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Growth-Dependent Expression of Dihydrofolate Reductase mRNA from Modular cDNA Genes

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Dihydrofolate reductase (DHFR) synthesis is regulated in a growth-dependent fashion. Dividing cells synthesize DHFR at a 10-fold-higher rate than do stationary cells. To study this growth-dependent synthesis, DHFR genes have been constructed from a DHFR cDNA segment, the adenovirus major late promoter, and fragments of simian virus 40 (SV40) which provide signals for polyadenylation. These genes have been introduced into Chinese hamster ovary cells. The DHFR mRNAs produced in different transformants are identical at their 5′ ends, but differ in sequences in their 3′ ends as different sites are utilized for polyadenylation. Three transformants that utilize either DHFR polyadenylation signals or the SV40 late polyadenylation signal exhibit growth-dependent DHFR synthesis. The level of DHFR mRNA in growing cells is approximately 10 times that in stationary cells for these transformants. This growth-dependent DHFR mRNA production probably results from posttranscriptional events. In contrast, three transformants that utilize the SV40 early polyadenylation signal and another transformant that utilizes a cellular polyadenylation signal do not exhibit growth-dependent DHFR synthesis. In these three cell lines, the fraction of mRNAs polyadenylated at different sites in a tandem array shifts between growing and stationary cells. These results suggest that the metabolic state of the cell is important in determining either the efficiency of polyadenylation at various sites or the stability of mRNA polyadenylated at various sites.

Mechanisms regulating synthesis of proteins required for cell division (e.g., housekeeping enzymes) have not been well studied. The synthesis of dihydrofolate reductase (DHFR), a crucial enzyme in DNA synthesis, is controlled by the growth state of the cell (4, 17). It has been shown that the level of DHFR mRNA is 10 to 20 times higher in growing cells than in resting cells. Treatment of stationary cells with cyclic AMP (cAMP) further reduces the level of DHFR mRNA (20, 24). Stimulation of cells by the addition of serum or virus infection results in increased synthesis of DHFR mRNA (19). Synthesis of DHFR mRNA appears to be specific to the early part of the S phase in the cell cycle (28), which perhaps explains the higher levels of this mRNA in growing cells.

Growth-dependent synthesis of DHFR has primarily been studied in cell lines with amplified copies of the endogenous DHFR gene. The cells are resistant to the folic acid analog methotrexate (MTX) because of the high level of DHFR expression. The linear relationship between the level of DHFR protein, mRNA, and genome DNA copies suggests that each gene in the amplified set is active and correctly regulated (3, 24). The growth-dependent regulation of DHFR mRNAs might result from posttranscriptional controls. The rate of transcription of DHFR genes as measured by short pulses of [^{3}H]uridine (5 min) revealed no difference in DHFR transcription rates between growing and stationary cells (27). However, another study that used longer pulses (20 min) did detect less DHFR mRNA synthesis in stationary cells (40). This latter measurement, however, could have been influenced by posttranscriptional degradation.

We have previously characterized the activity of several modular transcription units constructed around a mouse DHFR cDNA segment (22). These modular units contain segments specifying initiation of transcription, RNA splicing, and polyadenylation. In particular, these genes possessed (in a 5′ to 3′ polarity) DNA segments containing (i) the major late promoter (MLP) site of adenovirus 2, encoding the first leader of adenovirus 2 late mRNAs and its 5′ splice site,

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(ii) a 3′ splice site from an immunoglobulin gene, (iii) mouse DHFR cDNA, (iv) pBR322 vector, and (v) simian virus 40 (SV40). Plasmid DNA containing the modular DHFR gene was used to transform the Chinese hamster ovary (CHO) cells deficient in DHFR to a $DHFR^+$ phenotype. Independent clones were further selected for resistance to high concentrations of MTX to amplify the transfected and integrated gene several hundredfold. The structure of the integrated and amplified plasmid DNA (21), mRNA encoding DHFR (22), and chromosomes containing amplified DHFR genes (23) has been described for four independent transformants. DHFR mRNA is initiated at the MLP site and is spliced by joining the 5′ splice site and 3′ splice site in all the lines. The sites of polyadenylation of DHFR mRNAs vary among the four transformants (22).

We report here that DHFR mRNA synthesis from transfected cDNA genes can be growth dependent. Some of the sequences responsible for this regulation appear to be localized in the 3′ untranslated portion of the gene.

MATERIALS AND METHODS

Cell culture. DHFR-deficient (DUKX) CHO cells (12) were transformed to the $DHFR^+$ phenotype by calcium phosphate-mediated transfection of plasmid DNA in the absence of carrier DNA (21, 22). Four independent transformants derived after transfection with plasmid pASD11 or pASD12 were stepwise selected for increasing MTX resistance. Studies described here utilized transformants resistant to 50 μM MTX which contain several hundred copies of the plasmid DNA incorporated into the genome. (For an analysis of the karyotypic alterations associated with this amplification, see reference 23.) Three independent transformants (6A, 6B, 6C) were isolated after transformation of the $DHFR^-$ cells with pAdD26SV(A) no. 3 and were studied before MTX selection and amplification (22). The 3T3-R500 murine line has been described previously and was provided by P. Brown (9). Propagation of all these cell lines has been described previously (21). [See references 21 and 22 for construction of recombinants pASD11, pASD12, and pAdD26SV(A) no. 3.]

