Control of Dihydrofolate Reductase Messenger Ribonucleic Acid Production

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We used methotrexate-resistant mouse cells in which dihydrofolate reductase levels are approximately 500 times normal to study the effect of growth stimulation on dihydrofolate reductase gene expression. As a result of growth stimulation, the relative rate of dihydrofolate reductase protein synthesis increased threefold, reaching a maximum between 25 and 30 h after stimulation. The relative rate of dihydrofolate reductase messenger ribonucleic acid production (i.e., the appearance of dihydrofolate reductase messenger ribonucleic acid in the cytoplasm) increased threefold after growth stimulation and was accompanied by a corresponding increase in the relative steady-state level of dihydrofolate reductase ribonucleic acid in the nucleus. However, the increase in the nuclear level of dihydrofolate reductase ribonucleic acid was not accompanied by a significant increase in the relative rate of transcription of the dihydrofolate reductase genes. These data indicated that the relative rate of appearance of dihydrofolate reductase messenger ribonucleic acid in the cytoplasm depends on the relative stability of the dihydrofolate reductase ribonucleic acid sequences in the nucleus and is not dependent on the relative rate of transcription of the dihydrofolate reductase genes.

Messenger ribonucleic acid (mRNA) production or the rate of appearance of mRNA in the cytoplasm may be controlled by transcriptional or post-transcriptional events within the nucleus. Our current understanding of the control of mRNA production in animal cells is based largely on information concerning viral mRNA's in lytically infected cells and the highly abundant tissue-specific mRNA's found in certain types of differentiated cells. The large number of viral genomes and their transcripts present during lytic infections has provided an excellent opportunity to study the control of viral gene expression. For example, studies of the adenovirus late transcription unit have shown the importance of RNA splicing, cleavage, and polyadenylation in defining the structures and 3' boundaries of the late mRNA families (6, 26, 35). The available data concerning the tissue-specific distribution of specialized mRNA's suggest that the occurrence of these mRNA's is controlled at the level of transcription initiation (4, 8, 10, 24, 28, 30) and at the level of mRNA stability (14, 24, 27). Because tissue-specific mRNA's are important primarily in defining the properties of differentiated cells, these mRNA's constitute a special class of gene products that are present in only one cell type, are not required for the maintenance of normal cellular activities, and represent the transcription products of only a minute fraction of the active cellular genes. Estimates of the number of different mRNA's present in animal cells range from 10,000 to 20,000 (9). Most of these mRNA's are present in relatively low concentrations and code for proteins required by all types of cells (e.g., housekeeping enzymes). The factors that control the expression of these ubiquitous transcripts may differ markedly from the factors that control the metabolism of tissue-specific mRNA's and viral mRNA's.

Investigations concerning the structures and metabolism of ubiquitous mRNA's have been limited due to the low levels of these transcripts found in normal cells. We used a line of methotrexate-resistant mouse cells in which the level of one ubiquitous mRNA (the mRNA coding for dihydrofolate reductase [DHFR]) is approximately 500 times greater than the level found in normal cells. The greater concentration of DHFR mRNA in these cells is associated with a corresponding greater number of DHFR genes (2). DHFR is an enzyme that is required for the de novo biosynthesis of thymidylate and purine nucleotides and is found in all cells. Methotrexate-resistant cells are well suited as model systems for studying the control of DHFR gene expression because the large quantities of DHFR

gene products make it possible to quantitate DHFR protein and mRNA levels by using specific protein and nucleic acid probes (17, 23). In previous studies workers have used methotrexate-resistant cells to show that DHFR synthesis is induced by growth stimulation or by polyoma infections and is inhibited during growth arrest or by adenosine 3',5'-monophosphate (3, 13, 23, 33). In each case DHFR synthesis is proportional to the concentration of DHFR mRNA sequences, as determined by translation in a reticulocyte lysate system or by the kinetics of hybridization to a specific complementary deoxyribonucleic acid (DNA) probe (22, 23). All of the amplified DHFR genes appear to be active and to be subject to control by the same physiological parameters that control DHFR gene expression in normal cells (12, 13, 20, 23, 33). The increase in the DHFR mRNA level after growth stimulation is accompanied by a corresponding increase in DHFR mRNA production (19). We examined the effect of growth stimulation on several other aspects of DHFR mRNA metabolism. We found that the threefold increase in the relative rate of DHFR mRNA production after growth stimulation was accounted for by a corresponding increase in the relative steady-state concentration of DHFR RNA sequences in the nucleus. However, the increase in the nuclear level of DHFR RNA was not accompanied by an increase in the relative rate of DHFR gene transcription. These data suggested that the appearance of DHFR mRNA in the cytoplasm depends on the relative stability of DHFR RNA sequences in the nucleus and is not proportional to the relative rate of transcription of DHFR genes.

MATERIALS AND METHODS

Cell culture. Mouse S180-500R cells were derived from the previously characterized methotrexate-resistant cell line AT-300 (3, 16) by stepwise selection for the ability to grow in 500 μM methotrexate. Compared with the parental sarcoma-180 cell line, S180-500R cells are 30,000 times more methotrexate resistant and contain approximately 500 times more DHFR. Sarcoma-180 cells and the methotrexate-resistant S180-500R cells were grown in Eagle minimal essential medium containing Hanks salt solution and either fetal calf or calf serum (10%, wt/vol; GIBCO Laboratories). The S180-500R cells were maintained in a medium containing 500 μM methotrexate.

