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# THE CELL CYCLE REGULATION OF DIHYDROFOLATE REDUCTASE GENE EXPRESSION IN MOUSE FIBROBLASTS

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# THE CELL CYCLE REGULATION OF DIHYDROFOLATE REDUCTASE GENE EXPRESSION IN MOUSE FIBROBLASTS

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Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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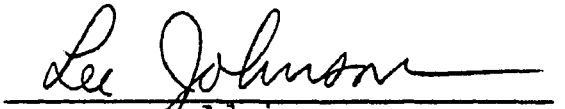

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I dedicate this work to my parents, who always have encouraged my crazy ambitions.

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## PUBLICATIONS

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## FIELD OF STUDY

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| BSA | bovine serum albumin |
| cAMP | cyclic adenosine 3', 5'-monophosphate |
| DB cAMP | dibutyryl cyclic AMP |
| DHFR | dihydrofolate reductase |
| DMSO | dimethylsulfoxide |
| DNA | deoxyribonucleic acid |
| mRNA | messenger ribonucleic acid |
| MTX | methotrexate |
| PBS | phosphate buffered saline |
| SDS | sodium dodecyl sulfate |
| THFA | tetrahydrofolic acid |

# INTRODUCTION

The expression of a eukaryotic gene and the concentration of the RNA and protein products of that gene can be controlled at various levels, including RNA transcription, RNA processing, RNA transport, translation, post-translational modification and RNA or protein degradation. The mechanisms which regulate these processes in eukaryotes are just beginning to be elucidated. In order to examine these processes in detail, it is necessary to use a system where there is differential expression of the genome, such as that occurring during the eukaryotic cell cycle.

The mammalian cell cycle is divided into stages or phases with respect to DNA replication and mitosis (Figure 1). These stages are: $G_1$, when RNA and protein are made to direct the synthesis of chromatin; S, when the chromatin synthesis occurs; $G_2$, when RNA and proteins are synthesized which are required for mitosis; and M, when mitosis and cell division occur. A specific array of biochemical events take place during each of these phases, including variations in cyclic nucleotide levels (Burger, et al., 1972) and deoxyribonucleoside triphosphate pools (Kit, 1976). How does the cell differentially control the levels of each of the

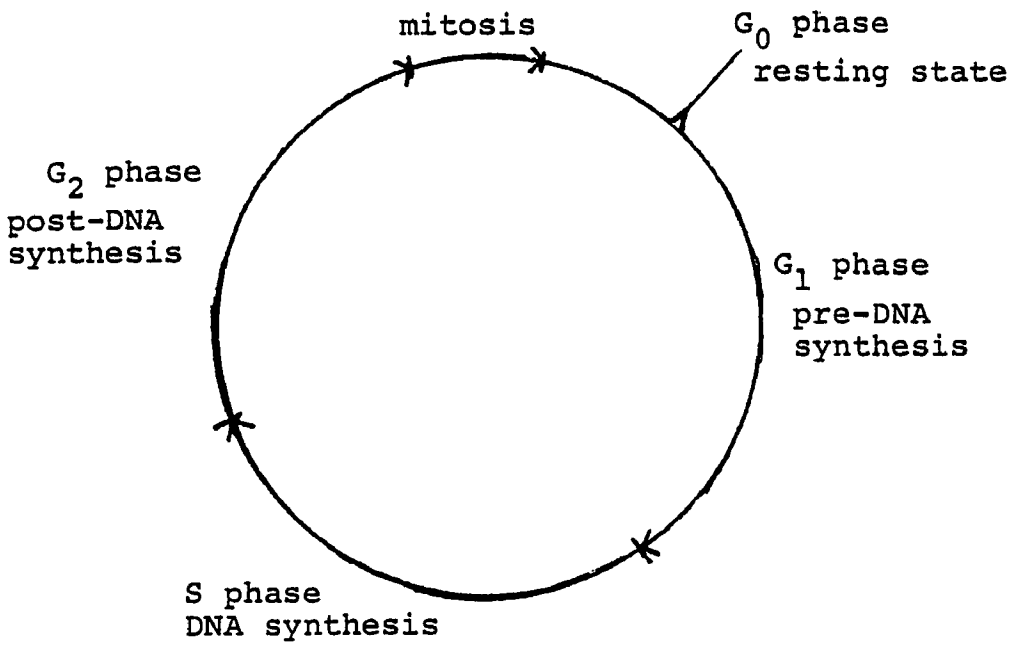


Figure 1. Phases in the life cycle of a typical mammalian cell.

vast number of substrates and enzymes, required during each stage, so effectively? A population of cells that are synchronous with respect to the cell cycle can be obtained to examine this control of gene expression during the cell cycle.

Most mammalian cultures require the presence of a factor found in serum, for growth and survival. In 1963, Todaro and Green published a paper detailing the isolation of several established cell lines from trypsinized mouse embryos. The cells were cultured and put on a rigid transfer schedule and inoculated at the same cell density. One of the established cell lines they isolated in this manner was designated 3T6. These cells were transferred every 3 days and inoculated at a cell density of $6 \times 10^5$ cells per 50 mm plate. After an initial decline in growth rate, there emerged a cell line capable of continual growth at low density; but at higher density, these cells exhibited a phenomenon referred to as contact inhibition or density dependent regulation of growth. These cells grew to a saturation density proportional to the concentration of the serum factors in the medium. When 3T6 cells are placed in 0.5% serum, they will grow until they reach confluence, and then become quiescent. In these quiescent or resting cells, there is no net synthesis of RNA or protein and the rate of DNA synthesis is less than 0.5% of that occurring in exponentially growing cells (Johnson, et al., 1974).

Normal cells make a choice between proliferation or quiescence when they reach $G_1$. According to one theory, quiescent cells have a lower probability of leaving the $G_1$ phase and continuing through the S, $G_2$ and M portions of the cell cycle (Brooks, 1976; Brooks, et al., 1980). These cells have the $G_1$ content of DNA and are said to be in a reversible branch of $G_1$ termed the $G_0$ (Pardee, et al., 1978) (Figure 1).

If the serum concentration of the medium on 3T6 resting cells is increased to 10%, these cells leave the resting state and synchronously pass through $G_1$, entering the S phase approximately 12 hours later. Upon serum stimulation of resting cells, a number of rapid changes take place. The rates of synthesis of messenger RNA (mRNA) and protein begin increasing linearly immediately after stimulation (Johnson, et al., 1974; Abelson, et al., 1974) and DNA synthesis increases dramatically about 12 hours after stimulation (Mauck and Green, 1973).

As these cells enter S phase, there is an increase in the rate of synthesis of various enzymes responsible for the synthesis of DNA precursors, including ribonucleotide reductase, thymidine kinase and thymidylate synthetase (Prescott, 1976). There are other enzymes which are required at low levels throughout the cell cycle, but for which the cell has an increased need during the period of DNA synthesis. An example of an enzyme of this nature is dihydrofolate reductase (DHFR).

DHFR is the enzyme responsible for the reduction of folic acid and dihydrofolic acid to tetrahydrofolic acid (THFA). THFA is an important co-factor in many single carbon transfer reactions, including several reactions during the de novo synthesis of thymidylic acid and purines which are precursors of DNA synthesis (Figure 2). The enzyme thymidylate synthetase catalyzes a reaction where $N^5$, $N^{10}$ -methylene THFA is oxidized to DHFA and the methyl group is transferred to dUMP, forming thymidylic acid. During rapid proliferation, when thymidylate is required for DNA synthesis, cells require the enzyme, DHFR, to replenish the depleted pools of THFA. DHFR is the target of a chemotherapeutic agent, methotrexate (MTX). This drug binds very strongly to the active site of DHFR ($K_i = 6.7 \times 10^{-10}$, Bertino, et al., 1964), destroying the enzymatic activity. It is believed that the depletion of THFA pools in rapidly proliferating cells results in starvation for thymidylic acid and death (Blakely, 1969).

A number of investigators have found that the level of DHFR varies with cell growth rate. The level of the enzyme is greater in rapidly proliferating cells, than in stationary phase cells which slowly traverse the cell cycle due to nutrient depletion (Hillcoat, et al., 1967; Chello, et al., 1977). Earlier studies in our laboratory had shown that exponentially growing mouse 3T6 fibroblasts were very sensitive to the toxic effects of MTX, while quiescent cells were

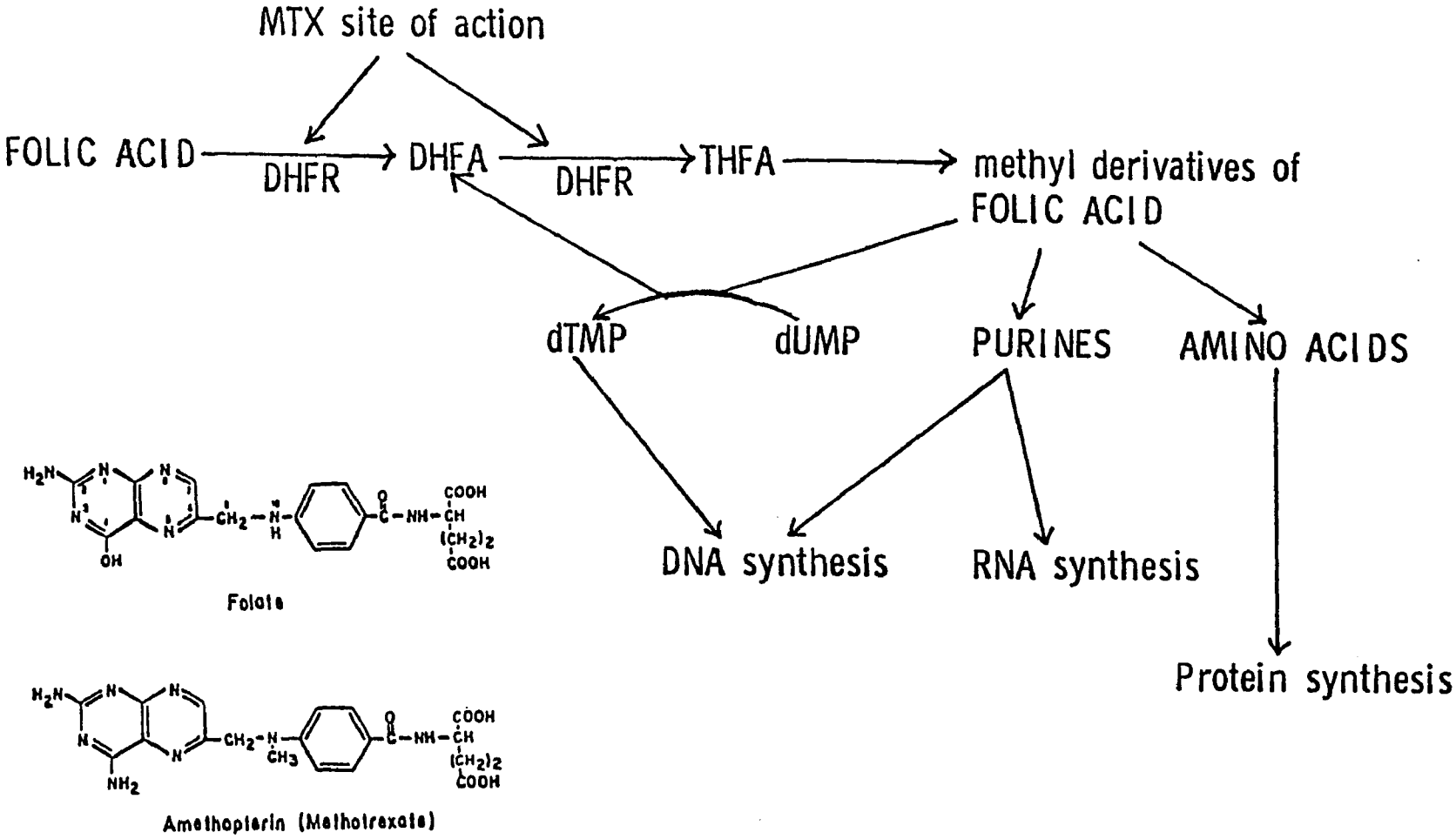


Figure 2. The biochemistry of DHFR.

completely resistant to MTX cytotoxicity (Johnson, et al., 1978a). These studies also showed that resting cells contained much lower levels of DHFR than exponentially growing cells.

Since the level of DHFR appeared to be closely related to the cell growth rate, my initial studies centered on the regulation of DHFR gene expression in resting and growing 3T6 cells, and in resting cells that had been serum stimulated to re-enter the cell cycle. I found that the rate of accumulation of DHFR is low in resting and high in growing 3T6 fibroblasts. About 10 hours after serum stimulation, DHFR accumulation increases rapidly, slightly preceding the onset of DNA replication (Johnson, et al., 1978b).

Due to the low levels of DHFR and DHFR mRNA, the direct quantitation of the rate of synthesis of DHFR and the amount of DHFR mRNA were extremely difficult in this system.

Most studies measuring the metabolism of specific proteins and mRNA sequences in eukaryotes have examined regulation of the gene expression for major proteins of a population of specialized cells, where a large percentage of the total messenger RNA is devoted to a single species of RNA. Examples of these include globin (Maniatis, et al., 1976), silk fibroin (Ohshima and Suzuki, 1977) and immunoglobulin (Schibler, et al., 1978). Because these specialized proteins are synthesized at such high levels, the isolation of the

protein and its mRNA are possible using the technology presently available.

These procedures are not as easily applied to the examination of the "housekeeping" enzymes, such as DHFR, which are normally present at low concentrations in the cell. A fortuitous series of events have resulted in an approach to this problem. In some cases, it has been observed that tumor cells become resistant to the cytotoxic effects of MTX, in cancer patients who have been treated for long periods of time with the drug (Bertino, et al., 1964). A similar resistance can be obtained in cultured cells exposed to low levels of MTX for many generations (Hakala, et al., 1961). Three mechanisms of acquired resistance to MTX have been well documented: an altered DHFR with decreased affinity to MTX (Bertino and Skeel, 1975; Albrecht, et al., 1972); impaired transport of MTX (Linquist, et al., 1976); and an elevated DHFR enzyme activity (Hakala, et al., 1961; Alt, et al., 1976). Increased levels of DHFR is by far the most common mechanism of resistance to MTX cytotoxicity. Studies from the laboratories of Schimke and Littlefield have clearly demonstrated that the increase in DHFR activity is due to an increased rate of synthesis of the enzyme (Alt, et al., 1976) and is associated with an increase in mRNA levels for this enzyme (Kellems, et al., 1976; Chang and Littlefield, 1978). I felt that the increased rate of DHFR synthesis and

increased DHFR mRNA levels in a MTX-resistant cell line would greatly facilitate further studies of the regulation of DHFR gene expression. Unfortunately, none of the cell lines which overproduced DHFR appeared to be sensitive to growth control. In order to study the regulation of DHFR gene expression during the transition from resting to growing cells, in more detail, I isolated and characterized a MTX-resistant 3T6 cell line, M50L3, which retains the ability to rest in medium containing 0.5% serum and overproduces DHFR.

I found that the regulation of DHFR gene expression in the overproducing cell line, M50L3, closely resembles that in the parent 3T6 cells and that there is a close correlation between the time of entry into S phase and the increase in DHFR content. I was also able to show that the increase in the rate of DHFR accumulation is due to an increase in the rate of DHFR synthesis (Wiedemann and Johnson, 1979) and that this is associated with an increase in the level of translatable DHFR mRNA (Wiedemann, Wu, Pratt and Johnson, manuscript in preparation). This cell line permits a more detailed analysis of the content and metabolism of DHFR and its mRNA.

# MATERIALS AND METHODS

## Cell Culture

The mouse 3T6 fibroblasts and MTX-resistant M50L3 cells were maintained on plastic petri dishes in Dulbecco-Vogt's modification of Eagles medium (Gibco) supplemented with 10% calf serum (Colorado Serum) and incubated at 37°C in an atmosphere of 10% carbon dioxide. Stock cultures of M50L3 were grown in the presence of 50 uM MTX. Routinely the medium was changed the day before a culture was to be trypsinized for passage.

Cultures of exponentially growing cells were prepared by seeding cells at low density in medium containing 10% serum. They were fed the next day and used for an experiment, 2 days after seeding. At the time of the experiment the cells were about 25% confluent.

Resting cultures were prepared by seeding cells on plastic petri dishes at a density of $7.0 \times 10^4$ cells/$cm^2$ in medium containing 0.5% calf serum, on day 1. The cells were fed on days 3 and 5, and used for the experiment on day 8 (Johnson, et al., 1974). Cultures of resting cells in roller bottles were prepared by seeding cells below confluence in medium containing 10% calf serum. When the cells reached

confluence, the medium was replaced with fresh medium containing 0.5% serum. The same feeding schedule was used as above, and the cells were used on day 8.

Resting cells were stimulated to re-enter the cell cycle by replacing the medium with fresh medium containing 10% serum. Serum withdrawal was accomplished by rinsing cells with serum-free medium and returning the cells to conditioned medium taken from replicate cultures of resting cells.

Determination of DHFR Levels

The rate of accumulation of newly synthesized DHFR was determined by the rapid and sensitive $^{3}$H-MTX binding assay (Kamen, et al., 1976). 3T6 cultures were pretreated with $10^{-6}$M MTX for 20 minutes to inactivate pre-existing DHFR activity. M50L3 cultures were exposed to $10^{-5}$M MTX in the presence of hypoxanthine (100 uM) and thymidine (30 uM) for a period of 2 days prior to the experiment. Before stimulation, excess MTX was removed by washing 3 times with serum-free medium at 37°C. The entire washing procedure should take at least 20 minutes to allow all unbound MTX to be removed (Goldman, et al., 1968). At various times after stimulation, the cultures were assayed for newly synthesized DHFR. Cultures were rinsed 3 times with phosphate buffered saline (0.137 M NaCl, 0.003 M KCl, 0.016 M $Na_2HPO_4$, 0.002 M $KH_2PO_4$, pH 7.4) and once with Buffer A (0.15 M KCl, 0.01 M $KH_2PO_4$, pH 6.0). They were lysed in Buffer A containing 0.5% Nonidet P-40 (Shell

Oil). Nuclei and cell debris were removed by centrifugation at 800 xg. An aliquot of the supernatant was added to 0.5 ml of Buffer A (final volume) containing 0.9 mg/ml bovine serum albumin (BSA), 4 pmoles $^{3}$H-MTX (Amersham 16 Ci/mMole) and 0.1 umole NADPH. The BSA used in this reaction mix was prepared as follows. 1 g of BSA was dissolved in 100 ml of $H_2O$. 0.7 g of Norit A was added. The suspension was stirred slowly for 20 minutes at 4°C and centrifuged for 20 minutes at 15,000 xg to remove the charcoal. The resulting supernatant was filtered through nitrocellulose to remove any remaining charcoal and stored frozen until needed.

The MTX-binding reaction was incubated for 5 minutes at 4°C to allow the formation of the $^{3}$H-MTX:DHFR complex. Excess $^{3}$H-MTX was removed by the addition of 1 ml of a suspension of 0.1 g dextran, 10 g Norit A, and 2.5 g BSA in 300 ml of Buffer A (pH 6.0). The mixture was centrifuged for 20 minutes at 7,000 rpm in an HB-4 rotor (Sorvall) and 1 ml of the clear supernatant was added to 8 ml of scintillation cocktail containing 4 g Omnifluor/liter of a toluene: Triton X-100 (2:1) mixture. The amount of radioactivity was determined using a Beckman Scintillation Counter.

A functional assay which measured the rate at which a cell extract reduced $^{3}$H-folic acid to $^{3}$H-tetrahydrofolid acid was performed essentially as described by Rothenberg (1966). $^{3}$H-folic acid (Amersham, 47 Ci/mMole) was purified prior to

use by chromatography on a DEAE-cellulose column. The column was eluted at room temperature with a linear gradient from 0.1 to 0.6 M ammonium bicarbonate (pH 8.3). The peak radioactive fractions were pooled, lyophilized and dissolved in 0.05 M Tris-Cl (pH 7.0).

The cells were lysed in 0.02 M citrate (pH 4.8) containing 0.5% Nonidet P-40 and centrifuged at 800 xg to remove nuclei and cell debris. Each 800 ul assay mixture contained 0.02 M citrate buffer (pH 4.8), 7.5 nmoles NADPH, 15 pmoles $^{3}$H-folic acid and 15 nmoles 2-mercaptoethanol. A 200 ul aliquot of cell extract was added to initiate the reaction. The reaction was stopped at the desired times by removing 400 ul of the reaction mixture and adding it to 400 ul of 0.014 M unlabeled folic acid. Then 400 ul of 0.088 M zinc sulfate was added to precipitate the folic acid leaving $^{3}$H-tetrahydrofolate in solution. Following centrifugation at 1000 xg for 10 minutes, the radioactivity remaining in the supernatant was determined using liquid scintillation methods. This assay suffered from high "backround levels" in reactions containing no DHFR, making it difficult to accurately quantitate low levels of the enzyme. To avoid this problem, I routinely used the $^{3}$H-MTX binding assay described above, to determine DHFR levels.

Determination of the Rate of DNA Synthesis

Cultures on 35 mm dishes were incubated in medium containing 1 uCi/ml $^{3}$H-thymidine (Schwarz-Mann, 36 Ci/ml) for 30

or 60 minutes. The cells were rinsed 4 times with ice cold phosphate buffered saline (PBS). The cells were lysed in 1 ml 0.1 N NaOH. The solution was neutralized with 100 ul of 1 M HCl and precipitated by the addition of an equal volume of 10% trichloroacetic acid. The samples were held at 0°C for 1 hour, and the precipitate was collected on GFA filters (Whatman). The precipitate was washed 3 times with ice cold 5% trichloroacetic and counted in a toluene based scintillation fluid containing 5% Protosol (New England Nuclear).

Cloning of Cells

50 cells were seeded on 100 mm plastic dishes. About 2 weeks after seeding, appropriate clones were chosen and their morphology noted. The medium was removed and the plate rinsed once with 2 ml of 0.01% trypsin. Sterile stainless steel rings were dipped in sterile vacuum silicon grease and carefully placed over the clone. The ring was pressed down gently to seal it against the dish, and 2 drops of fresh 0.01% trypsin solution were added. When the cells had detached from the plate, a few drops of medium containing 10% calf serum were added. The cells in the ring were gently suspended and transferred directly to a dish containing fresh medium.

