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Cell cycle regulation of dihydrofolate reductase mRNA metabolism in mouse fibroblasts

(gene expression/overproducing cells/nucleic acid hybridization)

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ABSTRACT **We have used the technique of DNA-excess filter hybridization to measure directly the content and metabolism of the mRNA for dihydrofolate reductase (DHFR; 5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase, EC 1.5.1.3). The studies were conducted with a methotrexate-resistant derivative of mouse 3T6 fibroblasts (M50L3) that overproduces the enzyme and its mRNA by a factor of 300 but regulates the level of the enzyme during the cell cycle in the same manner as normal 3T6 cells. We found that, when resting (G_0) M50L3 cells were serum-stimulated to reenter the cell cycle, the 10-fold increase in the rate of synthesis of DHFR that occurs at the beginning of S phase was the result of a corresponding increase in DHFR mRNA content. In pulse-labeling experiments, we found that there was a similar increase in the rate of production of the mRNA just prior to S phase. However, the half-life of the mRNA was the same (7.5 hr) in resting and exponentially growing cells. Therefore, the increase in DHFR mRNA content was due to an increase in the rate of production rather than an increase in the stability of the message. The delay between addition of [^{3}H]-uridine to the culture medium and the emergence of DHFR mRNA from the nucleus was 15–20 min for both resting and growing M50L3 cells. A similar delay was observed for total mRNA. Therefore, the time required for the processing of newly synthesized DHFR heterogeneous nuclear RNA into DHFR mRNA is about the same as that for the average mRNA.**

Dihydrofolate reductase (DHFR; 5,6,7,8-tetrahydrofolate: NADP$^+$ oxidoreductase, EC 1.5.1.3) is the enzyme responsible for the NADPH-dependent reduction of folic acid and dihydrofolic acid to tetrahydrofolic acid. Derivatives of tetrahydrofolic acid participate in various single-carbon transfer reactions, including the reductive methylation of deoxyuridylic acid to form thymidylic acid (1).

Previous studies have shown (2, 3) that the amount and rate of accumulation of DHFR were much lower in cultured mouse 3T6 fibroblasts that were resting in medium containing 0.5% serum in the G_0 state of the cell cycle than in exponentially growing 3T6 cells. When the resting cells were serum-stimulated to reenter the cell cycle, the rate of accumulation of the enzyme increased 10- to 20-fold as the cells entered S phase, about 11 hr after stimulation. The increase was due to *de novo* synthesis of the enzyme and was not affected when DNA synthesis was blocked by various inihibitors. Studies with actinomycin D suggested that the increase in DHFR gene expression was controlled at the level of transcription.

To facilitate our studies of DHFR gene expression, we isolated a methotrexate-resistant 3T6 cell line (M50L3) that overproduces DHFR and its mRNA by a factor of about 300 (4). Because DHFR gene expression appears to be regulated in the same manner in the overproducing cell line as in normal 3T6 cells, the former is an excellent model system for studying the molecular mechanism(s) for controlling the expression of the gene for this enzyme. In particular, direct studies of the content and metabolism of the mRNA [and heterogeneous nuclear RNA (hnRNA)] for the enzyme would be greatly facilitated by using this cell line.

Other studies with overproducing cell lines have shown that the rate of synthesis of DHFR is proportional to DHFR mRNA content (refs. 12 and 13; unpublished data). However, these studies did not determine the mechanism by which DHFR mRNA content is regulated. In this paper we show that the increase in DHFR mRNA content that occurs during the resting-to-growing transition is due to an increase in the rate of production rather than an increase in the stability of the mRNA.

MATERIALS AND METHODS

Cell Culture. Cultures of mouse 3T6 cells (5) and of their methotrexate-resistant derivative M50L3 (4) were maintained on 100-mm plastic petri dishes in the Dulbecco–Vogt modification of Eagle's medium (GIBCO) containing 10% calf serum (Colorado Serum, Denver, CO). The medium for M50L3 cells was supplemented with 50 μM methotrexate. Cultures of exponentially growing cells were prepared by seeding dishes at low density in medium containing 10% serum, replacing the medium on the next day, and using the cells for an experiment on the second day after seeding. At this time the cells were less than 50% confluent. Cultures of resting cells were prepared by seeding 7×10^4 cells per cm^2 in medium containing 0.5% serum. The medium was replaced 2 and 4 days after seeding, and the cultures were used for an experiment 7 days after seeding. Resting cells were stimulated to reenter the cell cycle by replacing the medium with fresh medium containing 10% calf serum (6).

