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Changes in Dihydrofolate Reductase (DHFR) mRNA Levels Can Account Fully for Changes in DHFR Synthesis Rates during Terminal Differentiation in a Highly Amplified Myogenic Cell Line

EDWARD E. SCHMIDT AND GARY F. MERRILL*

Department of Biochemistry and Biophysics, Weniger Hall 535, Oregon State University, Corvallis, Oregon 97331

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Dihydrofolate reductase (DHFR) enzyme is preferentially synthesized in proliferative cells. A mouse muscle cell line resistant to 300 μM methotrexate was developed to investigate the molecular levels at which DHFR is down-regulated during myogenic withdrawal from the cell cycle. H⁻αR300T cells contained 540 copies of the endogenous DHFR gene and overexpressed DHFR mRNA and DHFR protein. Despite DHFR gene amplification, the cells remained diploid. As H⁻αR300T myoblasts withdrew from the cell cycle and committed to terminal differentiation, DHFR mRNA levels and DHFR synthesis rates decreased with closely matched kinetics. After 15 to 24 h, committed cells contained 5% the proliferative level of DHFR mRNA (80 molecules per committed cell) and synthesized DHFR protein at 6% the proliferative rate. At no point during the commitment process did the decrease in DHFR synthesis rate exceed the decrease in DHFR message. The decrease in DHFR mRNA levels during commitment was sufficient to account fully for the decrease in rates of DHFR synthesis. Furthermore, DHFR mRNA remained polysomal, and the average number of ribosomes per message remained constant (five to six ribosomes per DHFR mRNA). The constancy of polysome size, along with the uniform rate of DHFR synthesis per message, indicated that DHFR mRNA was efficiently translated in postreplicative cells. The results support a model wherein replication-dependent changes in DHFR synthesis rates are determined exclusively by changes in DHFR mRNA levels.

Most replicative enzymes are expressed at very low levels. However, expression of dihydrofolate reductase (DHFR), an enzyme involved in DNA precursor biosynthesis, can be increased two to three orders of magnitude above the wild-type level by gene amplification. In highly amplified cell lines, DHFR constitutes as much as 10% of soluble cellular protein (1, 20); however, by all measured criteria, DHFR regulation appears to be identical in amplified and wild-type cells (17, 31, 36). Therefore, amplified cells are valuable for biochemical studies of the mechanisms regulating expression of the DHFR gene.

We recently developed the only reported amplified cell lines capable of undergoing terminal differentiation, mouse muscle cell lines H⁻αR50A and H⁻αR50T (31, 32). Using these cells, we demonstrated that levels of DHFR mRNA are transcriptionally down-regulated during myogenic withdrawal from the cell cycle (32). In the results that follow, a more highly amplified subline of H⁻αR50T, designated H⁻αR300T, was used to investigate whether a translational mechanism contributes to regulation of DHFR protein synthesis. The importance of this study is best illustrated by recent findings concerning S phase-dependent synthesis of thymidine kinase (TK). Numerous early studies had established a positive correlation between TK mRNA levels, TK activity levels, and the replicative state of the cell population (9, 12, 16, 25, 27, 30). However, more recent studies in which TK mRNA levels and TK synthesis rates were measured quantitatively revealed that, at least during myogenic withdrawal from the cell cycle, TK synthesis rates are not primarily limited by levels of TK mRNA in non-S phase cells (10, 11, 33). Rather, the translational efficiency of TK mRNA is reduced (11, 33). If DHFR was subject to the same translational regulation as TK, amplified cells could be used to facilitate biochemical analysis of the translational control mechanism. To test this possibility, we specifically tested whether the translational efficiency of DHFR mRNA, like that of TK message, decreased as muscle cells withdrew from the cell cycle during commitment.

MATERIALS AND METHODS

Parental muscle cell line H⁻α, a diploid hypoxanthine-guanosine phosphoribosyl-transferase-deficient substrain of the MM14D mouse skeletal myoblast line developed by Linkhart et al. (24), was used to derive methotrexate-resistant cell line H⁻αR50T (32). Cell line H⁻αR300T was derived from H⁻αR50T by further serial selection in 100, 200, and 300 μM methotrexate (A6770; Sigma). Cells were grown for at least 10 cell generations at each selective step. H⁻αR300T was maintained in growth medium (basal medium [0.5× Ham's F-10, 0.5× Dulbecco modified Eagle medium, 0.4 mM additional $CaCl_2$, 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.2, 1% glucose, 2.45 mg of NaH_2CO_3 per ml, 10 U of penicillin G per ml, 0.5 mg of streptomycin sulfate per ml] supplemented with 15% horse serum, fibroblast growth factor activity from bovine brain [7], and 300 μM methotrexate). Commitment was induced by rinsing cells twice with basal medium and then incubating them in differentiation medium (basal medium supplemented with 300 μM methotrexate and 1 μM insulin).