Preparation and analysis of [^{35}S]methionine-labeled cell extracts. Duplicate cultures of cells were established, and on reaching confluency, one dish was subcultured (1:6) into fresh medium and serum (α medium with 10% dialyzed fetal calf serum and 50 μM MTX were indicated). After 20 h, the cells were rinsed twice with phosphate-buffered saline at 37°C and incubated for 1 h at 37°C in Dulbecco modified Eagle medium (without methionine) supplemented with 2% dialyzed fetal calf serum and 25 μCi of [^{35}S]methionine (New England Nuclear Corp., Boston, Mass.) per ml. Cell extracts were prepared and analyzed by sodium dodecyl sulfate (SDS)-gel electrophoresis (25) as described previously (21). Cell pellets were suspended in 0.5 ml of 50 mM sodium phosphate (pH 7.0) with 1 mM phenylmethylsulfonyl fluoride and disrupted by sonication. Cell extracts were centrifuged for 1 h at 100,000 × *g*, and supernatants were taken for study. Incorporation of [^{35}S]methionine was measured by trichloroacetic acid precipitation, and equal numbers of incorporated counts (2×10^5 cpm) were applied to standard Laemmli SDS gels containing 15% (wt/vol) acrylamide and 0.086% (wt/vol) *N*′-methylenebisacrylamide (25). The gels were fixed in 10% (vol/vol) methanol–10% (vol/vol) acetic acid and were prepared for autoradiography by treatment with the fluor En-^{3}Hance (New England Nuclear Corp.) and drying. Dried gels were exposed to preflashed Kodak XR-5 film (8). Band intensities were quantitated with a Zeineh scanning densitometer.

RNA isolation. Duplicate cultures of cells were prepared, and on reaching confluency, one dish was subcultured (1:6) into fresh medium containing 10% dialyzed fetal calf serum. Where indicated, theophylline (1 mM) and dibutyryl cAMP (1 mM) were added to another confluent dish, without feeding. After 20 h, cytoplasmic RNA was prepared as previously described (21).

S1 nuclease mapping. A 3′ uniquely end-labeled probe was prepared by using either DHFR cDNA plasmids pDHFR26 or pDHFR12 (11) or the modular DHFR gene pAdD26SV(A) no. 3 by digesting these DNAs with restriction endonuclease *Taq*I. After phenol extraction, ether extraction, and ethanol precipitation, the digested DNA was treated with the Klenow fragment of *Escherichia coli* DNA polymerase I (New England Biolaboratories, Inc., Beverly, Mass.) in a 30-μl reaction volume with 100 μCi each of [^{32}P]dATP and [^{32}P]dCTP with cold 60 μM dGTP and 60 μM dTTP in 10 mM Tris (pH 7.8)–5 mM $MgCl_2$–3 mM dithiothreitol–50 mM NaCl for 30 min at 37°C. The reaction was stopped by adding EDTA to 10 mM and heating for 30 min at 68°C. After ethanol precipitation, the DNA was digested to completion with *Pst*I and electrophoresed on a 1.2% agarose (low melting temperature; Bethesda Research Laboratories, Gaithersburg, Md.) gel. The appropriate band was extracted by adding an equal volume of 10 mM Tris (pH 8.0)–1 mM EDTA, heating to 65°C for 10 min, extracting twice with phenol and twice with ether, and precipitating in ethanol with the addition of 0.3 M sodium acetate and 10 μg of yeast tRNA carrier. When necessary, the eluted fragment was repurified by repeating the gel electrophoresis described above. The specific activity of these probes varied between 0.5×10^6 and 2.0×10^6 cpm/μg.

RNA-DNA hybridization was carried out under R-loop conditions (10) with subsequent S1 nuclease digestion (6) as described previously (22). Hybridizations were performed in 80% formamide–0.4 M NaCl–0.04 M PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); pH 6.5]–1 mM EDTA for 3 to 5 h at 55°C (for the pDHFR26 probe), 51°C (for the pDHFR12 probe), or 45°C (for the pAdD26SV[A] no. 3 probe). These are the melting temperatures for each fragment in the hybridization buffer. After S1 nuclease digestion, products were analyzed by electrophoresis on glyoxal–2% agarose gels (30). Gels were dried and exposed using preflashed film and intensifying screens at −70°C (26).

Northern blot analysis. Approximately 3 μg of cytoplasmic polyadenylic acid [poly(A)]-containing [$poly(A)^+$] RNA was treated with glyoxal and electrophoresed on 1.4% agarose gels as previously described (30). RNA was transferred to nitrocellulose (38) and

was hybridized as previously described (22) in the presence of 10% dextran sulfate (39) to approximately 10^7 cpm of ^{32}P-labeled pDHFR26 (specific activity, 10^8 cpm/μg) prepared by nick translation (33). After hybridization, the filter was washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) with 0.5% SDS at 68°C. The filter was prepared for autoradiography with preflashed Kodak XR-5 film with intensifying screens at −70°C.