RNA Isolation. Cultures of cells were harvested and cytoplasmic extracts were prepared as described (6). RNA was purified from the cytoplasmic extracts by the phenol chloroform extraction procedure performed at room temperature (7). Poly(A)$^+$mRNA was isolated from total cytoplasmic RNA by chromatography on oligo(dT)-cellulose (Collaborative Research, T3) (6, 8).

Hybridization. The recombinant DNA plasmid pDHFR 21 (9) contained in *Escherichia coli* C-600 SR 1592 was kindly provided by Robert Schimke. This plasmid is a derivative of pBR322 that contains a 1500-base-pair insert of DNA complementary to mouse DHFR mRNA at the *Pst* I site. The insert represents the entire DHFR mRNA molecule except for about 100 nucleotides at the 5′ end. The plasmid-containing cells were grown in L broth supplemented with tetracycline (50 μg/ml) until they reached an A_{600} of 0.5. Then, chloramphenicol (200 μg/ml) was added and the plasmid was allowed to amplify for about 18 hr. The cells were collected and lysed, and a cleared

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Abbreviations: DHFR, dihydrofolate reductase; 2× NaCl/Cit, 0.3 M NaCl/0.03 M sodium citrate, pH 7.4; hnRNA, heterogeneous nuclear RNA.

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lysate was prepared essentially as described (10). The plasmid was purified from the cleared lysate by centrifugation in a cesium chloride/ethidium bromide gradient (10). Control pBR322 DNA was prepared in a similar manner. All work with recombinant DNA was conducted according to the December 1978 version of the National Institutes of Health guidelines for research involving recombinant DNA molecules.

Plasmid DNA (5 μg) was denatured in alkali and immobilized on a 13-mm nitrocellulose filter (Schleicher & Schuell, BA85) as described (11). Approximately 12% of the pDHFR21 DNA corresponds to DNA sequences able to hybridize to DHFR mRNA sequences (i.e., negative-strand cDNA). RNA to be hybridized was dissolved in 300 μl of 2× NaCl/Cit (0.3 M NaCl/0.03 M sodium citrate, pH 7.4) and added to a 14-mm siliconized glass vial. Unlabeled poly(A) was included in the hybridization solution at 500 μg/ml. The nitrocellulose filter was then added, and the solution was overlayered with mineral oil to prevent evaporation. The vials were capped and incubated at 65°C for 18 hr unless otherwise noted. After hybridization, the filters were washed with 2× NaCl/Cit for 5 min at room temperature, for 90 min at 65°C, and then for 5 min at room temperature. The filters were then incubated for 90 min at room temperature with 2× NaCl/Cit containing 20 μg of RNase A (Sigma) per ml and then washed several more times with 2× NaCl/Cit at room temperature. Finally, all filters were washed by suction filtration, dried, and assayed for in a toluene-based scintillation fluid. Control experiments with labeled plasmid DNA showed that there was no detectable loss of DNA from the filter during the hybridization and washing procedures.

RESULTS

Quantitation of DHFR mRNA Sequences. The minimal time required for complete hybridization of the DHFR mRNA sequences to the immobilized DHFR DNA was determined by incubating filters for various times with labeled cytoplasmic RNA from growing M50L3 cells. The amount of DHFR mRNA hybridized reached a plateau after 18 hr of incubation (Fig. 1). By plotting the $\log_2$ of DHFR mRNA not hybridized as a function of incubation time (Fig. 1 *Inset*), we found that the half-time for hybridization was about 3.5 hr. Thus, after 18 hr, only 3% of the DHFR sequences remained unhybridized. This was verified by adding a fresh filter to RNA that had previously been hybridized to DHFR DNA for 18 hr. About 1% of the radioactivity that hybridized to the first filter was bound to the second filter, indicating that hybridization was complete after 18 hr of incubation (data not shown).

