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Cell Biology

Regulation of dihydrofolate reductase synthesis in an overproducing 3T6 cell line during transition from resting to growing state

(gene expression/cell cycle/methotrexate)

LEANNE M. WIEDEMANN AND LEE F. JOHNSON*

Department of Biochemistry, The Ohio State University, Columbus, Ohio 43210

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ABSTRACT **We have isolated a methotrexate (MTX)-resistant clone of mouse 3T6 cells, designated M50L3, which grows normally in the presence or absence of 50 μM MTX and produces a level of dihydrofolate reductase (DHFR; 5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase, EC 1.5.1.3) that is increased about 300-fold compared to the parental 3T6 cells. The cells retain the ability to rest in the G_0 state when maintained in medium containing 0.5% calf serum and can be stimulated to reenter the cell cycle by increasing the serum concentration to 10%. The rate of accumulation of DHFR in resting M50L3 cells is about 1/25th of that in exponentially growing cells. When resting cells are stimulated to reenter the cell cycle, the rate of accumulation of DHFR starts to increase at about 8 hr and reaches a maximum (25-fold increase) at about 16 hr after stimulation. Pulse-labeling experiments show that the increase in DHFR accumulation is due to an increased rate of synthesis. This increase occurs at about the same time the cells enter S phase. However, inhibitors of DNA synthesis have no effect on the increase in DHFR accumulation after serum stimulation, indicating that there is no tight coupling of the two events. Actinomycin D inhibits the subsequent increase in DHFR accumulation if added 8 hr after stimulation but has no effect if added 16 hr after stimulation. This is consistent with the idea that the increase in DHFR gene expression depends on transcription of the gene and that DHFR mRNA synthesis begins at about the time the cell initiates DNA replication. DHFR gene expression appears to be regulated in the same manner in the overproducing cells as we found in the parental 3T6 cells [Johnson, L. F., Fuhrman, C. L. & Wiedemann, L. M. (1978) *J. Cell. Phys.* 97, 397–406]. Therefore, the alterations that are responsible for DHFR overproduction (presumably DHFR gene amplification) do not interfere with the ability of the cell to regulate the rate of synthesis of the enzyme after serum stimulation.**

Dihydrofolate reductase (DHFR; 5,6,7,8-tetrahydrofolate: NADP$^+$ oxidoreductase, EC 1.5.1.3) is responsible for the NADPH-dependent reduction of folic acid and dihydrofolic acid to tetrahydrofolic acid. Derivatives of tetrahydrofolate are required for many facets of single-carbon metabolism, including the biosynthesis of purines and thymidylic acid. The enzyme is also the target of the chemotherapeutic drug methotrexate (MTX) (1).

A number of laboratories have shown that the level of cellular DHFR is related to the growth rate. Exponentially growing cells contain severalfold higher levels of DHFR than do "stationary phase" cells, which grow slowly due to nutrient depletion (2, 3). The reduction in enzyme level is due primarily to a decreased DHFR mRNA level (4) which results in a lower rate of synthesis of the enzyme (5). Our own studies of DHFR accumulation in mouse 3T6 and 3T3 cells, which rest in the G_0 state as a result of density dependent (or contact) inhibition of growth, show that the rate of accumulation of DHFR in resting cells is about 1/40th that in exponentially growing cells. When the resting cells are induced to reenter the cell cycle by serum stimulation, the rate of accumulation of DHFR increases sharply about 10 hr later. This increase occurs at about the same time the cells enter S phase and is not affected when DNA synthesis is blocked by arabinofuranosylcytosine (cytosine arabinoside) or hydroxyurea. Results of studies with actinomycin are consistent with the possibility that the increase in DHFR accumulation depends on gene transcription and that DHFR mRNA is synthesized only between 7.5 and 15 hr after stimulation (6).