Nuclear transcription analysis. Nuclei were prepared from confluent monolayers of cells (ca. 10^8) or from confluent monolayers subcultured (1:6) 20 h earlier into fresh medium with serum. Nuclear transcription reactions were performed (29) and analyzed as previously described (18). Cells were rinsed with ice-cold phosphate-buffered saline, suspended in reticulocyte swelling buffer with 0.1 mM $CaCl_2$, and lysed by the addition of 0.5% Nonidet P-40 with vortexing. The nuclei were washed several times in ice-cold reticulocyte swelling buffer with 0.1 mM $CaCl_2$ and 0.5% Nonidet P-40. The nuclei were then suspended in 50 mM Tris (pH 8.3)–5 mM $MgCl_2$–0.1 mM EDTA with 40% glycerol at a DNA concentration of 1 to 2 mg/ml and, if not used immediately, stored at −70°C. Reactions (200 μl) contained nuclei with approximately 200 μg of DNA, 30% glycerol, 2.5 mM dithiothreitol, 1 mM $MgCl_2$, 70 mM KCl, 0.25 mM each of GTP and CTP, 0.5 mM ATP, and 200 μCi of UTP (α-^{32}P, 450 Ci/mmol, ICN). Reactions were incubated for 5 min at 26°C and were terminated by the addition of RNase-free DNase (Miles Laboratories, Inc., Elkhart, Ind., 7 μg per sample) and further incubated for 10 min at 26°C. EDTA was added to 5 mM, SDS was added to 1%, and reactions were incubated at 42°C with 30 μg of proteinase K for 30 min. After the addition of 100 μl of water, the reaction was phenol extracted (two times), chloroform extracted (two times), and ethanol precipitated. The ethanol pellet was suspended in 10 mM Tris (pH 8.0)–1 mM EDTA (200 μl), $MgCl_2$ was added to 15 mM, and 5 mg of RNase-free DNase was added again for 10 min at 37°C. Then EDTA was added to 20 mM, SDS was added to 1%, and 20 μg of proteinase K was added; the sample was then incubated for 1 h at 37°C. The sample was precipitated, after the addition of 100 μg of yeast tRNA, with ice-cold trichloracetic acid (30% final concentration with 30 mM sodium PP_i) and filtered onto nitrocellulose filters. Analysis of the counts indicated ca. 1.5×10^6 cpm incorporated (Cerenkov) per sample. With the inclusion of α-amanitin at 1 μg/ml, incorporation was reduced by approximately 30%. RNA was eluted from the filters by incubating them with 1.4 ml of 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; pH 7.5)–5 mM $MgCl_2$–1 mM $CaCl_2$–35 μg of RNase-free DNase for 30 min at 37°C. Then EDTA was added to 2 mM, SDS was added to 1%, and 35 μg of proteinase K was added; the sample was then incubated at 37°C for 30 min. The reaction was then heated to 68°C for 10 min, 200 μg of yeast tRNA was added, and the liquid was removed. Filters were rinsed with 0.5 ml of 10 mM Tris (pH 8.0)–1 mM EDTA with 1% SDS at 68°C for 10 min. This 0.5-ml volume was added to the previous 1.8 ml of aqueous solution and the sample was extracted with phenol (one time) and chloroform (one time), and ethanol precipitated. Greater than 90% of the incorporated counts were recovered.

Labeled RNA (10^6 cpm per sample) as prepared above was hybridized to strips of nitrocellulose prepared by Southern blotting (37). Nitrocellulose strips were prehybridized for at least 4 h in 5× SSC containing 25 mM sodium phosphate, 2 mM EDTA, 0.5% SDS, and 0.1% each of bovine serum albumin, polyvinylpyrrolidone, and Ficoll. Hybridizations were carried out in the same buffer with 10^6 cpm of incorporated RNA per sample per 0.5-ml volume, with the addition of 10% dextran sulfate (39) and 25 mM sodium PP_i after heating to 68°C for 5 min. Hybridizations were performed at 42°C for 48 h, and then the filters were washed repeatedly in 2× SSC–0.5% SDS–1 mM EDTA at 68°C. When the hybridization solution was rehybridized to a fresh filter, the signal obtained was only one-fifth that of the original filter, indicating that most of the specifically labeled RNA is hybridized under these conditions. The addition of α-amanitin (1 μg/ml) to the reaction reduced the signal by 80%, demonstrating that the products of the nuclear reaction are derived from RNA polymerase II.

Nitrocellulose strips for hybridization were prepared by *Pst*I digestion of plasmid pDHFR26 (2 μg per filter) and a plasmid encoding the chicken β-actin cDNA (pA1; 13) (2 μg per filter) and by electrophoresis on neutral 1.3% agarose gels with subsequent transfer to nitrocellulose as described previously (37) but with modifications (21). After the transfer, the filters were baked in vacuo for 2 h at 80°C and were treated with prehybridization solution. Before hybridization, strips were sectioned and hybridizations were performed in 1.5-ml Eppendorf tubes by submerging the filters in 0.5 ml of hybridization solution. Hybridized and washed nitrocellulose strips were prepared for autoradiography and exposed for various times to preflashed Kodak XR-5 film at −70°C with intensifying screens.

RESULTS

Growth-dependent DHFR synthesis. Modular DHFR genes containing the mouse DHFR cDNA and the adenovirus MLP were capable of transforming DHFR$^-$ CHO cells to DHFR$^+$ when SV40 DNA was introduced into the plasmid (21) (see Fig. 1 legend). Four independent transformants initially containing one to five copies of the transforming DNA were grown in increasing concentrations of MTX. Resistance to 50 μM MTX yielded a copy number of several hundredfold. This amplification facilitated the analysis of the DHFR mRNAs produced in these four lines (22). The major DHFR mRNA species in each transformant are depicted in Fig. 1 and will be discussed later.

These four transformants were tested for growth-dependent DHFR synthesis by electrophoresis of [^{35}S]methionine-labeled (1 h) extracts prepared from stationary resting cells or from growth-stimulated cells. Transformants 1B, 1D, and 2B all specifically induce (see Fig. 2 legend for quantitation) DHFR synthesis in growth-stimulated as compared with stationary-phase cells. DHFR is the only protein that

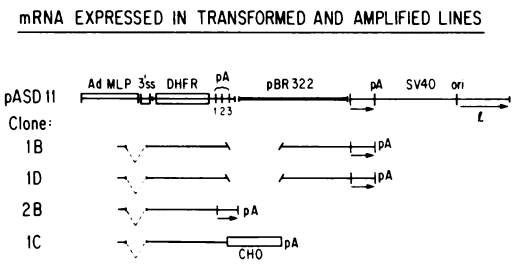


FIG. 1. Major DHFR mRNA in amplified lines. The major DHFR mRNA in independent transformants isolated by transformation with pASD11 are indicated. In all transformants, the adenovirus major late leader is properly spliced to the 3′ splice site. In 1B and 1D, an additional segment of RNA (approximately 1 kilobase) is removed, presumably by RNA splicing of sequences in pBR322. The mRNA is polyadenylated at the SV40 late polyadenylation signal. Transformant 2B has a rearrangement of the DNA sequences so that the 3′ end of the SV40 late transcription unit and the polyadenylation signal are positioned 3′ to the DHFR segment. The 3′ end of DHFR mRNA in transformant 1C is derived from cellular DNA sequences that are adjoined to the plasmid DNA sequences (open box). See reference 22 for details.

shows any significant change in synthesis after growth stimulation. The degree of growth stimulation is comparable to that observed for DHFR expression from amplified endogenous genes in 3T3 cells (data not shown). In contrast, transformant 1C shows negligible growth dependence of DHFR synthesis.