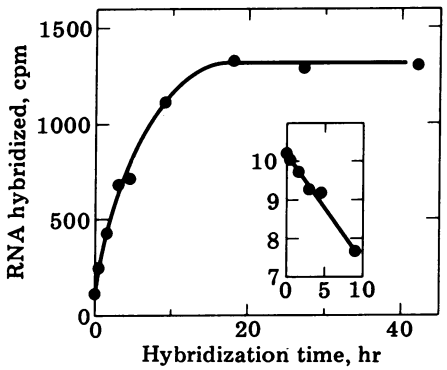

FIG. 1. Kinetics of hybridization. Growing M50L3 cells were labeled with $^{32}PO_4$ for 10 hr as described in Table 1. Total cytoplasmic RNA was prepared and hybridized for the indicated times with filters containing pDHFR 21 or PBR322 DNA. Each incubation mixture contained 10^6 cpm, and about 3 μg of total cytoplasmic RNA. After hybridization the filters were washed extensively, and the amount of labeled RNA bound to each filter was determined. Radioactivity associated with the pBR322 filters remained relatively constant at about 130 cpm and was subtracted from that bound to the pDHFR 21 filters. The results were plotted directly as a function of hybridization time. (*Inset*) Plot of the $\log_2$ of the difference between the maximal cpm hybridized (1300) and that hybridized at the indicated time.

Each filter should be capable of hybridizing about 0.6 μg of DHFR mRNA. Because less than 2% of cellular mRNA corresponds to DHFR mRNA, the capacity of the filters should be sufficient to hybridize the DHFR mRNA sequences from greater than 30 μg of total mRNA. In all of our experiments, the amount of total mRNA added to the hybridization solution was less than 3 μg, ensuring at least a 10-fold excess of DHFR DNA over DHFR mRNA.

Table 1 compares the level of DHFR mRNA sequences in resting and exponentially growing M50L3 cells and growing 3T6 cells. Because the cells had been labeled for at least 14 hr, the table gives a good approximation of the relative *content* of DHFR mRNA. We found that about 0.35% of poly(A)$^+$mRNA corresponds to DHFR mRNA in resting M50L3 cells. This percentage increases about 4-fold, to 1.25%, in growing M50L3 cells. Previous studies have shown that growing 3T6 cells contain about 3 times as much poly(A)$^+$mRNA as do resting 3T6 cells (6). If this were true for M50L3 cells as well, growing M50L3 cells would contain about 12 times as much poly(A)$^+$mRNA as do resting cells. Because the distribution of DHFR mRNA between the poly(A)$^+$- and poly(A)$^-$RNA fractions was the same in resting and growing cells, the content of *total* DHFR mRNA was also about 12-fold greater in growing than in resting cells. DHFR mRNA sequences were essentially undetectable in growing 3T6 cells. This is as expected because 3T6 cells contain about 0.3% of the DHFR (4) and DHFR mRNA (ref. 12; unpublished data) than do the overproducing cells.

The amount of radioactivity associated with control filters containing pBR322 DNA was extremely low. This demonstrates the effectiveness of the washing procedure and confirms that the radioactivity associated with the filters containing DHFR DNA sequences was due to the formation of specific DNA·RNA hybrids.

A previous study showed that about one-third of the translatable DHFR mRNA in mouse cells is unable to bind to poly(U)-Sepharose (13). On the basis of the amount of DHFR mRNA in the RNA fraction that did not bind oligo(dT)-cellulose, about 60% of total cytoplasmic DHFR mRNA was found in the poly(A)$^-$RNA fraction. These percentages were somewhat lower for pulse-labeled RNA (see below). We are not certain at present that the DHFR mRNA in the poly(A)$^-$ fraction completely lacks poly(A) or merely represents DHFR mRNA with poly(A) segments too short to bind to oligo(dT)-cellulose. Some of the DHFR RNA in the poly(A)$^-$ fraction may also represent untranslatable breakdown products of DHFR mRNA. Due to the higher input radioactivity (most of which is rRNA and tRNA), the amount of radioactivity bound to the pBR322 DNA filters was much greater when hybridizing poly(A)$^-$RNA. This led to a decrease in precision of the determination of DHFR mRNA level in the poly(A)$^-$RNA fraction compared to that for the poly(A)$^+$mRNA fraction.