Growth arrest of cells was achieved by plating cells at a density of 5×10^4 cells per cm^2 into plastic tissue culture flasks or Corning Glass roux bottles and waiting 7 days. Growth stimulation was achieved by brief trypsinization followed by a replating of the cells into fresh cell culture medium at a lower cell density.

Measurement of DHFR synthesis. The relative rate of DHFR synthesis was determined by using

direct immunoprecipitation of [^{3}H]leucine-labeled enzyme, as previously described (3). Incorporation into DHFR was expressed as a percentage of the trichloroacetic acid-precipitable protein in a high-speed supernatant fraction. From 2.5×10^7 to 5×10^7 cells were used for each experiment. Each value represents the average of at least three immunoprecipitations. Individual determinations varied from the average by no more than 25%.

[^{3}H]uridine labeling conditions. In vivo labeling of RNA was performed by incubating 5×10^7 cells with 600 μCi of [^{3}H]uridine (38 Ci/mmol; New England Nuclear Corp.) in 4 ml of Eagle minimal essential medium.

Preparation of RNA. Cytoplasmic RNA was prepared by phenol-chloroform extraction as previously described (22). Usually, nuclear RNA and total cellular RNA were prepared by the guanidine hydrochloride procedure described by Strohman et al. (32). In one experiment (see Fig. 7), nuclear RNA was prepared by the phenol-chloroform extraction procedure (22). RNA was separated into polyadenylic acid-containing [poly(A)$^+$] and poly(A)$^-$ fractions by oligodeoxythymidylic acid cellulose chromatography (4).

RNA-DNA hybridization. We are grateful to R. T. Schimke for providing *Escherichia coli* strain C-600 SR 1592, which contains the recombinant DNA plasmid pDHFR21, and to A. C. Chinault for providing a strain of *E. coli* containing plasmid pBR322. Plasmid pDHFR21 is a derivative of plasmid pBR322 which contains a 1,500-base pair insertion corresponding to all but approximately 100 nucleotides at the 5' end of the DHFR mRNA sequence (7). Both plasmids were isolated by standard procedures (21). Nitrocellulose filters (25 mm; type BA85; Schleicher & Schuell Co.) were loaded with 10-μg amounts of pDHFR21, pBR322, or *E. coli* DNA as described by Melli et al. (25). These filters were incubated with [^{3}H]uridine-labeled RNA at 45°C for at least 48 h in a reaction mixture containing 50% formamide, 20 mM tris(hydroxymethyl)aminomethane chloride (pH 7.7), 600 mM NaCl, 2 mM ethylenediaminetetraacetate, and 0.2% sodium dodecyl sulfate. After hybridization, the filters were washed four times in 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), incubated for 1 h at 37°C with 20 μg of ribonuclease A (Sigma Chemical Co.) per ml, washed several more times with 4× SSC, dried, and assayed for radioactivity. The amount of radioactivity bound to the pBR322 or *E. coli* filters (less than 0.005%) was considered nonspecific and was subtracted from the values for the pDHFR21 filters.

RESULTS

Measurement of [^{3}H]uridine-labeled DHFR mRNA sequences. (i) Hybridization of DHFR mRNA to pDHFR21. The assay used to measure DHFR mRNA sequences was based on specific hybridization to the recombinant DNA plasmid pDHFR21, a derivative of pBR322 which contains DNA corresponding to almost all of the DHFR mRNA sequence (7). Nitrocellulose filters containing 10-μg amounts

of either pDHFR21 or pBR322 were incubated with poly(A)⁺ cytoplasmic RNA from S180-500R cells that had been labeled for 1 h with [³H]uridine. The amount of radioactive RNA associated with pDHFR21 DNA (Fig. 1) was linearly related to the amount of RNA added over a wide range of input RNA and accounted for approximately 0.85% of the radioactive RNA added. The radioactivity associated with pBR322 filters (less than 0.005%) was considered nonspecific; in all experiments discussed below this value was subtracted from pDHFR21-associated radioactivity, which provided measurements that specifically reflected hybridization to the DHFR DNA sequence present in pDHFR21.

The length of time required for hybridization to reach a maximum value was determined by setting up a number of identical reactions, terminating the reactions after varying times, and determining the amount of radioactivity specifically associated with pDHFR21 DNA. We found that the reaction was more than 90% completed after 48 h of incubation and that at completion approximately 0.9% of the RNA was hybridized (data not shown).