Previous analysis of the 3′ ends of the mRNAs synthesized in these four lines revealed little polyadenylation at signals in the DHFR cDNA segment, even though this cDNA segment contained three of the four polyadenylation sites for cellular DHFR mRNAs (34). (These sites are labeled pA1, pA2, and pA3 in Fig. 1.) Instead, most DHFR mRNAs in these transformants are polyadenylated at sites downstream from the DHFR cDNA (Fig. 1). Two transformants (1B and 1D) produce similar DHFR mRNAs which contain pBR322 sequences and are polyadenylated at the SV40 late polyadenylation signal. The integrated DNA in transformant 2B has a rearrangement of the plasmid DNA so that most of the DHFR encoding mRNA is polyadenylated at the immediate flanking late SV40 site. Transformant 1C has integrated the plasmid DNA into cellular DNA so that most of the DHFR mRNA is polyadenylated in flanking CHO sequences. Thus, three transformants which utilize the polyadenylation signals from the SV40 late region show growth-dependent synthesis of DHFR; the single transformant that uses a cellular polyadenylation signal exhibits constitutive DHFR synthesis.

DHFR mRNA levels correlate with DHFR translation. S1 nuclease mapping in DNA probe excess demonstrates that the growth-dependent DHFR synthesis correlates with differences in DHFR mRNA levels. A 3′ end-labeled DNA probe was prepared from the DHFR cDNA segment which contains the entire 3′ untranslated sequence of the longest DHFR mRNA and thus encompasses the four polyadenylation sites at 750 (pA1), 1,000 (pA2), 1,200 (pA3), and 1,600 (pA4) bases present in the murine DHFR gene (11, 34, 35). When hybridized to cytoplasmic

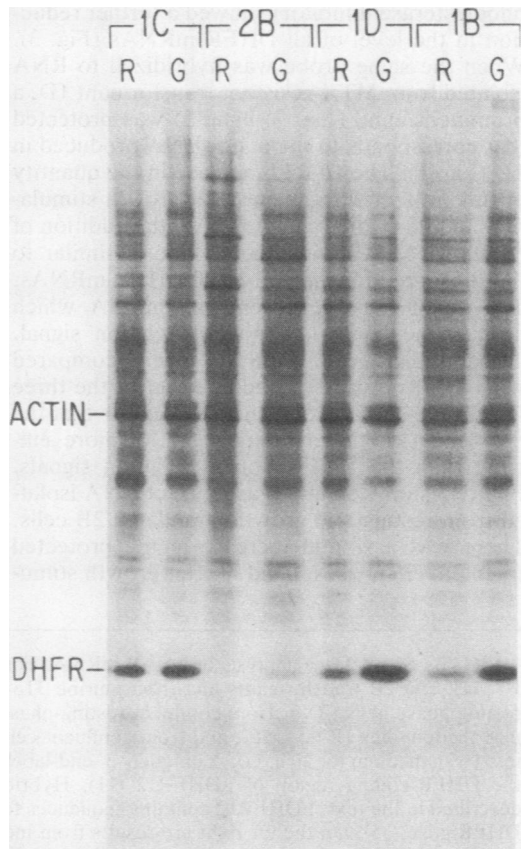


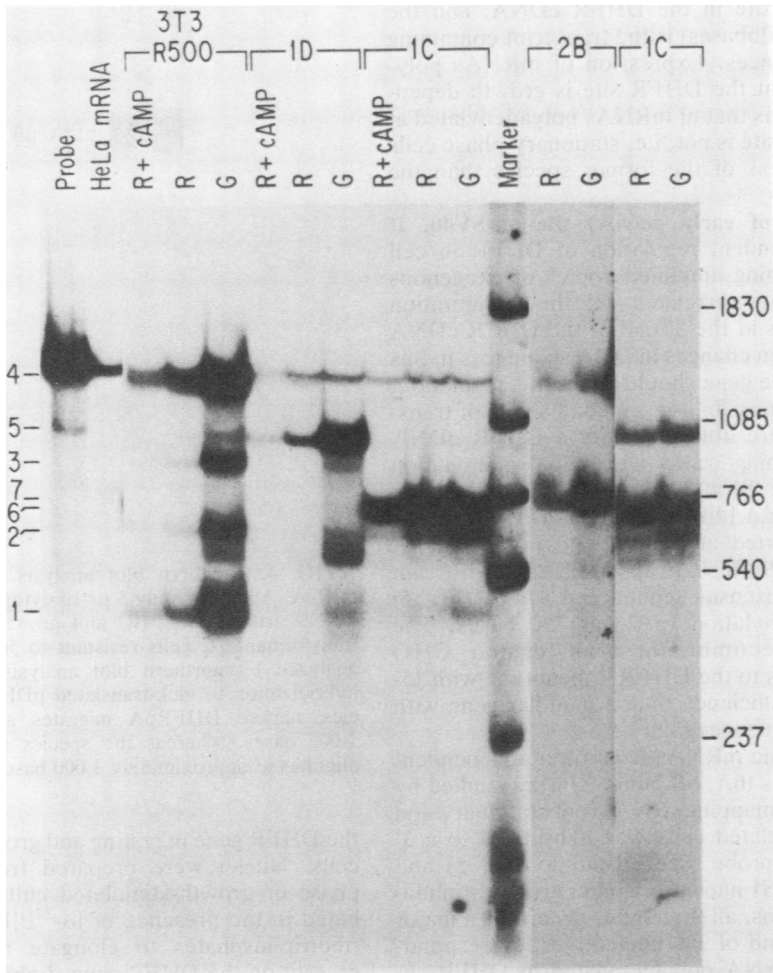
FIG. 2. Protein synthesis in resting and growth-stimulated amplified transformants. Duplicate confluent plates of transformants 1B, 1C, 1D, and 2B, resistant to 50 μM MTX, were either subcultured (1:6) into fresh medium (G) or left untreated (R) and incubated 20 h later with [^{35}S]methionine for 1 h. Extracts were prepared and analyzed as described in the text. After growth stimulation, transformants 1B and 1D exhibited a 10- and 15-fold increase in DHFR synthesis, respectively. Transformant 2B also exhibited a significant increase; however, due to its lower level of synthesis, it was not possible to quantitate the increase above background. Transformant 1C exhibited a two-fold increase in DHFR synthesis with growth stimulation.