DHFR mRNA Content in Serum-Stimulated M50L3 Cells. We examined DHFR mRNA content in serum-stimulated cells to determine if the content increased at about the same time the cells entered S phase, as expected from studies (ref. 4; unpublished data) on the rate of synthesis of the enzyme in stimulated cells. At their last change of medium, resting cells were fed with medium containing $^{32}PO_4$ at relatively low specific activity. After 3 days, the cells were serum-stimulated with medium containing $^{32}PO_4$ at the same specific activity. The

Table 1. Quantitation of DHFR mRNA

Cell status	RNA	Total, cpm × 10^{-6}	Input, cpm × 10^{-3}	pDHFR 21, cpm	pBR322, cpm	Δ, cpm	Δ, %	Total DHFR mRNA, cpm
				M50L3 cells				
Resting	Poly(A)$^+$	0.050	21.6	75	0	75	0.35	175 (37%)
	Poly(A)$^-$	2.93	863	105	17	88	0.0102	299 (63%)
Growing	Poly(A)$^+$	4.40	19.3	250	8	242	1.25	55,000 (40%)
	Poly(A)$^-$	156	1232	698	47	651	0.0528	82,400 (60%)
				3T6 cells				
Growing	Poly(A)$^+$	0.545	42.5	20	15	5	0.01	
	Poly(A)$^-$	22.1	1155	13	14	0	0	

Cultures of resting M50L3 cells were labeled in medium containing 10% of the normal amount of phosphate with 10 μCi of $^{32}PO_4$ (New England Nuclear) per ml for 3 days. Exponentially growing M50L3 cells or 3T6 cells were labeled for 14 or 18 hr, respectively, in phosphate-free medium containing 200 μCi of $^{32}PO_4$ per ml. A portion of the RNA ("input") was then incubated with filters containing pDHFR 21 DNA or pBR322 DNA and the amount of radioactivity bound to each filter was determined. The difference (Δ), representing labeled RNA hybridized specifically to the DHFR DNA, was determined and normalized to the amount of radioactive RNA in the hybridization mixture to give the percentage that was DHFR mRNA. This was multiplied by the total amount of labeled RNA to give the total amount of labeled DHFR mRNA in that RNA fraction.

3-day labeling period was adequate to label mRNA to equilibrium because the half-life of total mRNA was about 10 hr in resting (or growing) M50L3 cells (see below). At various times after stimulation, cultures were harvested and RNA was isolated. The amount of labeled DHFR mRNA (proportional to content of the mRNA) in the poly(A)$^+$- and poly(A)$^-$RNAs was then determined as in Table 1. DHFR mRNA content remained constant for at least 4 hr after stimulation and then increased 6- to 7-fold by the middle of S phase (16–20 hr after stimulation) (Fig. 2*a*). There were parallel increases in DHFR poly(A)$^+$- and poly(A)$^-$mRNA for at least 16 hr.

Total poly(A)$^+$mRNA content in serum-stimulated M50L3 cells increased in the same manner as in serum-stimulated 3T6 cells (6). Content increased linearly from the time of stimulation and doubled by about 14 hr (data not shown). The ratio of DHFR poly(A)$^+$mRNA content to total poly(A)$^+$mRNA content decreased slightly by 4 hr after stimulation (Fig. 2*b*), due to the fact that the increase in total mRNA preceded the increase in DHFR mRNA content. The ratio then increased at least 3-fold as the cells entered S phase. The maximal ratio in the experiment was somewhat lower than the ratio found in exponentially growing M50L3 cells. This may indicate that not all of the cells were stimulated to reenter the cell cycle.