Gel Electrophoresis and Fluorography

Slab gels (13 cm x 15 cm x 1mm) were prepared and electrophoresed using Laemmli's discontinuous buffer system (1970).

The running gel contained 11.3% acrylamide, 0.3% bis-acrylamide, 0.1% sodium dodecylsulfate (SDS), 0.375 M Tris-HCl Buffer (pH 9.2) and was polymerized using 0.025% by volume of tetramethylene diamine (TEMED) and 1 mg/ml ammonium persulfate. The upper gel contained 3.0% acrylamide, 0.08% bis-acrylamide, 0.1% SDS, 0.125 M Tris-HCl (pH 7.2) and was photopolymerized using 0.18 mg/ml riboflavin and 0.025% by volume of TEMED. The tray buffer contained 0.025 M Tris, 0.192 M glycine, and 0.1% SDS. Sample buffer contained (final concentration) 0.0625 M Tris-HCl (pH 7.2), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.001% bromophenol blue. Proteins were denatured in the sample buffer by being placed in a boiling water bath for 2 minutes. At times, protein samples were concentrated by precipitation with 9 volumes of acetone. When protein samples which were difficult to dissolve or dissociate, such as <u>in vitro</u> translation mixtures, they were to be electrophoresed, the concentrations of SDS and mercaptoethanol in the sample buffer were increased to 4% and 10% respectively.

Slab gels were fixed overnight in 9% acetic acid, 50% methanol, then stained with 0.25% Coomassie Brilliant Blue R in fixing solution for 1 hour. The gels were destained in 10% acetic acid for 1 hour, then 7% acetic acid, 20% methanol. After being soaked in 1% glycerol, slab gels were dried on filter paper using a Biorad gel drier.

Radioactive slab gels were soaked in destaining solution to remove radioactive amino acids. They were then immersed in dimethyl sulfoxide (DMSO) to remove the water, followed by immersion in 22.2% (weight/volume) of 2,5-diphenyl oxazole (PPO) in DMSO for 3 hours. The gel was then soaked in 10% acetic acid, 1% glycerol to remove DMSO and dried on filter paper, using the Biorad gel drier. Kodak X-omat film was exposed to the dried gel and placed at -70°C to allow the detection of labeled bands (Bonner and Laskey, 1974; Laskey and Mills, 1975).

Isolation of Polysomes

Total cytoplasmic RNA was isolated from 3T6 and M50L3 cells by a modification of the magnesium precipitation procedure described by Palmiter (1974). All operations were performed at 4°C. Solutions were treated with diethylpyrocarbonate and glassware was baked to eliminate RNase activity. Briefly, the cells were rinsed 3 times with ice cold PBS and harvested in PBS by scraping from roller bottles. They were collected by centrifugation at 800 xg and lysed in 3.3 mM NaCl, 1 mM $MgCl_2$, 3.3 mM Tris-HCl (pH 8.4), containing 1% Nonidet P-40 using a teflon homogenizer. Nuclei and cell debris were removed by centrifugation in an SS-34 rotor (Sorvall) at 15,000 rpm for 5 minutes. The supernatant was brought to 1 mg/ml heparin, 0.1 M $MgCl_2$, 0.025 M Tris-HCl (pH 8.4) and 25 mM NaCl. After an incubation for 1 hour at 0°C, the

solution was layered on 1/2 volume of 30% sucrose in 25 mM NaCl, 25 mM Tris-HCl (pH 7.5), 3.75 mM $MgCl_2$ and centrifuged for 35 minutes at 7,000 rpm in an HS-4 rotor (Sorvall). The pellet of precipitated polysomes was dissolved in SDS buffer and phenol-chloroform extracted.

Phenol-Chloroform Extraction

The material containing RNA and associated protein was dissolved in 2.5 ml SDS buffer (0.1 M NaCl, 0.01 M Tris-HCl, pH 7.4, 0.001 M EDTA, 0.5% SDS) and extracted at room temperature with phenol and chloroform (Penman, 1969) to remove the protein. A ml of distilled phenol and 1 ml of chloroform containing 1% isoamyl alcohol were added to the aqueous solution of dissolved polysomes, mixed vigorously, using a vortex mixer, and centrifuged at 800 xg. The organic phase was removed and discarded. This extraction was repeated twice more. The aqueous phase was then extracted twice with chloroform and transferred to another tube. The entire procedure was repeated until the aqueous-organic interphase was free of precipitated protein.

The RNA was precipitated with 2.5 volumes of ethanol, washed twice with 2 ml of 3 M sodium acetate (pH 6.0) (Palmiter, 1973), dissolved in 0.1 M potassium acetate and precipitated again with ethanol. The RNA was collected by centrifugation and dissolved in water at a concentration of 1 mg/ml (20 $A_{260}$ units/ml).

Determination of Poly (A)(+) mRNA Content

The total amount of poly (A)(+) mRNA was determined by the $^{3}$H-poly (U) hybridization procedure (Bishop, et al., 1974). Approximately 0.05 to 0.25 ug of poly (A)(+) RNA were added to a 0.5 ml reaction mixture containing about 15,000 cpm $^{3}$H-poly (U) in 0.3 M NaCl, 0.03 M sodium citrate, (pH 7.3) and incubated at 45°C for 10 minutes to allow the formation of a poly (A):poly (U) hybrid. After cooling to 0°C, approximately 0.1 mg of RNase A was added to degrade unhybridized $^{3}$H-poly (U). Carrier BSA (2 mg/ml) was added and the RNase-resistant hybrid was precipitated with 1 volume of 10% trichloroacetic acid. The precipitate was collected on GFC filters and the radioactivity determined using liquid scintillation techniques.

Translation of Polysomal RNA

The RNA was translated in a mRNA-dependent reticulocyte system (Pelham and Jackson, 1976) obtained from New England Nuclear. Between 5 and 20 ug of RNA were added to a 25 ul translation mixture containing about 20 uCi $^{3}$H-leucine and incubated for 60 minutes at 37°C. To determine the total amount of protein synthesized, a 2 ul aliquot was diluted by the addition of 100 ul of 0.1 N NaOH, 0.5% $H_2O_2$ and incubated at 37°C for 10 minutes. The base treatment discharges tRNAs and the $H_2O_2$ removes any color due to the heme group, allowing the accurate quantification of incorporation of $^{3}$H-leucine into macromolecules. This basic solution was neutralized with

10 ul of 1 M HCl, precipitated with 1 ml of 10% trichloroacetic acid and collected on glass fiber filters. The radioactivity on the filter was then determined. Backround values (protein synthesized in the absence of added RNA) generally ranged from 5% to 20% of the total incorporation and were subtracted from total incorporation. The amount of $^{3}$H-DHFR was determined by immunoprecipitation followed by gel electrophoresis.

Immunoprecipitation of DHFR

The amount of $^{3}$H-DHFR synthesized in vitro was determined by immunoprecipitation essentially as described (Alt, et al., 1976). A 10 ul aliquot of the translation assay was added to 54 ul of a premix containing 7.4% Triton X-100, 7.4% deoxycholate, 4 ug unlabeled DHFR, 0.4 umoles leucine and 500 cpm $^{14}$C-labeled DHFR. This was diluted to 300 ul with 0.1 M sodium phosphate (pH 7.0). 100 ul of DHFR antiserum were added and the samples were incubated for 1 hour at 37°C. The mixture was then diluted to 2 ml with 0.1 M sodium phosphate (pH 7.0) and the immunoprecititate was collected by centrifugation at 10,000 xg for 30 minutes. The precipitate was washed twice with the phosphate buffer, dissolved in electrophoresis sample buffer, and electrophoresed in SDS 12.5% poly acrylamide gels. The gels were fractionated into 2 mm slices which were held for 24 hours at 37°C in a toluene-based scintillation fluid containing 5% Protosol (New England

Nuclear) and counted in a scintillation counter. All data were corrected for recovery losses by monitoring the recovery of $^{14}$C-DHFR.

# RESULTS

## Regulation of DHFR in 3T6 Cells

Resting ($G_0$) cells are not synthesizing DNA, have no requirement of thymidylate and therefore do not deplete the cellular pool of THFA. These cells have little need for DHFR, while exponentially growing cells require the biosynthesis of purines and thymidylate for RNA and DNA synthesis and rapidly deplete their THFA pools (Figure 2) in the absence of DHFR. Previous studies in our laboratory had shown that resting cells contain only 17% as much DHFR activity as exponentially growing cells (Johnson, et al., 1978a). One method to control the levels of DHFR would be by regulation of the synthesis rate. Unfortunately, the percentage of protein synthesis devoted to the synthesis of DHFR is too small to measure directly, even in exponentially growing 3T6 cells. Assuming that the stability of the protein does not change, the rate of accumulation of DHFR activity should reflect the rate of DHFR synthesis. Preliminary results with cycloheximide, a protein synthesis inhibitor had indicated that DHFR was an extremely stable enzyme, both in resting and growing cells (Fuhrman and Johnson, unpublished). Alt, et al. (1976) had reported that the enzyme had a half life of 50 to 60 hours in mouse AT 3000 cells.

I compared the rate of accumulation of DHFR in resting and growing cells. Cultures were treated briefly with $10^{-6}$ M MTX to inactivate all pre-existing DHFR. The drug was then removed and the rate of recovery of DHFR enzyme activity was followed as a function of time. DHFR activity was quantitated by measuring the ability of a cell extract to bind $^{3}$H-MTX. I found that the rate of recovery of DHFR activity was much higher in exponentially growing cells than in non-cycling resting cells (Figure 3). This suggested that growing cells have more DHFR due to a higher rate of synthesis. If this is true, then the addition of cycloheximide should inhibit the increase in DHFR accumulation by inhibiting protein synthesis. Figure 3 shows that this was the case; the presence of cycloheximide prevented the increase in DHFR activity. Therefore, it appears that the increase in DHFR activity in growing cells is due to de novo synthesis of the enzyme and not the activation of a pro-enzyme. To determine the stability of DHFR mRNA, the RNA synthesis inhibitor actinomycin was added to growing cells. Figure 3 shows that DHFR continued to increase normally in the presence of the drug, indicating that DHFR mRNA is very stable, at least in the presence of actinomycin.

These results indicate that the gene for DHFR is not being expressed in resting cells, but is expressed in growing cells. This would suggest that when resting cells are stimulated to re-enter the cell cycle, the level of DHFR synthesis should increase. To test this, cultures of resting 3T6 mouse

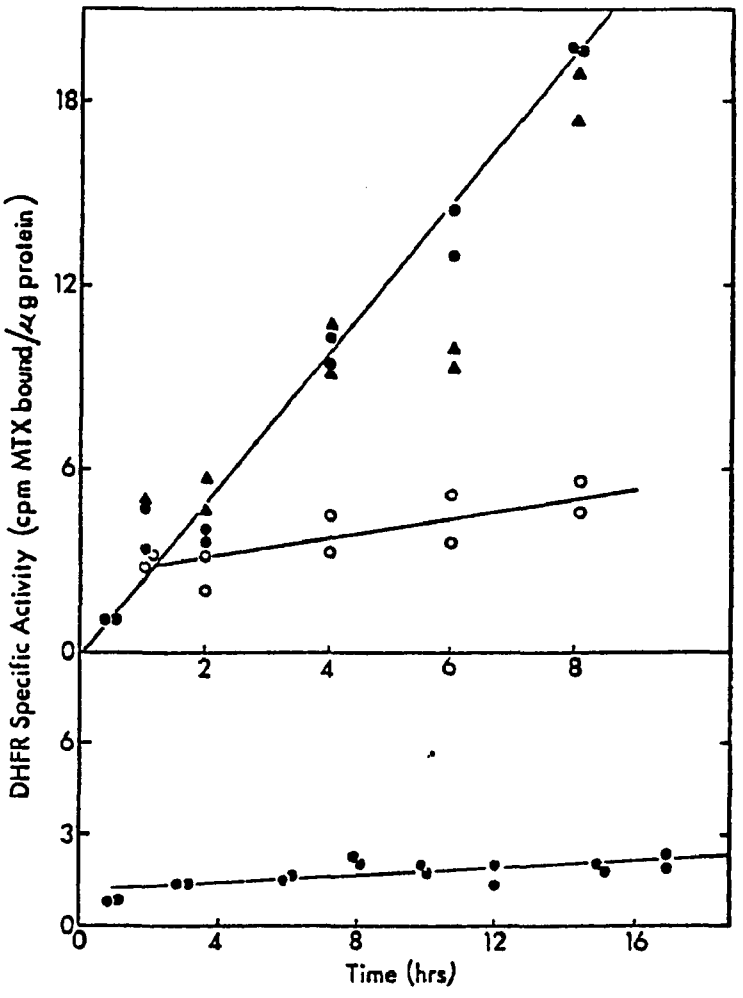


Figure 3. Rate of accumulation of DHFR in resting and growing 3T6 cells. Cultures of resting or growing 3T6 cells were pretreated with MTX to inactivate pre-existing DHFR. Resting cultures were fed with "conditioned medium" containing 0.5% serum, taken from resting 3T6 cells (lower panel). Growing cultures (upper panel) were fed with fresh medium containing 10% serum (●). Some growing cultures were fed with medium containing either 5 ug/ml cycloheximide (o) or 5 ug/ml actinomycin (▲). The level of DHFR as measured by the $^{3}$H-MTX binding assay was normalized to the amount of protein present (Lowry, et al., 1951).

fibroblasts were pretreated with $10^{-6}$ M MTX as before, and stimulated with fresh medium containing 10% calf serum. Resting control cultures were replaced in conditioned medium taken from sister resting plates. DHFR activity was determined at various times thereafter using the $^{3}$H-MTX binding assay. Figure 4 shows that the DHFR activity remained at the same level as in resting cells for the first 10 hours. At that time it began to increase in a linear fashion, until at least 20 hours after stimulation.

It was possible that newly synthesized DHFR can only be measured in stimulated cells after any residual MTX from the pretreatment is titrated. I tested this by mixing extracts from MTX pretreated resting cells and untreated growing cells and then assaying DHFR activity. If there is unbound MTX present in the resting cell extract, there should be a decrease in the total DHFR activity in the combined extracts. The results in Table 1 show that the rinsing procedure was effective and that no residual MTX was present in the pretreated resting cell extracts.

To determine if pretreatment with MTX had any effect on the regulation of the synthesis of DHFR after serum stimulation, I compared the levels of DHFR in 3T6 cells serum stimulated with and without the MTX pretreatment. The level of DHFR in cells pretreated with MTX was parallel to that found in control stimulated cells not pretreated with MTX (Figure 5).

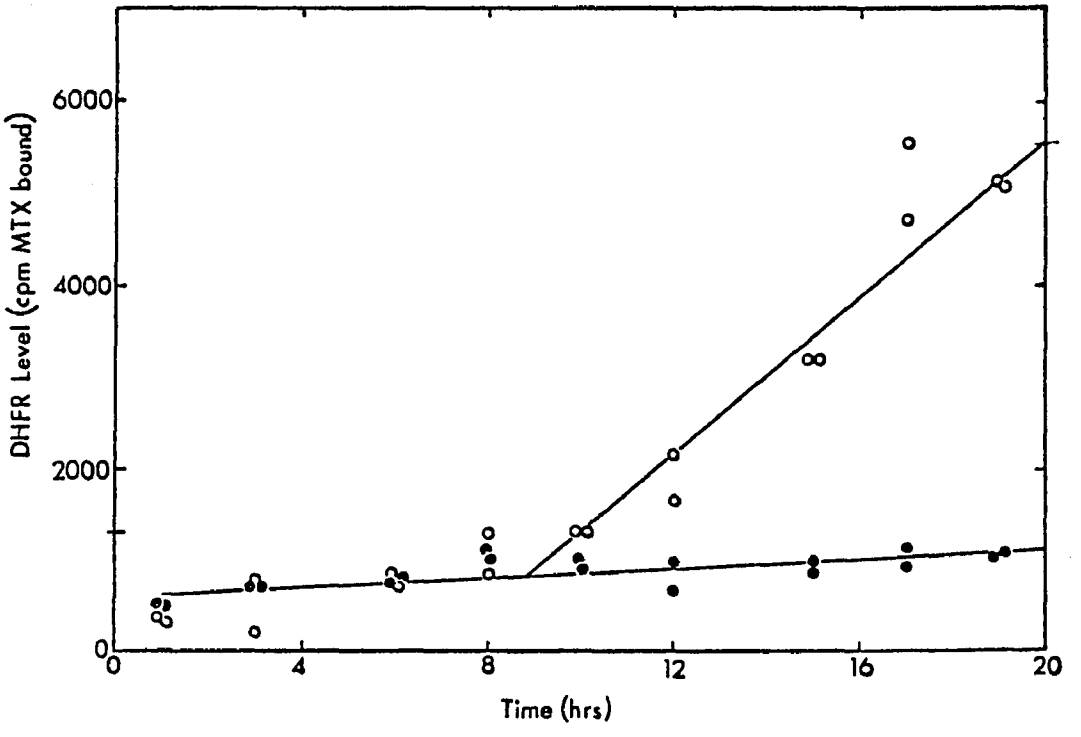


Figure 4. Increase in DHFR following serum stimulation of 3T6 resting cells. Cultures of resting 3T6 cells were pretreated with $10^{-6}$ M MTX to inactivate pre-existing reductase, then fed at time = 0 with conditioned medium containing 0.5% serum (●) or with fresh medium containing 10% serum (o). Cultures were harvested at various times; the level of active DHFR was determined by the $^{3}$H-MTX binding assay.

TABLE 1

THE EFFECT OF MIXING CELL EXTRACTS FROM RESTING AND GROWING CELLS ON THE TOTAL DHFR ACTIVITY

| Extract | cpm $^{3}$H-MTX bound | calculated |
|---|---|---|
| 25 ul growing | 3443.6 | -- |
| 200 ul resting | 460.0 | -- |
| mixture of 25 ul growing + 200 ul resting | 4168.1 | 3903.6 |
| mixture of 25 ul growing + 50 resting | 3555.2 | 3558.6 |

The level of active DHFR in each extract was determined by the $^{3}$H-MTX binding assay. The calculated values were obtained by calculation from the values determined before mixing.

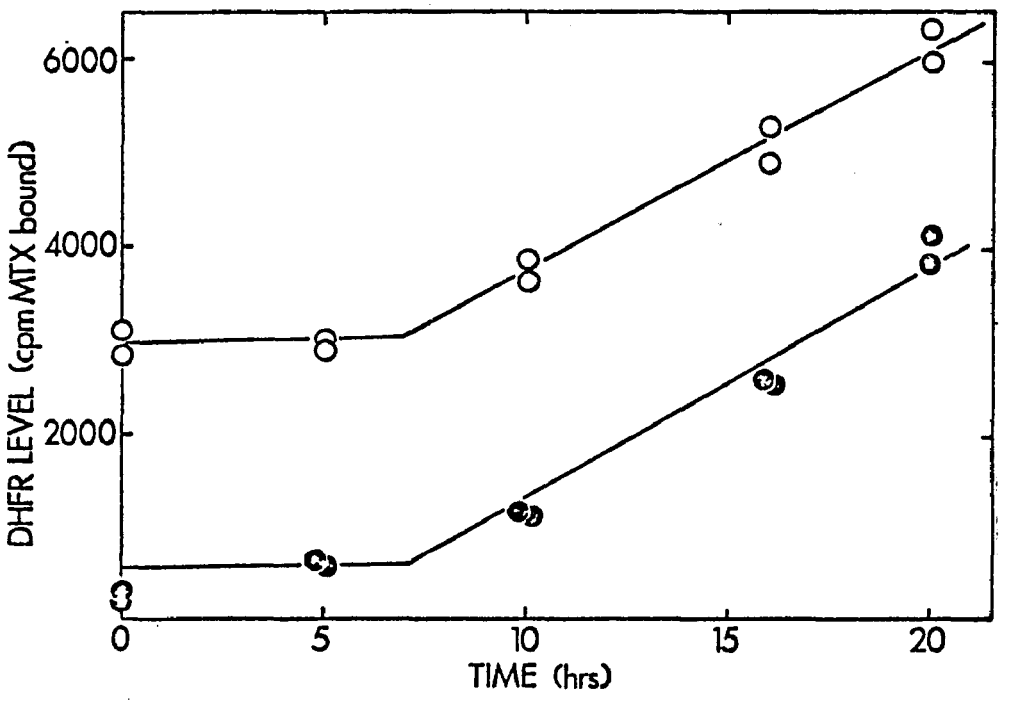


Figure 5. Increase in DHFR following serum stimulation of MTX-pretreated and control cultures of 3T6 cells. Cultures of resting 3T6 cells were pretreated with $10^{-6}$ M MTX, then stimulated with 10% serum at time = 0 (●). Control stimulated cultures received no MTX pretreatment (o). Cultures were harvested and the level of active DHFR was determined by the $^{3}$H-MTX binding assay.

This indicates that MTX pretreatment was an effective method to reduce the initial level of DHFR and did not affect the expression of the gene for this enzyme.

To be certain that I was measuring an increase in DHFR activity and not an increase in some other protein with an extremely high affinity for $^{3}$H-MTX, I measured DHFR levels at selected times after serum stimulation using a functional assay which measures the rate at which a cell extract reduces $^{3}$H-folic acid to $^{3}$H-THFA, as well as the $^{3}$H-MTX binding assay. Table 2 shows that I obtained very similar results with both assays.