(ii) Hybridization to pDHFR21 by using RNAs from parental and methotrexate-resistant cells. To demonstrate the specificity of the hybridization assay, parental and methotrexate-resistant sarcoma-180 cells were analyzed for the presence of [³H]uridine-labeled DHFR mRNA sequences (Fig. 2). The only known differences between the two cell lines are the 500-
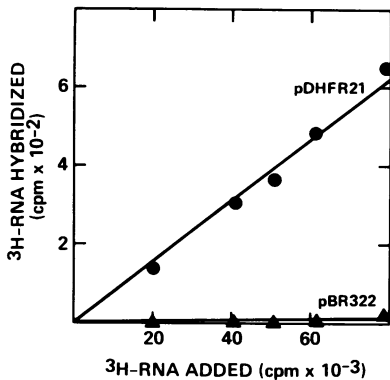

FIG. 1. *Hybridization of [³H]uridine-labeled RNA to pDHFR21 DNA. Cytoplasmic poly(A)⁺ RNA was prepared from S180-500R cells that had been labeled for 1 h with [³H]uridine. Different amounts of RNA were incubated with nitrocellulose filters containing 10-μg amounts of either pDHFR21 DNA or pBR322 DNA. The amounts of radioactivity associated with pDHFR21 filters (●) and pBR322 filters (▲) were determined and plotted as a function of the amount of [³H]RNA added.*
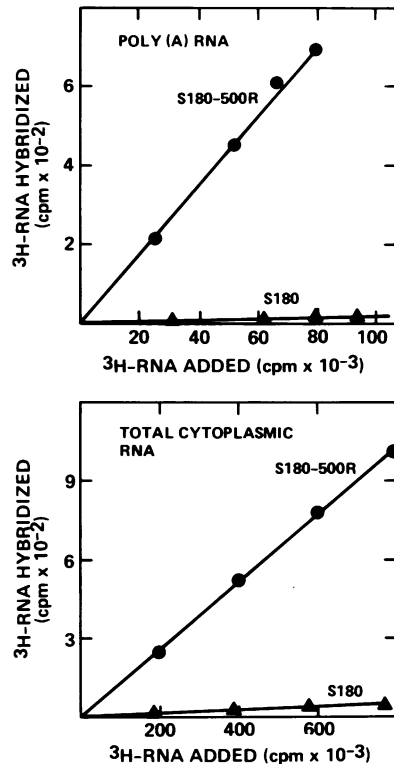

FIG. 2. *Hybridization of RNA from parental and methotrexate-resistant cells to pDHFR21 DNA. Poly(A)⁺ RNA and total cytoplasmic RNA were prepared from sarcoma-180 (S180; parental) and S180-500R (methotrexate-resistant) cells that had been labeled for 1 h with [³H]uridine. Specific hybridization to pDHFR21 DNA was measured and plotted as a function of the amount of radioactive RNA added. Symbols: ▲, sarcoma-180 cells; ●, S180-500R cells.*

fold greater concentrations of DHFR genes, mRNA, and protein in the drug-resistant cell line (2). The amount of hybridization to pDHFR21 was much greater with RNA from the methotrexate-resistant cells (S180–500R) than with RNA from the parent cells (sarcoma-180). The extent of specific hybridization observed with RNA from the sarcoma-180 cells was too low to make an accurate comparison with RNA from the S180-500R cells. Experiments with other overproducing cell lines and their parental lines gave similar results.

Effect of growth stimulation on DHFR RNA metabolism. (i) Relative rates of DHFR mRNA production in resting and growing cells. Previously, we showed that the relative concentration of DHFR RNA is three-fold greater in growing cells than in resting cells. To determine whether this was associated with a corresponding greater relative rate of DHFR

mRNA production, we performed the following experiment. Resting and growing cells were labeled for 1 h with [³H]uridine, cytoplasmic RNA was prepared, and the incorporation of radioactivity into DHFR RNA was measured by hybridization to pDHFR21. The amount of radioactive RNA hybridized was plotted as a function of the amount of radioactive RNA added (Fig. 3). The slopes of the resulting lines are proportional to the percentage of in vivo labeled RNA that consisted of DHFR RNA sequences. When either total cytoplasmic RNAs or poly(A)⁺ cytoplasmic RNAs from resting and growing cells were examined in this way, the relative incorporation of radioactivity into DHFR mRNA was approximately three times greater in growing cells (Fig. 3). Assuming that no significant degradation of DHFR RNA occurred during the 1-h labeling time, these values indicated that the relative rate of DHFR mRNA production (i.e., the appearance of DHFR mRNA in the cytoplasm) was approximately three times greater in

growing cells than in resting cells. This rate was associated with a twofold increase in total [³H]uridine incorporation per cell in growing cells (Fig. 4 and Table 1). We do not know whether this represented an actual increase in RNA synthesis in growing cells or resulted from a difference in [³H]uridine uptake or pool-specific activity.

More than 95% of the radioactive DHFR RNA that entered the cytoplasm during the 1-h [³H]uridine labeling period was poly(A)⁺ RNA. This was shown by labeling cells for 1 h with [³H]uridine, fractionating the total cytoplasmic RNA into poly(A)⁺ and poly(A)⁻ fractions by oligodeoxythymidylic acid cellulose chromatography, and assaying each fraction for the presence of radioactive DHFR RNA (Fig. 5).