RNA isolated from growth-stimulated 3T3-R500 mouse MTX-resistant cells (a cell line containing amplified endogenous DHFR genes), four prominent DNA fragments representative of 750-, 1,000-, 1,200-, and 1,600-base mRNA species were observed after S1 nuclease digestion and gel electrophoresis (Fig. 3, bands 1 through 4). RNA from stationary-phase cells also generated the four bands in the same proportion, but at 1/10 to 1/20 the abundance (Fig. 3). Thus, all four DHFR mRNAs increase in level after growth stimulation. In addition, stationary-phase cells that were treated for 24 h with 1 mM dibutyryl cAMP and 1 mM theophylline (a phosphodiesterase inhibitor) showed a further reduction in the level of all DHFR mRNAs (Fig. 3). When the same probe was hybridized to RNA isolated from MTX-resistant transformant 1D, a prominent band (Fig. 3, band 5) was protected that corresponds to the major RNA produced in 1D (Fig. 1). The 10-fold increase in the quantity of this protected fragment after growth stimulation and its 4-fold reduction with the addition of dibutyryl cAMP and theophylline is similar to that observed for the 3T3-R500 DHFR mRNAs. The major band represents an mRNA which utilizes the SV40 late polyadenylation signal. The abundance of this mRNA species compared with those polyadenylated at either of the three DHFR polyadenylation signals suggests that the SV40 late polyadenylation signal is more efficient than the DHFR polyadenylation signals. Figure 3 shows a similar analysis of RNA isolated from resting and growth-stimulated 2B cells. There was a sixfold increase in the protected fragment (760 bases, band 7) after growth stimulation. Thus, for the 1D and 2B cell lines, the growth-dependent change in DHFR protein synthesis is due to a change in the DHFR mRNA level.

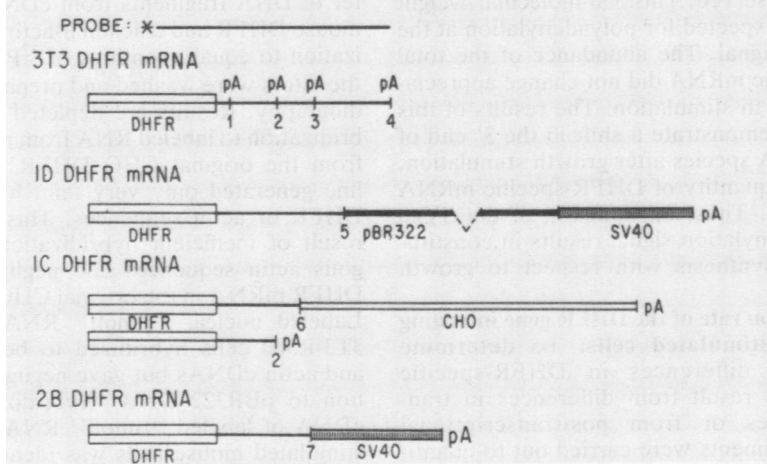
Hybridization of the same probe to RNA isolated from MTX-resistant transformant 1C yielded a doublet after S1 nuclease digestion and gel electrophoresis (Fig. 3). The slower-migrating band (band 6) represents the mRNA species with polyadenylation in flanking CHO sequences. Its level only increases twofold with growth stimulation and is reduced only twofold in stationary-phase cells treated with dibutyryl cAMP and theophylline. In transformant 1C, a second fragment (band 2) was generated from mRNA polyadenylated at a site in the DHFR cDNA (pA2 at 1,000 bases, Fig. 3). The amount of this mRNA increased fourfold with growth stimulation and was repressed fourfold after the addition of cAMP and theophylline.