To determine if the increase in DHFR accumulation depended on gene transcription, I examined the effect of addition of actinomycin at various times after serum stimulation. I had already seen that the presence of actinomycin had very little effect on the synthesis of DHFR in growing 3T6 cells (Figure 3). Cultures of resting 3T6 cells were serum stimulated and 5 ug/ml actinomycin was added to cultures at various times. The subsequent rate of accumulation was then determined. Figure 6 shows that the increase in DHFR activity was completely inhibited when actinomycin was added at 7.5 hours following serum stimulation, indicating that little if any DHFR mRNA had accumulated during the first 7.5 hours (upper most panel, Figure 6). Accumulation of DHFR was only partially affected when the actinomycin was added at 12.5 hours

TABLE 2

INCREASE IN DIHYDROFOLATE REDUCTASE FOLLOWING SERUM STIMULATION: COMPARISON OF FUNCTIONAL AND BINDING ASSAYS

| Sample | | $^3$H-MTX Binding Assay | | Functional Assay | |
|---|---|---|---|---|---|
| | | $^3$H-MTX bound (cpm) | normalized[1] | Initial rate (arbitrary units) | normalized* |
| resting (not pretreated) | 1 | 446 | .95 | 315 | .99 |
| | 2 | 498 | 1.05 | 319 | 1.01 |
| resting (9 hrs after pretreatment) | 1 | 123 | .26 | 78 | .25 |
| | 2 | 96 | .20 | 64 | .20 |
| resting (21 hrs after pretreatment | 1 | 138 | .29 | 95 | .30 |
| | 2 | 140 | .30 | 83 | .26 |
| stimulated (9 hrs after pretreatment) | 1 | 150 | .32 | 100 | .32 |
| | 2 | 131 | .28 | 92 | .29 |
| stimulated (21 hrs after pretreatment | 1 | 610 | 1.29 | 342 | 1.07 |
| | 2 | 548 | 1.16 | 304 | .96 |

Duplicate 100 mm dishes of resting 3T6 cells were treated and harvested at the indicated times as described in Figure 4. The level of active DHFR in each cell extract was determined by both the $^3$H-MTX binding assay and the functional assay as described in Materials and Methods.

*The levels of active reductase (cpm $^3$H-MTX bound or the initial rate of synthesis of $^3$H-tetrahydrofolate) were normalized to the average level found in untreated resting cells to facilitate comparison of the results of the two assays.

Figure 6. Effect of inhibition of RNA synthesis on DHFR gene expression in 3T6 cells. Cultures of resting 3T6 cells were serum stimulated at time = 0. At time = 7.5 hours (upper panel), 12.5 hours (middle panel), or 15 hours (lower panel), experimental cultures (▲) were exposed to $10^{-6}$ M MTX to inactivate pre-existing DHFR and 5 ug/ml actinomycin to inhibit RNA synthesis. After 30 minutes, the cultures were rinsed extensively to remove excess MTX, and fed with fresh medium containing 10% serum and 5 ug/ml actinomycin. Control stimulated cultures (●) were treated the same way except that actinomycin was omitted. Control resting cultures (o) were pretreated with MTX, then fed with conditioned medium containing 0.5% serum. Cultures were harvested at later times and the level of active DHFR was determined for each by the $^{3}$H-MTX binding assay.

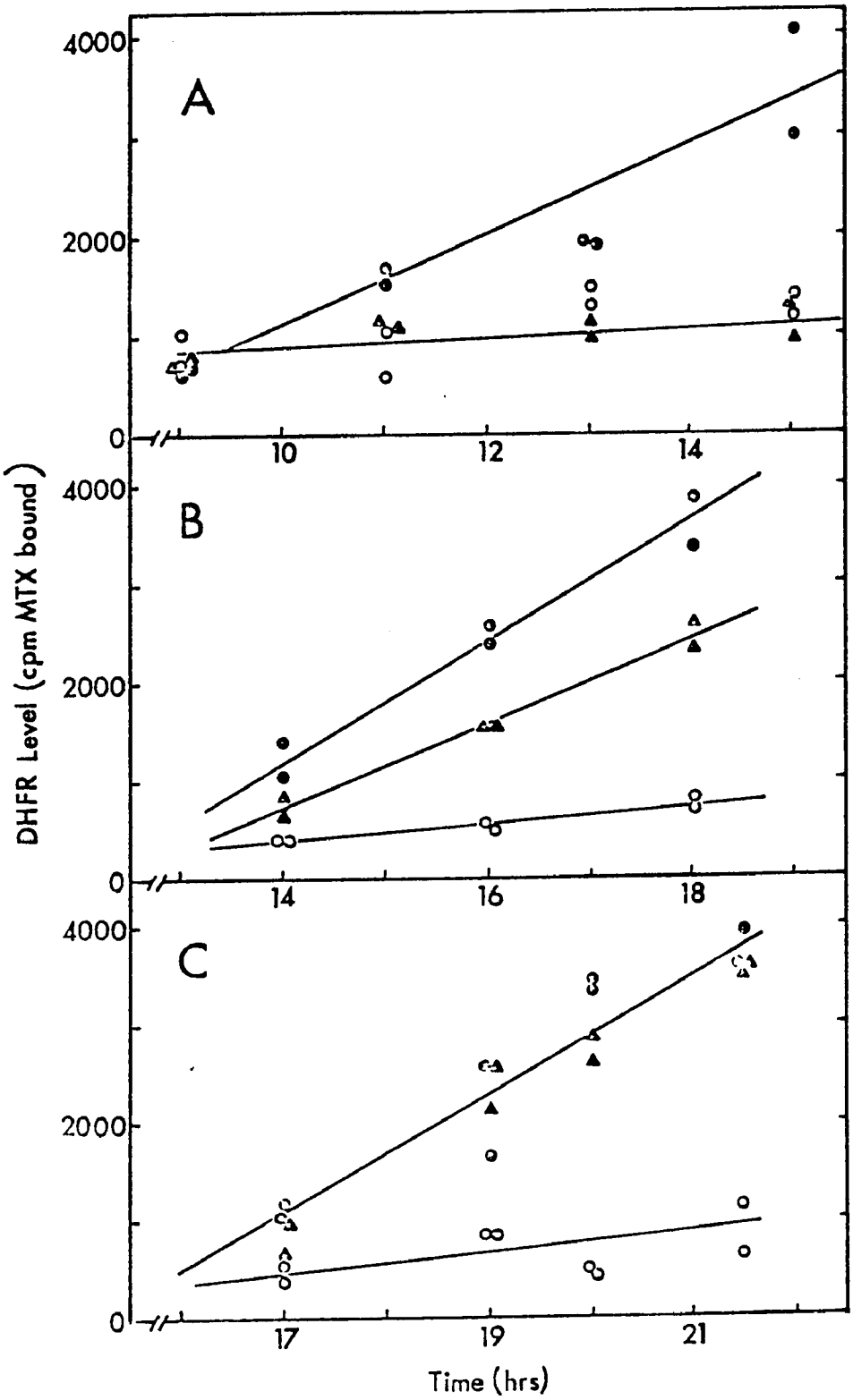
A
B
C
4000
2000
0
10
12
14
16
18
17
19
21
DHFR Level (cpm MTX bound)
Time (hrs)

Figure 6.

following serum stimulation (middle panel, Figure 6). There was little effect when actinomycin was added at 15 hours following serum stimulation (lowest panel, Figure 6). These results suggest that DHFR mRNA is synthesized in stimulated 3T6 cells between 7.5 and 15 hours after stimulation.

It was previously shown that DNA synthesis begins 10 to 12 hours after serum stimulation of resting 3T6 mouse fibroblasts (Mauck and Green, 1973). To determine if MTX pretreatment has any inhibitory effect on the ability of stimulated cells to enter S phase, I compared the profile of $^{3}$H-thymidine incorporation into DNA in serum stimulated cells with or without MTX pretreatment. Figure 7 shows that the pretreatment had no effect on the ability of these cells to enter into S.

The increase in the rate of DHFR accumulation occurs at about the same time as DNA replication. This is logical since THFA is required for the production of thymidylic acid. THFA is oxidized to dihydrofolic acid during thymidylic acid synthesis (Figure 2), so the cellular need for DHFR is greatest during DNA replication. One might expect that the inhibition of DNA synthesis would reduce the need for DHFR and therefore reduce the rate of DHFR synthesis. I studied the effect of blocking DNA synthesis on DHFR gene expression. Resting 3T6 cells were pretreated with MTX and serum stimulated in the presence of cytosine arabinoside or hydroxyurea.

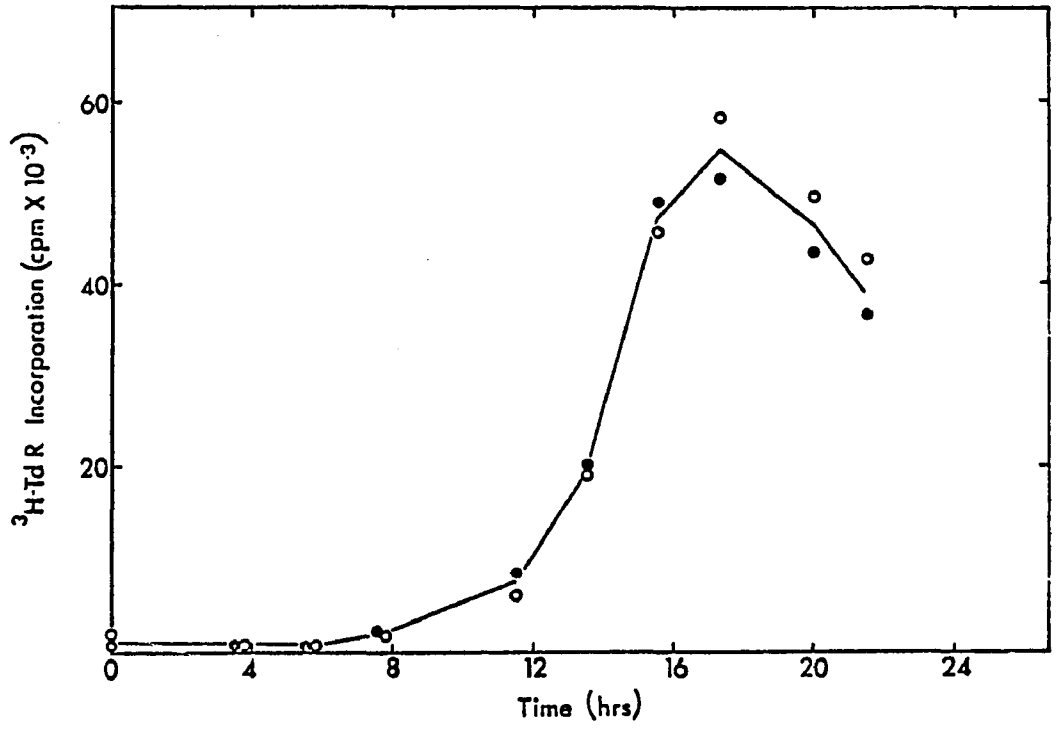


Figure 7. Rate of incorporation of $^3$H-thymidine into DNA in MTX-treated and control serum stimulated cells. Cultures of resting 3T6 cells were pretreated with $10^{-6}$ M MTX as before (o). Control cultures (●) were not pretreated. All cultures were fed at time = 0 with fresh medium containing 10% serum. The rate of incorporation of $^3$H-thymidine into DNA was measured by exposing cultures to $^3$H-thymidine for 60 minutes and measuring incorporation into TCA insoluble materials.

The drug levels used were sufficient to inhibit greater than 98% of DNA synthesis. Figure 8 shows that the presence of either of these drugs had no effect on the increase in the rate of DHFR accumulation in stimulated cells. Therefore, there does not appear to be a direct coordination between DHFR gene expression and DNA replication. This is in contrast to the synthesis of histones which is blocked when DNA synthesis is blocked (Borun, et al., 1967; Stein and Borun, 1972). These results do not rule out the possibility of a common signal occurring prior to entry into the S phase of the cell cycle, regulating DHFR gene expression as well as DNA synthesis.

These results showed for the first time that DHFR was the product of a gene whose expression was differentially regulated during the cell cycle. DHFR synthesis was low in resting cells and increased at the $G_1$-S boundary in growing cells. The expression appears to be controlled at the level of transcription. The DHFR gene appears to be a member of a family of genes whose expression are required during DNA replication.

## Isolation of MTX-Resistant Cell Line

It became apparent that obtaining detailed information about the molecular mechanisms of the regulation of DHFR level would be difficult due to the low levels of the DHFR and DHFR mRNA present in the cell. The literature contained

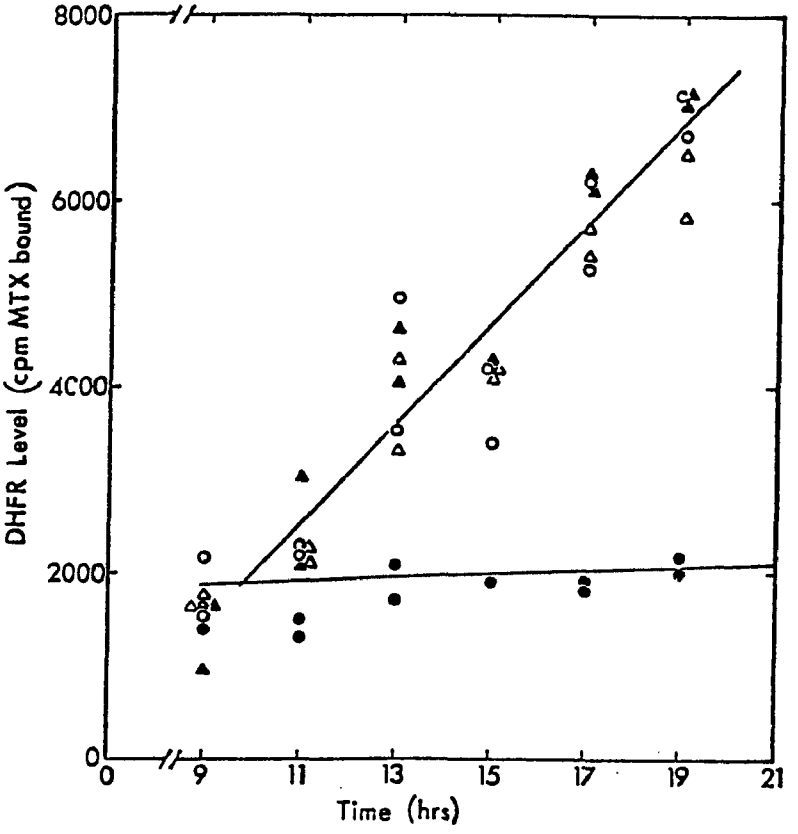


Figure 8. Effect of inhibitors of DNA synthesis on DHFR gene expression. Cultures of resting 3T6 cells were pretreated with $10^{-6}$ M MTX as before, then fed at time = 0 with fresh medium containing 10% serum (o). In some cases the medium also contained 5 ug/ml cytosine arabinoside (▲) or 30 ug/ml hydroxyurea (Δ). Resting cultures were fed with conditioned medium containing 0.5% serum (●). Cultures were harvested at various times; the level of active DHFR was determined by the $^{3}$H-MTX binding assay.

many reports of cell lines, resistant to high levels of MTX, which produced increased levels of DHFR (Hakala, et al., 1961; Littlefield, 1969) and its mRNA (Kellems, et al., 1976). Unfortunately, none of these cell lines appeared to be particularly susceptible to growth control. Therefore, I set about to isolate a cell line derived from 3T6, which would not only overproduce DHFR, but would retain the ability to rest in 0.5% serum and maintain the regulation of DHFR gene expression in the same manner as the parental 3T6 cells.

This cell line would serve as a model system for more detailed studies of the regulation of DHFR gene expression. A cell line which produces higher levels of DHFR and its mRNA would permit direct analysis of the rate of DHFR synthesis and quantification of the amount and rate of transcription of DHFR mRNA.

If cultured cells are exposed to low levels of MTX for many generations, they become resistant to the toxic effects of the drug (Littlefield, 1969). By gradually increasing the drug level over prolonged periods, resistance to high levels of the drug can be achieved (Hakala, et al., 1961). I used this method to obtain MTX-resistant cells from mouse 3T6 fibroblasts. Mouse 3T6 fibroblasts were initially seeded in medium containing 0.02 uM MTX and supplemented with 30 uM thymidine. This concentration of MTX was sufficient to kill at least 50% of the cells. The remaining cells grew

noticeably slower and they appeared flatter than cells not in the presence of the drug. When the surviving cells began growing more rapidly (after about 2 weeks) the MTX concentration was increased to 0.1 uM. After the remaining cells adapted to this new level of drug (2-5 weeks), the MTX concentration was doubled. This process was repeated until the level of 50 uM MTX was reached, and took about 7 months. These cells were seeded at a density of 50 cells per 100 mm plates. After about 2 weeks a series of 13 clones were isolated and each assigned a letter. Some of these were later subcloned and designated a number also (e.g. L3). Table 3 presents some characteristics of a few of the clones. The morphologies and sizes of the cells varied widely, as did the degree of contact inhibition of growth. Some clones grew slower, while others had doubling times similar to the parental 3T6 cells. Many clones were unable to survive in 0.5% calf serum (e.g. H2,I) when I attempted to make resting cells. Of those capable of survival in 0.5% serum, none appeared to rest quite as well as the original 3T6 parent line (e.g. L3, B), as measured by $^{3}$H-thymidine incorporation. The M50L3 clone was chosen for more detailed studies due to the higher levels of DHFR activity and its ability to experience density dependent growth regulation. I deleted the supplemental thymidine from the medium. After a short period of adaptation (2 weeks) the cells grew just as well as in the presence of the nucleoside.

TABLE 3

PROPERTIES OF METHOTREXATE-RESISTANT CELLS

| | | 3T6 | M50L3 | M50B | M50H2 | M50I |
|---|---|---|---|---|---|---|
| doubling time: | no drug | 19 | 19 | 27 | ND* | ND |
| | + 50 uM MTX | -- | 21 | 29 | 20 | 24 |
| DHFR level (relative) | | 1 | 300 | 70 | 60 | 11 |
| rate of DNA synthesis (resting/growing) | | 0.005 | 0.038 | 0.038 | ND | ND |

Doubling time was determined by plating cells on a series of dishes at low density. Duplicate dishes were trypsinized and cell density determined as a function of time after plating. Relative DHFR level in exponentially growing cells was determined by measuring the specific activity of DHFR (cpm $^{3}$H-MTX bound per mg cell protein in the cell line of interest and normalizing this value to that obtained for normal 3T6 cells. Rate of DNA synthesis was determined for resting and exponentially growing cultures and expressed as a ratio. Clones M50H2 and M50 I was not able to survive in 0.5% serum, therefore comparisons of rates of DNA synthesis could not be made.

*ND = not determined.

The cellular proteins of M50L3 and the parent 3T6 cells were compared by electrophoretic separation on SDS 11.3% polyacrylamide slab gels. The only obvious difference was the presence of a protein band in M50L3, co-migrating with authentic purified DHFR, having an apparent molecular weight of 21,000. The band co-migrated with purified DHFR that was isolated by Jin-Shyun R. Wu from M50L3 cells by affinity chromatography on folate sepharose. The band was undetectable in electrophoresed 3T6 cell extracts (Figure 9).

Hakala, et al. (1961) and Alt., et al. (1976) had reported that some MTX-resistant cells must be cultured continuously in the presence of the drug in order to maintain high levels of DHFR activity. I examined the stability of the DHFR-overproducing trait in M50L3 cells cultured in the absence of the MTX selective pressure. The cells were maintained in medium lacking MTX and assayed at various times for the amount of DHFR relative to that in the sensitive 3T6 cells. Cultivation of the M50L3 cells in the absence of MTX resulted in a marked decrease in the relative level of DHFR activity (Figure 10). Within 2 months, DHFR levels stabilized about 10-fold above that found in the MTX-sensitive 3T6 cells. The instability of the overproduction trait in M50L3 is similar to that observed in AT 3000 cells (Alt, et al., 1976).


Figure 9. Comparison of the cytoplasmic proteins of 3T6 and M50L3 cells. Cultures of exponentially growing 3T6 or M50L3 cells were harvested and cytoplasmic extracts were fractionated by SDS 11.3% polyacrylamide slab gel electrophoresis. The gels were then stained with Coomassie blue. Lanes: A, 3T6 extract; B, M50L3 extract; C, pure DHFR isolated from M50L3 cells.

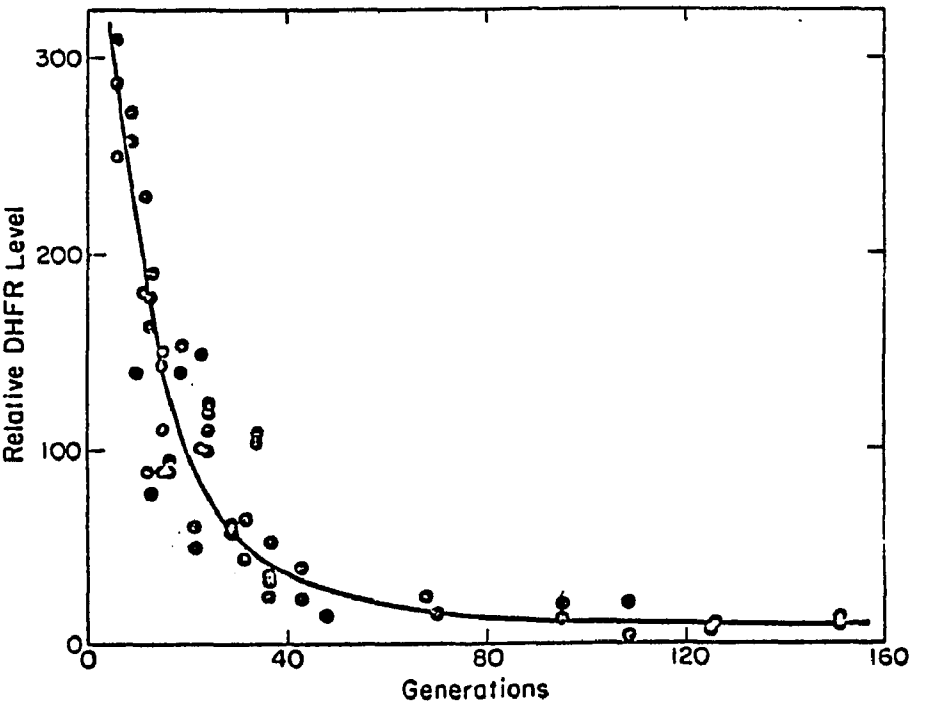


Figure 10. Loss of overproduction trait in cells cultured in the absence of MTX. Cultures of M50L3 cells were grown in the absence of MTX beginning on day 0. At various times, cells were harvested and the DHFR specific activity was determined. This value was normalized to the specific activity of DHFR in enponentially growing 3T6 cells (22.5 cpm of $^{3}$H-MTX bound per ug of cytoplasmic protein).