A Southern blot was used to determine DHFR gene copy in the amplified cells. Cultures of amplified or parental cells were harvested by scraping in 350 μl of 1× TES (10 mM Tris [pH 7.5] at 25°C, 5 mM EDTA, 1% sodium dodecyl sulfate

* Corresponding author.

[SDS]) containing 200 μg of proteinase K per ml, and lysates were incubated for 1 h at 55°C. Sodium acetate was added to 0.3 M, lysates were extracted with phenol-chloroform and chloroform, and nucleic acids were precipitated by adding an equal volume of isopropanol. Pellets were washed with 70% ethanol and resuspended in 400 μl of 1× RD (10 mM Tris [pH 7.5] at 25°C, 5 mM EDTA, 0.3 M NaCl), RNase A was added to 100 μg/ml, and mixtures were incubated for 1 h at 37°C. Proteinase K was added to 50 μg/ml, and samples were incubated for 30 min at 55°C. After extraction with phenol-chloroform and chloroform, DNA was precipitated by adding 2.5 volumes of ethanol and incubating for 2 h at −20°C. DNA pellets were collected by centrifugation, washed with 70% ethanol, and resuspended in 400 μl of TE (10 mM Tris [pH 7.5] at 25°C, 1 mM EDTA). Sodium acetate was added to 0.3 M, DNA was precipitated and washed in 70% ethanol as above, and pellets were resuspended in 100 μl of TE. DNA concentrations were determined in duplicate by a fluorometric dye-binding assay (21) and by A_{260}.

Amplified cell DNA or p4D3000 plasmid DNA (32) (1 amol = 3.9 pg of the 5.9-kb plasmid) was mixed with 30 μg of chicken liver DNA and was incubated with 2 U of EcoRI/μg of DNA for 2 h at 37°C in 100-μl volumes. Reactions were stopped by adding 300 μl of TE, 30 μl of 3 M sodium acetate, and 1 ml of ethanol. DNA was precipitated for 1 h at −80°C, collected by centrifugation, and washed with 70% ethanol. DNA pellets were resuspended in 25 μl of TE, 5 μl of dye buffer (50% glycerol, 100 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol) was added, and samples were separated on a 1% agarose gel in 40 mM Tris-acetate (pH 8.0 [at 25°C])–2 mM EDTA. The gel was stained with ethidium bromide to visualize the markers and was photographed. DNA in the gel was denatured, neutralized, and blotted onto nitrocellulose as described previously (32). The blot was washed for 1 min in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), baked for 2 h at 80°C in a vacuum oven, and prehybridized for 4 h at 42°C in Stark's buffer (5× SSC, 25 mM sodium phosphate [pH 6.5 at 25°C], 250 μg of denatured sheared salmon sperm DNA per ml, 0.02% bovine serum albumin, 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone 40, 50% deionized formamide). The blot was hybridized for 36 h at 42°C in a mixture of 0.8× Stark's buffer and 10% dextran sulfate containing 5×10^6 cpm of denatured DHFR probe (random hexamer-labeled 3.4-kb EcoRI genomic insert from pDR34 [5]), washed as described previously (32), and exposed to X-ray film for 20 h with an intensifying screen.

Metaphase-chromosome spreads were prepared from $H^-\alpha$ and $H^-\alpha$R300T cells. Proliferative cultures were arrested in metaphase by incubation for 2 h in growth medium containing Colcemid at a final concentration of 0.5 μg/ml. Cells were harvested with collagenase and collected by centrifugation. Cell pellets were dispersed, resuspended in 3 ml of 75 mM KCl, and incubated at room temperature for 15 min. Cells were collected by centrifugation as above, dispersed, resuspended in fixative (75% methanol, 25% glacial acetic acid), and incubated at room temperature for 10 min with constant gentle agitation. Cells were collected as above, dispersed, resuspended in 3 ml of fresh fixative, and incubated for an additional 10 min. Cells were collected, dispersed, resuspended in 0.5 ml of fresh fixative, and dripped onto wet chilled slides. Slides were air-dried, flooded with hematoxylin (7.5 mg/ml, HHS-1; Sigma), rinsed with water, and air-dried. For fluorescent DNA detection, slides were stained for 10 min with 1 μg of ethidium bromide per ml in 50 mM sodium phosphate, pH 6.5, counter-stained with hematoxylin as above, and observed by fluorescence microscopy with a 515- to 565-nm filter.

