

Dihydrofolate Reductase Gene Expression in Cultured Mouse Cells Is Regulated by Transcript Stabilization in the Nucleus

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ABSTRACT Methotrexate-resistant cells, which contain a 500-fold amplification of dihydrofolate reductase (DHFR) genes, were used as a model system for studying the regulation of DHFR gene expression during growth stimulation. We have shown that a threefold increase in DHFR mRNA levels following growth stimulation results from a corresponding increase in DHFR mRNA production (i.e., delivery to the cytoplasm) and is not the result of a change in DHFR mRNA half-life. We previously showed that the increase in DHFR mRNA production during growth stimulation is not accompanied by an increase in the relative rate of transcription of the DHFR gene. This suggested that changes in DHFR mRNA production during growth stimulation are due to changes in the stability of DHFR transcripts in the nucleus. Using continuous labeling experiments *in vivo* comparing the stability of DHFR RNA with specific reference sequences, we show that in growing cells most DHFR transcripts were converted to mRNA, whereas in resting cells the majority of DHFR transcripts were rapidly degraded in the nucleus. There was no significant difference in the rate of processing and transport of stable DHFR transcripts. Therefore, changes in the stability of DHFR RNA in the nucleus control the amount of mRNA available for translation in the cytoplasm.

The production of a eucaryotic mRNA is a complex process involving transcriptional and posttranscriptional events. Control may be exerted at transcription initiation or at any of the other events necessary for maturation and delivery of the RNA to the cytoplasm (13, 29). Although a great deal is known about the synthesis and processing of viral transcripts (42), current understanding of cellular mRNA metabolism is based largely on information concerning the highly abundant mRNAs found in specific types of differentiated cells. The available evidence indicates that tissue-specific or qualitative regulation of gene expression is controlled primarily at the level of transcription initiation. Although prevalent mRNAs are important in defining the properties of specialized cells, they are unusual in several respects: they are not required for the maintenance of normal cellular activities, they are normally present in only a single type of cell, and they represent the transcription products of a very small fraction of the active cellular genes. Most of the active genes within a cell specify transcripts that are present at relatively low levels and code

for proteins required by all cells. The factors controlling the synthesis and turnover of ubiquitous transcripts may be markedly different from those controlling the metabolism of the highly abundant mRNAs present in only one or a few specific types of cells. Detailed investigations concerning the structure and metabolism of ubiquitous mRNAs have been severely limited owing to the small quantity of such transcripts. To circumvent this problem, we have used a methotrexate-resistant mouse cell line with a 500-fold amplification of dihydrofolate reductase (DHFR)¹ genes (2) to examine the metabolism of an ubiquitous mRNA normally expressed at levels too low to measure accurately.

DHFR is required for the *de novo* biosynthesis of thymidylate and purine nucleotides, and belongs to a group of enzymes often referred to as housekeeping enzymes, which are present in small quantities in all cells. Methotrexate-

¹Abbreviations used in this paper: DHFR, dihydrofolate reductase; poly(A), polyadenylic acid; TCA, trichloroacetic acid.

resistant cells have been used to show that DHFR protein synthesis is (a) cell cycle regulated, (b) stimulated by cell growth, (c) induced by lytic infection with polyoma or adenovirus, and (d) inhibited by cyclic AMP (17, 23, 26, 40). In each case DHFR protein synthesis is proportional to the concentration of DHFR mRNA as judged by translation in a reticulocyte lysate system or by hybridization to a DHFR cDNA probe (23, 26). Two features of methotrexate-resistant cells make them well suited as model systems for studying the control of DHFR mRNA metabolism. First, the large quantity of DHFR gene products makes it relatively easy to quantitate DHFR protein and mRNA levels using specific protein and nucleic acid probes. Second, available data indicate that most of the amplified DHFR genes are active and subject to control by the same physiological parameters controlling DHFR gene expression in normal cells (17, 21, 23, 25, 26, 38).

We have used DHFR gene amplification mutants as a model system for studying the effect of growth stimulation on DHFR gene expression. We found that growth stimulation results in a threefold increase in the relative rate of DHFR mRNA production. However, this increase in DHFR mRNA production is not accompanied by an increase in the relative rate of transcription of DHFR genes. These observations suggest that growth regulation of DHFR gene expression is controlled at the level of transcript stability within the nucleus. To test this possibility directly, we conducted a series of [³H]-uridine continuous labeling experiments *in vivo* in which the stability of DHFR primary transcripts (exon portions only) was compared with total RNA as well as with specific reference sequences. DHFR intron sequences were used as a reference for sequences that turn over rapidly in the nucleus. Furthermore, the comparison of DHFR exon with intron stability also served as a comparison of one part of the DHFR transcription unit with another. We also compared the stability of DHFR RNA with 18S rRNA, a stable reference sequence derived from RNA polymerase I transcription units. The results of these experiments indicated that in growing cells most DHFR primary transcripts were converted to mature mRNA, whereas in resting cells the majority of DHFR transcripts were rapidly degraded in the nucleus. In contrast to DHFR exon sequences, the stability of rRNA and DHFR intron sequences did not change as a result of growth stimulation. Thus growth regulation of DHFR gene expression is controlled at the level of transcript stability within the nucleus.