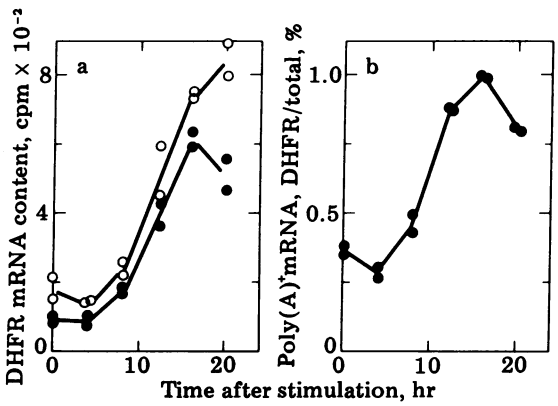

FIG. 2. Quantitation of DHFR mRNA content in stimulated cultures. (*a*) Cultures of resting M50L3 cells were labeled for 3 days with medium containing 10% of the normal amount of phosphate with 10 μCi of carrier-free $^{32}PO_4$ per ml. The cultures were serum-stimulated at time 0 with medium containing $^{32}PO_4$ at the same concentration and specific activity and were harvested at various times thereafter. Poly(A)$^+$mRNA (●) and poly(A)$^-$mRNA (○) fractions were isolated, and the amount of labeled DHFR mRNA in each fraction was determined as in Table 1. (*b*) The amount of DHFR poly(A)$^+$mRNA was normalized to the amount of total poly(A)$^+$-mRNA. Sucrose gradient analysis showed that the level of rRNA contamination of each preparation of poly(A)$^+$mRNA was 2–5%, which was ignored in our calculations.

Synthesis and Turnover of DHFR mRNA in Resting and Growing Cells. The increase in DHFR mRNA content could be the result of an increase in the rate of production of DHFR mRNA or of an increase in its stability. We determined the rate of labeling of DHFR poly(A)$^+$mRNA [relative to total poly(A)$^+$-mRNA] in resting and growing M50L3 cells. About 15 min after the addition of [^{3}H]uridine to the culture medium, labeled poly(A)$^+$mRNA began to emerge from the nucleus (Fig. 3). A similar lag has been observed for normal 3T6 cells (14). The slope of the curve for labeling of total mRNA decreased significantly over the 3-hr labeling period. We attribute this to the turnover of unstable species of mRNA that are labeled preferentially during a brief exposure to [^{3}H]uridine. The curvature also may be due in part to a reduction in the specific activity of the intracellular [^{3}H]UTP pool over the course of the labeling period. We found that DHFR [^{3}H]mRNA also began to emerge from the nuclei of growing cells about 15 min after addition of [^{3}H]uridine (Fig. 3*b*). The labeling of DHFR mRNA was linear for at least 3 hr, which is consistent with the idea that DHFR mRNA is relatively stable (see below). The emergence of labeled DHFR mRNA from the nuclei of resting cells (Fig. 3*a*) appeared to begin about 20–25 min after addition of [^{3}H]uridine, which may indicate a slightly lower rate of processing of the DHFR mRNA precursor in resting cells than in growing cells. Subsequent labeling of DHFR mRNA in resting cells was linear.

It took at least 3 hr (in other experiments, 4–6 hr) for the ratio of labeled DHFR poly(A)$^+$mRNA to total poly(A)$^+$mRNA (Fig. 3*c*) to reach a steady-state value that approximated the equilibrium ratios shown in Table 1. We attribute this delay to the fact that the specific activity of unstable mRNA is greater than that of the more stable mRNA species during a brief exposure to [^{3}H]uridine. This difference in specific activities diminished with longer labeling times. At each labeling time, the ratio was about 4 times greater in growing than in resting M50L3 cells. With the assumption that the rate of production of poly(A)$^+$mRNA is 2–3 times greater in growing M50L3 cells than in resting cells [as is the case in 3T6 cells (14)], the rate of synthesis of DHFR mRNA is about 8–12 times greater in growing than in resting M50L3 cells.