DHFR synthesis rates were determined by in vivo labeling. Cultures were incubated for 1 or 2 h with 50 to 100 μCi of [^{35}S]Met (641 Ci/mmol; New England Nuclear) per ml in methionine-free Ham's F12-Dulbecco modified Eagle medium (50%, vol/vol) (GIBCO) supplemented with 1 μM insulin. Cultures were rinsed twice with ice-cold phosphate-buffered saline and stored at −80°C. Cells were scraped from dishes and sonicated in either 10 mM piperazine-*N*-*N*′-bis(2-ethanesulfonic acid (PIPES; pH 7.0)–150 mM KCl or 5 mM Tris (pH 7.8)–10 mM KCl–10 mM $MgCl_2$ (both buffers gave identical results). DNA concentration was analyzed by the method of Labarca and Paigen (21) and was used to calculate the number of cell equivalents in each lysate (10 pg of DNA per cell). Extracts were clarified and proteins were separated by electrophoresis through 12% polyacrylamide-SDS gels as described by Laemmli (22). Gels were fixed, stained with Coomassie blue, and destained as described previously (10). Destained gels were photographed, washed for 30 min in water, and incubated for 1 h in 1 M salicylic acid (S-3007; Sigma). Gels were rinsed with water, dried, and exposed to X-ray film with an intensifying screen. After exposure, Coomassie-blue-stained DHFR bands were excised, incubated for 12 to 24 h in 0.2 ml of 30% hydrogen peroxide at 65°C, mixed with 2.0 ml of scintillation fluid (2 parts toluene, 1 part Triton X-100, 4.0 mg of Omnifluor per ml), and assayed by liquid scintillation for 50 min.

DHFR mRNA levels were determined by RNase protection. The hybridization and digestion protocol, including a description of probes and DHFR pseudo-mRNA, has been published elsewhere (31, 32). Protected fragments were analyzed by denaturing gel electrophoresis and autoradiography or by liquid scintillation. In the latter case, RNase digestions were terminated by adjusting reactions to 0.5% (wt/vol) sodium pyrophosphate and 5% (wt/vol) trichloroacetic acid, 0°C. Samples were incubated on ice for >30 min, and nucleic acids were collected by washing samples through pre-wet 1-cm diameter GF/C filters (Whatman) with 5% trichloroacetic acid. Filters were rinsed with 70% ethanol, solubilized with 0.2 ml of Soluene, mixed with 2.0 ml of 4 mg/ml Omnifluor in toluene, and assayed by liquid scintillation for 50 min.

Polysomal RNA preparations were isolated by velocity sedimentation by using a modification of the methods of Gross and Merrill (11). All manipulations were performed on ice or at 4°C unless otherwise noted. Cell cultures on 10-cm dishes were rinsed with phosphate-buffered saline containing 10 μg of cycloheximide per ml and were either frozen at −80°C or harvested immediately (both procedures gave identical results). Cells were harvested by scraping in 300 μl of 1× (fresh dishes) or 150 μl of 2× (frozen dishes) lysis mix (1× = 50 mM Tris [pH 7.5 at 25°C], 250 mM NaCl, 25 mM $MgCl_2$, 0.5% Triton X-100, 0.1 U of RNasin [Promega] per ml, 20 μg of cycloheximide per ml, 2 mM dithiothreitol). Lysates were transferred to 15-ml glass Corex tubes and clarified by centrifugation for 10 min at 13,000 rpm in a Sorvall SS-34 rotor. Clarified lysates were either stored frozen at −80°C or used immediately (both procedures gave identical results). Clarified lysates were carefully layered on 12-ml sucrose gradients (15 to 50% linear gradients in 50 mM Tris [pH 7.5 at 25°C], 250 mM NaCl, 25 mM $MgCl_2$, 20 μg of cycloheximide per ml). Sedimentation was performed in a Beckman SW40 rotor at 32,000 rpm for 130 min. Gradients were fractionated by injecting a 65% sucrose solution into the bottom of the centrifuge tubes (0.375 ml/min) and col-