MATERIALS AND METHODS

Cell Culture: The S180-500R cell line used for these experiments is a mouse sarcoma cell line that was isolated by stepwise selection for the ability to grow in 500 μ M methotrexate (1, 19, 27). This cell line contains approximately 500 times more DHFR genes per diploid genome than the parent S180 cells. S180-500R cells were grown in Eagle's minimum essential medium containing Hank's balanced salt solution, 10% calf serum, and 500 μ M methotrexate.

We performed growth stimulation by trypsinizing confluent monolayers of cells and replating them in fresh medium at a lower density (1, 25). For most of the experiments, cells were replated at a density of 1:2. The density of replating does not significantly affect the level of induction of DHFR gene expression (R. E. Kellems, unpublished observations).

Isolation of Nuclei: Cells were disrupted with 1% Triton X-100-deoxycholate as previously described (27), and the nuclei were isolated by sedimentation through sucrose (28).

Labeling of RNA: *In vivo* labeling of RNA was done as previously described (27). For *in vitro* labeling experiments, isolated nuclei were incubated at 27°C in a solution containing 16% glycerol, 20 mM Tris HCl, pH 8.0, 5 mM MgCl₂, 150 mM KCl, 0.4 mM ATP, GTP, and CTP, and 100 μ Ci [α -³²P]UTP

in a final volume of 200 μ l. Incorporation of radioactivity was linear for >30 min. We quantitated *in vivo* as well as *in vitro* labeled RNA by spotting 5 or 10 μ l onto Whatman 540 filter paper circles (Whatman Laboratory Products Inc., Clifton, NJ), rinsing them several times with 10% trichloroacetic acid (TCA), once with 95% EtOH, and drying and counting them for radioactivity.

Isolation of RNA: Cytoplasmic RNA was prepared by phenol-chloroform extraction as previously described (27). Total RNA and nuclear RNA labeled *in vivo* were isolated by the guanidine HCl-cesium chloride method described by Glisin et al. (16).

For experiments using isolated nuclei, the nuclei labeled with [³²P]UTP were treated for 30 min with proteinase K (100 μ g/ml) in 1X SET (10 mM Tris, 5 mM EDTA and 1% SDS). After adding 25 μ g tRNA as carrier, we extracted the mixture two times with equal volumes of phenol-chloroform. Nucleic acids were then precipitated in 70% ethanol and redissolved in 1X SET. After two precipitations with 3.0 M sodium acetate, the RNA was washed with 10% cold TCA containing 30 mM Na₃P_O₇, and dissolved in 1X SET.

RNA was separated into polyadenylic acid containing [poly(A)+]RNA and poly(A)- RNA by oligo-d(T) cellulose chromatography (5). Incorporation of labeled nucleotides into RNA was measured by precipitation with 10% TCA onto Whatman filters. After several rinses with 10% TCA and one rinse with 95% ethanol, the filters were dried and counted.

Hybridization: DNA-excess filter hybridizations were performed as previously described (27). Hybridizations were normally done in triplicate with increasing amounts of input RNA so that after subtraction of background, 300–1,500 cpm were specifically hybridized. Association of labeled RNA with blank filters, used as background measurements, was normally 5–100 cpm. We thank Dr. R. T. Schimke (Stanford University) for providing the recombinant plasmid pDHFR21 which contains all but approximately 100 nucleotides of the cDNA to DHFR mRNA (8) and Dr. N. Arnhem (State University of New York at Stony Brook) for providing the ribosomal sequence rib 1.9 (4). pDRH23 is a plasmid with a 2.3-kilobase (kb) EcoRI/HindIII fragment from the third intron of the DHFR gene inserted into the EcoRI/HindIII sites of pBR327. The efficiency of hybridization was determined to be 34% by rehybridization of DHFR mRNA melted off of nitrocellulose filters after hybridization to pDHFR21.

Pool Specific Activity: Cells were labeled as described above. At various times monolayers were rinsed with PBS (8 g/l NaCl, 0.2 g/l KCl, 0.1 g/l CaCl₂, 0.1 g/l MgCl₂, 6 H₂O, 1.15 g/l Na₂HPO₄·H₂O, 0.2 g/l KH₂PO₄) and then 1.5 ml of 0.4 N HClO₄ was added. The mixture was scraped into a 15-ml corex tube and 0.75 ml of 1 N KOH was added (final pH 5.5–7.5). After vortexing the mixture was centrifuged in a JS-13 rotor at 3,000 rpm for 5 min. The supernatant was then lyophilized and dissolved in 200 μ l of 0.35 M K₃PO₄, pH 4.2. The samples were analyzed by high pressure liquid chromatography with a Zorbax-Sax column (E. I. DuPont de Nemours & Co., Newtown, CT) equilibrated with 350 mM K₃PO₄, pH 4.2. Nucleoside triphosphates were eluted isocratically with a flow rate of 1.0 ml/min. The UTP peak was collected and counted to determine its radioactive content. There was little or no radioactivity associated with the CTP peak. The mass of the peak was determined by comparison to UTP standards monitored at 262 nm.