We compared the stability of DHFR mRNA in resting and growing cells. Cultures were labeled with [^{3}H]uridine for 4 hr

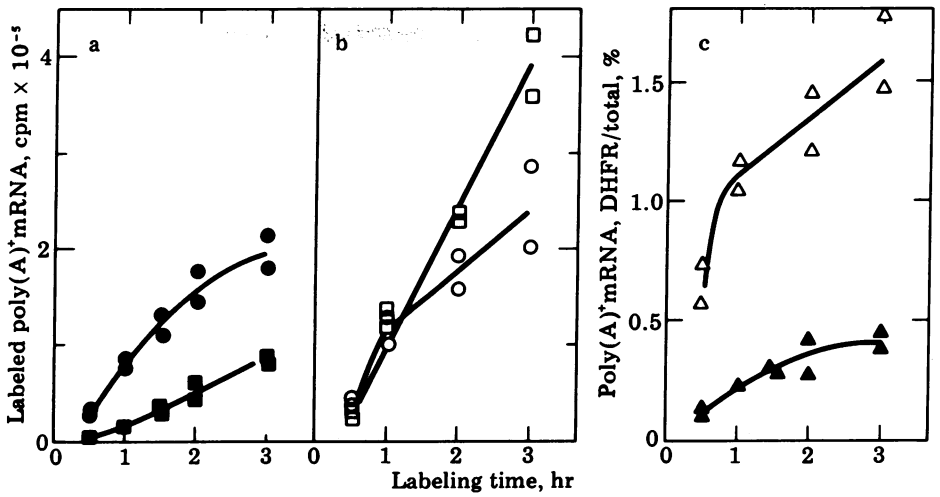

FIG. 3. Emergence of DHFR mRNA from the nucleus. Cultures of resting (*a*) or exponentially growing (*b*) M50L3 cells were labeled at time 0 with [^{3}H]uridine (New England Nuclear, 36.8 Ci/mmol) at 50 μCi/ml. At the indicated times, cultures were harvested and poly(A)$^+$-mRNA was isolated. The amount of radioactivity in total mRNA (●, ○) and in DHFR mRNA (■, □; values shown represent actual values multiplied by 100) was determined and plotted as a function of labeling time. (*c*) Ratio of DHFR poly(A)$^+$mRNA to total poly(A)$^+$mRNA was determined for resting (▲) and growing (△) cells and plotted as a function of labeling time.

and then chased with a vast excess of unlabeled uridine and cytidine as described (15). It is not possible to detect mRNA with an extremely short (1–2 hr) half-life in this type of experiment. The half-life of total poly(A)$^+$mRNA in resting or growing M50L3 cells was about 10.5 hr (Fig. 4), similar to the values obtained for total mRNA in resting or growing 3T6 cells (15). The half-life of DHFR poly(A)$^+$mRNA was about 7.5 hr in both resting and growing M50L3 cells. The half-life of DHFR poly(A)$^-$mRNA was also 7.5 hr for both resting and growing cells (data not shown).

If the increase in the cellular content of DHFR mRNA in growing cells were due entirely to an increase in stability, the half-life of DHFR mRNA would have to be less than 30 min in resting cells and 7.5 hr in growing cells to account for the 12-fold difference in DHFR mRNA content (16). Because we found that DHFR mRNA half-life was 7.5 hr in resting as well as growing cells, we conclude that the increase in DHFR mRNA content in growing cells must be due primarily, if not exclusively, to an increase in the rate of production of the mRNA.

We next studied the rate of labeling of DHFR mRNA in serum-stimulated cells. Cultures were labeled for 2-hr periods at various times after stimulation. The amounts of labeled DHFR mRNA in the poly(A)$^+$- and poly(A)$^-$RNA fractions were determined and normalized to the amount of labeled total poly(A)$^+$mRNA. The normalization procedure eliminated the need to correct for differences in the specific activity of RNA precursors which may result from different rates of transport of $^{32}PO_4$ at different times after stimulation. There was a 4-fold increase in the relative rate of synthesis of DHFR poly(A)$^+$-

