electrophoresis sample buffer to give 100,000 cpm per 40 µl of solution. 40 µl was then applied to each gel run.

APPENDIX 4 (cont.)

Nuclear and Cytoplasmic Extracts:

The media was removed and the monolayer rinsed gently two times with 4°C PBS. 1.0 ml Lysis Buffer B was then added and the monolayer incubated for fifteen minutes at 4°C, periodically checking to see whether nuclei had extruded from the cells. After fifteen minutes, the plate was scraped and then tilted in the cold to collect the lysate. The lysate was transferred to a 12 ml conical tube and held on ice for six minutes with vortexing at the #4 setting for 15 seconds every two minutes. The extract was then centrifuged at 2000 rpm for fifteen minutes in the International centrifuge at 4°C to pellet the nuclei. After centrifugation, the supernatant was decanted and saved on ice as the cytoplasmic fraction. The nuclear pellet was resuspended in 1.0 ml Lysis Buffer B and vortexed on the #4 setting for fifteen seconds. This solution was centrifuged again for fifteen minutes at 2000 rpm, 4°C in the International centrifuge to repellet the nuclei. This wash supernatant was pooled with the previous supernatant and the nuclear pellet was dissolved in 1.0 ml Lysis Buffer A. This nuclear fraction was then adjusted to 0.01 M $MgCl_2$, and 5 µg deoxyribonuclease I and 1 µg ribonuclease A was made. Both the nuclear and cytoplasmic extracts were incubated for thirty minutes at 37°C. At the end of this time, they were adjusted to 0.01 M Na_2EDTA and dialyzed against 0.1% SDS for at least eight hours. 10 µl of each extract was spotted on a glass fibre filter and counted. The remainder of the solution was lyophilized and redissolved in electrophoresis sample buffer to give 100,000 cpm per 40 µl solution. 40 µl was applied to each gel.

Histone Nuclear Fractions:

The media was disposed of and the monolayer rinsed gently two times with cold PBS (4°C). 2.0 mls Lysis Buffer B was pipetted onto the cells

APPENDIX 4 (cont.)

and the dish was incubated at 4°C for fifteen minutes. After fifteen minutes, the dish was scraped and tilted to collect the extract. The lysate was put into a 12 ml conical tube and held on ice. The plate was rinsed once with 2.0 mls cold Lysis Buffer B and this rinse was then pooled with the initial cell suspension. The solution was then vortexed for fifteen seconds at a setting of 4 and centrifuged at 4°C for fifteen minutes at 2000 rpm. The supernatant was decanted, the nuclei washed once in 1 ml Lysis Buffer B and pelleted again by centrifuging at 2000 rpm for fifteen minutes at 4°C. The supernatant was again decanted and the nuclear pellet resuspended in 0.1 ml Lysis Buffer B without NP-40. 5 µg deoxyribonuclease I was added and the solution incubated for thirty minutes at 37°C. After thirty minutes, 0.1 ml electrophoresis sample buffer was added and 20 µl BME. 100 µl of this mixture were analyzed per gel.

APPENDIX 5. Procedure for making gels.

The following reagents were mixed together in the amounts indicated, evacuated to remove air bubbles, and then the gels were poured.

Reagents	Amounts
RESOLVING GEL:	
Water	2.5 mls
1.5 M Tris-HCl, Ph 8.8	2.5 mls
Acrylamide: N'-N'-bis-methylene acrylamide	5.0 mls
TEMED	10 µl
10% Ammonium persulfate	25 µl
STACKING GEL:	
Water	7.4 mls
0.5 M Tris-HCl, pH 6.8	1.25 mls
Acrylamide: N'-N'-bis-methylene acrylamide	1.33 mls
TEMED	10 µl
10% Ammonium persulfate	25 µl

REPLICATION OF A MAMMALIAN GENOME: THE ROLE OF *DE NOVO* PROTEIN BIOSYNTHESIS DURING G1 PHASE

by

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ABSTRACT

Previous experiments carried out in our laboratory have shown that protein biosynthesis must occur through mid-G1 phase before CHL cells initiate S phase (4). These studies were extended to CHO cells in order to answer several questions that deal with: 1) the exact time of synthesis during G1 phase of the proteins involved in replication, 2) the number of proteins synthesized, 3) whether a chemical assay could be developed to assess the synthesis of these cycle-specific proteins independent of continued growth in culture, 4) the function of these proteins, and 5) regulation of their synthesis during G1 phase. Through the use of the protein synthetic inhibitors, cycloheximide and emetine, I found that S phase DNA replication in CHO cells becomes resistant to the inhibition of protein biosynthesis by the end of the fifth hour of G1 phase. Thus, in CHO cells, proteins necessary for cellular entry into S phase are synthesized by the end of the fifth hour of G1 phase. Experiments with α-amanitin (a transcriptional inhibitor) demonstrated that entrance into S phase required transcription until the end of the fourth hour of G1 phase.

Therefore, it appears that a messenger RNA required for the initiation of S phase is synthesized by the fourth hour of G1 phase and is subsequently translated into proteins necessary for cellular entry into S phase by the fifth hour of G1 phase. Thus, regulation of the synthesis of proteins involved in programmed DNA replication during G1 phase is at the transcriptional level. SDS polyacrylamide gel electrophoresis of double label crude cellular protein extracts was used to assay for these cycle-specific proteins. These studies led to the conclusion that G1 phase "replicative" proteins were synthesized in less