Regulation of DHFR in Overproducing Cells

To determine if DHFR gene expression in M50L3 cells was regulated in the same manner as in the normal parent 3T6 cells, I repeated many of the experiments previously described, using the overproducing cell line. I first compared the accumulation of DHFR in resting and growing cells. Due to the high level of DHFR, a short MTX-pretreatment was not sufficient to inactivate all of the enzyme. I found it more convenient to include $10^{-5}$ M MTX along with 30 uM thymidine and 100 uM hypoxanthine in the final medium change of the resting feeding schedule, or 2 days prior to use of the growing cells. This allowed for more complete inactivation of DHFR. The presence of hypoxanthine and thymidine during the prolonged exposure to MTX eliminates any deleterious effect MTX may have during the pretreatment. The cells were then washed extensively to remove unbound MTX. I found that the rate of accumulation was very low in resting M50L3 cells and about 25-fold higher in exponentially growing cells (Figure 11). Pre-incubation with MTX had no effect on the rate of accumulation of DHFR other than reducing the pre-existing DHFR activity. The presence of cycloheximide inhibited the increase in DHFR activity (Figure 11), indicating that the increase was due to de novo synthesis of the enzyme. Actinomycin had little effect on the accumulation of DHFR activity for at least 6 hours in growing cells, suggesting

Figure 11. Rate of accumulation of DHFR in resting and growing M50L3 cells. Cultures of resting or growing M50L3 cells on 35 mm culture dishes were prepared from stocks grown in the absence of MTX for 7 days. Cultures were incubated with 50 uM MTX for 2 days to inactivate essentially all of the pre-existing DHFR. Excess unbound MTX was then removed by rinsing the cultures extensively with serum-free medium and feeding them at time = 0 with medium appropriate for the experiment. Resting cultures were fed with conditioned medium containing 0.5% serum (lower panel). Growing cultures (upper panel) were fed with fresh medium containing 10% calf serum (●), 10% calf serum plus 5 ug/ml cycloheximide (Δ) or 10% calf serum plus 5 ug/ml actinomycin (▲). Control growing cells, which had not been pretreated with MTX but which had been rinsed with serum-free medium and fed at time = 0 with fresh medium containing 10% calf serum (o), were also analyzed. The rate of accumulation of DHFR was determined by harvesting cultures at various times and measuring the level of active DHFR, and normalized to the amount of protein present at time = 0 so that the rate of accumulation of DHFR in resting and growing cells could be compared. The normalized DHFR level in resting cultures not pretreated with MTX was 380 cpm/ug protein. Protein concentration was determined by the procedure described by Lowry, et al. (1951) using bovine serum albumin as the standard.

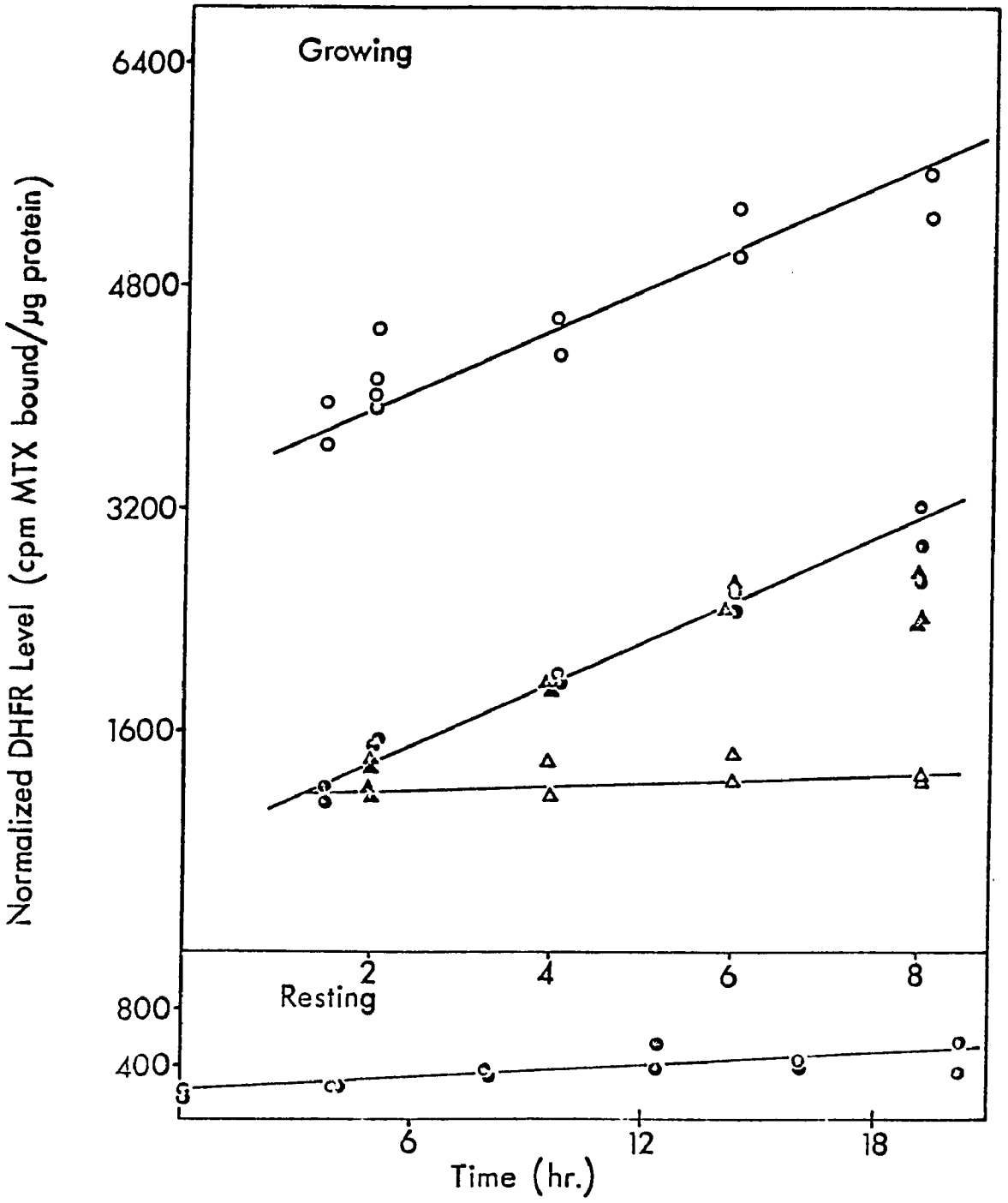
Growing
6400
4800
3200
1600
800
400
Resting
2
4
6
8
6
12
18
Time (hr.)
Normalized DHFR Level (cpm MTX bound/µg protein)

Figure 11.

that DHFR mRNA is not turning over rapidly and that its translation is not inhibited under these conditions (Figure 11).

In order to determine if resting M50L3 cultures responded to serum stimulation in a manner similar to normal 3T6 cells, I examined the accumulation of DHFR in resting M50L3 cells that were serum stimulated to re-enter the cell cycle. As shown in Figure 12, the level of DHFR remained low for the first 10 hours, and then increased sharply. Figure 12 also shows that MTX pretreatment had no effect on DHFR gene expression.

To determine if the increase in the rate of accumulation of DHFR depended on the transcription of mRNA, I repeated the experiments examining the effect of addition of actinomycin at various times after stimulation. Addition of actinomycin at 8 hours after stimulation blocked the increase of DHFR activity (Figure 13). However, addition of actinomycin at 16 hours after stimulation had little effect on subsequent accumulation for at least 5 hours. As in 3T6, these results suggest that little DHFR mRNA is synthesized prior to 8 hours of stimulation, and that its synthesis occurs between 8 and 16 hours of stimulation, coinciding with the time at which the rate of accumulation is increasing rapidly.

In 3T6 cells, the inhibition of DNA synthesis with hydroxyurea or cytosine arabinoside had no effect on the

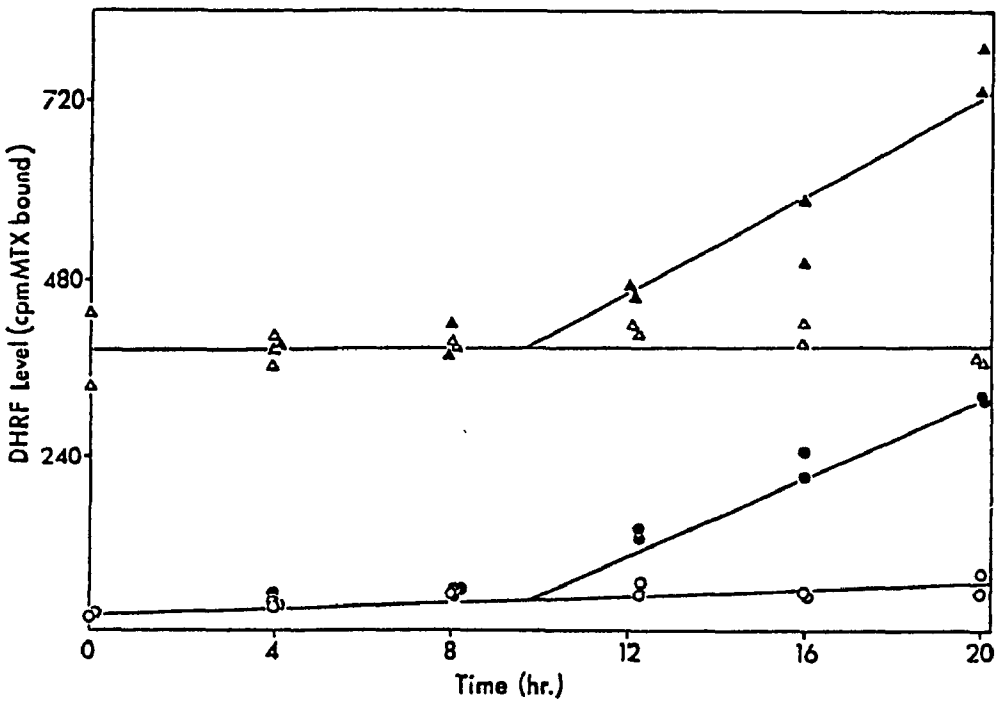


Figure 12. Increase in DHFR following serum stimulation of MTX-pretreated and control cultures of M50L3 cells. Cultures of resting 3T6 cells were pretreated with $10^{-5}$ M MTX as described in Figure 11, then stimulated with 10% serum at time = 0 (●), while resting cells were fed with conditioned medium containing 0.5% serum (o). Control resting (Δ) and stimulated (▲) received no MTX pretreatment. Cultures were harvested and the level of active DHFR was determined by the $^{3}$H-MTX binding assay.

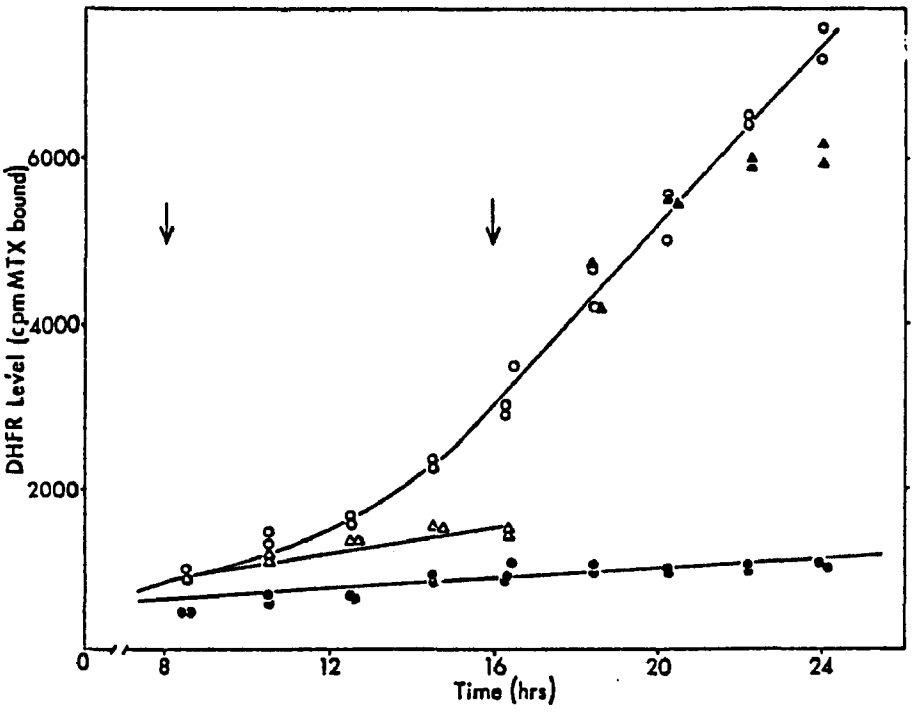


Figure 13. Increase in rate of accumulation of DHFR in serum stimulated M50L3 cells in the presence of actinomycin. Cultures of resting M50L3 cells on 35 mm dishes were pretreated with 50 uM MTX for 2 days as in Figure 11 and then rinsed extensively with serum-free medium and fed at time = 0 with fresh medium containing 10% calf serum (o). Control cultures were fed with conditioned medium containing 0.5% calf serum (●). At time = 8 hours (Δ) or 16 hours (▲) actinomycin D was added to the stimulated cultures at a final concentration of 5 ug/ml. Duplicate cultures were harvested at various times and assayed for DHFR activity using the $^{3}$H-MTX binding assay.

expression of DHFR (Figure 8). Figure 14 shows that direct inhibition of DNA synthesis also had no effect on the increase in DHFR synthesis in M50L3 cells, indicating a lack of direct coupling of these two events.

The cells used in this experiment were also stimulated in the presence of 30 uM thymidine and 100 uM hypoxanthine, which does not affect the rate of synthesis of DHFR. Figure 15 shows that this is also true for the 3T6 cells stimulated in the presence of thymidine and hypoxanthine. I also found that the presence of all 4 deoxynucleosides (30 uM each) in the culture medium had no effect on the increase in DHFR gene expression after serum stimulation in M50L3 cells (Figure 16) or 3T6 (data not shown). Therefore, the cell is probably not increasing DHFR gene expression in response to a decrease in the size of the cellular pools of purines, thymidine or other deoxynucleosides.

I examined the regulation of DHFR synthesis in the presence of leucovorin (5-formyl tetrahydrofolate) a reduced folate commonly used in rescue treatment after administration of MTX during chemotherapy. If the translation or transcription of DHFR was regulated by product inhibition, one might expect the presence of reduced folate to inhibit the increase in DHFR synthesis following serum stimulation. Experiments with either the M50L3 cells (data not shown) or 3T6 cells (Figure 17) showed that this was not true.

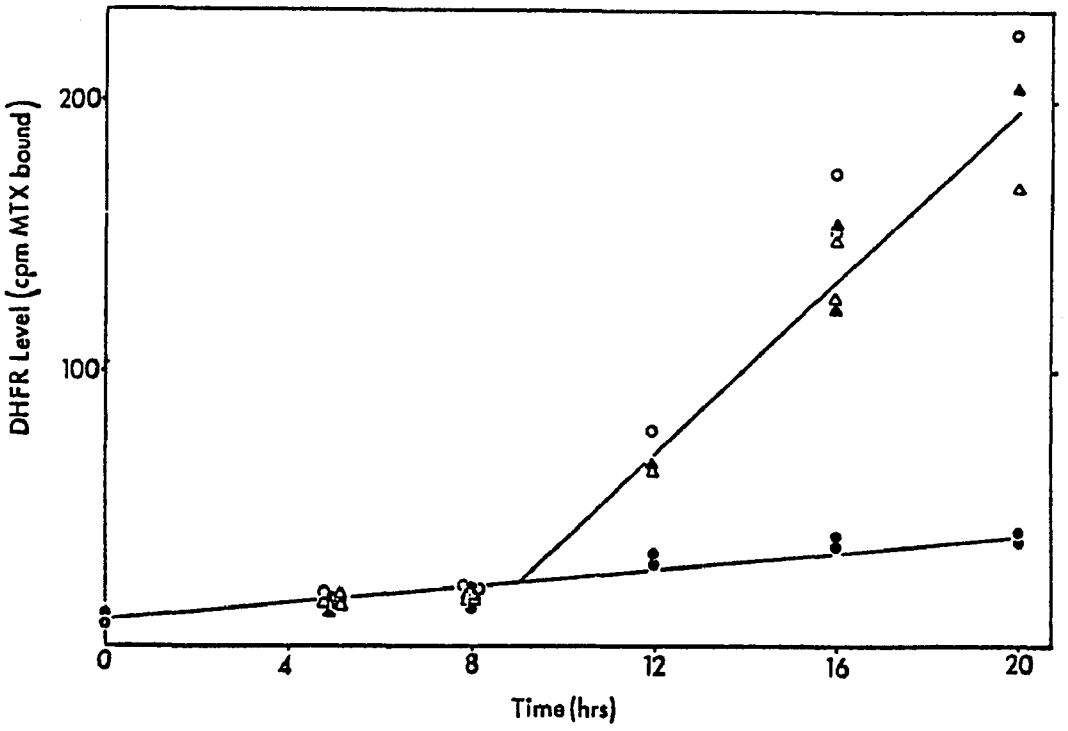


Figure 14. Increase in rate of DHFR accumulation in the absence of DNA synthesis in M50L3 cells. Cultures of resting M50L3 cells were pretreated with MTX and serum stimulated at time = 0 as in Figure 12. In this experiment both pretreatment and serum stimulation were performed in the presence of 30 uM thymidine and 100 uM hypoxanthine. Some cultures were stimulated in the presence of cytosine arabinoside (5 ug/ml) (▲) and others, in the presence of hydroxyurea (30 ug/ml (Δ). Control cultures were stimulated in the absence of drugs (o) or they were fed at time = 0 with conditioned medium containing 0.5% serum (●). Cultures were harvested at various times and the amount of active (newly synthesized) DHFR was determined by the $^{3}$H-MTX binding assay.

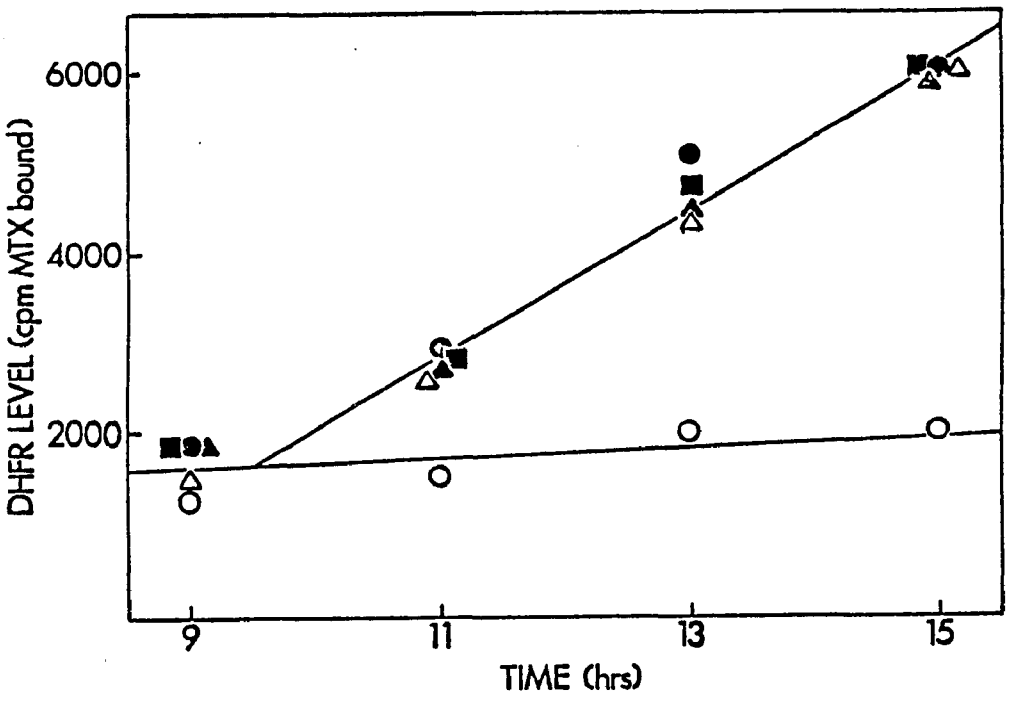


Figure 15. Increase in the rate of DHFR accumulation in the presence of hypoxanthine and thymidine in 3T6 cells. Cultures of resting 3T6 cells were pretreated with $10^{-6}$ M MTX and serum stimulated at time = 0. Control cultures were stimulated with medium containing 10% calf serum (●) or with conditioned medium containing 0.5% serum (o). Some cultures were stimulated in the presence of 30 uM thymidine (▲), 100 uM hypoxanthine (Δ), or both (■). Cultures were harvested at various times and the amount of DHFR activity was determined using the $^{3}$H-MTX binding assay.