Because DHFR and DHFR-mRNA are present at low levels in normal 3T6 cells, the investigation of the molecular mechanism for regulating DHFR gene expression in such cells would be difficult. However, it has been observed that, when cells become resistant to high levels of MTX, the level of DHFR (7), its mRNA (4, 8), and even the DHFR gene (9) are increased several hundred-fold. If DHFR gene expression were regulated in overproducing cells in the same manner as in normal cells, the overproducing cells would be an ideal system for studying the regulation of DHFR gene expression. Unfortunately, none of the MTX-resistant cell lines isolated previously appeared to be particularly susceptible to growth control. Because this property was critical as far as the regulation of DHFR gene expression was concerned, we decided that we would attempt to isolate a MTX-resistant 3T6 cell line that not only would overproduce DHFR but also would retain the ability to enter the resting state and regulate DHFR gene expression in the same manner as the parental 3T6 cells. This paper reports the isolation and characterization of such a cell line.

MATERIALS AND METHODS

Cell Culturing. Cultures of cells were maintained on plastic petri dishes in the Dulbecco–Vogt modification of Eagle's medium (GIBCO) supplemented with 10% calf serum (Colorado Serum). A line of 3T6 cells resistant to 50 μM MTX was developed from mouse 3T6 fibroblasts (10) by a modification of a described procedure (7). Cells were initially plated in medium supplemented with 30 μM thymidine and 0.02 μM MTX. After the surviving cells had adapted to the drug level and were growing rapidly (usually about 2 weeks), the level of MTX was doubled. This process was repeated until a level of 50 μM MTX was achieved. This culture was plated at low density and a series of clones were picked. Clones that grew well and had high levels of DHFR activity were studied further (see *Results*). Because the cells grew equally well in medium containing or lacking 30 μM thymidine, the nucleoside was omitted. Stocks of M50L3 cells were maintained in medium containing 50 μM MTX and 10% calf serum. When cells containing

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Abbreviations: DHFR, dihydrofolate reductase; MTX, methotrexate; $NaDodSO_4$, sodium dodecyl sulfate.

* To whom reprint requests should be addressed.

active DHFR were required, stocks of cells were grown in medium lacking drug for 8–10 cell doublings prior to use.

Cultures of exponentially growing cells were prepared by seeding dishes at low density in medium containing 10% serum and feeding the cultures the next day; these cells were used for an experiment 2 days after seeding. At the time of the experiment, the cells were less than 20% confluent. Cultures of resting cells were prepared by seeding 7×10^4 cells per cm^2 in medium containing 0.5% calf serum. The cultures were fed on the second and fourth days after seeding and were used for an experiment on the seventh day after seeding (11). Resting cells were stimulated to reenter the cell cycle by replacing the medium with fresh medium containing 10% calf serum.

Determination of DHFR Levels. The level and rate of accumulation of DHFR were determined by the rapid and sensitive [^{3}H]MTX binding assay as described (6, 12).

RESULTS

Isolation and Characterization of MTX-Resistant 3T6 Cell Line. We isolated a number of clones of 3T6 cells that were able to grow normally in medium containing 10% serum in the presence of 50 μM MTX and to enter the resting state when kept in medium containing 0.5% serum. Two such clones, designated M50L3 and M50B, are described in Table 1. Both produced high levels of DHFR and were able to enter the resting state at about the same density as did parental 3T6 cells. The resistant cells did not appear to rest quite as well as normal 3T6 cells, as judged by a higher rate of DNA synthesis and the presence of occasional mitotic cells in the MTX-resistant resting cultures. When resting M50L3 cells were serum stimulated, the cells began synthesizing DNA about 9 hr after stimulation (data not shown) as do 3T6 cells (6). Because M50L3 produced a higher level of DHFR than did M50B, we concentrated most of our efforts on the former.

To determine if M50L3 cells would continue to overproduce DHFR in the absence of selective pressure, M50L3 cells were cultured in medium lacking MTX and assayed at later times for the amount of DHFR per cell. The DHFR level decreased rapidly ($t_{1/2} \approx 7$ days) and stabilized at a level about 10-fold above that found in 3T6 cells within a period of 2 months (Fig. 1). The instability of the overproduction trait was similar to that observed previously for mouse AT3000 cells (5) but quite different from the situation in hamster A5 cells in which the overproduction trait is a stable characteristic (13).