DHFR mRNA Turnover: Resting and growing cells were labeled for 1 h as described above. The monolayers were then washed with medium containing 5 mM uridine and 2.5 mM cytidine and then refed with medium containing uridine and cytidine until harvested.

RESULTS

The Relative Rate of DHFR Transcriptional Activity Does Not Change during Growth Stimulation

We have previously shown by *in vivo* pulse-labeling with [³H]uridine that the relative rate of DHFR transcription does not change during growth stimulation (see Materials and Methods) (27). To confirm this unexpected observation we measured the incorporation of ³²P-labeled UTP into nascent transcripts in nuclei isolated from cells harvested at various times after growth stimulation. No new initiation of transcription occurs in nuclei isolated under these conditions and by limiting the amount of UTP available, each nascent transcript is elongated at most a few hundred nucleotides (12). Therefore the amount of radioactivity incorporated into a specific RNA sequence is proportional to the number of nascent transcripts present at the time the nuclei were isolated. Nuclei were

incubated in vitro for 30 min with [³²P]UTP, the RNA was extracted, and the incorporation of radioactivity into DHFR transcripts was determined by hybridization to a DHFR cDNA (pDHFR21). Data were expressed as a percentage (or ppm) of total radioactivity added to each hybridization reaction (Fig. 1A). The relative incorporation of ³²P into DHFR transcripts did not change significantly as a result of growth stimulation (Fig. 1A, solid circles). Shown for comparison is the level of DHFR message production during growth stimulation, as judged by [³H]uridine incorporation into cytoplasmic DHFR mRNA after a 1-h in vivo labeling. These measurements confirm our previous observations which indicated that the increase in DHFR mRNA production after growth stimulation is not accompanied by an increase in the relative transcription rate.

The relative incorporation of [³H]uridine into DHFR transcripts in vivo depends on the labeling time (reference 27; see also Fig. 5). To determine if this is also observed in vitro, we measured the incorporation of [³²P]UTP into DHFR transcripts of isolated nuclei labeled for various times. Nuclei from resting (before growth stimulation) and growing (24 h after growth stimulation) cells were incubated in the presence of [³²P]UTP for various times, and the incorporation of radioactivity into total RNA and DHFR RNA was measured. Incorporation of [³²P]UTP into total RNA increased linearly

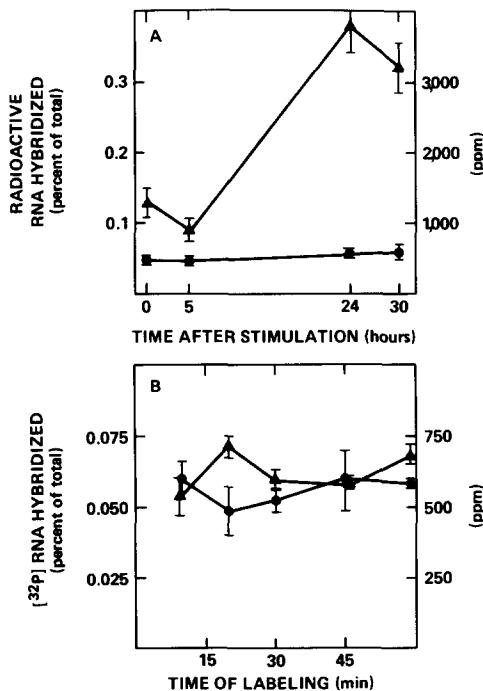


FIGURE 1 (A) In vitro measurement of relative transcription rates at various times after growth stimulation. Nuclei were isolated at various times after growth stimulation and labeled with [³²P]UTP for 30 min. RNA was isolated and hybridized to DHFR cDNA immobilized on nitrocellulose (●). Each point is an average of at least four experiments with three determinations per experiment. Shown for comparison is the effect of growth stimulation on DHFR mRNA production (▲). To measure DHFR mRNA production, cells were labeled for 1 h with [³H]uridine at various times after growth stimulation, and cytoplasmic RNA was isolated and hybridized to DHFR cDNA. (B) The effect of labeling time on the relative incorporation of [³²P]UTP into DHFR transcripts. Isolated nuclei from resting (●) and growing (▲) cells were labeled for various times with [³²P]UTP. RNA was isolated and hybridized to DHFR cDNA. Shown is the average of two experiments with three determinations each.

for at least 30 min (data not shown). However, the labeling time had no effect on the relative incorporation of [³²P]UTP into DHFR transcripts of nuclei prepared from resting or growing cells (Fig. 1B). These results indicate that posttranscriptional events affecting the relative level of DHFR transcripts in vivo did not occur in the isolated nuclei.