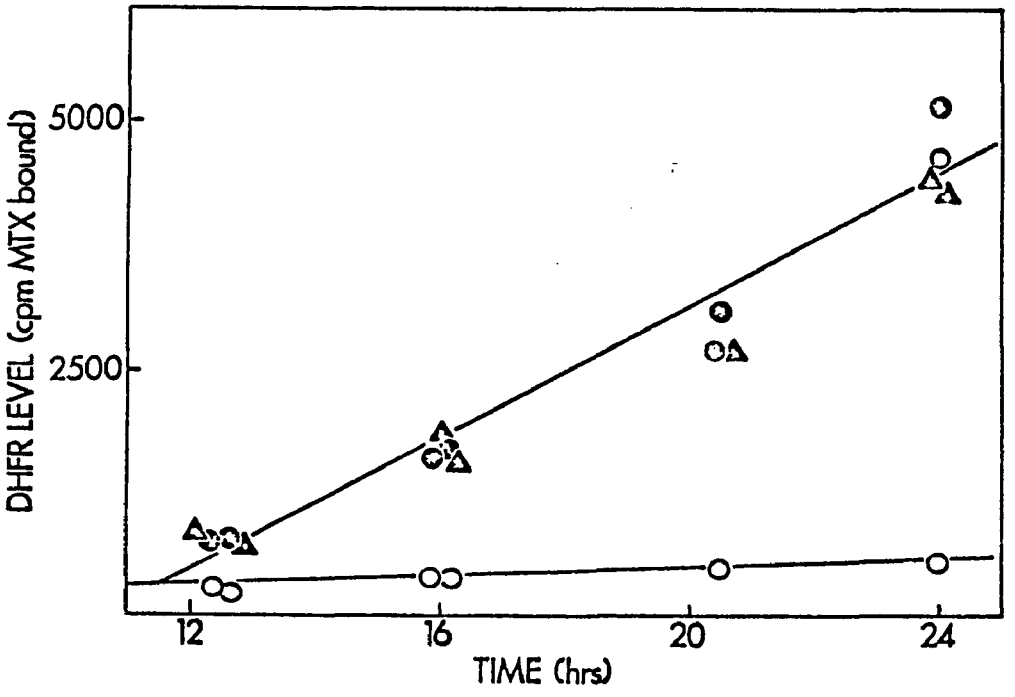


Figure 16. Increase in the rate of accumulation in M50L3 cells stimulated in the presence of deoxynucleosides. Cultures of resting M50L3 cells were pretreated with MTX as described in Figure 11 and stimulated in the presence (▲) or absence (●) of the 4 deoxynucleosides ( 30 uM). Control resting cultures were pretreated with MTX and returned to conditioned medium containing 0.5% serum. Cultures were harvested at various times after stimulation and the level of DHFR was determined by the $^{3}$H-MTX binding assay.

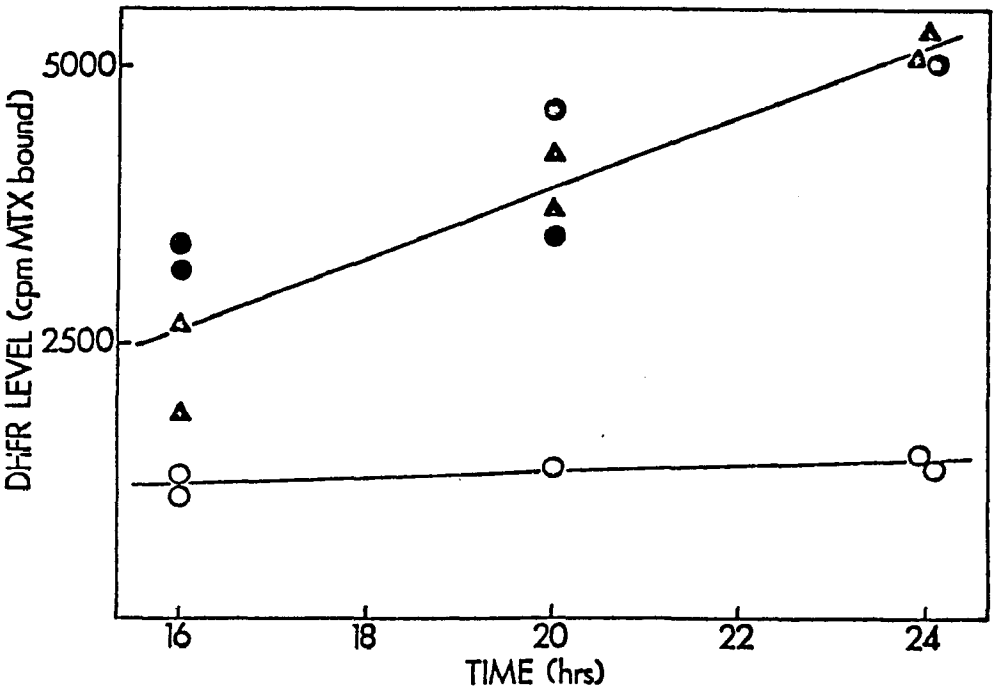


Figure 17. Increase in the rate of DHFR accumulation in 3T6 cells stimulated in the presence of leucovorin. Cultures of resting 3T6 cells were pretreated with MTX as described in Figure 15 and stimulated in the presence (▲) or absence (●) of $10^{-6}$ M leucovorin. Control resting cultures were pretreated with MTX and returned to conditioned medium containing 0.5% serum (o). Cultures were harvested at various times after stimulation and the level of DHFR was determined by the $^{3}$H-MTX binding assay.

## Further Studies of the Regulation of DHFR Gene Expression

Despite the overproduction of DHFR, the gene for this enzyme is regulated in a similar manner in M50L3 as in the parental 3T6. Somehow the alteration which results in DHFR overproduction does not interfere with the regulation of its expression. Therefore, M50L3 cells were an excellent model system for studying the molecular details involved in controlling DHFR gene expression.

Using the M50L3 cells, I was able to study the rate of DHFR synthesis directly. I exposed cultures of M50L3 cells to $^{3}$H-leucine for 1 hour at various times after serum stimulation. Due to the overproduction of DHFR in these cells, the location and relative labeling of the DHFR band could be visualized by fluorography after cytoplasmic extracts were electrophoresed on SDS slab gels. There was little labeling of the DHFR band in resting cells or cells stimulated for less than 8 hours. The relative intensity of the DHFR band then increased until 16 hours after stimulation, where it appeared to level off (Figure 18).

I quantitated the rate of synthesis of DHFR by cutting out the region of the fluorographed gel corresponding to DHFR. The piece of gel was minced, then swelled in water and incubated for 24 hours at 37°C in a toluene based scintillation fluid, containing 5% Protosol (New England Nuclear). The value obtained was normalized to the amount of radioactivity

Figure 18. Relative labeling of DHFR after serum stimulation. Cultures of resting M50L3 cells were serum stimulated at time = 0. At the times indicated below, the culture medium was replaced with fresh "labeling medium" containing 10% calf serum, 0.5% of the normal amount of leucine, and $^{3}$H-leucine (25 uCi/ml; final specific activity, 6 Ci/mmol). The cultures were incubated at 37°C for 1 hour and then harvested. Labeled proteins were fractionated by SDS slab gel electrophoresis; 2.0 (± 0.1) x $10^{5}$ cpm of labeled protein was applied to each slot of the gel. The fluorogram of the gel is shown here. Labeling medium was added as follows: Lane A, 0 hours; B, 4 hours; C, 8 hours; D, 12 hours; E, 16 hours; F, 20 hours; G, 24 hours. Lanes H and I contained labeled proteins from growing M50L3 and growing 3T6 cells, respectively, that had been labeled under the same conditions as the stimulated cultures. Lane J contained pure $^{14}$C-DHFR isolated from M50L3 cells.

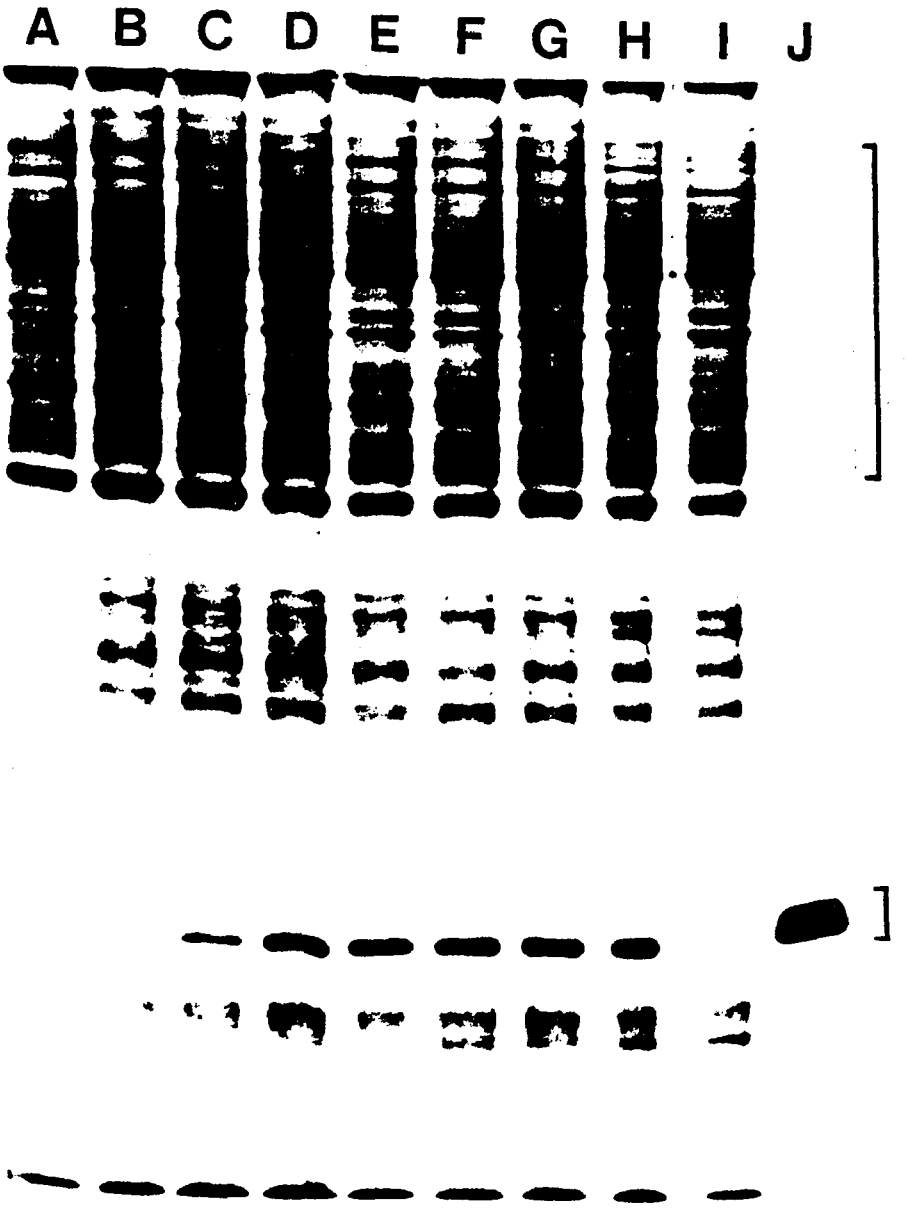


Figure 18.

found in the "high molecular weight proteins" represented by the upper bracket in Figure 18. These normalized values, which represent the relative rate of DHFR synthesis at various times after serum stimulation, are shown in the lower panel of Figure 19. The relative rate increased about 5-fold between 8 and 16 hours after stimulation and then remained constant. The absolute rate of DHFR synthesis (upper panel, Figure 19) is obtained by multiplying the relative rate of DHFR synthesis (lower panel) by the rate of total protein synthesis (rate of $^{3}$H-leucine incorporation). The rate of protein synthesis began increasing linearly at the time of stimulation and doubled about 12 hours later. The rate of DHFR synthesis began increasing linearly at 8 hours after serum stimulation. This correlated well with the increase in accumulation of DHFR. I concluded that the increase in the rate of accumulation of DHFR is due primarily, if not entirely, to an increased rate of synthesis.

Experiments with cycloheximide had indicated that DHFR is stable in resting, growing and serum stimulated 3T6 cells (Furhman and Johnson, unpublished). J. R. Wu (unpublished) has measured the half-life of DHFR in M50L3 resting cells and found it to be a particularly stable enzyme with a half-life of 41 hours. Her results agree with those of Alt, et al. (1976) who reported a half-life of about 50 hours in exponentially growing mouse AT 3000 cells.

Figure 19. Rate of synthesis of DHFR in serum stimulated M50L3 cells. Exponentially growing or serum stimulated cells were labeled for 1 hour as described in Figure 18. The labeled proteins were subjected to SDS slab gel electrophoresis and the dried gels were fluorographed. The region corresponding to labeled DHFR (lower bracket in Figure 18) was cut from the gel and allowed to swell in water. Radioactivity was determined by scintillation spectrometry using a toluene-based scintillation fluid containing 5% Protosol and was normalized to the amount of radioactivity found in "high molecular weight proteins" (upper bracket in Figure 18). This region contains approximately 70% of the labeled protein on the gel. The same regions were cut from a gel of labeled 3T6 proteins to determine the relative amount of radioactivity in proteins other than DHFR with the same electrophoretic mobility. This value was subtracted from the values obtained for stimulated or growing M50L3 cells to give the corrected values for relative labeling of DHFR (lower panel). The rate of DHFR synthesis is the product of the relative rate of DHFR and the rate of total protein synthesis (upper panel). The rate of protein synthesis was determined at various times after serum stimulation by measuring the rate of incorporation of $^{3}$H-leucine into trichloroacetic acid-insoluble radioactivity.

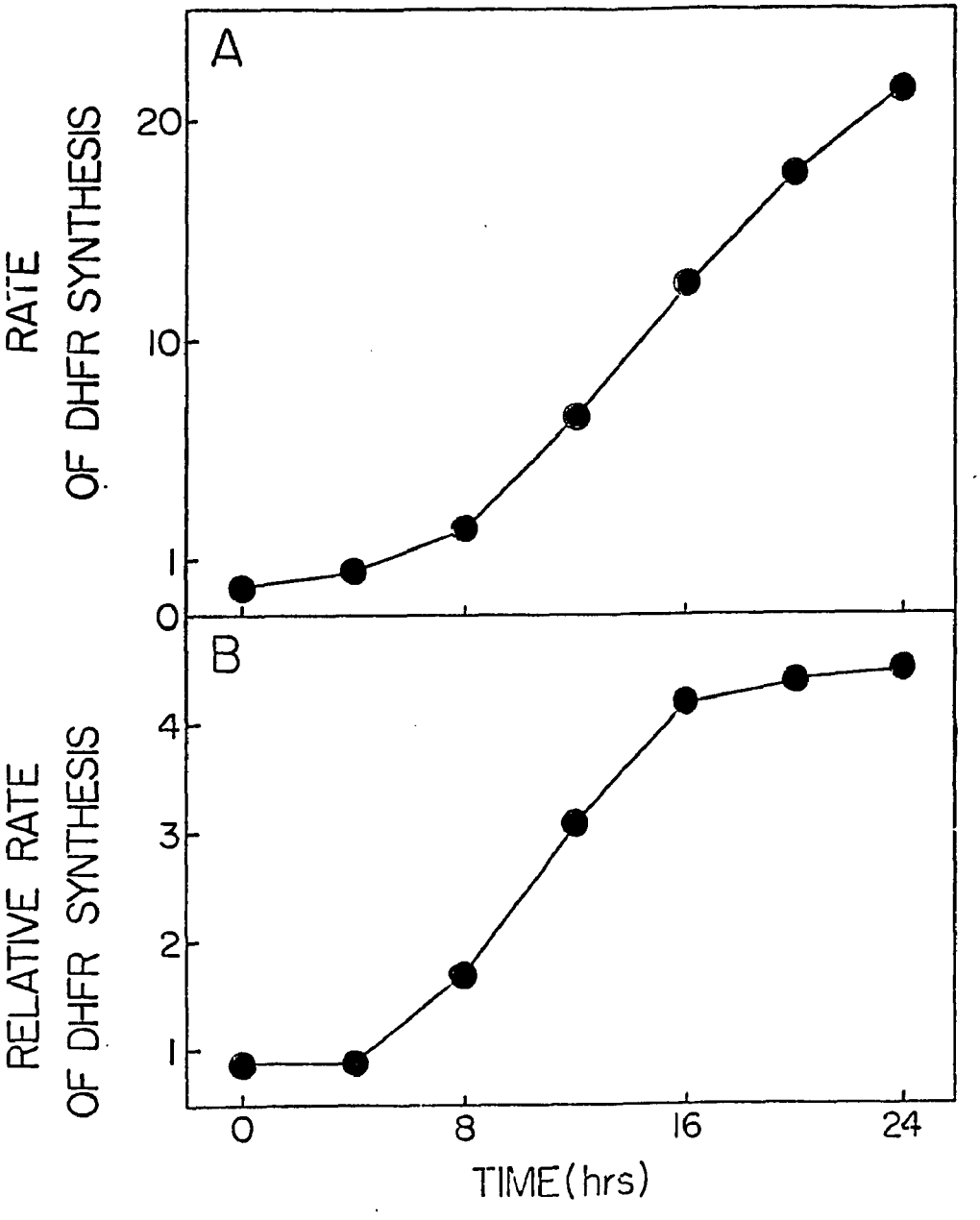
A
B
RATE
OF DHFR SYNTHESIS
RELATIVE RATE
OF DHFR SYNTHESIS
20
10
1
0
4
3
2
1
0
8
16
24
TIME(hrs)

Figure 19.

## Relationship between Cyclic AMP Levels and DHFR Gene Expression

The role of cyclic adenosine 3', 5'-monophosphate (cAMP) in growth regulation has been examined by many investigators. Their findings have been consistent with the idea that cAMP regulates the specific stages in the cell cycle, particularly the growth arrest in $G_1$ (Burger, et al., 1972; Coffino, et al., 1975). It has been reported that increased intracellular levels of cAMP inhibit the cell's entry into the S phase of the cell cycle (Kram, et al., 1973). Recently, Kellems, et al. (1979) have presented evidence that both the entry into S phase and the increase in DHFR synthesis were blocked when MTX-resistant mouse fibroblasts were serum stimulated in the presence of dibutyryl cAMP (DB cAMP) and theophylline. I also found that when resting M50L3 cultures were serum stimulated in the presence of 0.2 uM DB cAMP and 1 mM theophylline, both the increase in DNA synthesis and DHFR accumulation were prevented (Figure 20). However, when 3T6 cells were treated in the same manner, these processes were only partially inhibited (Figure 20). This was true even if the concentrations of DB cAMP and theophylline were doubled (data not shown).

This unexpected observation may be due to an increased sensitivity of MTX-resistant cells to elevated levels of cAMP, while the parental 3T6 are only partially affected by these conditions. It is also possible that the 3T6

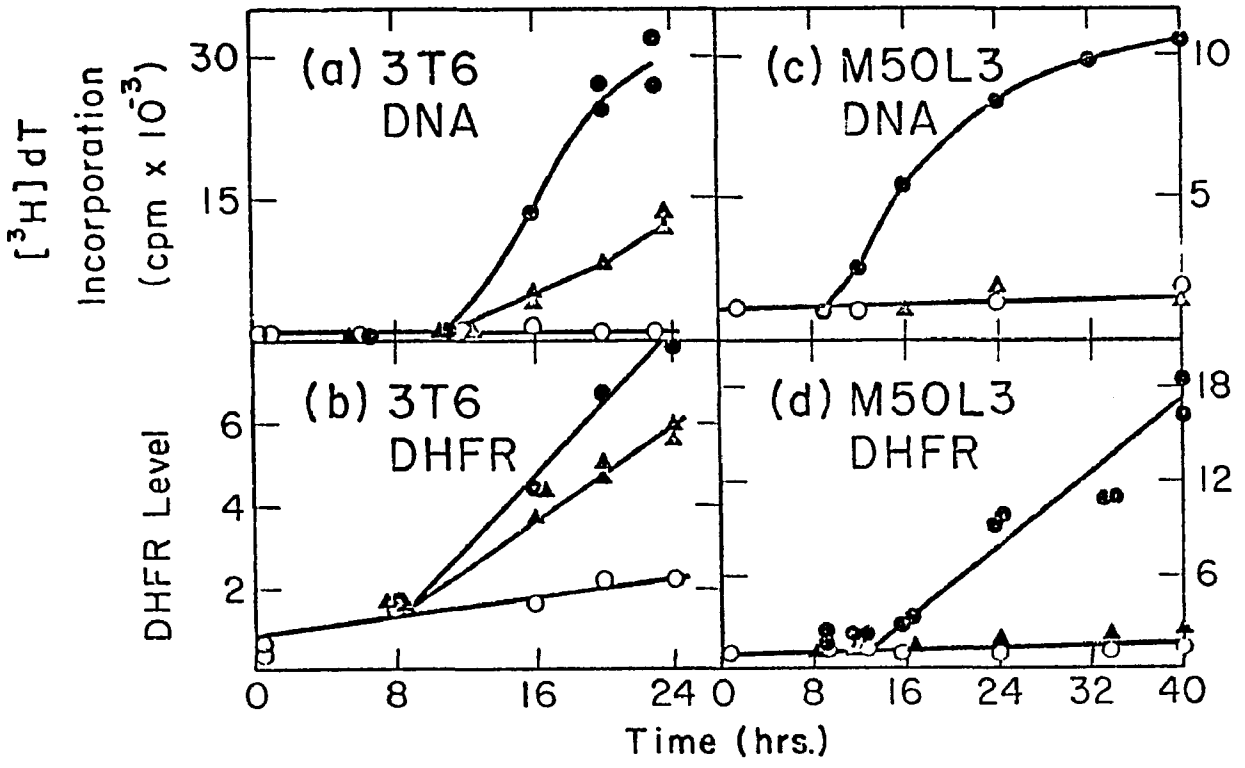


Figure 20. Effect of dibutyryl cAMP on DNA synthesis and DHFR accumulation. Cultures of resting 3T6 or M50L3 cells were serum stimulated at time = 0 in the presence (▲) or absence (●) of 0.2 mM DB cAMP and 1 mM theophylline. Control resting cultures were maintained in medium containing 0.5% serum. At various times cultures were assayed for the rate of DNA synthesis ($^3$H-thymidine incorporation) (upper panels) and the level of active DHFR (lower panels). Cultures assayed for DHFR activity were treated with unlabeled MTX prior to stimulation to inactivate pre-existing DHFR.

population contains two or more classes of cell phenotype, some of which are sensitive to elevated cAMP levels, while others are insensitive. Since the M50L3 cell line was originally derived and cloned from the 3T6 population, an isolate containing the sensitivity to high cAMP may have been chosen.