Table 1. Properties of MTX-resistant cells

	3T6	M50L3	M50B
Doubling time, hr:			
No drug	19	19	27
+50 μM MTX	—	21	29
DHFR level (relative)	1.0	300	70
Saturation density, resting cells/cm^2	9×10^4	6×10^4	9×10^4
Rate of DNA synthesis, resting/growing	0.005	0.038	0.038

Doubling time was determined by plating cells on a series of dishes at low density. Duplicate dishes were trypsinized and cell density was 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 of [^{3}H]MTX bound per mg of cell protein) in the cell line of interest and normalizing this value to that obtained for normal 3T6 cells. Saturation density was determined for resting cultures that had been kept in 0.5% serum for 7 days. Rate of DNA synthesis ([^{3}H]thymidine incorporation) per cell was determined (6) for resting and exponentially growing cultures and expressed as a ratio.

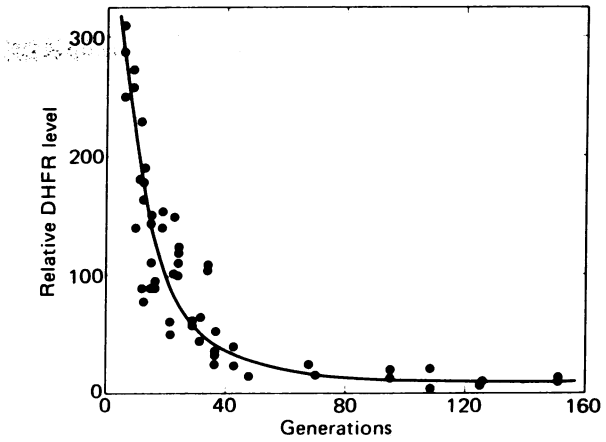

FIG. 1. Loss of overproduction trait in cells cultured in the absence of MTX. Cultures of M50L3 cells were grown in the absence of MTX beginning on day 0. At various times, cells were harvested and the DHFR specific activity was determined. This value was normalized to the specific activity of DHFR in exponentially growing 3T6 cells (22.5 cpm of [^{3}H]MTX bound per μg of cytoplasmic protein).

The cellular proteins of M50L3 cells and 3T6 cells were compared by subjecting cytoplasmic extracts to sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide slab gel electrophoresis. The only obvious difference was a protein with a molecular weight of about 21,000 that was prominent in the M50L3 pattern but undetectable in 3T6 (Fig. 2). This protein comigrated with authentic DHFR isolated from M50L3 cells by affinity chromatography on folate-Sepharose and represented about 4% of total cytoplasmic protein (Ruth Wu and L. J., unpublished data).

Regulation of DHFR Gene Expression. To determine if DHFR gene expression was regulated in the same manner in the overproducing cells as in the normal 3T6 cells, we first compared the rate of accumulation of DHFR in resting and growing M50L3 cells by using a procedure we developed for normal 3T6 cells (6). Cultures were incubated with 50 μM MTX to inactivate essentially all of the preexisting DHFR. The cultures were then rinsed extensively to remove unbound MTX and fed with medium appropriate for the experiment. We then measured the rate of increase of active (newly synthesized)

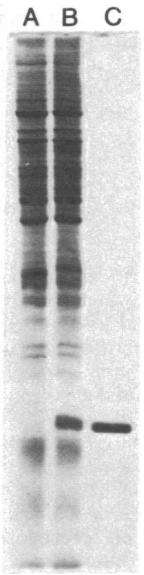

FIG. 2. Comparison of the cytoplasmic proteins of 3T6 and M50L3 cells. Cultures of exponentially growing 3T6 or M50L3 cells were harvested and cytoplasmic extracts were fractionated by $NaDodSO_4$ 11.3% polyacrylamide slab gel electrophoresis (14). The gels were then stained with Coomassie blue. Lanes: A, 3T6 extract; B, M50L3 extract; C, pure DHFR isolated from M50L3 cells.

DHFR. The rate of accumulation of DHFR was very low in resting M50L3 cells and about 25-fold higher in growing cells (Fig. 3). Incubation with MTX had no effect on the rate of accumulation of DHFR in growing cells other than reducing the "background level" of preexisting DHFR. When cycloheximide was included in the medium, the increase in DHFR activity was inhibited, indicating that the increase was due to *de novo* synthesis of the enzyme. Actinomycin D had little effect on the accumulation of DHFR for at least 6 hr in growing cells, suggesting that DHFR mRNA is not turning over rapidly and that its translation is not inhibited under these conditions.