The Half-life of DHFR mRNA Is the Same in Resting and Growing Cells

To ascertain whether changes in stability are involved in regulating the cytoplasmic level of DHFR mRNA, we determined the half-life of DHFR mRNA in resting and growing cells. Cells were labeled for 1 h with [³H]uridine and then chased in the presence of excess unlabeled uridine and cytidine (see Materials and Methods). At various times cytoplasmic RNA was isolated and hybridized to pDHFR21. Fig. 2 shows the amount of radioactivity present in DHFR mRNA per flask of cells after various chase times. The half-life of DHFR mRNA was the same in resting and growing cells, ~6 h. However, any DHFR mRNA with a half-life that is short relative to the 1-h labeling time would not have been measured. The value of 6 h is very similar to the half-life of DHFR mRNA obtained by pulse-labeling methotrexate-resistant mouse 3T6 cells for much longer times (21).

DHFR Transcripts Are Less Stable in the Nuclei of Resting Cells Than in Those of Growing Cells

To examine the effect of growth stimulation on the stability of newly made DHFR transcripts, we compared the incorporation of [³H]uridine into DHFR sequences with other specific reference sequences. In previously reported data (27), as well as in that shown in Fig. 1, the incorporation of radioactive precursors into DHFR RNA was expressed relative to total or poly(A)+ RNA. For the experiments described below, we compared the incorporation of [³H]uridine into DHFR mRNA sequences with the incorporation into ribosomal 18S RNA and a 2.3-kb sequence corresponding to part of the third intervening sequence of the DHFR gene. These sequences served as references representing stable and unstable transcripts.

Fig. 3 shows a comparison of incorporation of [³H]uridine

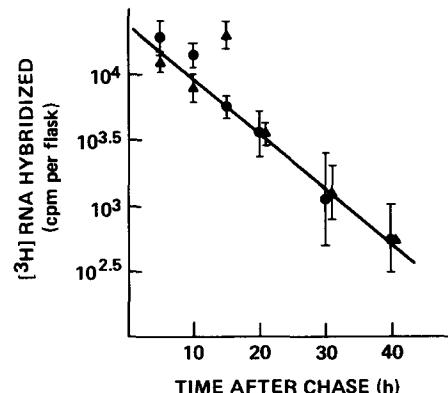


FIGURE 2 Half-life of DHFR mRNA in resting (●) and growing (▲) cells. Resting and growing cells were labeled with [³H]uridine for 1 h and then chased with cold uridine and cytidine. Cells were harvested after various chase times and the amount of radioactivity incorporated into DHFR mRNA was measured and plotted as a function of chase time. Each point is an average of three determinations.

into DHFR exon sequences with incorporation into 18S rRNA. Resting and growing cells were labeled for various times with [³H]uridine, total RNA was isolated, and incorporation into DHFR exons (Fig. 3A) as well as 18S rRNA (Fig. 3B) was determined by hybridization to the DHFR cDNA or to rib 1.9 (a probe containing sequences complementary to 18S rRNA). There was very little difference in the relative level of incorporation of [³H]uridine into 18S rRNA between resting and growing cells at each of the time points measured. The value at 5 min when corrected for the size of the transcription unit and efficiency of hybridization (34%, see Materials and Methods) gave a value of ~40% for the percentage of total transcriptional activity devoted to ribosomal RNA synthesis in resting and growing cells. Fig. 3C shows the ratio of radioactivity incorporated into DHFR mRNA relative to 18S rRNA sequences for various labeling times. In growing cells the ratio did not change with labeling time, indicating that DHFR transcripts were as stable as 18S rRNA transcripts over the times measured (90 min). In resting cells the ratio decreased with time, showing that in these cells DHFR transcripts were less stable than 18S rRNA transcripts.

To compare the stability of DHFR exon sequences with DHFR intervening sequences, we labeled cells for various times *in vivo* with [³H]uridine and isolated total RNA. The amount of radioactivity present in exon and intron sequences