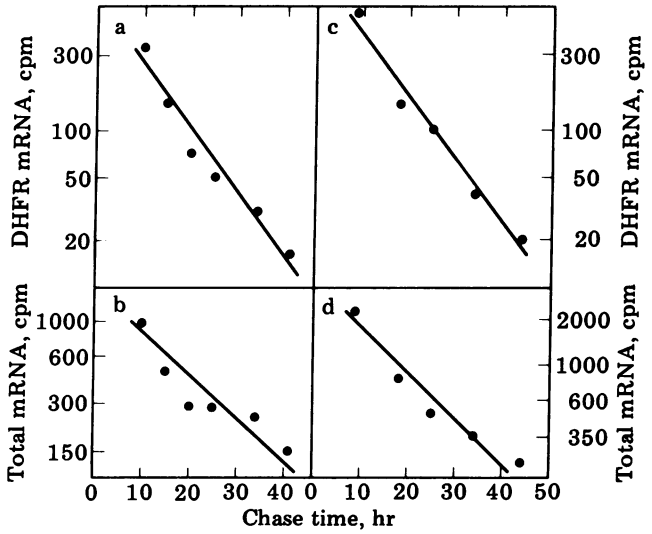

FIG. 4. Stability of DHFR mRNA. Cultures of resting (*c* and *d*) or exponentially growing (*a* and *b*) M50L3 cells were labeled for 4 hr with [^{3}H]uridine (36.8 Ci/mmol) at 50 μCi/ml. At time 0, the medium was replaced with medium containing 5 mM uridine and 2.5 mM cytidine and serum at the appropriate level. At the indicated times, cultures were harvested and the amounts of radioactivity in total poly(A)$^+$mRNA (*b* and *d*) and DHFR poly(A)$^+$mRNA (*a* and *c*) were determined. These values were plotted on a logarithmic scale as a function of chase time. [The values for total poly(A)$^+$mRNA represent 1/60th of the entire sample.] For each graph, a straight line was drawn to a reasonably good fit to the data. The half-lives described by these lines are: *a*, 7.5 hr; *b*, 10.5 hr; *c*, 7.5 hr; *d*, 10.5 hr. In some cases, the data may have been better described by more than one decay component. The density of the growing cells at the end of the chase period was about 65% of confluence.

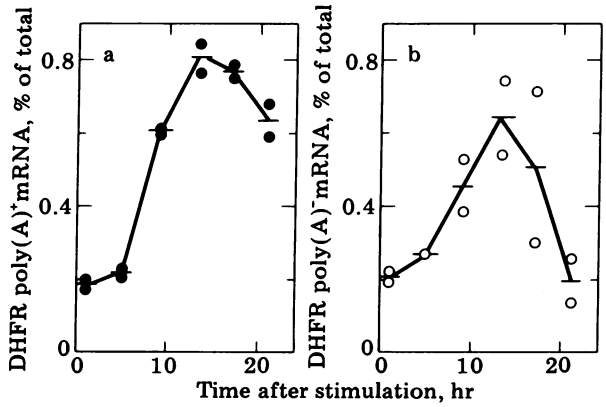

FIG. 5. Rate of production of DHFR mRNA after serum stimulation. Cultures of resting M50L3 cells were serum-stimulated at time 0. At various times, cultures were labeled for 120 min with $^{32}PO_4$ (200 μCi/ml) in phosphate-free medium containing 10% serum. After the labeling period, the cultures were harvested and poly(A)$^+$- and poly(A)$^-$RNAs were isolated. The amount of radioactivity in DHFR mRNA in each fraction was determined and normalized to the amount of radioactivity in total poly(A)$^+$mRNA to give the rate of synthesis of DHFR poly(A)$^+$mRNA (*a*) and DHFR poly(A)$^-$mRNA (*b*) relative to the rate of synthesis of total poly(A)$^+$mRNA. The horizontal lines represent the labeling intervals and are plotted at the average of the two determinations.

mRNA as the cells progressed from the resting state through S phase (Fig. 5). The relative rate of labeling reached a maximum between 12 and 18 hr after stimulation and then decreased. A similar pattern was observed for DHFR poly(A)$^-$-mRNA except that the decrease in the ratio at 20 hr was much more pronounced. The precision of the data for the DHFR poly(A)$^-$mRNA was lower than that for the DHFR poly(A)$^+$-mRNA due to the increased background correction (for nonspecific binding of RNA to the filters) that had to be made for the former.