We next examined the accumulation of DHFR in resting M50L3 cells that were serum stimulated to reenter the cell cycle. At about 9 hr after stimulation, the rate of accumulation of DHFR began to increase (Fig. 4). By 15 hr after stimulation, the rate of DHFR accumulation was about 25 times greater (by comparison of slopes) than in resting cells. The same increase in rate of accumulation was observed in the presence or absence of MTX pretreatment (data not shown), again showing that pretreatment was an effective method for reducing the activity of preexisting DHFR without affecting DHFR gene expression.

To determine if the increase in DHFR synthesis depended on gene transcription, we examined the effect of addition of

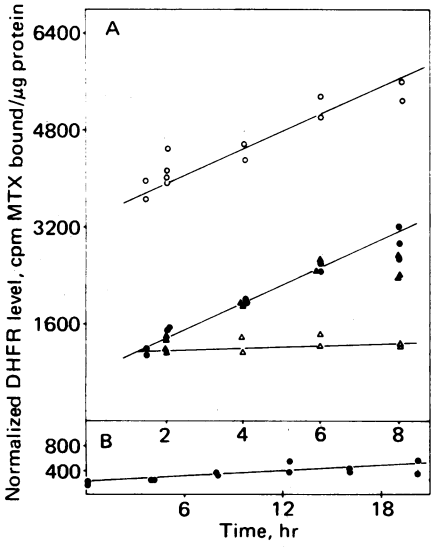

FIG. 3. Rate of accumulation of DHFR in growing (*A*) and resting (*B*) M50L3 cells. Cultures of resting or growing M50L3 cells on 35-mm culture dishes were prepared from stocks grown in the absence of MTX for 7 days. Some cultures were incubated with 50 μM MTX for 2 days prior to the experiment to inactivate essentially all of the preexisting 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 taken from sister cultures of resting M50L3 cells. Growing cultures were fed with fresh medium containing 10% calf serum (●), 10% calf serum plus cycloheximide (5 μg/ml) (△), or 10% calf serum plus actinomycin (5 μg/ml) (▲). 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 (○), were also analyzed. The rate of accumulation of DHFR was determined by harvesting cultures at various times and measuring the level of active (newly synthesized) DHFR by the [^{3}H]MTX binding assay. This level was 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/μg of protein. Protein concentration was determined by the procedure of Lowry *et al.* (15) with bovine serum albumin as the standard.

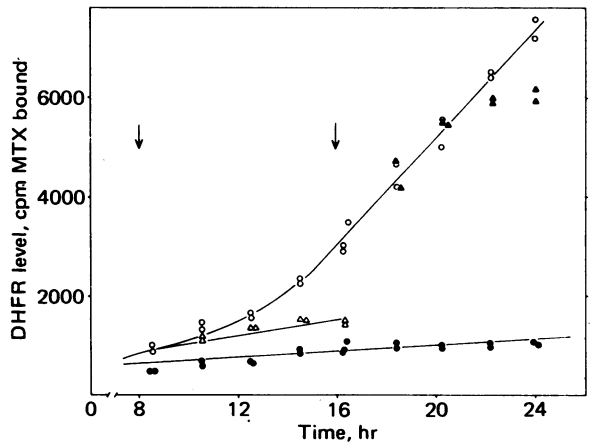

FIG. 4 Increase in rate of accumulation of DHFR in serum-stimulated M50L3 cells. Cultures of resting M50L3 cells on 35-mm dishes were pretreated with 50 μM MTX for 2 days as in Fig. 3 and then rinsed extensively with serum-free medium and fed at time = 0 with fresh medium containing 10% calf serum (○). Control cultures were fed with conditioned medium containing 0.5% calf serum (●). At time = 8 hr (△) or 16 hr (▲) actinomycin D was added to the stimulated cultures at a final concentration of 5 μg/ml. Duplicate cultures were harvested at various times and 20-μl samples were assayed for DHFR level by the [^{3}H]MTX binding assay. The level of DHFR in resting cells not pretreated with MTX was 3850 cpm.

actinomycin D, at various times after stimulation, on the subsequent rate of accumulation of DHFR. Addition of actinomycin D at 8 hr after stimulation blocked the subsequent increase in DHFR activity (Fig. 4). However, addition of actinomycin D at 16 hr after stimulation had little effect on subsequent accumulation of DHFR for at least 5 hr. These data are consistent with (but do not prove) the idea that DHFR mRNA is not present in resting cells or in cells stimulated for less than 8 hr. DHFR mRNA synthesis begins about 8 hr after stimulation, which coincides with the time at which the rate of accumulation of DHFR is increasing rapidly.