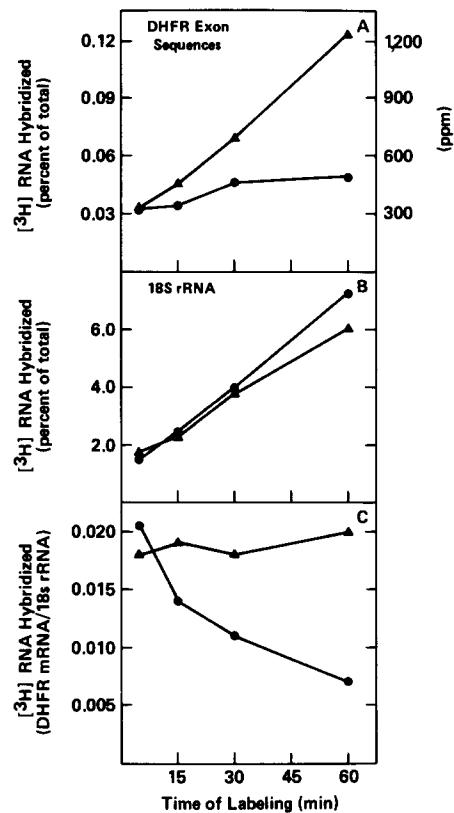


FIGURE 3 Comparison of [³H]uridine incorporation into DHFR exon sequences with 18S ribosomal RNA. Resting (●) and growing (▲) cells were labeled with [³H]uridine for various times and total cellular RNA was isolated. The percentage of [³H]uridine incorporated into DHFR exons (A) and 18S rRNA (B) was determined by hybridization to specific DNA probes immobilized on nitrocellulose filters. Each point is the average of four experiments with three determinations each. (C) The ratio of [³H]uridine incorporated into DHFR mRNA specific sequences versus 18S sequences was determined at various labeling times for resting (●) and growing (▲) cells.

was determined by hybridization to specific DNA probes. Fig. 4 shows the relative rate of incorporation of [³H]uridine into DHFR mRNA (Fig. 4A) and RNA corresponding to a 2.3-kb piece of DNA from the third intervening sequence of the DHFR gene (Fig. 4B). This intron sequence does not contain any detectable repetitive elements (11). Both resting and growing cells incorporated the same relative level of [³H]uridine into this intervening sequence for at least 90 min (Fig. 4B), indicating that this DHFR intron sequence had the same relative stability in resting and growing cells. Similar results were obtained using a 1.8-kb sequence from the fifth intron of the DHFR gene (data not shown). After adjustment for the size of the probes, the ratio of incorporation into exon versus intron sequences was determined (Fig. 4C). A ratio of 1.0 at short labeling times indicated that, as expected, introns were transcribed at the same relative rate as exons in resting and growing cells. With increased labeling times, the ratio of radioactivity incorporated into exons versus introns increased more rapidly in growing cells, indicating that exons were more stable relative to introns in growing cells than in resting cells.

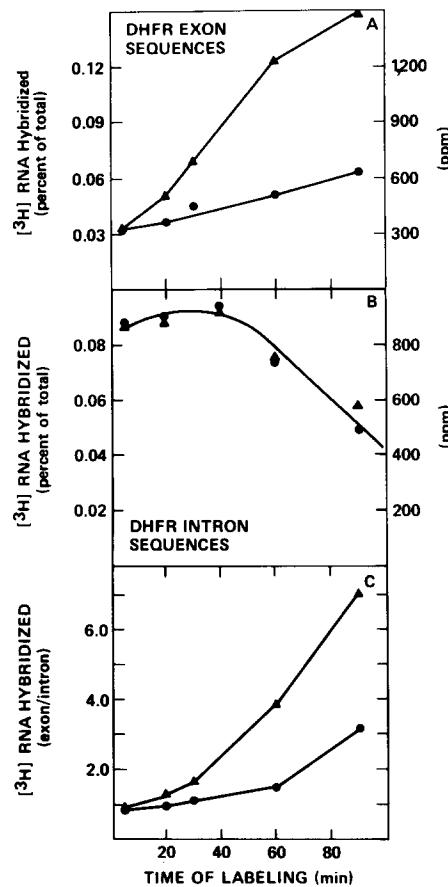


FIGURE 4 Comparison of [³H]uridine incorporation into DHFR exon sequences with DHFR intron sequences. Resting (●) and growing (▲) cells were labeled for various times with [³H]uridine and total cellular RNA was isolated. The percentage (or ppm) of [³H]uridine incorporated into DHFR exons (A) or an intervening sequence (B) was determined by hybridization to DNA immobilized on nitrocellulose filters. Each point represents the average of three experiments with three determinations for each experiment. (C) The ratio of radioactivity incorporated into DHFR mRNA specific sequences (exons) versus intron sequences was determined as a function of increased labeling times. The data are adjusted for the length of the DNA probes.

Stable DHFR RNA Is Transported to the Cytoplasm at the Same Rate in Resting and Growing Cells

In order to determine if processing and transport of stable DHFR transcripts occurred at the same rate in resting and growing cells, the distribution of labeled RNA between the nucleus and cytoplasm was determined as a function of increasing labeling times. Cells were labeled for various times with [³H]uridine, nuclear and cytoplasmic RNA were isolated, and the radioactivity incorporated into DHFR RNA was determined (Fig. 5). Since unstable DHFR transcripts are very short lived and are degraded rapidly during the isolation of nuclei (27), Fig. 5 mainly shows radioactivity incorporated into stable DHFR RNA. The distribution of labeled DHFR RNA between the nucleus and cytoplasm was similar in resting (Fig. 5B) and growing cells (Fig. 5A) for each labeling time, although the absolute amount of radioactivity incorporated was greater in growing cells. After ~1 h of continuous labeling, the radioactive DHFR RNA was equally distributed between the nucleus and cytoplasm in both resting and growing cells. By measuring the approach to steady-state of labeled DHFR RNA in the nucleus (30), we estimated the half-life for transport of DHFR RNA to the cytoplasm to be ~20 min in both resting and growing cells. This indicated that there is no difference in the processing and transport time of the stable DHFR transcripts as a result of growth stimulation.