(ii) Effect of growth stimulation on the relative rates of DHFR mRNA production and DHFR protein synthesis. Cell growth was stimulated by replating confluent cells at a lower density in fresh medium. At varying times after growth stimulation, cells were labeled for 1 h with either [³H]uridine or [³H]leucine. Poly(A)⁺ cytoplasmic RNA was prepared from [³H]uridine-labeled cells, and incorporation into DHFR RNA was determined by hybridization to pDHFR21. The values obtained were expressed as percentages of the amount of radioactive RNA added to the hybridization assay
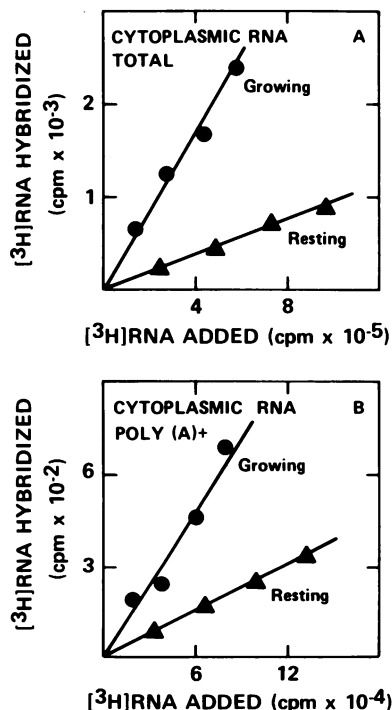

FIG. 3. *Relative rates of DHFR mRNA production in resting cells (▲) and growing cells (●). Cytoplasmic RNAs were prepared from resting and growing cells that had been labeled for 1 h with [³H]uridine. A portion of each cytoplasmic RNA was further fractionated into poly(A)⁺ RNA. Specific hybridization of total cytoplasmic RNA (A) and poly(A)⁺ cytoplasmic RNA (B) to pDHFR21 was measured and plotted as a function of the amount of radioactive RNA added.*
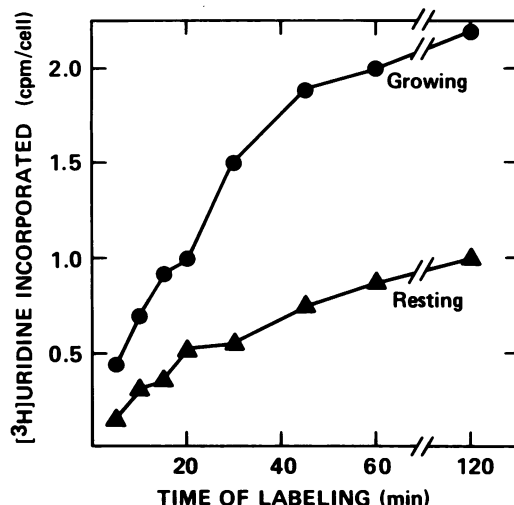
FIG. 4. *Incorporation of [³H]uridine into resting cells (▲) and growing cells (●). Cells (10⁷ cells per determination) were labeled for increasing lengths of time with [³H]uridine, the total incorporation of radioactivity into trichloroacetic acid-precipitable material per cell was measured, and the values were plotted as a function of labeling time. The values represent averages of two independent experiments, each based on three determinations.*

TABLE 1. *Distribution of ^3H-labeled DHFR RNA in nuclear and cytoplasmic RNAs in resting and growing cells* [a]

Type of cells	Amt of [^3H]RNA (cpm/10^6 cells)			Amt of ^3H-labeled DHFR RNA (cpm/10^6 cells)			Amt of ^3H-labeled DHFR RNA (%)		
	Total	Nuclear	Cytoplasmic	Total	Nuclear	Cytoplasmic	Total	Nuclear	Cytoplasmic
Resting	1.1×10^6	9.1×10^5	1.9×10^5	530	260	270	0.048	0.029	0.142
Growing	1.7×10^6	1.4×10^6	2.7×10^5	2,070	1,030	1,040	0.122	0.074	0.385

[a] Resting and growing cells were labeled for 1 h with [^3H]uridine (600 μCi/4 ml containing 5×10^7 cells), and either total, nuclear, or cytoplasmic RNA was prepared. The incorporation of radioactivity into DHFR RNA was measured by hybridization to pDHFR21 and is expressed as a percentage of the trichloroacetic acid-precipitable radioactivity present in each RNA preparation.
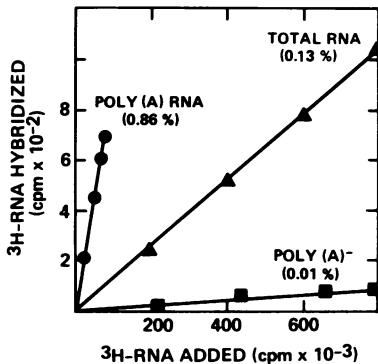

FIG. 5. *DHFR RNA fractionates with poly(A)+ RNA. Cytoplasmic RNA was prepared from exponentially growing S180-500R cells that had been labeled for 1 h with [^3H]uridine. The RNA was fractionated into a poly(A)+ fraction and a poly(A)− fraction by oligodeoxythymidylic acid cellulose chromatography and was assayed for the presence of DHFR RNA sequences by hybridization to pDHFR21. Specific hybridization was plotted as a function of the amount of radioactive RNA added. The values in parentheses indicate the precentages of radioactivity hybridized. Symbols: ▲, total cytoplasmic RNA; ●, poly(A)+ RNA; ■, poly(A)− RNA.*
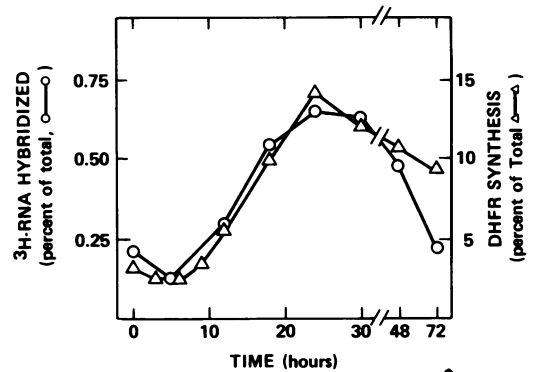