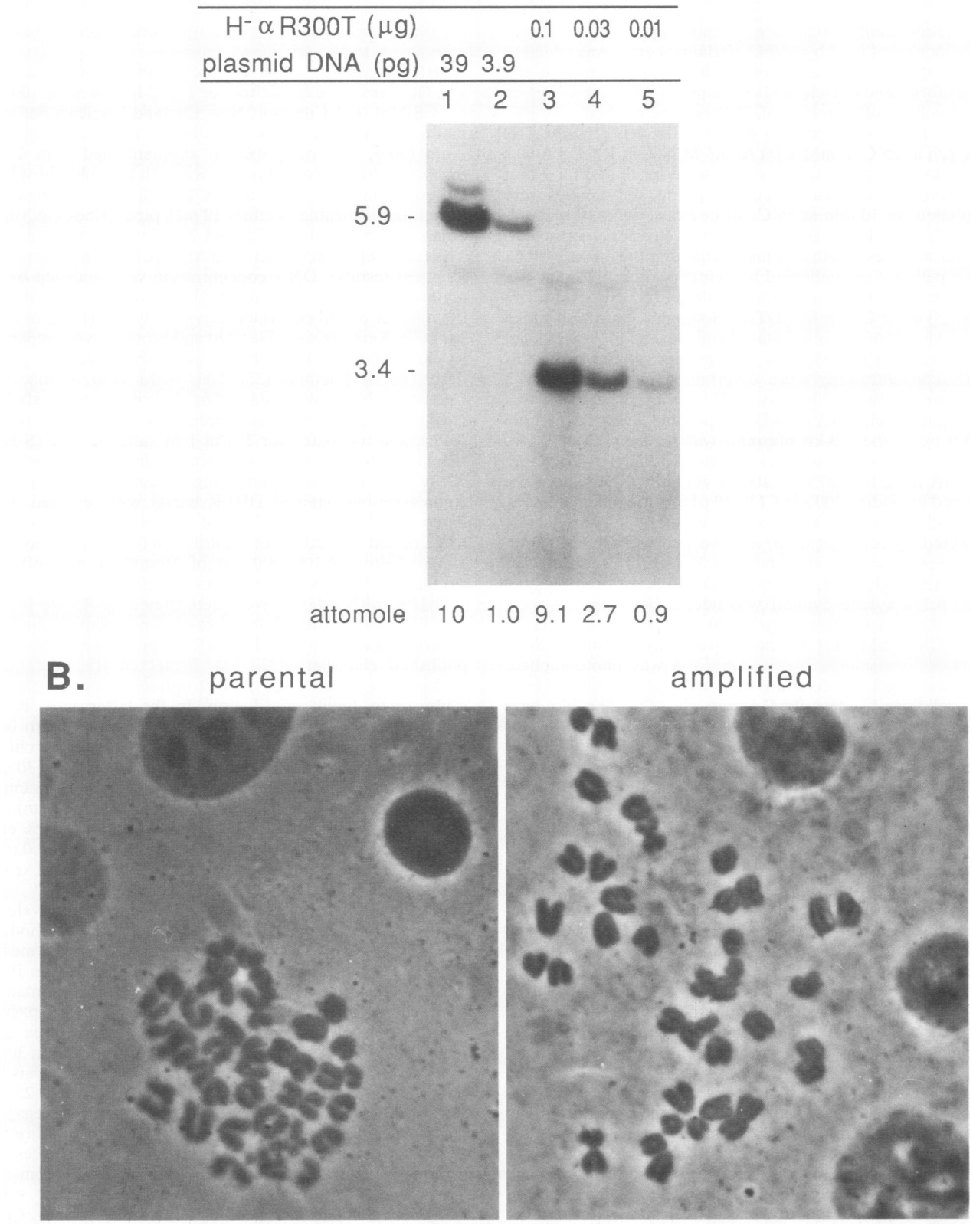

FIG. 1. Southern blot and metaphase analysis of amplified cell line H$^-$αR300T. (A) DHFR gene copy number in cell line H$^-$αR300T. Samples containing indicated amounts of cellular DNA or plasmid p4D3000 DNA were mixed with 30 μg of chicken liver DNA, digested with *Eco*RI, and analyzed on a Southern blot. The blot was probed with a 3.4-kb *Eco*RI genomic DNA fragment spanning the *dhfr* transcription initiation site. Band sizes (in kilobase pairs) are indicated; band intensities were determined by laser densitometry and are represented as molar amounts of DHFR DNA per sample (bottom). (B) Phase-contrast micrographs of parental and amplified cell metaphase-chromosome spreads. Mouse chromosomes are acrocentric. In both H$^-$α and H$^-$αR300T cells, fusion of acrocentric chromosomes frequently gave chromosomes with a metacentric appearance. Both cell lines exhibited a diploid number of chromosomes (2N = 40).

lecting the gradient at the top with an Isco model 185 density gradient fractionator. An Isco type 6 optical unit (λ = 254 nm) and UA-5 recorder (calibrated immediately before each use) were used to monitor absorbance during fractionation. Aliquots of each gradient (0.5 ml) were collected and stored at −20°C. The time delay between a sample passing the optical unit and being recovered was determined and used to align each aliquot with the scan. Aliquots were thawed and pooled to represent different polysomal and nonpolysomal fractions. Subsequent steps were performed at room temperature except as noted. Each pooled fraction was adjusted to 2.5 ml with water and received 280 μl of 10× TES. *Saccharomyces cerevisiae* RNA was added to 25 μg/ml, and proteinase K (Boehringer) was added to 20 μg/ml. Samples were incubated for 45 min at 55°C, adjusted to 250 mM NaCl, and were extracted with phenol-chloroform and chloroform and precipitated with ethanol at −20°C. RNA was collected by centrifugation. RNA pellets were washed with 70% ethanol, resuspended in 20 μl of water at 65°C, and assayed by RNase protection (10 μl per assay) (31, 32).

RESULTS