Growing Cells Synthesize Twice as Much RNA as Resting Cells

For the experiments described above, as well as those

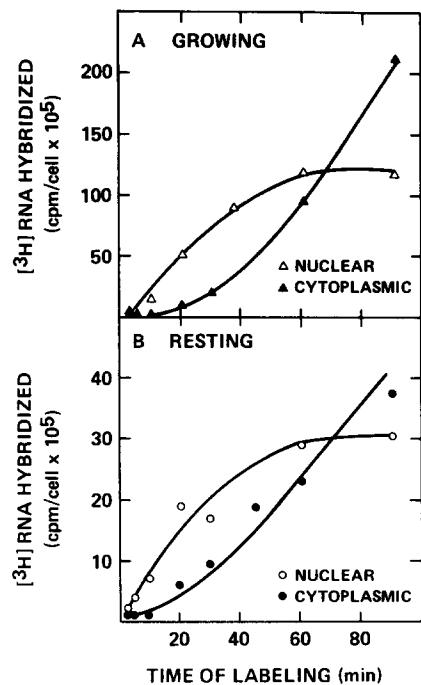


FIGURE 5. Accumulation of [³H]uridine into nuclear and cytoplasmic DHFR RNA. Growing (A) and resting (B) cells were labeled for various times. Cells were fractionated into nuclear and cytoplasmic compartments and RNA was extracted from each. [³H]Uridine incorporation into DHFR RNA was determined and expressed as cpm incorporated per cell. Shown is the average of three experiments with three determinations for each point in each experiment.

reported previously (27), the amount of radioactivity incorporated into DHFR RNA was expressed relative to either total RNA or specific reference sequences. To compare the actual amount of RNA synthesis in resting and growing cells, we measured the UTP pool specific activity at various times after labeling with [³H]uridine (see Materials and Methods). Fig. 6 shows that the UTP pool specific activity was the same in resting and growing cells throughout the labeling period. After the introduction of [³H]uridine, the UTP pool specific activity increased very rapidly, reached a maximum after ~15 min, and then declined slowly. Since the pool specific activities were the same in resting and growing cells, the amount of [³H]uridine incorporated into RNA served as a direct indication of the amount of RNA synthesized (Fig. 7B). RNA was isolated from cells labeled for various times, and the incorporation of radioactivity into total RNA as well as DHFR RNA was determined. Growing cells incorporated approximately twice as much [³H]uridine into total RNA as resting cells at all labeling times (Fig. 7C), indicating that growing cells synthesized twice as much RNA.

DHFR Genes Are Transcribed at a Minimum Frequency of Once Every 50 min

An estimate of the minimum required frequency of synthesis of DHFR transcripts can be made using the values obtained for the half-life of the mRNA and the relative level of DHFR mRNA. If we assume there are about 500,000 mRNAs in an average mouse cell (20, 41), and that in growing cells ~3% are DHFR messengers in S180-500 cells (27), then there are approximately 15,000 DHFR mRNAs per cell. With a half-life of 6 h, each cell must replace 7,500 DHFR messengers in 6 h or 1,250 per hour. Since these cells have approximately 1,000 DHFR genes, each gene must provide a minimum of one new mRNA every 50 min. Therefore, assuming a transcription rate of approximately 5 kb/min (13), most of the time a DHFR gene (35 kb) would be inactive, and rarely would more than one nascent transcript be present on a DHFR gene. For these calculations we made the assumption that in growing cells every DHFR transcript is utilized as an mRNA. Fig. 3C shows that the percentage of DHFR transcripts that form stable messenger RNAs is the same as the percentage of 18S transcripts that are converted to stable 18S rRNA. If we assume the 18S transcripts are completely conserved, then DHFR transcripts are also completely conserved

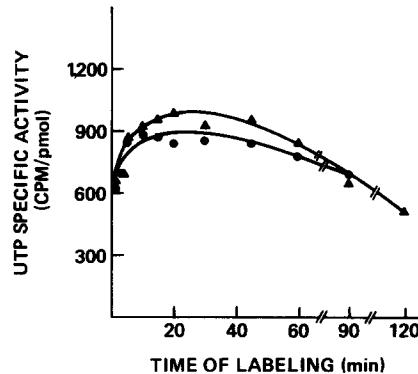


FIGURE 6. Specific activity of the UTP pool at various times after labeling with [³H]uridine. At various times after the initiation of labeling, resting (●) and growing (▲) cells were harvested and the acid-soluble fraction was assayed for the specific activity of the UTP pool. Each point is an average of from three to eight measurements.