DISCUSSION

Our results confirm several predictions we made previously that were based on the effects of actinomycin D on DHFR gene expression. In particular, we show that the increase in DHFR synthesis is controlled at the level of DHFR mRNA production, that the increase in mRNA production begins about 8 hr after stimulation, and that the mRNA does not have a short half-life.

Our studies were made possible by the availability of a methotrexate-resistant 3T6 cell line that overproduces both DHFR and its mRNA by a factor of about 300 and that appears to regulate DHFR gene expression in the same manner as normal 3T6 cells do (4). Because DHFR mRNA represents less than 0.01% of total cellular mRNA in normal 3T6 cells, it is extremely difficult to quantitate by DNA-excess filter hybridization (Table 1). However, we had no difficulty quantitating DHFR mRNA in the overproducing cells by this procedure. Our studies were also greatly facilitated by the availability of a recombinant DNA plasmid containing sequences corresponding to DHFR mRNA (9), which was used in our filter hybridization studies.

We show that the increase in the rate of synthesis of DHFR, which occurs at the beginning of S phase, is due to a corresponding increase in DHFR mRNA content. A similar correspondence between the rate DHFR synthesis and DHFR mRNA level has been observed by others (e.g., refs. 12, 13, and 17). We also show that the increase in DHFR mRNA content is due to an increase in the rate of production rather than to an increase in stability of the mRNA.

A number of studies have shown that a given mRNA molecule may be found in both the poly(A)$^+$- and poly(A)$^-$RNA fractions (e.g., ref. 18). We found that DHFR mRNA was distributed more or less evenly between these two fractions under all conditions examined in this study. Furthermore, the stability of DHFR poly(A)$^+$mRNA was the same as that of the message lacking poly(A). Further studies are required to determine if the DHFR mRNA sequences in the poly(A)$^-$RNA fraction completely lack poly(A) and if they are derived from DHFR poly(A)$^+$mRNA or are exported from the nucleus without the modification.

It has been reported that the DHFR gene is at least 42 kilobases long (19). If the initial transcription product of the gene (i.e., DHFR hnRNA) is the same length as the gene, as found for a number of hnRNA molecules (e.g., refs. 20 and 21), >95% of the original transcript must be removed to produce mature DHFR mRNA, which is 1.6 kilobase long (19). We observed that labeled DHFR mRNA began to appear in the cytoplasm about 20 min after the addition of [^{3}H]uridine to the culture medium. A similar lag was observed for total poly(A)$^+$mRNA. Therefore, the time required for processing of a newly synthesized DHFR mRNA precursor into cytoplasmic DHFR poly(A)$^+$mRNA is the same as that for the "average" mRNA molecule. It is not possible to determine from these experiments if splicing occurs as the DHFR hnRNA is being transcribed or after the entire precursor has been synthesized. It will be important to determine if full-length transcripts as well as processing intermediates can be detected in the nuclei.

We found that DHFR mRNA represented about 1.25% of total poly(A)$^+$mRNA in growing M50L3 cells. However, other studies have shown that the protein itself represents about 4% of the soluble protein in growing M50L3 cells (ref. 4; unpublished data). This may be explained by the fact that *soluble* protein represents less than one-third of *total* cell protein (22). Therefore, DHFR probably represents about 1.5% of *total* cellular protein in M50L3 cells. Furthermore, at least half of the DHFR mRNA sequences are found in the poly(A)$^-$RNA fraction; DHFR mRNA may represent a larger percentage of poly(A)$^-$mRNA than poly(A)$^+$mRNA.

Further studies will be required to determine how the production of DHFR mRNA is regulated during the cell cycle. The control could be exerted at the level of transcription or at the level of DHFR hnRNA processing and export to the cytoplasm. Analysis of the rate of labeling of nuclear DHFR sequences and the efficiency of export of these sequences to the cytoplasm will clarify this issue. Such studies should be feasible by using the overproducing cell line.

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