In order to distinguish between these two possibilities, I isolated clones from the 3T6 population and examined their sensitivity to high cAMP levels during serum stimulation. Resting cells were made from each of the 11 clones isolated and stimulated in the presence or absence of 0.2 mM DB cAMP and 1 mM theophylline. At 20 and 26 hours after stimulation, cultures were harvested and assayed for DHFR activity. The incorporation of $^{3}$H-thymidine into DNA was also determined at these times. I found that there was indeed variation among the various clones. There were three distinct phenotypes present. In 2 clones, the drugs had no effect on the expression of the gene for DHFR or DNA synthesis (lowest panel, Figure 21), while 6 clones still exhibited a partial inhibition of both activities in the presence of the increased cAMP levels (middle panel, Figure 21). In 3 clones, the presence of DB cAMP completely inhibited the increase in the rate of accumulation of DHFR and the increase in the synthesis of DNA (uppermost panel, Figure 21). It is, therefore, quite possible that the M50L3 clone was derived from a cell which already

Figure 21. Accumulation of DHFR in several clones of 3T6 mouse fibroblasts in the presence of DB cAMP. Resting cultures of clones of 3T6 were pretreated with MTX and serum stimulated in the presence (▲) or absence (●) of 0.2 mM DB cAMP and 1 mM theophylline. Control resting cultures (o) were pretreated with MTX and then returned to conditioned medium containing 0.5% serum. At 20 and 26 hours, the cultures were assayed for the level of DHFR. Cultures were also assayed for rate of DNA synthesis at these times. The inhibition of the synthesis of DNA was similar to that observed for the inhibition of DHFR.

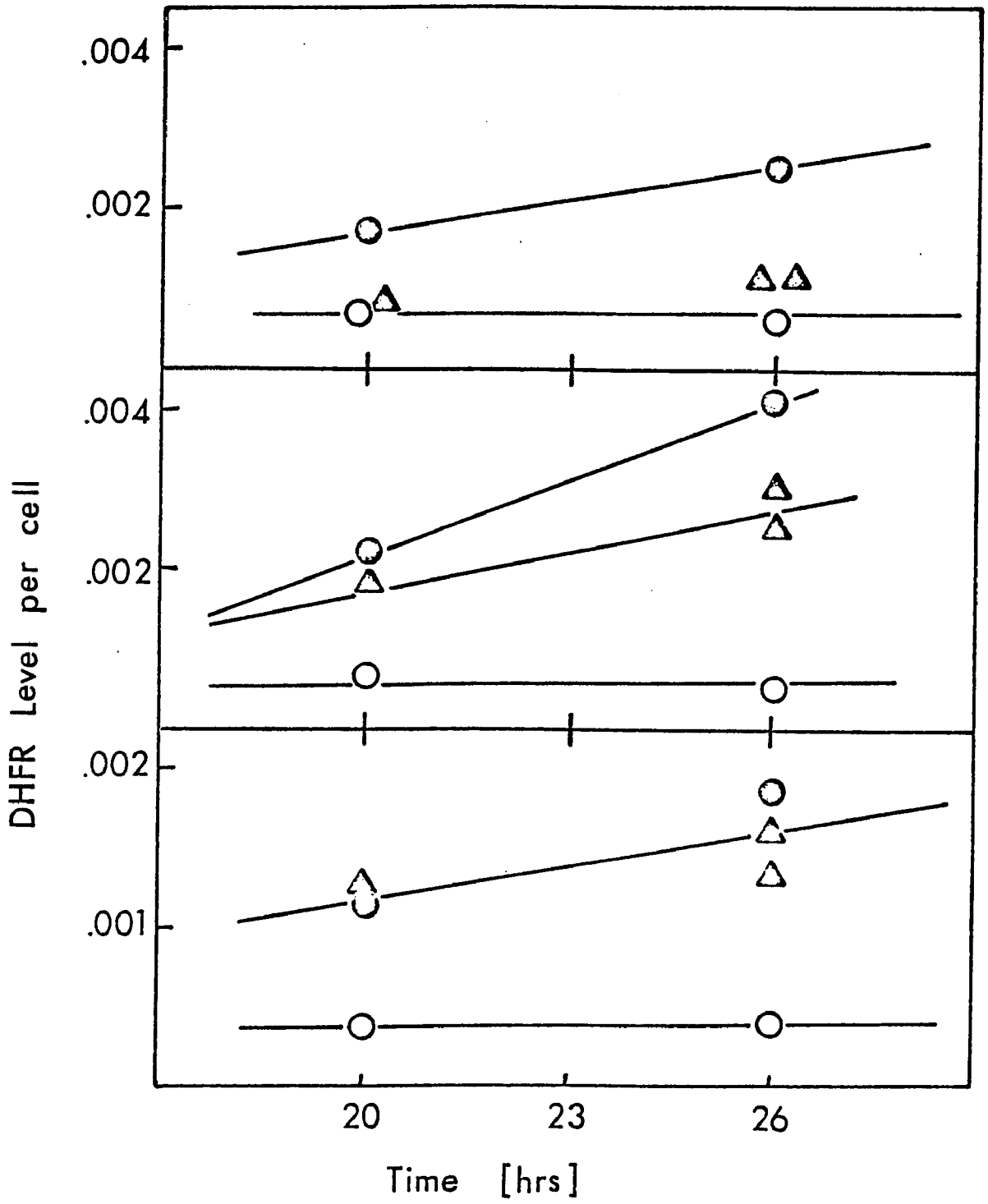


Figure 21.

had a phenotype where the regulation of the cell cycle was sensitive to high levels of cAMP. But, there are still some cells in the 3T6 population which are partially sensitive to high levels of cAMP.

In order to determine if the cAMP sensitive regulatory event occurred early or late in the $G_0$-$G_1$ phase of cell cycle, I also examined the effect of addition of DB cAMP and theophylline at 6 hours after stimulation. Figure 22 shows that the increase in DHFR was almost completely inhibited in the presence of these drugs. The increase in the rate of DNA synthesis was also much reduced (data not shown). This would suggest that the signal turning on the genes required for DNA synthesis, such as DHFR probably occurs late in $G_1$.

## Turning off of the Expression of the DHFR Gene

Since the regulation of cell cycle events requires the "turning off" as well as "turning on" of genes such as DHFR, I have also investigated the reversal of the stimulation process. Returning stimulated cells to conditioned medium taken from resting cultures will cause the reversal of the stimulation and return the cells to a resting state. Figure 23 shows that when the serum stimulus is withdrawn from M50L3 cells 20 hours after stimulation, the rate of DNA synthesis returned to the resting level within 15-20 hours (upper panel, Figure 23). The lower panel of Figure 23 shows

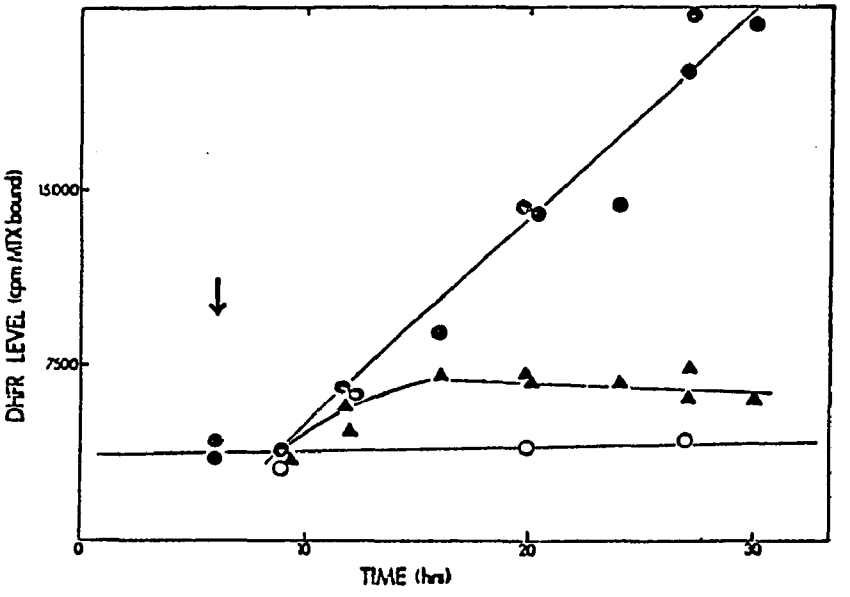


Figure 22. Accumulation of DHFR in M50L3 cells that were serum stimulated and treated with DB cAMP. Cultures of resting cells were serum stimulated at time = 0 (●). At 6 hours (arrow), some cultures were treated with 0.2 mM DB cAMP and 1 mM theophylline (▲). At various times, the level of active DHFR was determined. These cultures were pretreated with unlabeled MTX prior to stimulation to inactivate pre-existing DHFR. DHFR accumulation in control resting cells is also shown (o).

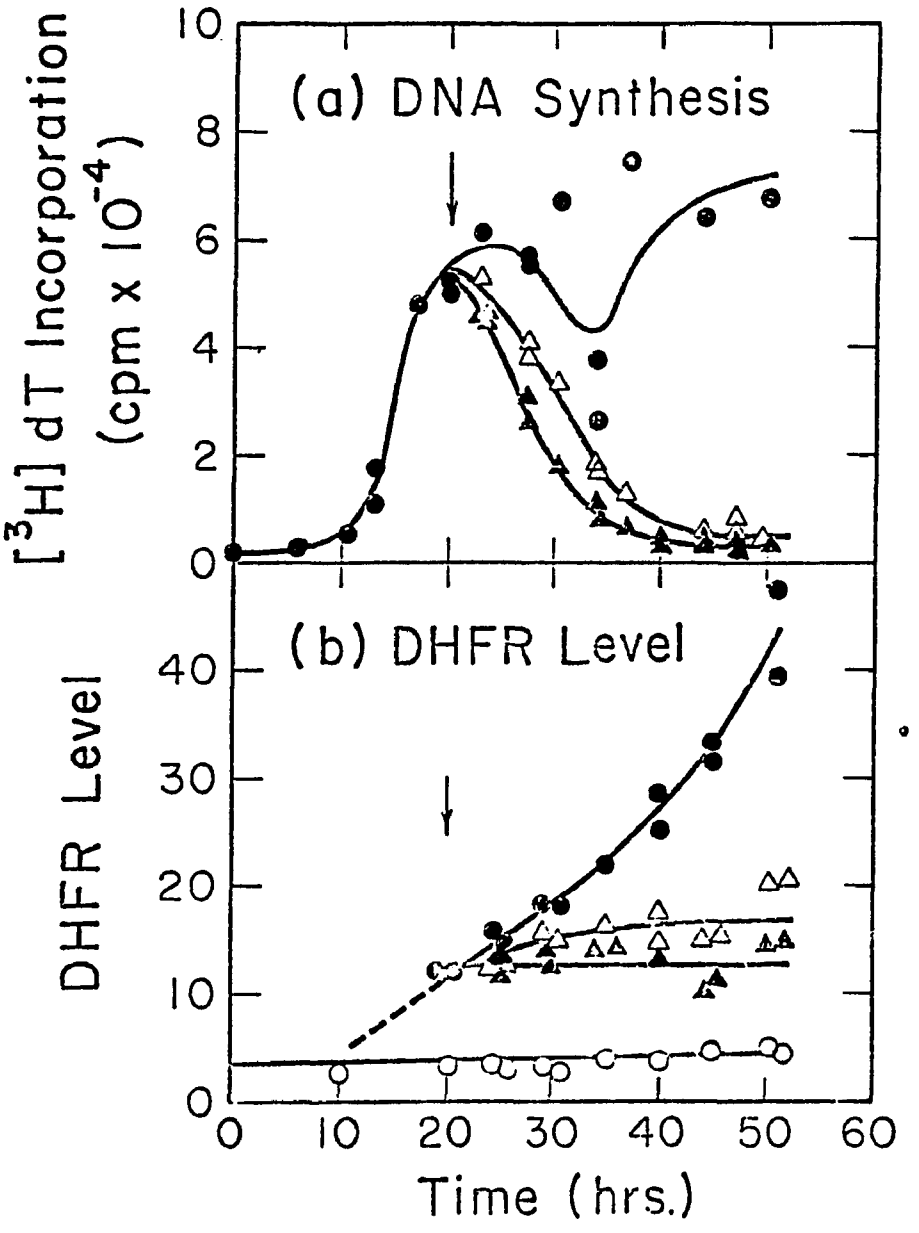


Figure 23. Accumulation of DHFR in M50L3 cells that were serum-withdrawn or treated with DB cAMP 20 hours following serum stimulation. Cultures of resting cells were serum stimulated at time = 0 (●). At 20 hours (arrow), the serum stimulus was withdrawn from some of the cultures (Δ). Other cultures were treated with 0.2 mM DB cAMP and 1 mM theophylline (▲). At various times, the rate of DNA synthesis (upper panel), and the level of active DHFR (lower panel) were determined. Cultures assayed for DHFR level were treated with unlabeled MTX prior to stimulation to inactivate pre-existing DHFR. Accumulation of DHFR in control resting cells is also shown (o). (The data in the upper panel of this figure were kindly supplied by J. R. Wu.)

that either serum withdrawal or the addition of DB cAMP results in an inhibition of further DHFR accumulation. This evidence would suggest that the gene for DHFR is not being expressed due to some signal related to growth state. This signal probably has some relation to cAMP levels.

I also looked at the effects of serum withdrawal at 20 hours after stimulation, on DHFR accumulation in the MTX-sensitive 3T6 cells. Figure 24 shows that not only is DHFR accumulation inhibited, but that there is a decrease in DHFR activity, which returned to the resting level within 20 hours. This would indicate that DHFR becomes unstable under these cellular conditions in 3T6 cells; whereas, the enzyme appears stable under the same conditions in M50L3.

Regulation of DHFR Translatable mRNA Levels

I had shown that the increase in the rate of accumulation was due to an increase in the rate of synthesis of DHFR. In order to determine if the rate of synthesis was dependent on the level of translatable mRNA present in M50L3 cells, I used a mRNA-dependent *in vitro* translating system. I chose to use a system prepared from rabbit reticulocytes available commercially from New England Nuclear. Figure 25 shows a typical plot representing the rate of total protein synthesis as measured by $^{3}$H-leucine incorporation, as a function of time of incubation. The reaction mixture contained total RNA from growing M50L3 cells and was incubated

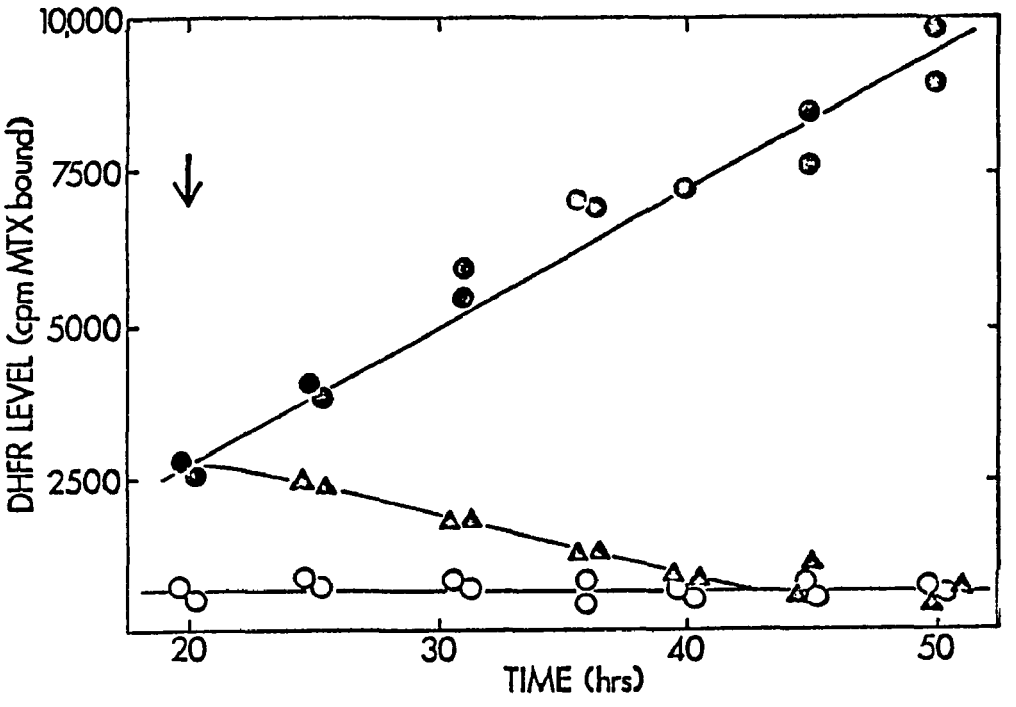


Figure 24. Accumulation of DHFR in 3T6 cells that were serum-withdrawn 20 hours following stimulation. Cultures of resting cells were pretreated with MTX and serum stimulated at time = 0 (●). At 20 hours (arrow), the serum stimulus was withdrawn (▲). Resting control cultures were pretreated with MTX and returned to conditioned medium containing 0.5% serum (o). At various times, the level of active DHFR was determined by the $^{3}$H-MTX binding assay.

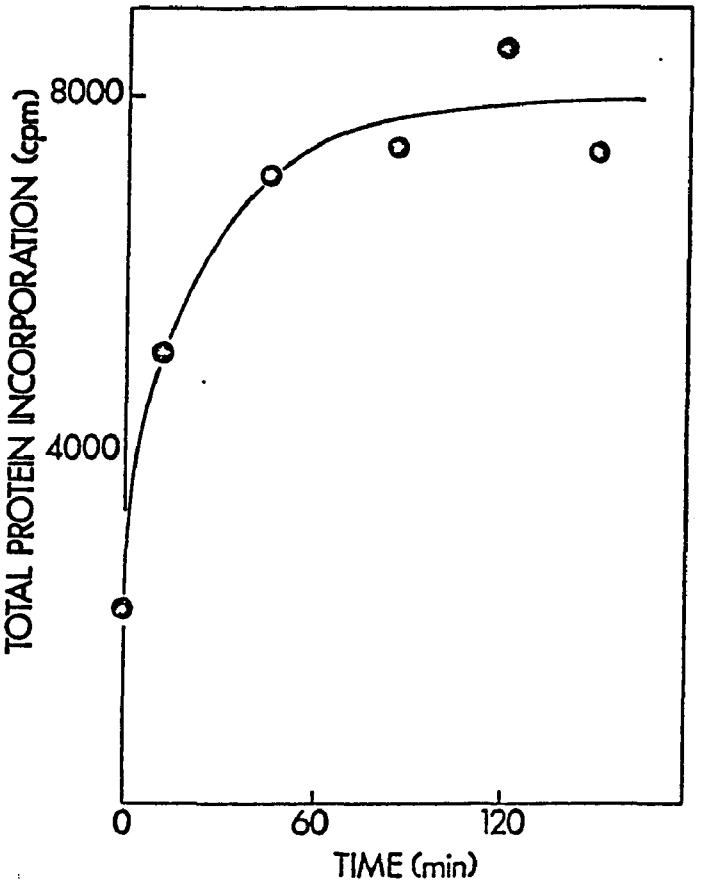


Figure 25. The mRNA directed incorporation of $^{3}$H-leucine into protein in an in vitro translation system. A reaction mixture containing 20 uCi of $^{3}$H-leucine and 0.03 ug of polysomal RNA from 3T6 cells was incubated at 37°C. At various times, 2 ul aliquots were removed and assayed for base stable, acid insoluble material labeled with $^{3}$H-leucine.

at 37°C. Since the reaction appeared to be complete by 60 minutes, all subsequent reactions were terminated at this time.

In order to compare the amount of DHFR synthesis directed by RNA isolated from growing M50L3 cells, resting M50L3 cells and growing 3T6 cells, I isolated total polysomal RNA from roller bottle cultures of each. Total RNA was translated rather than (A)(+) mRNA, since I was not certain that all DHFR mRNA would be polyadenylated under all conditions. The relative amount of poly (A)(+) RNA in each RNA batch was determined by hybridization to $^{3}$H-poly (U). The amount of RNA corresponding to 1000 cpm (1 unit) or 2000 cpm (2 units) of $^{3}$H-poly (U) (about 0.03 -0.06 ug of poly (A)(+) RNA) were added to each assay mixture. An assay mixture was incubated without any added RNA to measure the endogenous activity of the lysate. The endogenous incorporation was usually represented about 5-20% of the total incorporation.

Antibody specific of DHFR was prepared by J. R. Wu in the following manner: DHFR was purified from M50L3 cells by affinity chromatography on folate-Sepharose and injected into rabbits. Antiserum was isolated, and the specificity of this antibody preparation was determined as shown in Figures 26 and 27. Figure 26 shows a fluorogram of the electrophoretic profiles of these translation assay mixtures, before and after immunoprecipitation.

Figure 26. Pattern of $^{3}H$-labeled proteins synthesized in an *in vitro* translation system before and after immunoprecipitation. Total polysomal RNA was isolated from roller bottle cultures of growing M50L3, resting M50L3 and growing 3T6 cells and used to direct the translation of proteins in an mRNA dependent *in vitro* translation system derived from rabbit reticulocytes. An identical reaction mixture without any added RNA was also incubated to measure endogenous incorporation. One half of each sample was precipitated with DHFR specific antiserum. The other half of the reaction mixture was acetone precipitated. The immunoprecipitated and acetone precipitated materials were dissolved in electrophoresis sample buffer, and subjected to electrophoretic separation on SDS 12.5% polyacrylamide slab gels. A negative of the fluorogram of the gel is shown here. Lane A contains labeled $^{14}C$-DHFR isolated from M50L3 cells. The source of RNA for *in vitro* translation: Lane B, growing M50L3; C, resting M50L3; D, growing 3T6; E, no added RNA. After immunoprecipitation: Lane F, growing M50L3; G, resting M50L3; H, growing 3T6; I, no added RNA.