FIG. 6. *Effects of growth stimulation on DHFR protein synthesis and mRNA production. At different times after growth stimulation, S180-500R cells were labeled for 1 h with either [^3H]uridine or [^3H]leucine. Cytoplasmic poly(A)+ RNA was prepared from the [^3H]uridine-labeled cells, and incorporation into DHFR mRNA was measured by hybridization to pDHFR21. The relative rate of DHFR protein synthesis was determined by direct immunoprecipitation of the enzyme from extracts of [^3H]leucine-labeled cells. Each value represents the mean of at least three determinations. Symbols: △, DHFR protein synthesis; ○ [^3H]RNA hybridized.*

and were plotted as a function of time after growth stimulation (Fig. 6). After an initial decline, the relative rate of incorporation of radioactivity into DHFR mRNA (i.e., the relative rate of DHFR mRNA production) increased to a maximum at approximately 25 h after stimulation. The relative rates of DHFR protein synthesis were determined by direct immunoprecipitation of the enzyme from extracts of [^3H]leucine-labeled cells and were plotted as a function of time after growth stimulation. After an initial decrease during the first 4 to 6 h, the relative rate of DHFR protein synthesis increased from 4% to a maximum of approximately 15% of the soluble protein at 25 h. Thus, the threefold increase in the relative rate of DHFR mRNA production discussed above was proportional to the increase in the relative rate of DHFR protein synthesis (Fig. 6).

(iii) Incorporation of [^3H]uridine into nuclear DHFR RNA. The incorporation of [^3H]uridine into the nuclear RNAs of resting and growing cells was measured by labeling cells for 1 h, preparing the nuclear RNA, and quantitating the radioactive DHFR RNA by hybridization to pDHFR21. The amount of radioactive RNA hybridized was plotted as a function of the amount of radioactive RNA added (Fig. 7), and the results indicated that there was a threefold greater relative incorporation of radioactivity into nuclear DHFR RNA from growing cells. It is very likely that the relative amount of radioactive DHFR RNA present in the nuclear RNA at the end of 1 h of [^3H]uridine labeling represented a steady-state balance among synthesis, turnover, and transport from the nucleus. To test this possibility, resting and growing cells
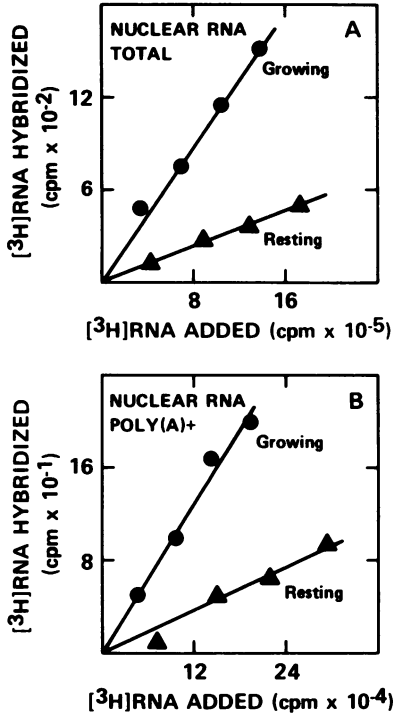
FIG. 7. *Incorporation of [³H]uridine into the nuclear RNAs of resting cells (▲) and growing cells (●). Nuclear RNAs were prepared from resting and growing cells that had been labeled for 1 h with [³H]uridine. A portion of each nuclear RNA was further fractionated into poly(A)⁺ RNA. Specific hybridization of total nuclear RNA (A) and poly(A)⁺ nuclear RNA (B) to pDHFR21 was measured and plotted as a function of the amount of radioactivity added.*

were labeled continuously with [³H]uridine for varying times (5, 10, 20, and 60 min), nuclear RNA was prepared, and the incorporation of radioactivity into DHFR RNA was measured (Fig. 8). In resting cells the percentage of incorporated radioactivity present in the nuclear DHFR RNA did not depend on the labeling time, indicating that steady-state labeling was achieved within 5 min. In growing cells the relative incorporation into nuclear DHFR RNA reached half-maximal values within 10 min and by 60 min approached a steady-state level that was approximately fourfold greater than the level found in resting cells.

We compared the relative rate of DHFR mRNA production with the relative steady-state level of DHFR RNA in the nucleus at varying times after growth stimulation by labeling cells for 1 h with [³H]uridine and measuring the incorporation of radioactivity into nuclear and cytoplasmic DHFR RNAs. The resulting values

(Fig. 9) were expressed as percentages of the radioactivity present in total nuclear and cytoplasmic RNAs, and these values provided measures of the relative steady-state level of DHFR RNA in the nucleus and the relative rate of DHFR mRNA production (i.e., appearance in the cytoplasm), respectively. At 5 h after growth stimulation, the relative levels of incorporation of [³H]uridine into nuclear and cytoplasmic DHFR RNAs during 1 h of labeling were slightly less than the levels observed in confluent cells. By 24 h incorporation into both nuclear and cytoplasmic DHFR RNA sequences reached a maximum, which was approximately three times greater than the incorporation observed in resting cells. Thus, at varying times after growth stimulation, the relative rate of DHFR mRNA production was proportional to the relative steady-state level of DHFR RNA in the nucleus (Fig. 9 and Table 1).