To determine if the increased rate of DHFR accumulation was due to an increased rate of synthesis, we exposed cultures of M50L3 cells to [^{3}H]leucine for 60 min at various times after serum stimulation. Cytoplasmic extracts were subjected to $NaDodSO_4$ slab gel electrophoresis followed by fluorography to reveal the location and relative labeling of the protein bands. There was little labeling of the DHFR band in resting cells or in cells stimulated for less than 8 hr (Fig. 5). The relative intensity of the DHFR band increased markedly after 8 hr and then appeared to level off by 16 hr after stimulation.

The rate of synthesis of DHFR was quantitated by cutting out the region of the fluorogram corresponding to DHFR and determining the amount of radioactivity. This value was normalized to the radioactivity in higher molecular weight proteins to obtain the *relative* rate of DHFR synthesis at various times following stimulation. The relative rate increased about 5-fold between 8 and 16 hr after stimulation and then remained constant (Table 2). To obtain the rate of synthesis of DHFR per cell, the relative rate was multiplied by the rate of total protein synthesis (rate of [^{3}H]leucine incorporation per cell). We found that rate of synthesis of DHFR began increasing about 8 hr after stimulation and was about 12-fold greater than in resting cells at 16 hr and about 20-fold greater at 24 hr following serum stimulation. Because the increase in rate of synthesis of DHFR correlated fairly well with the increase in the rate of accumulation, we conclude that the increase in the rate of accumulation of DHFR is due primarily, if not entirely, to an increased rate of synthesis.

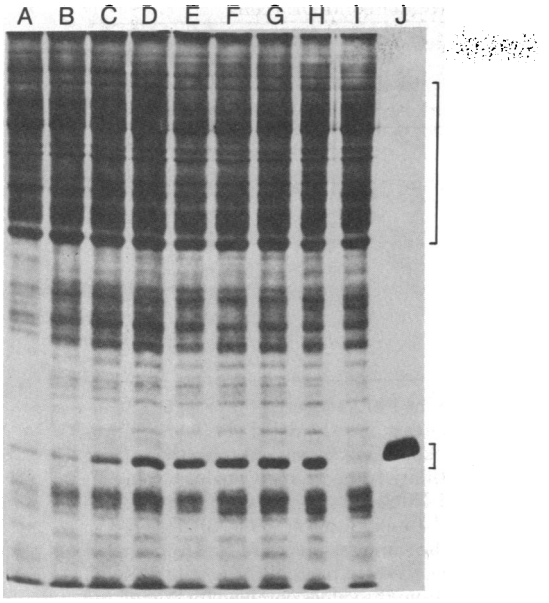

FIG. 5. Relative labeling of DHFR after serum stimulation. Cultures of resting M50L3 cells were serum stimulated at time = 0. At the times indicated below, the culture medium was replaced with fresh "labeling medium" containing 10% calf serum, 0.5% of the normal amount of leucine, and [^{3}H]leucine (25 μCi/ml; final specific activity, 6 Ci/mmol). The cultures were incubated at 37°C for 1 hr and then harvested. Labeled proteins were fractionated by $NaDodSO_4$ slab gel electrophoresis; 2.0 ($\pm$0.1) $\times$ 10^5 cpm of labeled protein was applied to each slot of the gel. The fluorogram (16) of the gel is shown here. Labeling medium was added as follows: lane A, 0 hr; B, 4 hr; C, 8 hr; D, 12 hr; E, 16 hr; F, 20 hr; and G, 24 hr. 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.