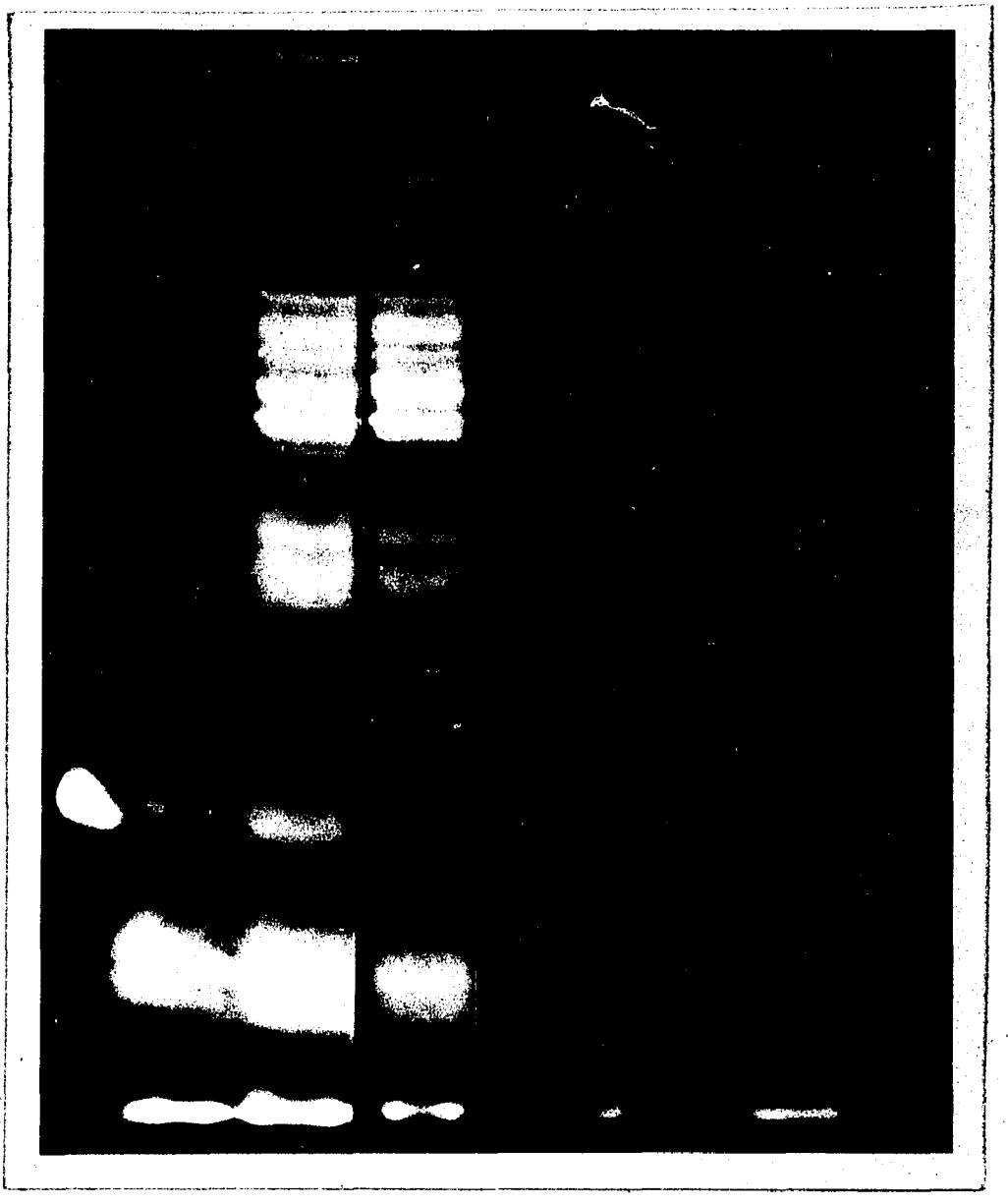


Figure 26.

Figure 27. Immunoprecipitation of *in vitro* translation products of polysomal RNA from 3T6 and M50L3 cells. Polysomal RNA from growing 3T6 and M50L3 cells was translated *in vitro*. Samples of the translation assay mixture were mixed with 500 cpm of $^{14}C$-labeled DHFR and acetone precipitated or immunoprecipitated as described in Figure 26. Proteins were fractionated on SDS 12.5% polyacrylamide tube gels, which were sliced into 2 mm sections, incubated for 24 hours in a toluene-based scintillation fluid containing 5% Protosol and counted in a scintillation counter. The direction of migration is left to right.

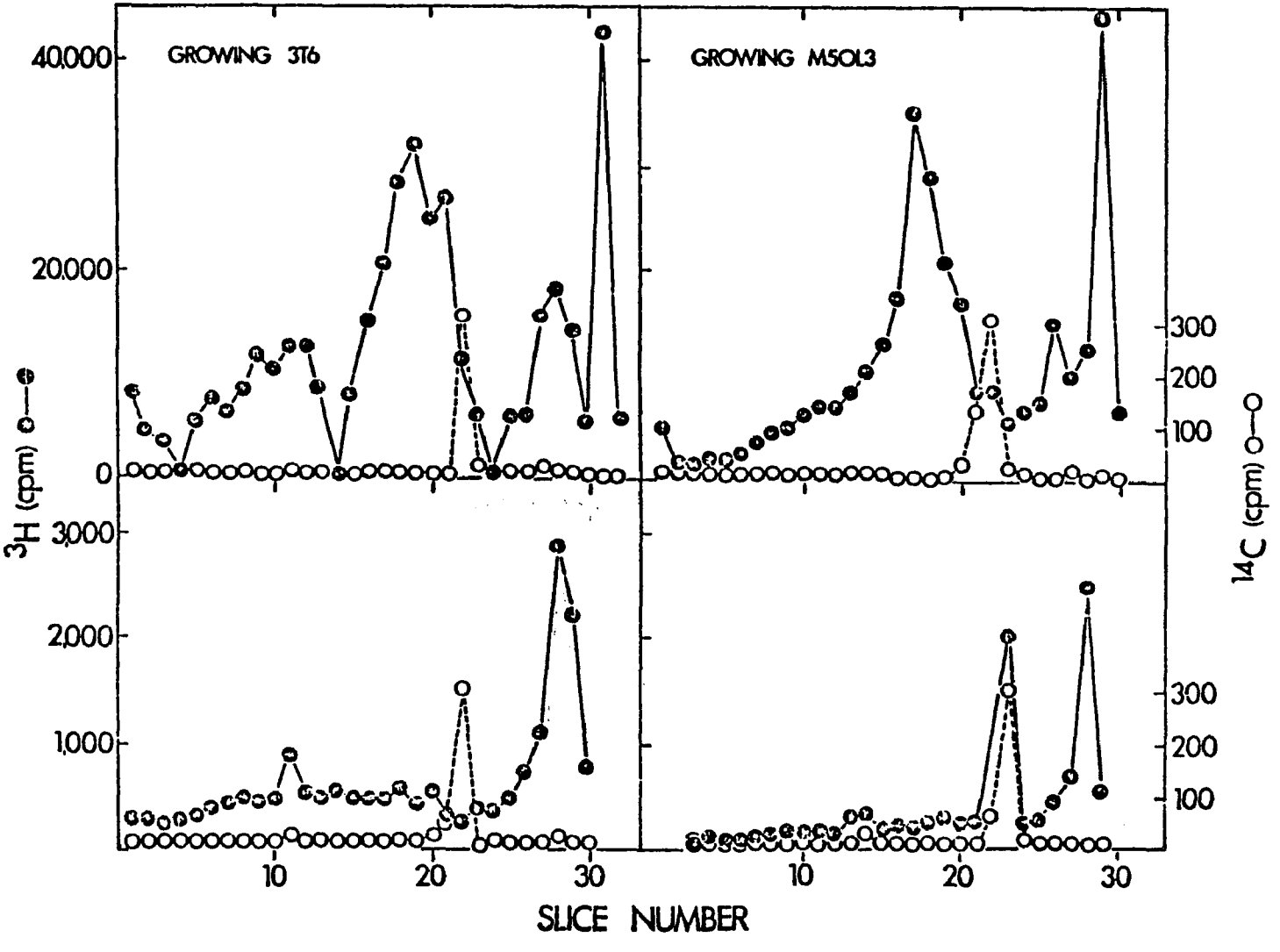
GROWING 3T6
GROWING M5OL3
40,000
20,000
0
3,000
2,000
1,000
300
200
100
10
20
30
$^3H$ (cpm)
$^{14}C$ (cpm)
SLICE NUMBER

Figure 27.

When I compare the proteins synthesized in vivo (Figure 18, Lanes A, H, and I) to those synthesized in vitro (Figure 26, Lanes B, C, and D), I found the electrophoretic pattern very similar. There were no protein bands observed in the backround sample (Figure 26, Lane E). Although some of the larger proteins were synthesized in reduced amounts, it is clear that the system was capable of synthesizing proteins larger than DHFR. One can see that DHFR is the only major band of labeled protein found in the immunoprecipitates (Figure 26, Lanes F, G, H, and I). Although other bands are present they are well separated from DHFR. These additional bands may represent proteins which co-purify with DHFR, indicating that the antiserum is not entirely specific. Since only the portions of the gel containing DHFR are analyzed for radioactivity, these bands should not interfere with the analysis of labeled DHFR.

In order to quantitate the amount of DHFR synthesized, $^{14}$C-labeled DHFR was included in the immunoprecipitation mixture, as a recovery marker. Figure 27 shows the radioactivity in fractionated electrophoretic gels of in vitro labeled extracts from exponentially growing 3T6 and M50L3 cells, before and after immunoprecipitation. Again, the only significant peak in the immunoprecipitates co-migrates with the $^{14}$C-labeled DHFR. Since the level of DHFR in 3T6 cells is only 1/300 of that in the M50L3 cells, it was not

surprising that little if any labeled DHFR was detected in the immunoprecipitate of the *in vitro* translation of 3T6 RNA.

To determine if the amount of RNA has any effect on the relative amount of DHFR synthesis, I analyzed the effect of increased amounts of RNA on total protein synthesis and total DHFR synthesis. Figure 28 shows that these two quantities increased as the level of RNA increased until about 2 units of RNA, where it then leveled off. I found that the relative amount of DHFR remains about the same, although there may be a slight decrease as the level of RNA increases (Figure 29). All assays to determine relative amount of DHFR were performed in duplicate using between 1 and 2 units of RNA.

To determine if the changes in the rate of accumulation were the result of changes in DHFR mRNA content, I isolated total mRNA from roller bottle cultures of M50L3 cells at various times after serum stimulation and subsequent serum withdrawal. The labeled DHFR synthesized was quantitated following immunoprecipitation and compared to the total amount of protein synthesized in the translation mixture. The ratio should be an accurate reflection of the relative amount of DHFR mRNA in a given RNA preparation. The lowest panel of Figure 30 shows that the percentage of DHFR mRNA is approximately 0.8% in resting M50L3 cells. Due to the fact that poly (A)(+) mRNA synthesis begins to increase immediately upon stimulation (middle panel, Figure 30), the relative level

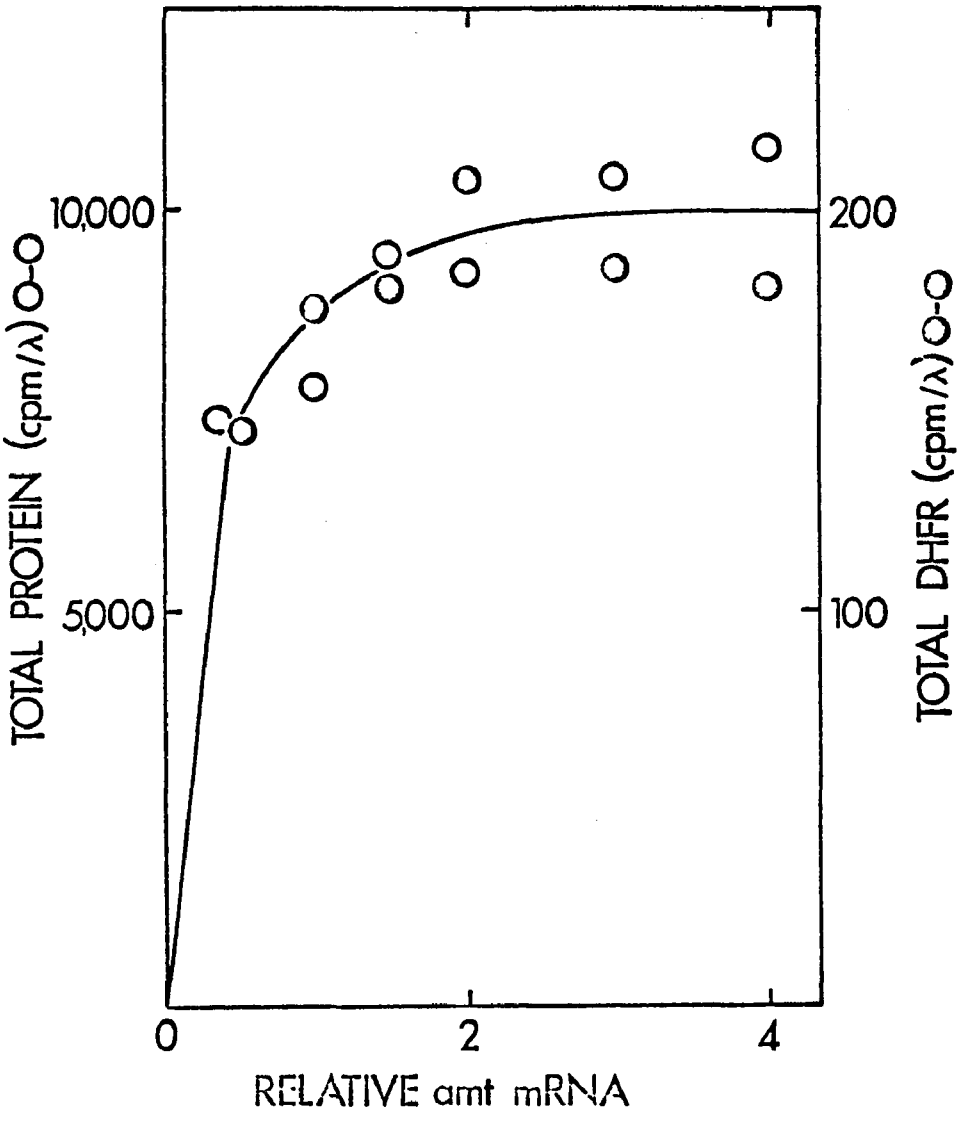


Figure 28. Amount of $^{3}$H-leucine incorporated into total protein and DHFR in an in vitro translation system, as a function of units of RNA added. Increasing amounts of total polysomal RNA from M50L3 cells were added to a series of in vitro translation reaction mixtures. After a 60 minute incubation, the reaction was terminated and an aliquot was assayed for total base stable, TCA precipitable $^{3}$H-leucine incorporation (o). The amount of $^{3}$H-labeled DHFR (●) was determined by immunoprecipitation. 1 unit of RNA will hybridize about 1000 cpm of $^{3}$H-poly (U) and represents about 0.03 ug of poly (A) + RNA. The amount of total RNA will vary with growth state.

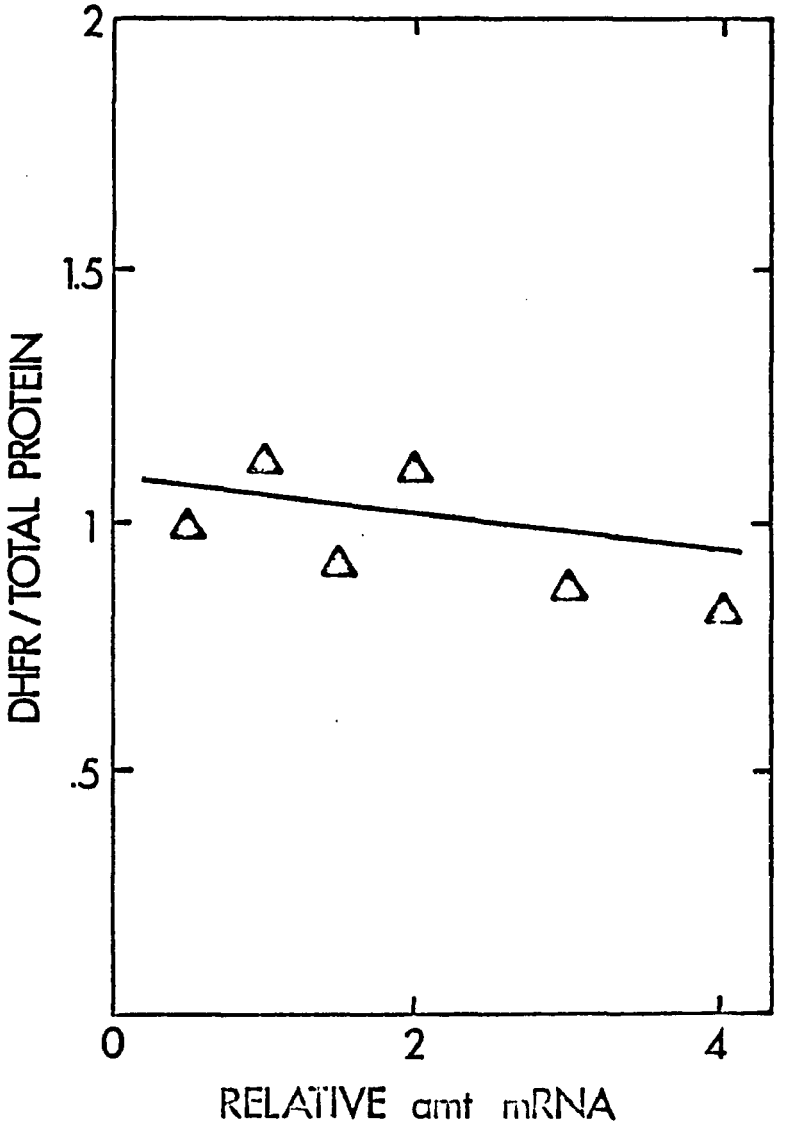


Figure 29. The relative amount of DHFR synthesized in vitro as a function of units of RNA added. The amount of DHFR synthesized in vitro was normalized to the total amount of protein synthesized in the reaction (arbitrary units). Details are described in Figure 28. The average amount of DHFR synthesis directed by the growing M50L3 mRNA preparations was 1.2%.

Figure 30. DHFR mRNA content in serum stimulated and serum withdrawn M50L3 cells. Roller bottle cultures of resting M50L3 cells were serum stimulated at time = 0 (●). At 18 hours (arrow) the serum stimulus was withdrawn from some of the cultures (▲). At various times, cultures were harvested and polysomal RNA isolated. The RNA was translated in an in vitro system, and the amount of $^{3}$H-DHFR synthesized was normalized to the total amount of $^{3}$H-protein synthesized to give the relative DHFR mRNA content (lowest panel). Each RNA preparation was translated several times at different RNA concentrations. Each result is plotted as a separate point.

Total poly (A)(+) mRNA content was determined in cultures of cells growing on 60 mm petri dishes (middle panel). Resting cultures were serum stimulated at time = 0 in the presence (■) or absence (●) of 0.2 mM DB cAMP and 1 mM theophylline. At 20 hours (arrow) the serum stimulus was withdrawn from some cultures (Δ). Other cultures were treated with DB cAMP and theophylline (▲). At various times cultures were harvested and poly (A)(+) mRNA content was determined by the $^{3}$H-poly (U) assay. The content of mRNA was normalized to the average value obtained for resting M50L3 cells (o).

The total content of DHFR mRNA (uppermost panel) in serum stimulated (o) and serum withdrawn (Δ) cells was the product of the relative DHFR mRNA content (lowest panel) and the total mRNA content (middle panel). The values were normalized to the average amount of DHFR mRNA present at 6 hours following stimulation.

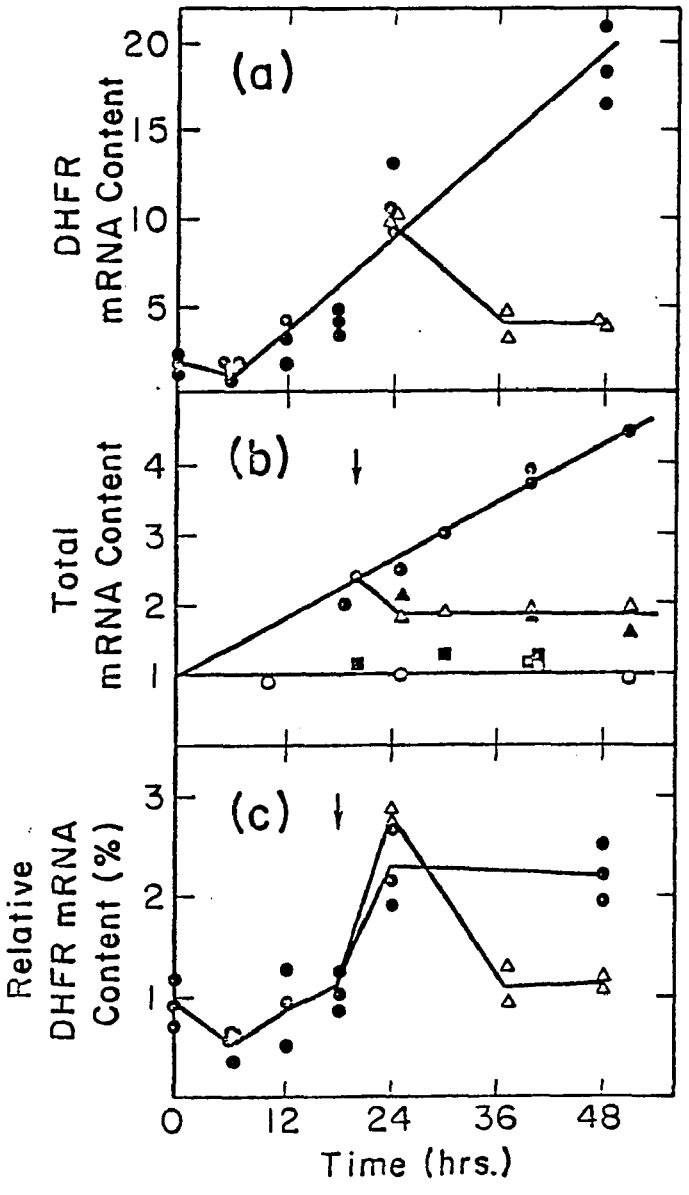
(a)
DHFR mRNA Content
20
15
10
5
(b)
Total mRNA Content
4
3
2
1
(c)
Relative DHFR mRNA Content (%)
3
2
1
0
12
24
36
48
Time (hrs.)

Figure 30.

of DHFR mRNA decreases during the first few hours following serum stimulation, and then begins to increase to about 5-fold by 24 hours after stimulation. In other experiments (lower panel, Figure 31) the maximum was observed by 16 to 20 hours after stimulation. The total amount of DHFR mRNA was determined by multiplying the relative amount of DHFR mRNA (lower panels, Figures 30 and 31) by the total amount of poly (A)(+) mRNA present in the cell at that time (Figure 30, middle panel). This is plotted in the upper panel of Figures 30 and 31. A 10-fold increase in DHFR mRNA content was observed by 24 hours following serum stimulation and this value is less than that observed for the in vivo rate of DHFR synthesis (Figure 19) although the profiles appear very similar. In the in vivo labeling experiment, the level of DHFR is normalized to the soluble protein which represents only 20-30% of the total protein synthesized (Alt, et al., 1978). Therefore, one might expect the percentage of DHFR synthesized to appear greater than in the in vitro system, where I have translated total RNA.