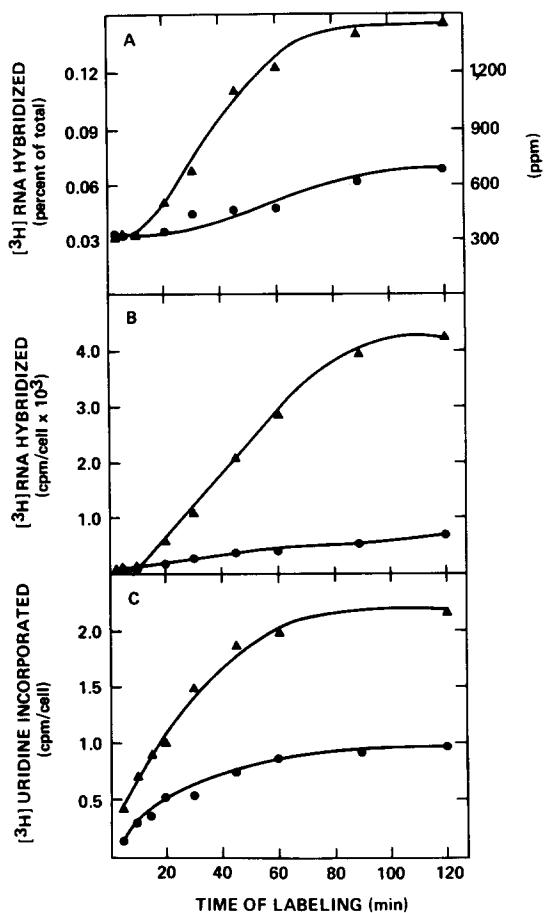


FIGURE 7 Effect of labeling time on the incorporation of [³H]-uridine into DHFR RNA in resting (●) and growing (▲) cells. Cells were labeled with [³H]uridine for various times and total cellular RNA was isolated. (A) The amount of radioactivity present in DHFR was determined and expressed as a percentage (or ppm) of the radioactivity in total RNA. (B) The amount of radioactivity incorporated into DHFR per cell at various labeling times. (C) The amount of total [³H]uridine incorporation per cell. Each point represents an average of four experiments with three determinations for each.

in growing cells.

It is also possible to estimate the fraction of RNA polymerase II activity devoted to transcription of the DHFR gene. By adjusting for the difference between the length of the cDNA probe (1,500 bases) and the DHFR gene (~35 kb) (11) and the efficiency of hybridization, 34% (data not shown), we determined that the DHFR genes account for ~2.5% of the transcriptional activity in the S180-500 cells. Because ribosomal transcription accounts for ~40% of transcriptional activity in these cells (Fig. 3) DHFR transcriptional activity would account for ~4.2% of the polymerase II activity. Since these cells have an approximate 500-fold increase in the number of DHFR genes, each DHFR gene should account for 0.008%, or 1/12,000 of the polymerase II activity. If there are 10,000–20,000 active genes in a cell (14), these data would indicate that the DHFR gene is transcribed at an average frequency.

DISCUSSION

One approach that has been commonly used to study messenger RNA metabolism is the use of blot hybridization techniques in which unlabeled RNA is fractionated by gel electro-

phoresis, transferred to filter paper, and detected by hybridization with specific radiolabeled nucleic acid probes (3). While this approach gives information concerning the size and steady-state level of the mRNA being studied, it does not provide direct information concerning the transcriptional activity of a gene. Nor can blot hybridization experiments usually be used to detect and study the fate of short-lived intermediates that are generated during the metabolism of mRNA. In vivo or in vitro labeling experiments are generally necessary to obtain this type of information. For example, two methods are currently used to measure the transcription rate of a gene. One method is to pulse-label cells in vivo with [³H]uridine for labeling times short enough to minimize posttranscriptional events (10). The other method is to elongate nascent transcripts in isolated nuclei in the presence of [³²P]UTP and cold nucleoside triphosphates (12, 22). With either method the labeled RNA is hybridized to an unlabeled probe and the hybridized radioactivity is expressed as a percentage (or ppm) of the total radioactivity incorporated. We have used both methods to examine the effect of growth stimulation on the transcriptional activity of DHFR genes. We have previously shown by in vivo pulse-labeling of RNA with [³H]uridine for <5 min that the relative increase in DHFR mRNA during growth stimulation is not due to an increase in the relative rate of transcription (27). In this report, we confirmed this result by measuring the incorporation of [³²P]UTP into nascent DHFR transcripts labeled in vitro using isolated nuclei.

Continuous labeling experiments *in vivo* comparing the stability of DHFR RNA with total RNA and with specific reference sequences indicated that DHFR transcripts were more stable in growing than in resting cells. DHFR intron sequences were used as a reference for sequences that turn over in the nucleus. The comparison of DHFR exon with intron stability was also a comparison of the stability of one part of the DHFR transcription unit with another. In addition, we compared the stability of DHFR RNA with 18S rRNA, a stable reference sequence which is not part of the DHFR transcription unit. The results of these experiments indicated that in growing cells, most DHFR transcripts were converted to mRNAs, whereas in resting cells most were rapidly degraded in the nucleus. Continuous labeling experiments indicated that stable transcripts took the same length of time to reach the cytoplasm in resting and growing cells. By measuring the approach to steady-state (30), we estimated that the half-life for transport of stable transcripts to the cytoplasm was ~20 min in resting and growing cells. An increase in the fraction of stable DHFR transcripts in growing cells resulted in an increase in the nuclear steady-state level of DHFR RNA. The amount of DHFR mRNA that entered the cytoplasm was proportional to the nuclear steady-state levels resulting in a threefold increase in DHFR mRNA production following growth stimulation. This increase in DHFR mRNA production fully accounted for the increase in protein synthesis since there was no change in the half-life of the DHFR mRNA during growth stimulation.