Amplified cell line H⁻αR300T. To determine the translational efficiency of a message, one must accurately measure both the cellular concentration of the mRNA and the rate of synthesis of the protein. We recently described a sensitive RNase protection assay for quantitating DHFR mRNA (31, 32); an equally quantitative means of measuring DHFR synthesis rates was needed. Recovery of [^{3}H]methotrexate binding activity after blockage of existing sites with nonlabeled methotrexate (17, 31, 36) was judged to be too indirect, inaccurate, and insensitive a measure of DHFR synthesis. We therefore investigated whether DHFR was sufficiently abundant in amplified muscle cells to allow direct electrophoretic analysis. The previously described amplified muscle cell line H⁻αR50T (32) did not produce an electrophoretically distinguishable DHFR protein band. Therefore, a more highly amplified substrain, H⁻αR300T, resistant to 300 μM methotrexate, was isolated.

A Southern blot was used to determine the DHFR gene copy number in H⁻αR300T (Fig. 1A). Comparison of the DHFR-specific band intensities from the amplified cells (lanes 3 to 5) with the signal arising from a standard curve generated with plasmid p4D3000 DNA (lanes 1 and 2) indicated that cell line H⁻αR300T contained 90 amol of DHFR genes per μg of DNA. On the basis of an estimate of 10 pg of DNA per cell, cells contained 540 DHFR genes per cell (270-fold amplification).

Karyotypic examination of cell line H⁻αR300T was used to determine whether amplification of the DHFR gene was accompanied by any gross chromosomal aberrations (Fig. 1B). Surprisingly, the cells remained diploid (2N = 39.9 ± 1.9; n = 33) despite drug selection, large-fold gene amplification, and isolation from the parental cell line for over 100 cell generations. Karyotypic examination also revealed no double-minute chromosomes (Fig. 1B). This finding was unexpected inasmuch as Kaufman et al. (18) have reported that amplified copies of DHFR genes are preferentially located in double-minute chromosomes in mouse cell lines. As a more sensitive test for double minutes, slides bearing metaphase-chromosome spreads were stained with ethidium bromide and observed by fluorescence microscopy (18). No extrachromosomal DNA was detected (n = 12 metaphase spreads; data not shown).