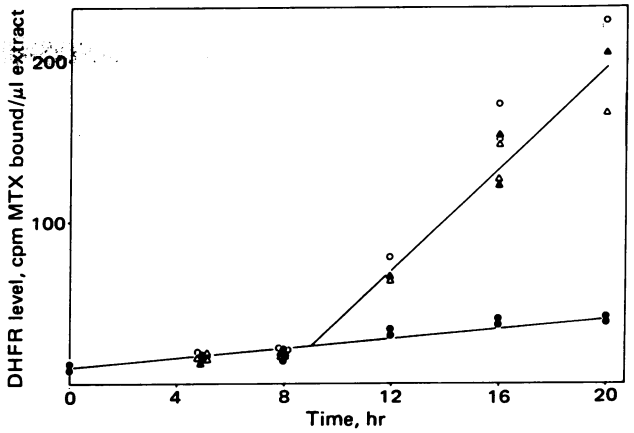

FIG. 6. Increase in rate of DHFR accumulation in the absence of DNA synthesis. Cultures of resting M50L3 cells were pretreated with MTX and serum stimulated at time = 0 as in Fig. 3. In this experiment, both pretreatment and serum stimulation were performed in the presence of 30 μM thymidine and 100 μM hypoxanthine. Some cultures were stimulated in the presence of arabinofuranosylcytosine (5 μg/ml) (▲) and others, in the presence of hydroxyurea (30 μg/ml) (△). Control cultures were stimulated in the absence of drugs (○) or were fed at time = 0 with conditioned medium containing 0.5% calf serum (●). Cultures were harvested at various times and the amount of active (newly synthesized) DHFR was determined by the [^{3}H]MTX binding assay. Because different volumes (20–100 μl) of cell extract were assayed in this experiment, DHFR levels are expressed as cpm of [^{3}H]MTX bound per μl of cell extract assayed.

The increase in the rate of DHFR synthesis occurred at about the same time the cells began DNA replication. To determine if these two events were tightly coupled, we studied the effect of blocking DNA synthesis on DHFR gene expression. Resting M50L3 cells were serum stimulated in the presence of arabinofuranosylcytosine (5 μg/ml) or hydroxyurea (30 μg/ml). These drug levels inhibited the incorporation of [^{3}H]thymidine into DNA in growing 3T6 cells by greater than 98%. Inhibition of DNA synthesis had no effect on the increase in DHFR synthesis (Fig. 6), indicating a lack of coupling of the two events. Fig. 6 also shows that the increase in DHFR synthesis was not affected by the presence of 30 μM thymidine plus 100 μM hypoxanthine. We also found that the presence of all four deoxynucleosides (30 μM each) in the culture medium had no effect on the increase in DHFR gene expression after serum stimulation (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 or thymidylic acid or other deoxynucleotides.

DISCUSSION

This paper describes the isolation and characterization of a line of 3T6 cells, designated M50L3, that is able to grow normally in the presence of 50 μM MTX as well as enter the resting (G_0) state when kept in medium containing 0.5% serum. The mechanism of MTX resistance in these cells, as in other such lines (17), is massive overproduction of DHFR, the target enzyme for the drug. M50L3 cells contain 300 times as much DHFR as does the 3T6 cells from which they were derived. We have also found, by *in vitro* translation experiments, that there is a similar increase in the level of DHFR mRNA (to be published elsewhere). The enzyme is one of the major translation products, accounting for approximately 4% of cytoplasmic protein.

The regulation of DHFR gene expression appears, by all criteria examined, to be the same in M50L3 cells as we reported earlier for the parental 3T6 cells (6). The rate of accumulation of DHFR in resting M50L3 cells is about 4% of that in exponentially growing cells. Because about 4% of the cells also appear to be traversing the cell cycle (as judged by the rate of DNA synthesis in resting compared to growing M50L3 cells), it is possible that within a truly noncycling cell the rate of DHFR accumulation is near zero. When resting M50L3 cells are serum stimulated to reenter the cell cycle, they begin DNA synthesis about 9 hr later, as do 3T6 cells. The rate of accumulation of DHFR also begins to increase about 8–9 hr later and reaches a maximum about 16 hr after stimulation. The increase in rate of accumulation is due primarily (if not entirely) to an increased rate of synthesis of the enzyme.