The apparent shift in the ratio of the two DHFR mRNA species in transformant 1C as a result of growth stimulation was further characterized. Figure 3 shows an identical S1 nuclease analysis with another independently isolated RNA preparation and probe. It also revealed a change in the ratio of the two DHFR mRNA species but little change in total DHFR mRNA levels. In addition, equal amounts of poly(A)$^+$ RNA from stationary-phase and growth-stimulated cells were electroporesed on a denaturing agarose gel, transferred to nitrocellulose, and hybridized to a nick-translated DHFR cDNA plasmid probe (Fig. 4). Two size classes of DHFR-specific mRNAs were present in growth-stimulated cells. The shorter species (~1,000

FIG. 3. S1 nuclease analysis of DHFR mRNA. Total cytoplasmic RNAs (30 μg) from 50 μM MTX-resistant 1C, 1D, and 2B transformants and from murine 3T3-R500 MTX-resistant cells were prepared from confluent resting-phase cells (R) or from confluent resting-phase cells treated for 20 h with 1 mM dibutyryl cAMP and 1 mM theophylline (R+cAMP), and from confluent cells which had been growth stimulated by subculturing 1:6 into fresh medium for 20 h (G). A uniquely 3′ end-labeled DNA probe was prepared by flush-ending a *Taq*I site in the DHFR-coding region of pDHFR12 (11). Hybridization and S1 nuclease digestion were carried out as described in the text. pDHFR12 contains sequences for the four major polyadenylation sites in the 3′ end of the DHFR gene (35). On the far right are results from independent RNA preparations from growth-stimulated and resting-phase 1C cells which were hybridized to a probe similarly prepared from a DHFR cDNA clone (pDHFR26 of reference 11). This probe is approximately 250 bases shorter than the probe used in the other lanes. Also shown are results from hybridization to HeLa RNA which contains no homologous DHFR sequences and also the undigested probe from pDHFR12 (1/10 the amount used in these experiments). Sizes of molecular weight markers (*Hinf*I SV40) are indicated on the right. The diagram below shows the homology of the probe to the various mRNA species. The DHFR-coding regions (box) are identical in all mRNA species. 3T3-R500 DHFR mRNA contains four major species (labeled 1 through 4), distinguished by differences in polyadenylation, which correspond to the 750-, 1,000-, 1,200-, and 1,600-base mRNAs described in references 34 and 35. This mRNA protects four different-sized probe DNAs from S1 nuclease digestion. 1D DHFR mRNA produces a major band which maps to the position at which DHFR sequences end and enter pBR322 DNA in the recombinant pASD11 (band 5). 1C DHFR mRNA diverges at the position (band 6) where the plasmid DNA has integrated into the CHO genome. It also produces a slightly smaller protected fragment derived from hybridization to an mRNA produced by polyadenylation at site pA2 in the 3′ end of the DHFR cDNA. This fragment comigrates with the respective fragment protected after hybridization of the probe to murine 3T3-R500 RNA (band 2). DHFR mRNA from 2B produces a single band (band 7) that corresponds to the rearrangement in the 3′ untranslated sequences of the plasmid.



S1 Analysis of DHFR mRNA



bases) corresponds to polyadenylation at the 1,000 base site in the DHFR cDNA, and the larger (~3 kilobases) is the transcript containing CHO sequences. Expression of mRNAs polyadenylated at the DHFR site is growth dependent, whereas that of mRNAs polyadenylated at the cellular site is not; i.e. stationary-phase cells contained less of the former species than the latter.

Insertion of early poly(A) site of SV40. If growth-dependent regulation of DHFR in cell lines containing amplified copies of exogenous modular genes correlates with the configuration of sequences at the 3′ end of the DHFR cDNA segment, then changes in DNA sequences in this portion of the gene should affect this regulation. To test this possibility, a second series of transformants were obtained from a DHFR cDNA gene containing other tandem polyadenylation signals. The SV40 early polyadenylation site (*Bcl*I [0.20 m.u.] to *Pst*I [0.04 m.u.]) was selected and inserted at a *Bgl*II site in the DHFR cDNA. The *Bgl*II site maps 25 bases downstream from the consensus sequence AUAAA (Fig. 5) for polyadenylation (pA1) at 750 bases. The resulting recombinant transformed CHO DHFR$^-$ cells to the DHFR$^+$ phenotype with 15-fold-higher efficiency than a modular gene with no SV40 sequences (22).

Cytoplasmic mRNAs from three independent transformants (6A, 6B, and 6C) were studied by S1 nuclease mapping. RNA from stationary and growth-stimulated cells was hybridized to a 3′ end-labeled probe (see legend to Fig. 5) and treated with S1 nuclease. Under growth stimulation conditions, all three lines generated a major protected band of 390 nucleotides, corresponding to an mRNA polyadenylated in DHFR sequences. When stationary-phase RNA was used, a major band at 550 nucleotides and a dramatic reduction in the band at 390 nucleotides were observed. This 550-molecular-weight band is that expected for polyadenylation at the SV40 early signal. The abundance of the total DHFR-specific mRNA did not change appreciably with growth stimulation. The results of this experiment demonstrate a shift in the 3′ end of DHFR mRNA species after growth stimulation, but the total quantity of DHFR-specific mRNA is not altered. Thus, introduction of the SV40 early polyadenylation signal results in constitutive DHFR synthesis with respect to growth phase.

Transcription rate of the DHFR gene in resting and growth-stimulated cells. To determine whether the differences in DHFR-specific mRNA levels result from differences in transcription rates or from posttranscriptional events, experiments were carried out to quantitate the number of transcription complexes on the DHFR gene in resting and growth-stimulated cells. Nuclei were prepared from stationary-phase or growth-stimulated cultures and incubated in the presence of [α-^{32}P]UTP and other ribotriphosphates to elongate nascent RNAs present on the DHFR gene. Labeled transcripts were isolated and hybridized to nitrocellulose strips which contained three different DNAs. These strips were prepared from Southern transfer of DNA fragments from cDNA plasmids of mouse DHFR and chicken β-actin. After hybridization to equal quantities of ^{32}P-labeled RNA, the filters were washed and prepared for autoradiography. Results are depicted in Fig. 6. Hybridization to labeled RNA from nuclei prepared from the original CHO DHFR$^-$ (DUKX) cell line generated only very faint hybridization to DHFR or actin sequences. This is probably a result of inefficient hybridization by heterologous actin sequences and negligible levels of DHFR mRNA in the original CHO DHFR$^-$ line. Labeled nuclear "runoff" RNA from murine 3T3-R500 cells hybridized to both the DHFR and actin cDNAs but gave negligible hybridization to pBR322 DNA. Hybridization to actin cDNA of labeled "runoff" RNA from growth-stimulated mouse cells was identical to that of resting-phase cells. Hybridization to DHFR

FIG. 4. Northern blot analysis of 1C poly(A)$^+$ mRNA. Approximately 3 μg of cytoplasmic poly(A)$^+$ mRNA from resting (R) and growth-stimulated (G) transformant 1C cells resistant to 50 μM MTX was analyzed by northern blot analysis (see text) and hybridization to nick-translated pDHFR26. The species marked DHFRpA migrates at approximately 1,000 bases, whereas the species marked CHOpA migrates at approximately 3,000 bases.