(iv) Relative rates of transcription of DHFR genes. With progressively shorter labeling times the incorporation of [³H]uridine into nuclear DHFR RNA appeared to approach the same value in resting and growing cells (Fig. 8), suggesting that the relative transcription rate of DHFR genes was the same in each case. To
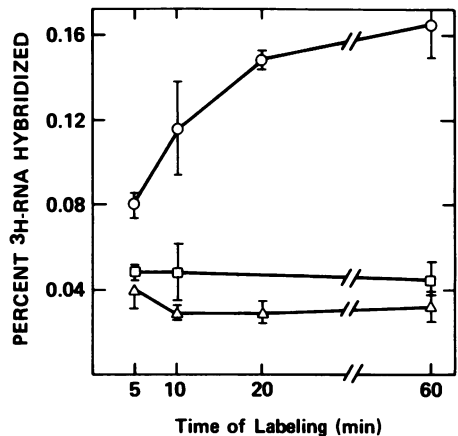

FIG. 8. *Effect of labeling time on the incorporation of [³H]uridine into the nuclear DHFR RNAs of resting and growing cells. Resting cells and cells harvested at 5 and 24 h after growth stimulation were labeled with [³H]uridine for the times indicated, and nuclear RNAs were prepared by the phenol-chloroform extraction procedure. The amount of radioactivity present in each DHFR RNA was determined by hybridization to pDHFR21 and was expressed as a percentage of the amount of radioactivity present in total nuclear RNA. Each value represents the mean and standard deviation of three determinations from a single experiment. Symbols: □, resting cells; △, cells examined 5 h after growth stimulation; ○, cells examined 24 h after growth stimulation.*
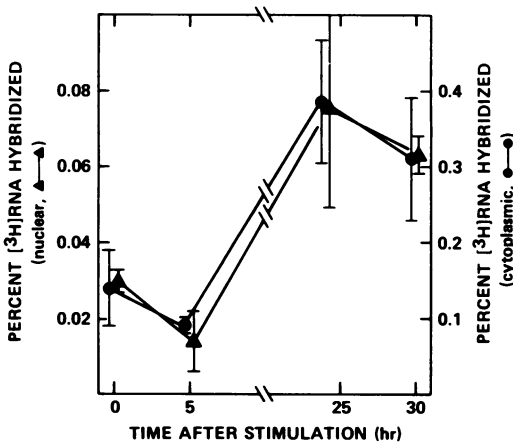
FIG. 9. *Effects of growth stimulation on the relative steady-state level of nuclear DHFR RNA and the relative rate of DHFR mRNA production. Cells were labeled for 1 h with [³H]uridine at different times after growth stimulation, nuclear and cytoplasmic RNAs were prepared, and the incorporation into DHFR RNA was measured by hybridization to pDHFR21. Incorporation into nuclear and cytoplasmic DHFR RNAs is plotted as a percentage of [³H]uridine incorporation into total nuclear and cytoplasmic RNAs. Each point represents the mean and standard deviation of four independent experiments, and the values from each independent experiment were based on at least three measurements. Symbols: ▲, nuclear RNA; ●, cytoplasmic RNA.*

estimate the in vivo transcriptional activity, we prepared total RNAs from resting and growing cells that were pulse-labeled for 1, 2, 3, and 5 min with [³H]uridine. The use of total RNA rather than nuclear RNA should have provided more accurate measurements of transcriptional activity by minimizing the effects of any post-transcriptional events that occurred during the isolation of nuclei. The incorporation of radioactivity into DHFR RNA was measured by hybridization to pDHFR21 and was expressed as a percentage of incorporation into total RNA (Fig. 10). There was no significant difference between the relative rates of incorporation of [³H]uridine into the DHFR RNAs of resting and growing cells for labeling times of 5 min or less. Assuming that no appreciable degradation of DHFR pre-mRNA sequences occurred during such a short labeling time, these results suggested that there was no significant difference in the relative rate of DHFR gene transcription between resting and growing cells.

We also determined the relative rates of synthesis of DHFR RNA at varying times after growth stimulation by labeling cells for 5 min with [³H]uridine, extracting total RNA, measuring the incorporation of radioactivity into

DHFR RNA, and expressing the resulting values as percentages of total incorporation. Our results indicated that no significant increase in DHFR RNA synthesis occurred as a result of growth stimulation (Fig. 11). The same fraction of total radioactivity was associated with poly(A)⁺ RNA at each time point (approximately 35%), indicating no substantial change in the fraction of poly(A)⁻ RNA synthesized (e.g., rRNA, small nuclear RNA, histone RNA). For comparison, Fig. 11 shows the effect of growth stimulation on the relative rate of DHFR mRNA production. Thus, the increase in the relative rate of DHFR mRNA production after growth stimulation was not accompanied by a significant increase in the relative rate of transcription of the DHFR genes.