The increase in DHFR synthesis occurs at the time the cells begin synthesizing DNA. This temporal relationship is not unexpected because various methyl derivatives of tetrahydrofolate are required for the *de novo* synthesis of purines and thymidylic acid. However, studies with inhibitors of DNA synthesis show that there is no direct coupling of the two processes. Furthermore, the cell does not increase DHFR synthesis in response to depletion of the cellular pools of DNA precursors because stimulation of M50L3 cells in the presence of hypoxanthine and thymidine, or of all four deoxynucleosides, has no effect on the increase in DHFR synthesis. Therefore, it appears that the expression of the DHFR gene is controlled by a cell cycle-specific event.

Studies using actinomycin D to block RNA synthesis indicate that the increase in DHFR synthesis depends on transcription, probably of the DHFR gene, which begins about 8 hr after serum stimulation. However, this conclusion is based in part on experiments using actinomycin D, which may exert secondary

Table 2. Rate of synthesis of DHFR in serum-stimulated M50L3 cells

Labeling conditions, hr after stimulation	Relative labeling of DHFR, %		Rate of total protein synthesis (normalized)	Rate of DHFR synthesis (normalized)
	Uncorrected	Corrected		
Growing 3T6	1.7	0		
Growing M50L3	5.9	4.2		
Stimulated M50L3, 0–1	2.6	0.9	1.0	1.0
Stimulated M50L3, 4–5	2.6	0.9	1.6	1.6
Stimulated M50L3, 8–9	3.4	1.7	1.7	3.2
Stimulated M50L3, 12–13	4.8	3.1	2.1	7.2
Stimulated M50L3, 16–17	5.9	4.2	2.5	12
Stimulated M50L3, 20–21	6.1	4.4	3.2	16
Stimulated M50L3, 24–25	6.2	4.5	4.2	21

Exponentially growing or serum-stimulated cells were labeled for 1 hr as described in Fig. 5. The labeled proteins were subjected to $NaDodSO_4$ slab gel electrophoresis and the dried gels were fluorographed. The region corresponding to labeled DHFR (lower bracket in Fig. 5) 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 (New England Nuclear) and was normalized to the amount of radioactivity found in "high molecular weight proteins" (upper bracket in Fig. 5). 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. The value for growing 3T6 cells is the average of seven determinations (range, 1.5–2.0). The value for cells stimulated for 8 hr is the mean of two determinations (3.3, 3.4); the value for cells stimulated for 16 hr is the mean of three determinations (range, 5.6–6.3). 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 (11). Duplicate values (which differed by <10% in each case) were averaged and normalized to the value obtained for cells stimulated at time = 0 (11,300 cpm/60-min incubation). The rate of DHFR synthesis is the product of the relative rate of DHFR synthesis and the rate of total protein synthesis. This value has also been normalized to the rate in cells stimulated at time = 0 to show the fold increase in the rate of DHFR synthesis at various times after stimulation.

effects, and will have to be regarded as tentative until verified by direct measurements of the synthesis, processing, and cytoplasmic accumulation of DHFR mRNA. Clearly, such measurements will be greatly facilitated by the use of this cell line.

When this paper was in preparation, we became aware of a study by Kellems *et al.* (18) which showed that infection of an overproducing 3T6 cell line with polyoma virus resulted in a 4–5-fold increase in the relative rate of DHFR synthesis and a corresponding increase in mRNA abundance. They also reported that addition of fresh serum to such cultures in stationary phase resulted in a 2-fold increase in DHFR synthesis and that inhibitors of DNA synthesis did not block the increase in DHFR gene expression. The difference between their results and ours in the fold increase in DHFR synthesis after growth stimulation may be due to a difference in the method used to arrest the growth of the cells or to a fundamental difference in the susceptibility of the cell lines to growth control.

Overproduction of DHFR has been shown to be the result of DHFR gene amplification in every cell line examined to date (17), and we assume that our cell line will be no exception. Because DHFR gene expression appears to be controlled in the same manner in overproducing M50L3 cells as in the parental cells, the ability of the cell to control the expression of the multiple gene copies has not been lost as a result of the amplification process. Therefore, if specific regulatory molecules are responsible for controlling the level of DHFR gene expression by controlling the transcription of the gene, the processing/export of the mRNA, or the translation of the mRNA, they must either be amplified in parallel with the structural gene or be present in great excess in normal cells.

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