When serum stimulus is withdrawn, I have shown that the accumulation of DHFR ceases. To determine if this is caused by a decrease in the level of translatable DHFR mRNA, I withdrew the medium containing 10% serum from cells which had been stimulated for 18 hours. The relative DHFR mRNA content returned to the resting value within 24 hours (lowest panel,

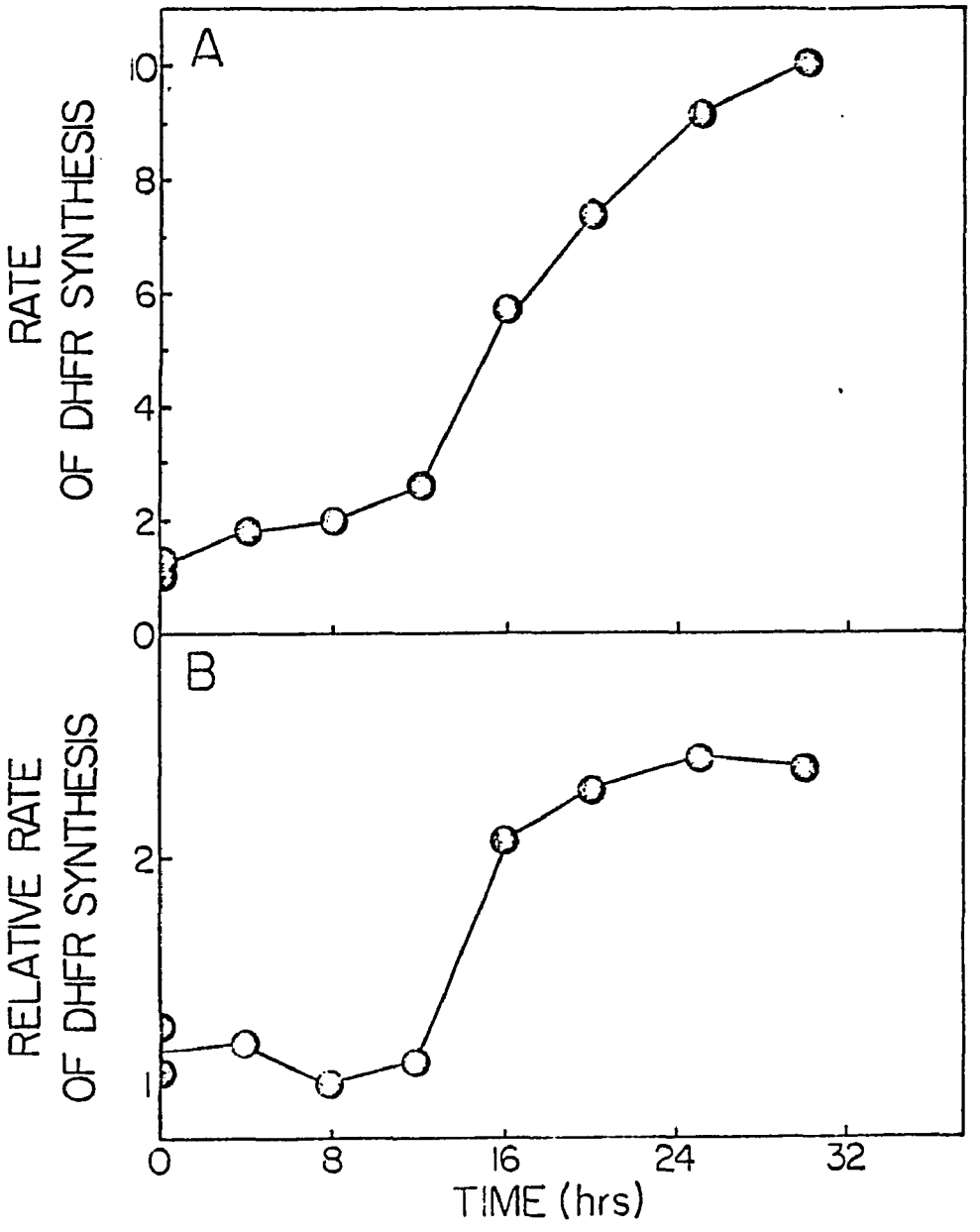


Figure 31. DHFR mRNA content in serum stimulated M50L3 cells. Polysomal RNA was isolated from serum stimulated M50L3 cells at various times. The RNA was translated in an in vitro system and the amount of $^{3}$H-DHFR was normalized to the total amount of $^{3}$H-protein synthesized to give the relative DHFR mRNA content (lower panel).

The total content of DHFR mRNA (upper panel) was the product of the relative DHFR mRNA content (lower panel) and the total mRNA content (middle panel of Figure 30). The values were normalized to the average amount of DHFR mRNA present in resting M50L3 cells.

Figure 30). A corresponding decrease in total DHFR mRNA was also observed (uppermost panel, Figure 30). This correlates well with the rapid decrease in the rate of accumulation after serum withdrawal (Figure 23).

# DISCUSSION

In this dissertation I describe the cell cycle regulation of the enzyme dihydrofolate reductase (DHFR) in mouse 3T6 fibroblasts and a DHFR-overproducing cell line, M50L3, derived from 3T6 fibroblasts.

## Regulation of DHFR Gene Expression

I found that the rate of DHFR synthesis is related to the cell cycle and increases during the $G_1$-S boundary. Although direct perturbation of DNA synthesis has no inhibitory effect on DHFR gene expression, the rate of DHFR synthesis does parallel the increases and decreases in the rate of DNA synthesis during serum stimulation and serum withdrawal. This would suggest that although there is not a direct coupling between the increase in DHFR accumulation and DNA synthesis, the two events may be coordinated by a common signal. Recent studies of the expression of the genes for thymidylate synthetase (Navalgund, et al., 1980) and thymidine kinase (Muench and Johnson, in preparation) have shown a similar pattern of cell cycle regulation. It is probable that DHFR, thymidylate synthetase and thymidine kinase are members of a family of genes which are expressed coordinately when required for the synthesis of DNA precursors during DNA replication.

Using the DHFR overproducing cell line, M50L3, I have been able to show that the changes in DHFR accumulation are due to corresponding changes in the rate of DHFR synthesis. These changes are due to increases and decreases in the levels of translatable DHFR mRNA and account for the variations in the rate of DHFR synthesis in vivo.

These observations have recently been confirmed by another study in our laboratory, where the DHFR mRNA has been quantitated by DNA-excess filter hybridization. In addition, Hendrickson, Wu and Johnson (submitted) have found that the increase in DHFR mRNA content is the result of an increased rate of production of DHFR mRNA.

It is important to note that DHFR is synthesized in resting and $G_1$ phase cells, although at a much lower rate than in S phase cells. This is logical since THFA is required for a variety of reactions besides the production of thymidylate. However, since THFA is oxidized to dihydrofolate during thymidylate synthesis, whereas the oxidation state remains unchanged in all other reactions, the cellular need for DHFR is greatest during the S phase. One might expect that the rate of synthesis of enzymes which are required exclusively for the synthesis of precursors of DNA would be much lower in resting cells than in growing cells. Detailed studies in this laboratory of the S phase enzymes thymidylate synthetase (Navalgund, et al., 1980) and thymidine kinase (Muench

and Johnson, in preparation) have shown this to be the case. This implies that the control of DHFR gene expression is not as tight as that for other S phase enzymes.

## Cyclic AMP Regulation of the Cell Cycle

It has been reported that cAMP levels fluctuate during the cell cycle (Zeilig and Goldberg, 1977) and that decreased levels of cAMP correlate well with growth stimulation (Burger, et al., 1972). Findings have been consistent with the idea that cAMP regulates specific stages in the cell cycle, particularly during growth arrest in $G_1$ (Coffino, et al., 1975). Kellems, et al. (1979) have reported that the entry into S phase and the increase in DHFR synthesis are blocked when MTX-resistant mouse fibroblasts are serum stimulated in the presence of DB cAMP and theophylline. I also found this to be true in the M50L3 cells. Rates of DHFR synthesis and DNA replication in resting cells which were serum stimulated for 20 hours and then exposed to DB cAMP and theophylline responded in a manner similar (if not more rapid) to that in serum withdrawn cultures. The rate of accumulation of DHFR and the levels of DHFR mRNA decreased dramatically.

The literature contains many models attempting to explain the role of cAMP as a regulator in cell cycle control. The most popular model points to the cAMP-dependent protein kinase present in the cytosol. These kinases have been shown to contain two catalytic subunits and two regulatory subunits.

When the regulatory subunits are combined with the catalytic subunits, the protein kinase has low catalytic activity (Erlichman, et al., 1973). The generation of free active catalytic units generally requires the addition of cAMP which combines with the regulatory subunits allowing the catalytic subunits to dissociate (Soderling, et al., 1973). The activity of one type of cAMP-dependent protein kinase increases in relation to the initiation of DNA synthesis in S phase (Costa, et al., 1976) It has also been shown that the microinjection of the catalytic subunit of a cAMP-dependent protein kinase into Xenopus oocytes will block the progesterone stimulation of meiotic cell division, while microinjection of the regulatory subunit induced the cell division sequence in the absence of progesterone (Maller and Krebs, 1977). These findings suggest that cell cycle events may be regulated by a phosphoprotein subject to control by cAMP-dependent protein kinase.

Other evidence suggests that cAMP binds specifically to a cytoplasmic receptor protein (possibly the regulatory subunit of protein kinase). This complex then moves to the nucleus, where it is found in association with DNA and protein (Kallos, 1977). The association is similar to that described for steroid hormones and their receptors (Yamamoto and Alberts, 1976). These interactions with the genome may trigger a biological response relating to cell cycle control.

It should be noted that I have isolated clones from 3T6 which are partially or fully insensitive to high cAMP levels.

Insel, et al. (1975) have isolated similar DB cAMP insensitive cells from S49 mouse lymphoma cells. They have found that these mutant lines have a defect in the cAMP binding proteins and associated protein kinase. Since both the S49 mutant lines and my own 3T6 clones maintain a normal cell cycle (Coffino, et al., 1975), even in the presence of high cAMP levels, the cyclic nucleotide can only be considered a "nonessential regulator" of the cell cycle and may act as a negative modulator of cell cycle progression.

Frearson, et al. (1966) have reported that DHFR activity increased in mouse kidney cell cultures infected with polyoma virus. Kellems, et al. (1979) were able to show that the increase in DHFR activity was due to an increase in the rate of DHFR synthesis and a corresponding increase in the level of DHFR mRNA, using a MTX-resistant mouse fibroblast cell line. However, the polyoma virus induction of DHFR synthesis and DHFR mRNA levels were unaffected by increased cAMP levels, unlike the induction caused by serum stimulation of resting cells (Kellems, et al., 1979).

A model, taking these observations into account, is shown in Figure 32. The model suggests that the control of DHFR and possibly other S phase enzymes is regulated by at least two regulatory pathways: one involving serum components and the other involving cAMP levels. The induction caused by polyoma virus may represent a third regulatory pathway, or may cause a modification of the others.

Figure 32. Hypothetical model for functional involvement of cyclic AMP and serum components in the regulation of the gene for DHFR and other S phase enzymes. Protein kinase, in its inactive form is represented as $R_2C_2$. When present in this form, the cell is in the replicative portions of the cell cycle. If cAMP concentrations increase, the $R_2C_2$ complex dissociates forming 2 active catalytic subunits and 2 regulatory subunits, bound to cAMP. The active subunit of protein kinase may phosphorylate a component which has some effect on gene regulation. The cAMP:R complex may bind to a short control region of the DNA, turning off the expression of DHFR and other S phase enzymes. When R is defective or cAMP levels are low, transcription may occur.

Since DHFR is still regulated in resting and serum stimulated cells which are insensitive to high levels of cAMP (see text), another control region responding to serum components may exist.

Polyoma viral infection may interfere with any of the steps in the figure, or may produce a modification of the RNA polymerase recognizing a new promoter unaffected by these regulatory sites.

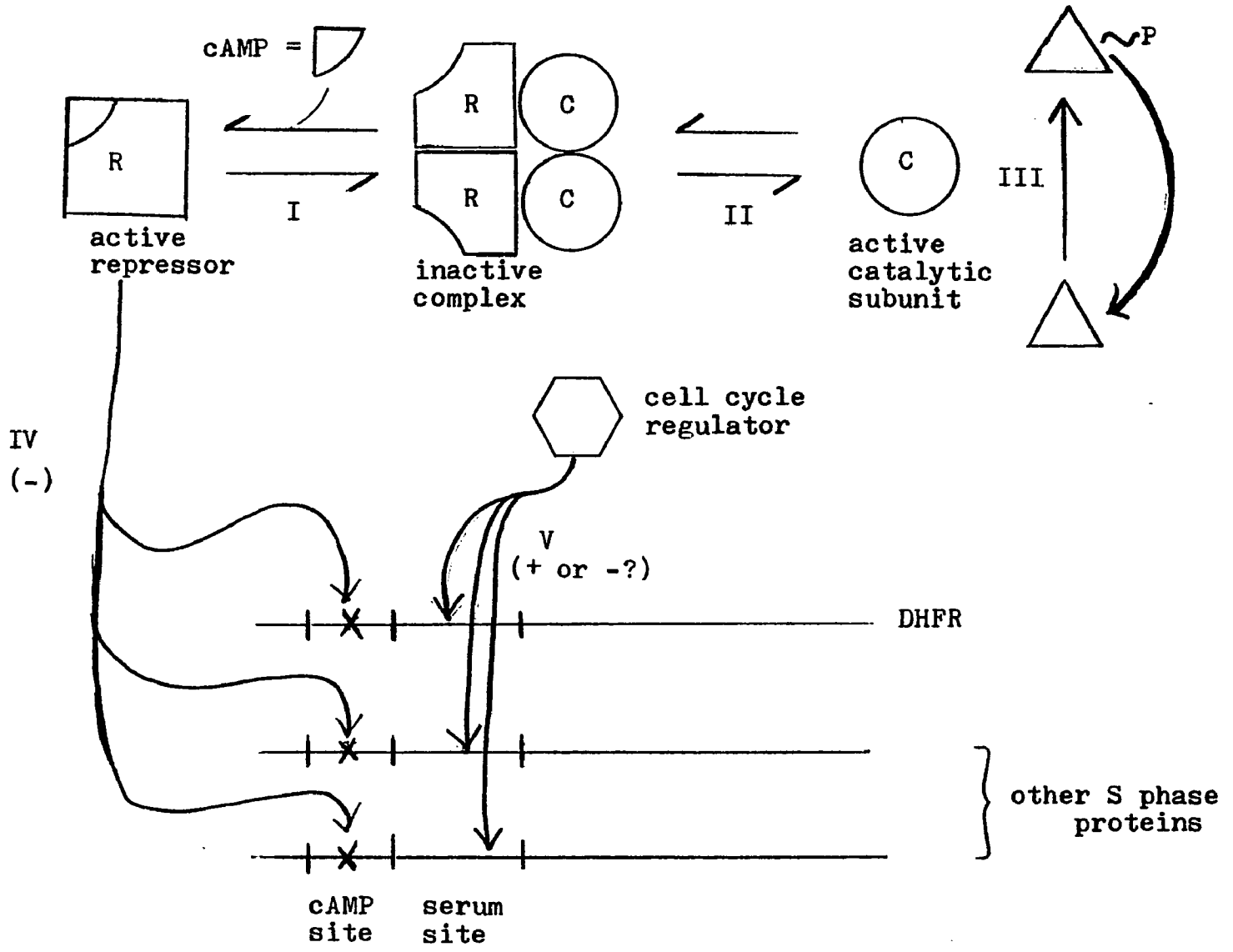
cAMP =
R
active
repressor
I
R
C
R
C
inactive
complex
II
C
active
catalytic
subunit
III
~P
IV
(-)
cell cycle
regulator
V
(+ or -?)
DHFR
other S phase
proteins
cAMP
site
serum
site

Figure 32.

Mechanism of DHFR Overproduction

In order to examine the detailed regulation of DHFR, I isolated and characterized a MTX-resistant cell line which overproduced DHFR by a factor of 300 over normal 3T6 cells.

The mechanism of the overproduction of a specific gene product is the focus of research in a number of laboratories. In 1961 Hakala and co-workers (Hakala, et al., 1961) found that resistance to high levels of MTX was due to increased levels of the enzyme DHFR. Schimke's laboratory was able to show that the increased levels of DHFR were the result of corresponding increases in the relative rate of synthesis of DHFR (Alt, et al., 1976) and due to parallel increases in the level of translatable DHFR mRNA (Kellems, et al., 1976).

It has since been shown that the DHFR gene is selectively multiplied in the mutant cell lines (Alt, et al., 1978). The first suggestion that this was the case was reported by Biedler and Spengler (1976). They observed large, homogeneously staining regions in the chromosomes of Chinese hamster cells, which were stably resistant to high levels of MTX. These regions were absent in the MTX-sensitive parent cell chromosomes. There occur both stable and unstable lines of MTX-resistant cells. I have shown that the trait in M50L3 cells is retained only when cells are maintained continually in the presence of MTX. It has recently been shown by *in situ* hybridization that amplified DHFR sequences in unstably

MTX-resistant cells are associated with small paired chromosomal elements, called double minute chromosomes (Kaufman, et al., 1979), while stable amplified sequences are located in the homogeneously staining region of the chromosome (Nunberg, et al., 1978; Dolnick, et al., 1979). Since double minutes do not appear to associate with the spindle apparatus at mitosis (Kaufman, et al., 1979), they may segregate randomly and unequally into daughter cells. Loss of DHFR overproduction could be accounted for by the overgrowth of a culture by cells containing fewer double minute chromosomes.

Future of Overproducing Cell Lines

Regardless of how these amplified DHFR sequences are acquired or where they are located, the fact that they still experience a similar response to cell cycle regulation indicates that these genes are under the control of their original regulatory sequences. This would suggest that these regulatory regions might also be amplified. These results have demonstrated a new approach that can be taken to permit detailed studies of the structure and expression of the gene for DHFR and genes for other "housekeeping" proteins.

The occurrence of gene amplification is not entirely new. In the oocytes of many species the genes for ribosomal RNA are specifically amplified, presumably to allow production of large numbers of ribosomes which are incorporated into the egg (Brown and Dawid, 1968). Recently, Spradling and

Mahowald (1980) have reported a 10-fold amplification of the genes for chorion proteins during oogenesis in Drosophilia melanogaster. This may suggest that the occurrence of specific gene amplification is of general significance.

There are also other examples of drug resistance due to the increase of a specific protein level. These proteins include ribonucleotide reductase (Meuth and Green, 1974), hydroxymethyl glutaryl Co A reductase (Sinensky, 1977) and aspartate transcarbamylase (Kempe, et al., 1976). In the last case, the overproduction has been shown to be due to accumulation of the mRNA coding for aspartate transcarbamylase (Padgett, et al., 1979) and a corresponding increase in the number of gene copies (Wahl, et al., 1979). It is possible that overproduction of a target protein, as a means of developing resistance to a toxic agent, may prove to be a general mechanism, if proper selective conditions are used.

Overproducing cell lines can be used to examine the control of the various enzymes expressed at the same time during the cell cycle, in molecular detail. Recently, I have assisted in the isolation of a series of cell lines which overproduce thymidylate synthetase. These cells overproduce this enzyme by at least 200-fold when compared to the sensitive cell line even when they are resistant to relatively low levels of 5-fluorodeoxyuridine (i.e. 3 uM) (Rossana, Wiedemann, Navalgund and Johnson, in preparation). One might speculate

that the control regions of the genes for DHFR and thymidylate synthetase may resemble one another, allowing for their coordinated control as members of a class of unlinked genes required for the cell's entry into S phase.

## SUMMARY

Dihydrofolate reductase (DHFR) is the target enzyme for the chemotherapeutic drug, methotrexate (MTX). I have examined the regulation of the expression of the gene for this enzyme in cultured fibroblasts during various growth states.

The level of DHFR gene expression is very low in resting ($G_0$) 3T6 mouse fibroblasts, but increases sharply in serum stimulated cells, just prior to DNA replication (Johnson, et al., 1978b). Because DHFR and DHFR mRNA are present at low levels in normal 3T6 cells, the investigation of the molecular mechanism for the regulation of DHFR gene expression in these cells is difficult. However, it has been observed that when cells become resistant to high levels of MTX, the level of DHFR and its mRNA are increased several hundred-fold. I have isolated a MTX-resistant 3T6 cell line, M50L3, which overproduces DHFR as well as DHFR mRNA. The cells also retain the ability to rest in medium containing 0.5% serum. The regulation of DHFR gene expression in M50L3 appears to be the same as in normal 3T6 cells. In particular, in vivo labeling studies show that the rate of synthesis is very low in resting cells and during the first 8 hours following serum stimulation, but increases at least 10-fold by 16 hours following stimulation (Wiedemann and Johnson, 1979).

Studies with inhibitors of RNA or DNA synthesis indicate that DHFR mRNA is transcribed at the beginning of the S phase, but that the increase in DHFR gene expression is not tightly linked to DNA replication. The in vitro translation studies show that DHFR mRNA is present in low levels in resting cells and increases rapidly between 8 and 16 hours after stimulation.

Addition of DB cAMP to M50L3 cultures inhibits the serum stimulation of DNA replication and DHFR synthesis. This indicates that although DHFR gene expression is not directly linked to DNA synthesis, these two events may be regulated by a common control signal that responds to changes in cAMP levels. DHFR gene expression is turned off following the addition of DB cAMP or the removal of serum stimulus at 20 hours after stimulation (during the S phase). These decreases are also closely correlated with decreases in the level of DHFR mRNA. These results suggest that phosphorylated proteins may play a role in coordinating DHFR gene expression with the cell's entry into the S phase.

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