DISCUSSION

Growth stimulation is a convenient experimental procedure which we have used to study the control of DHFR gene expression. We have shown previously that growth stimulation is accompanied by the entry of quiescent cells into the S-phase and a threefold increase in the relative rate of DHFR protein synthesis. In this work we studied the effect of growth stimulation on various parameters of DHFR RNA metabo-
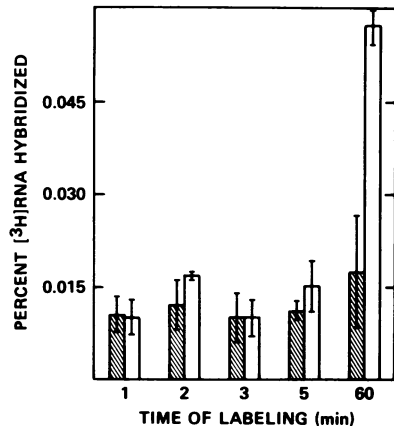

FIG. 10. *Relative rates of transcription of DHFR genes in resting cells (cross-hatched bars) and growing cells (open bars). Resting and growing cells were labeled with [³H]uridine for 1, 2, 3, and 5 min, total RNAs were prepared, and the incorporation into DHFR RNA was measured by hybridization to pDHFR21. For comparison, some resting and growing cells were labeled for 60 min, nuclear RNA was prepared, and the incorporation into DHFR RNA was measured by hybridization to pDHFR21. Incorporation into DHFR RNA is plotted as a percentage of [³H]uridine incorporation into total or nuclear RNA. The information shown represents the means and standard deviations of three independent experiments, each based on multiple measurements.*
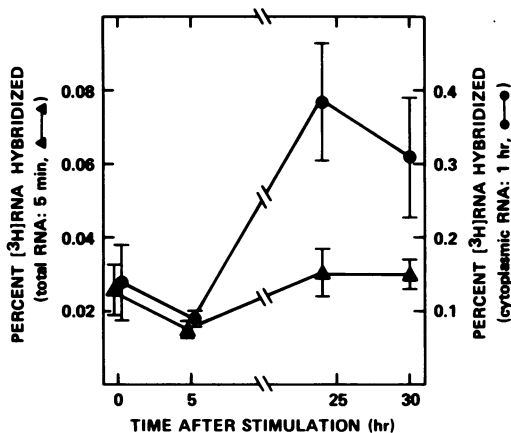
FIG. 11. *Effect of growth stimulation on the relative rate of transcription of DHFR genes. Cells were labeled with [³H]uridine for 5 min at different times after growth stimulation, total RNA was prepared, and the incorporation of radioactivity into DHFR was measured by hybridization to pDHFR21. Incorporation into DHFR RNA was plotted as a percentage of total [³H]uridine incorporation. Shown for comparison is the effect of growth stimulation on DHFR mRNA production (see Fig. 9). Each point represents the mean and standard deviation of four independent experiments, and the values from each independent experiment were based on multiple measurements. Symbols: ▲, relative rate of DHFR gene transcription (i.e., total RNA, 5 min of labeling); ●, relative rate of DHFR mRNA production (cytoplasmic RNA, 1 h of labeling).*

lism, including the relative rate of DHFR mRNA production, the relative level of DHFR RNA in the nucleus, and the relative rate of transcription of DHFR genes. For each type of measurement, in vivo [³H]uridine-labeled DHFR RNA was measured by hybridization to plasmid pDHFR21, a derivative of pBR322 which contains a 1,500-base-pair insertion corresponding to most of the DHFR mRNA sequence (7). Specific hybridization to the DHFR DNA insertion present in pDHFR21 was due to a poly(A)⁺ RNA which was present at much higher levels in methotrexate-resistant cells than in the parent cell lines, indicating that hybridization was specific for DHFR RNA. Since the probe consisted of DNA complementary to DHFR mRNA and because the filters were treated with ribonuclease before the amount of radioactivity hybridized was measured, the assay detected only those sequences which were present in mature DHFR mRNA. The linear relationship between the amount of RNA hybridized and the amount added to the reaction mixture showed that the reactions contained an excess of DNA and that all DHFR mRNA or a constant percentage of

DHFR mRNA was hybridized over a wide range of input RNA. We found that there was two times more [³H]uridine incorporation into growing cells than into resting cells, but do not know at this time whether this represented an actual increase in RNA synthesis or resulted from changes in [³H]uridine uptake or pool specific activity. Therefore, in this study the values for incorporation of [³H]uridine into DHFR RNA were expressed as relative values (as percentages) rather than as absolute values, eliminating any effect of variation in uridine pool sizes or differences in the specific activities of in vivo labeled RNAs. This allowed us to compare the relative incorporation rates of [³H]uridine into DHFR mRNA sequences accurately in different RNA preparations and under different physiological conditions.

We examined the effects of growth stimulation on DHFR mRNA production (the relative rate of appearance of DHFR mRNA in the cytoplasm) and the relative steady-state level of DHFR RNA in the nucleus by labeling cells for 1 h with [³H]uridine and measuring the incorporation of radioactivity into cytoplasmic and nuclear DHFR RNAs, respectively. We found that a threefold increase in DHFR protein synthesis after growth stimulation of methotrexate-resistant sarcoma-180 cells was accompanied by a corresponding increase in the relative rate of DHFR mRNA production. Since the half-life of DHFR mRNA is much longer than the 1-h labeling time used for these experiments (19), these measurements should be an accurate reflection of the relative rate of DHFR mRNA production. Steady-state nuclear levels of DHFR RNA were also determined at different times after growth stimulation by labeling cells with [³H]uridine until the incorporation of radioactivity into nuclear DHFR RNA approached a constant percentage of total nuclear RNA. The constant percentage reflected the relative level of DHFR RNA in the nucleus and was considered to be the steady-state level. The threefold increase in DHFR mRNA production after growth stimulation was accompanied by a corresponding increase in the steady-state level of DHFR RNA in the nuclei of growing cells.