To compare the level of transcriptional activity between resting and growing cells, it is necessary to relate radioactivity incorporated into pulse labeled RNA to actual synthesis of RNA. To make this comparison, we determined the UTP pool specific activity throughout the labeling period. Since the pool specific activity of UTP at various times after labeling with [³H]uridine was the same in resting and growing cells,

the amount of radioactivity incorporated could be used directly as an indication of the amount of RNA produced. Continuous labeling experiments showed that growing cells synthesized twice as much RNA as resting cells. Thus, we observed a sixfold increase in the amount of DHFR mRNA produced as a result of growth stimulation. This was accounted for by a twofold increase in total cellular RNA synthesis and threefold increase in the relative stability of nuclear DHFR RNA.

The frequency at which new DHFR transcripts are initiated was estimated to be very low, even in growing cells, approximately one transcript per gene per hour. The actual frequency could be greater if not all transcripts are utilized as mRNA. However, in growing cells, the same fraction of DHFR and 18S rRNA transcripts were converted into mature stable RNAs. If all 18S transcripts are completely conserved, then all DHFR transcripts must also be conserved. Furthermore, radioactivity is incorporated into cytoplasmic DHFR mRNA at the same rate as incorporation into nascent DHFR transcripts during a pulse-labeling experiment, indicating that all DHFR transcripts are utilized for mRNA. This low frequency of transcription of the DHFR gene (one transcript per gene every 50 min) is contrasted with the high frequency of transcription of several tissue-specific genes. For example, two β -globin genes account for approximately the same amount of transcriptional activity in induced murine erythroleukemic cells (34) as 1,000 DHFR genes in S180-500 cells, indicating that each globin gene can generate 500 times the number of transcripts per hour than a DHFR gene (over 10 per min). Also electron micrographs have shown that certain genes can have many nascent transcripts associated with them at any given time (31). Although the frequency of DHFR transcription is very low compared with genes expressed at high levels in certain specialized cells, our calculations suggest that each DHFR gene is transcribed at an average frequency accounting for about 1/12,000 of total polymerase II transcriptional activity in a cell.

We have studied the effect of growth stimulation on DHFR gene expression in a cultured cell line that contains a 500-fold amplification of DHFR genes. When quiescent cells are growth stimulated by trypsinization and replating in fresh medium, numerous cellular enzymes involved directly or indirectly in DNA synthesis are induced. The level of DHFR (which is necessary for the production of purine and pyrimidine nucleotides) increases threefold during growth stimulation. This type of regulation, a modulation of gene expression in response to different physiological states of the cells, is different than the tissue-specific or "on or off" type of regulation that has been studied extensively in other systems (6, 7, 18, 32-37). Although tissue specific gene expression is transcriptionally controlled, DHFR gene expression represents the first example of posttranscriptional stabilization of nuclear transcripts being involved in the regulation of gene expression. Kaufmann and Sharp (24) have recently shown that the changes in the expression of mouse DHFR minigenes introduced into hamster cells is not associated with changes in the level of transcriptional activity. Their data suggest that sequences in the 3' noncoding region of DHFR mRNAs, possibly polyadenylation sites, may be involved in regulating DHFR mRNA production. Contrasting results have been reported by Wu et al. (39) who detected a change in DHFR transcriptional activity after serum stimulation. The use of different cell lines or experimental procedures may account

for this difference. Other examples of nontranscriptional regulation of gene expression have recently been reported. The decrease in the appearance of host mRNAs in the cytoplasm of adenovirus infected cells is not accompanied by a decrease in transcription of the host genes (reference 15 and S. S. Yoder and S. M. Berget, personal communication). And finally, changes in the production of tubulin mRNA due to changes in the pool of nonpolymerized tubulin subunits are not accompanied by changes in the transcriptional activity of tubulin genes (9). It is important to examine the expression of other housekeeping genes to see if regulation at the level of transcript stabilization in the nucleus is commonly observed.

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Note Added in Proof: M. Groudine and C. Casimir have recently provided another example of a housekeeping gene that is controlled posttranscriptionally. In a paper titled "Post-transcriptional regulation of the chicken thymidine kinase gene" (1984, *Nucleic Acids Res.* 12:1427-1446), they demonstrate that thymidine kinase mRNA levels in logarithmically growing cells are 5-10-fold higher than in confluent cells whereas transcriptional activity of the thymidine kinase gene is the same.

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