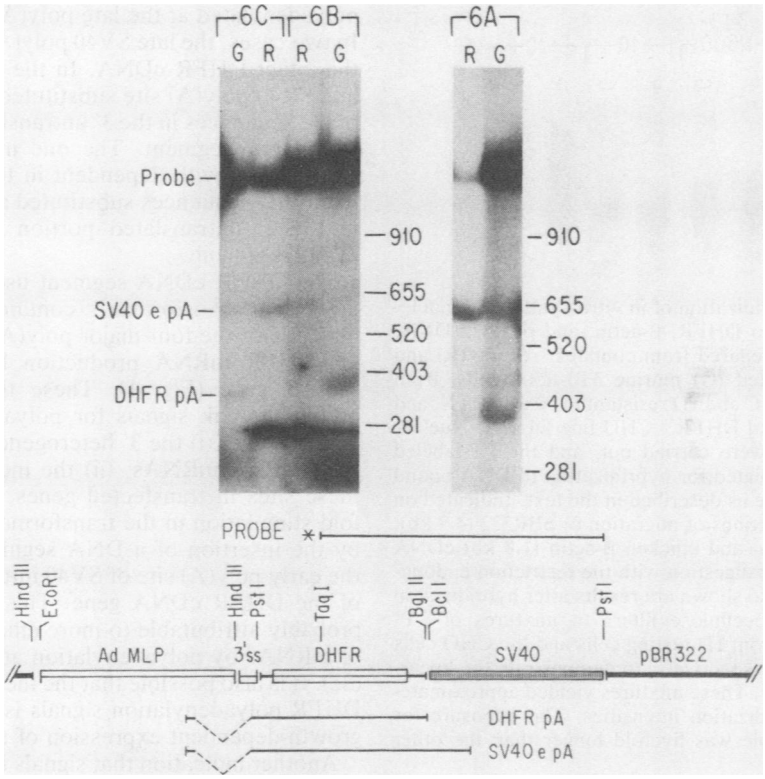


FIG. 5. S1 analysis at DHFR mRNA in transformants utilizing the SV40 early polyadenylation signal. Transformants 6A, 6B, and 6C were obtained by transformation of DHFR-deficient cells to the DHFR$^+$ phenotype by pAdD26SV(A) no. 3 (see text; 22). Total cytoplasmic RNA (30 μg for 6B and 6C and 100 μg for 6A) was hybridized to a *Taq*I-to-*Pst*I DNA fragment from pAdD26SV(A) no. 3 which had been 3′ end labeled at the *Taq*I site. The migration of Alu-digested pBR322 marker DNA is indicated. The diagram at the bottom depicts the probe utilized in these experiments and the structure of pAdD26SV(A) no. 3 with the two potential DHFR mRNA species derived by polyadenylation at a DHFR polyadenylation signal (DHFR pA) or at the SV40 early polyadenylation signal (SV40 epA). mRNA from growth-stimulated cells produces a protected fragment of 390 nucleotides, approximately 60 nucleotides shorter than expected if pAI in the DHFR cDNA were being utilized. Approximately 20 bases upstream from this putative 3′ end is an AAGAAA sequence which may function as a polyadenylation signal at a fivefold-lower efficiency (C. Montell, E. Fisher, M. Caruthers, and A. J. Berk, submitted for publication) than the consensus hexanucleotide AAUAAA (32).

cDNA of labeled "runoff" was only twofold greater in growth-stimulated mouse cells than in resting-phase cells. Thus, although cytoplasmic DHFR mRNA differs at least 10-fold in abundance (see Fig. 3), there is only 2-fold change in the transcription rate of the DHFR gene.

When labeled nuclear runoff transcripts from growth-stimulated and resting 1D and 1C cells were analyzed for DHFR sequences, no difference was observed. In transformant 1D, efficient hybridization occurs to pBR322 sequences because these sequences are present in the 3′ untranslated portion of the DHFR mRNA (Fig. 1). We conclude that both transformants 1C and 1D demonstrate a negligible variation in the transcription rate of the modular DHFR gene in growth-stimulated versus resting-phase cells. However, the steady state of DHFR mRNA in the 1D line changes 10-fold with growth stimulation, whereas that of the 1C line remains constant (Fig. 3). Therefore, the increase in DHFR mRNA levels with growth stimulation must in part result from posttranscriptional events for both endogenous and transfected DHFR genes.