When the cells were pulse-labeled for progressively shorter periods of time, the relative rate of [³H]uridine incorporation into nuclear DHFR RNA approached the same value in resting and growing cells, suggesting that the relative rate of transcription of DHFR genes was the same in each case. The most commonly used method for determining the relative transcription rates of specific genes in procaryotic and eucaryotic cells involves the hybridization of in vivo pulse-la-

beled RNA to specific sequences of immobilized DNA (10). In general, the hybridization reactions are carried out in the presence of an excess of DNA and the amount of radioactive RNA hybridized is expressed as a percentage of the radioactive RNA added. More recently, in an effort to incorporate more radioactivity into RNA, a number of investigators have used isolated nuclei to determine relative transcription rates in vitro (15). Derman et al. (10) compared the in vivo and in vitro methods and found that they provide quite similar results. Although both methods provide similar results, we used the in vivo method since it avoids potential artifacts that may result from the cellular fractionation procedures inherent in the in vitro approach involving isolated nuclei. Furthermore, we did not need to use isolated nuclei in order to obtain a sufficient quantity of radioactive DHFR RNA since in vivo pulse-labeled DHFR RNA was readily detected due to the very large number of transcriptionally active DHFR genes. Therefore, to obtain accurate measurements of transcriptional activity and to minimize the effects of any post-transcriptional events that might occur during the isolation of nuclear RNA, we prepared RNA from cells that were pulse-labeled for 5 min or less at varying times after growth stimulation. No significant increase in the relative rate of [^{3}H]uridine incorporation into DHFR RNA occurred as a result of growth stimulation, indicating that the relative rates of transcription of DHFR genes were the same in resting and growing cells. Therefore, the threefold increase in the steady-state level of DHFR RNA in the nuclei of growing cells must have resulted from an increase in the relative stability of the DHFR gene transcripts. The increase in the relative stability of nuclear DHFR RNA after growth stimulation may have resulted from an increase in DHFR RNA stability, a decrease in the stability of other transcripts, or a combination of both. In any case, these data suggest that the relative increase in DHFR mRNA production after growth stimulation was controlled primarily by post-transcriptional events within the nucleus. It is possible that the DHFR gene transcripts in resting cells were in some way different (and consequently less stable) than the DHFR transcripts in growing cells.

The relative rate of appearance of an mRNA in the cytoplasm may be controlled by transcriptional or post-transcriptional events within the nucleus. Post-transcriptional events may include the metabolism of mRNA precursors and the transport of mRNA to the cytoplasm. Evidence favoring transcriptional control of mRNA production in animal cells is based mainly on the expression of genes that code for specialized products of highly differentiated cells. The available data pertaining to this small but prominent class of structural genes indicate that large increases in the transcription of specific genes occur during differentiation (5, 8, 24, 31) and in response to certain hormonal stimuli (28, 30). The structural genes coding for these prevalent mRNA's represent such a minute fraction of active genes that it is not difficult to imagine that, although these genes may be controlled at the level of transcription, other genes may be controlled by selection of initial transcripts for processing or nucleoplasmic transport or both. One event in the selection of a transcript for further processing is polyadenylation. Recent studies indicated that polyadenylation plays an important role in controlling the expression of immunoglobulin genes in the development of the immune response (1, 11, 29) and in controlling what portion of the adenovirus late transcript is converted into mature mRNA (26). Additional evidence supporting post-transcriptional control of mRNA production has been presented by Davidson and Britten, who found that RNA sequences that are present on polysomes only in certain types of cells during sea urchin development are nevertheless found in the nuclear RNA of all cells (9, 34). These authors suggested that the steady-state concentrations of particular mRNA's depend on the fractions of nuclear mRNA precursors that are processed and exported. Other workers have shown that in mammalian cells the relative cytoplasmic levels of several specific mRNA's are considerably different from their relative rates of nuclear synthesis (18, 25). These investigators also concluded that post-transcriptional events are involved in determining the cytoplasmic concentrations of at least some mRNA's. However, these studies did not distinguish between post-transcriptional events that occurred in the nucleus and in the cytoplasm (i.e., mRNA turnover). Changes in the stabilities of specific mRNA's play a major role in controlling the cytoplasmic concentrations of hormonally induced mRNA's (14, 24, 27).

We studied some parameters which control the production of an mRNA that codes for an enzyme found in all cells. This study was possible primarily because of two important characteristics of methotrexate-resistant cells. First, the large quantity of DHFR mRNA produced by these cells (500 times normal) allowed us to measure the synthesis and accumulation of DHFR RNA in the nucleus and the appearance of mRNA in the cytoplasm. Second, all of the amplified DHFR genes in methotrexate-resist-

ant cells are active and are subject to control by the same physiological parameters that control DHFR gene expression in normal cells. Using these cells, we found that the increase in the relative rate of DHFR mRNA production after growth stimulation resulted from an increase in the relative stability of DHFR gene transcripts in the nucleus.

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