Rates of DHFR synthesis during commitment. Proliferating

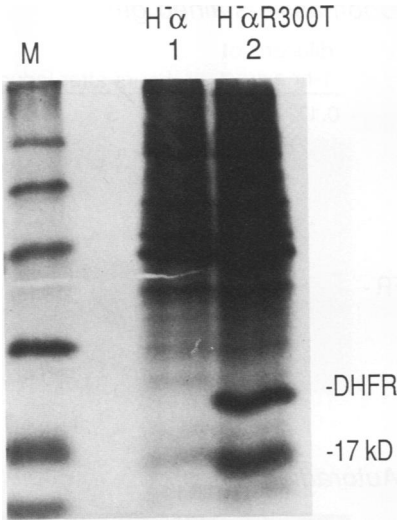

FIG. 2. Synthesis of DHFR in proliferative muscle cells. Proliferative cultures of parental H⁻α (lane 1) and amplified H⁻αR300T (lane 2) cells were incubated for 1 h in 50 μCi of [^{35}S]Met per ml. Labeled proteins in clarified lysates (10^5 cell equivalents per lane) were separated by electrophoresis through a 12% polyacrylamide-SDS gel and visualized by autoradiography. The 23-kDa DHFR protein band visible in H⁻αR300T cells is indicated, as is a 17-kDa protein of unknown identity. Molecular weight markers (lane M) were (from top) 200, 97, 68, 43, 29, 18, and 14 kDa. kD, kilodalton.

H⁻αR300T muscle cells were labeled briefly with [^{35}S]Met, and proteins in clarified whole-cell lysates were separated by SDS-gel electrophoresis. As shown in the autoradiogram in Fig. 2, amplified H⁻αR300T cells (lane 2) gave a strong band at 23 kDa, which corresponded to the molecular mass of DHFR protein. Nonamplified H⁻α parental cells showed no band at 23 kDa (lane 1). The presence of a 23 kDa band in amplified cells, and its absence in nonamplified cells, was also evident when Coomassie-blue staining was used to visualize steady-state protein levels (data not shown). In both the autoradiogram and the Coomassie-blue-stained gel, all other protein bands were of similar intensity and mobility in amplified and nonamplified cells, with the exception of a band at 17 kDa. The band at 17 kDa was enriched in H⁻αR300T extracts and may have been due to another protein encoded within the *dhfr* amplicon.

On the basis of its mobility and its absence in control cells, we concluded that the prominent 23-kDa band present in amplified cells represented DHFR protein. Published results have established that DHFR protein has a half-life of 50 to 100 h in proliferative cells (1, 28, 31) and that there is no detectable change in the half-life of DHFR during muscle cell commitment (31). Because DHFR degradation is negligible during short labeling periods, DHFR-associated radioactivity after a 1- to 2-h incubation with [^{35}S]Met accurately represents the rate of [^{35}S]Met incorporation into DHFR protein. Furthermore, the rate of [^{35}S]Met incorporation into total protein changes only slightly with commitment (10), indicating that the specific activity of the [^{35}S]Met pool remains relatively constant. By normalizing DHFR-specific radioactivity to total [^{35}S]Met incorporation, the rate of DHFR protein synthesis can be accurately determined.

Having established that DHFR synthesis rates could be directly measured in H⁻αR300T cells, we asked whether rates of DHFR synthesis changed during myogenic with-