DISCUSSION

Some modular DHFR genes exhibit growth-dependent expression in CHO cells, as demonstrated by an approximately 10-fold elevation in DHFR mRNA level with growth stimulation of stationary-phase cells. In addition, we found that treatment of stationary cells with dibutyryl cAMP and theophylline further reduced the level of DHFR mRNA. Endogenous DHFR genes

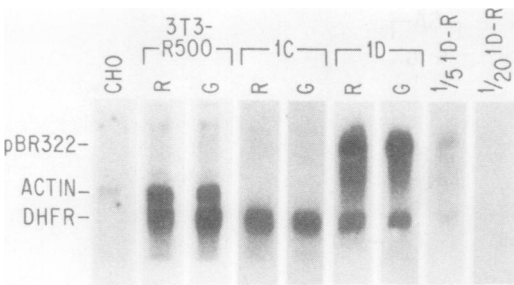


FIG. 6. Hybridization of in vitro-synthesized nuclear [^{32}P] RNA to DHFR, β-actin, and pBR322 DNA. Nuclei were prepared from confluent resting (R) and growth-stimulated (G) murine 3T3-R500 cells, from transformants 1C and 1D resistant to 50 μM MTX, and from the original DHFR$^-$ CHO line DUKX. Nuclear transcriptions were carried out, and the ^{32}P-labeled RNAs were isolated for hybridization to DNA bound to nitrocellulose as described in the text. Indicated on the left are positions of migration of pBR322 (4.3 kb), DHFR (1.2 kb), and chicken β-actin (1.8 kb) cDNA sequences after digestion with the restriction endonuclease *Pst*I. Also shown are results after hybridization of similar nitrocellulose filters to mixtures of ^{32}P-labeled RNA from 1D resting cells and the CHO cells (1:5 and 1:20, respectively) to demonstrate the linearity of this assay. These mixtures yielded approximately linear hybridization intensities. The exposure for the CHO sample was fivefold longer than the other exposures.

have previously been shown to be growth dependent for expression over a similar range and have a similar response to increases of cAMP (20, 24). Whether the phenotypic similarities between the growth-dependent regulation of endogenous and exogenous DHFR genes reflect similar mechanisms is difficult to prove. In addition, overlapping mechanisms for growth-dependent regulation may control expression of the endogenous gene, whereas only a subset of these might regulate expression of any transfected modular DHFR gene.

Previous work has suggested that the growth-dependent regulation of DHFR is the consequence of posttranscriptional changes (27). In vitro measurements of the number of elongating polymerases on either endogenous DHFR genes of mouse or transfected DHFR genes in CHO cells did not indicate a difference in the level of transcription between resting and growing cells.

Analysis of the first set of four transformants containing modular DHFR genes suggested that their growth-dependent regulation is largely mediated by signals 3′ to the protein-coding sequences in the DHFR cDNA segment. All of the modular genes had common sequences 5′ of the DHFR coding sequences. However, the three DHFR modular genes that were growth dependent for expression had the common feature of being transcribed to yield a prominent mRNA polyadenylated at the late poly(A) site of SV40. In two cases, the late SV40 poly(A) site followed the intact DHFR cDNA. In the third case, the late SV40 poly(A) site substituted for about half of the sequences in the 3′ untranslated portion of the cDNA segment. The one modular DHFR gene, not growth dependent in this set of four, had CHO sequences substituted at a similar site in the 3′ untranslated portion of the DHFR cDNA segment.

The DHFR cDNA segment used to generate these four transformants contained sequences for three of the four major poly(A) sites utilized for DHFR mRNA production by the mouse cellular gene (Fig. 1). These three sites are probably weak signals for polyadenylation, as evidenced by (i) the 3′ heterogeneity of endogenous DHFR mRNAs, (ii) the inefficient use of these sites in transfected genes, and (iii) a 15-fold stimulation in the transformation efficiency by the insertion of a DNA segment containing the early poly(A) site of SV40 into the 3′ portion of the DHFR cDNA gene. This stimulation is probably attributable to more efficient synthesis of mRNAs by polyadenylation at the SV40 site (22). It is also possible that the inefficiency of the DHFR polyadenylation signals is related to the growth-dependent expression of this gene.

Another indication that signals in the 3′ region of DHFR genes influence the synthesis of mRNAs in a growth-dependent fashion follows from the shift in the fraction of mRNA polyadenylated at a series of sites. Growing cells synthesize mRNAs polyadenylated in DHFR sequences, whereas the same cells accumulate mRNAs polyadenylated at downstream sites in their stationary phase. For example, genes with the SV40 early poly(A) site inserted downstream of the DHFR segment produce, in growing cells, approximately equal amounts of mRNAs polyadenylated at the DHFR and SV40 sites. However, the same cell line under stationary conditions accumulates mRNAs polyadenylated at the downstream SV40 site. The total DHFR mRNA level is not growth dependent in these cell lines. The shift in the fraction of mRNA polyadenylated could be due to changes either in the efficiency of polyadenylation or in the rate of degradation of the shorter mRNAs. In either case, accumulation of DHFR mRNAs seems regulated by posttranscriptional events affected by sequences in the 3′ noncoding portion of the gene.

These limited results suggest that the rates of synthesis and degradation of mRNAs with polyadenylation sites in the DHFR gene and in the SV40 late region might be growth dependent. In both cases, growth dependence could be explained if the physiological state of cells in S phase favored accumulation of these mRNAs.

The early functions of SV40 stimulate cells into S phase before late mRNAs accumulate. This would imply that part of the restriction on the accumulation of late SV40 mRNA at early times after infection is posttranscriptional, a supposition consistent with the previous detection of transcription of the late region at this time (7, 15).

Alternate utilization of polyadenylation signals may modulate gene expression. With the mitogenic stimulation of resting B-cell lymphocytes, there is a shift from a downstream polyadenylation signal specifying an mRNA for a membrane-bound mu heavy-chain immunoglobulin to an alternate upstream polyadenylation signal that generates a differently spliced mRNA specifying a secreted mu heavy chain (2, 14). This effect is very analogous to the results described in this paper. Shifts in the sites of polyadenylation are also important in the maturation of transcripts from the adenovirus MLP (1, 31, 36) and possibly also in the developmental specific generation of alternate, processed RNA transcripts from the calcitonin gene (5).

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