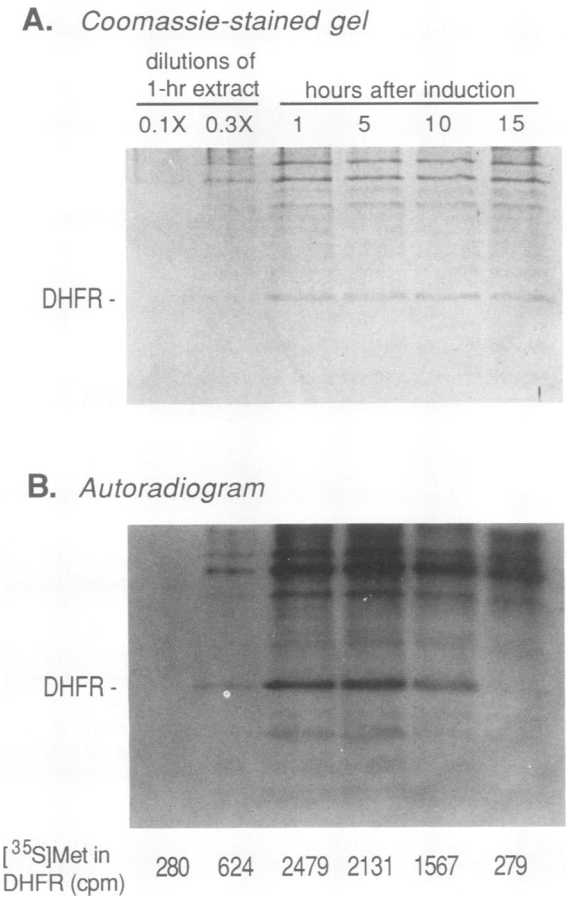

FIG. 3. DHFR synthesis rates during commitment. At indicated times after commitment was induced, H⁻αR300T cells were incubated for 2 h in 100 μCi of [35S]Met per ml. Proteins in clarified lysates (10^5 cell equivalents per lane for undiluted sample) were separated by electrophoresis through 12% polyacrylamide-SDS gels. Three gels were run; a typical gel is shown. (A) Coomassie-blue-stained gel. (B) Autoradiogram of the gel in panel A. The DHFR bands were excised and analyzed by liquid scintillation. Radioactivity data (in counts per minute) represent the average of all three gels. The DHFR band is indicated to the left of each panel.

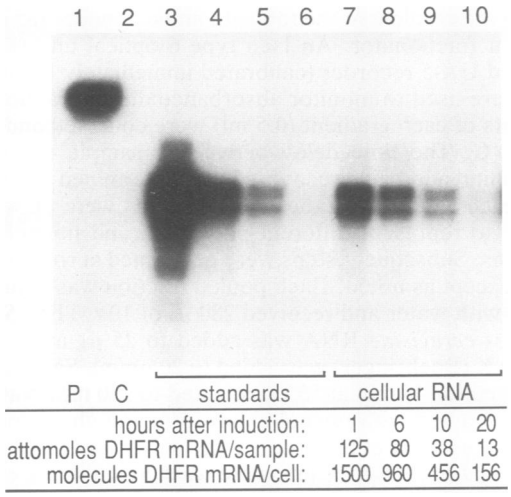

FIG. 4. DHFR mRNA levels during muscle cell commitment. RNA was harvested from H⁻αR300T cells at specified times after commitment was induced, and DHFR mRNA levels were quantitated by RNase protection. Experimental samples contained 5×10^4 cell equivalents (0.5 μg) of RNA (lanes 7 to 10). Standard curve samples contained 640, 160, 40, and 10 amol of DHFR pseudo-mRNA (lanes 3 to 6). All hybridization mixtures contained 10 fmol of probe and 50 μg of RNA (cellular or standard curve RNA was supplemented with yeast RNA to 50 μg). Lane C was a control that showed no signal arose from yeast RNA. Lane P contained 300 amol of nondigested probe. After RNase digestion, half of each sample was run on the gel shown. The remainder was precipitated with trichloroacetic acid and assayed by liquid scintillation. Standard curve signals were used to determine the number of attomoles of DHFR mRNA in each sample, which in turn were converted to DHFR mRNA molecules per cell on the basis of the number of cell equivalents in each sample.

drawal from the cell cycle. Figure 3A shows a Coomassie-blue-stained gel containing labeled H⁻αR300T cell extracts harvested 1, 5, 10, and 15 h after inducing commitment. (No cells withdraw from the cell cycle during the first 2 h after induction, and virtually all cells withdraw by 15 h [31]; thus, the 1-h cells were fully proliferative and the 15-h cells were fully committed.) Roughly equivalent amounts of the 23-kDa DHFR band were present at all time points, consistent with the previous finding that, because of the extreme stability of the protein, absolute levels of DHFR enzyme do not decrease significantly during commitment (31). An autoradiogram of the gel is shown in Fig. 3B. The intensity of the 23-kDa DHFR signal decreased significantly during commitment. To relate the difference in intensity of the autoradiographic signal to the magnitude of change in DHFR synthesis rate, 0.1× and 0.3× dilutions of the proliferative (1 h) cell extract were run in parallel lanes. On the basis of a comparison of autoradiographic signal intensities, DHFR synthesis decreased to 10% that of proliferative cells by 15 h after induction. A more quantitative measure of DHFR synthesis was obtained by liquid scintillation analysis of excised bands. Results (in counts per minute) are listed below each lane. On the basis of liquid scintillation results, the magnitude of the decrease in DHFR synthesis was 7.6-fold by 15 h.

Translational efficiency of DHFR mRNA during commitment. To measure DHFR mRNA levels, an RNase protection assay was used (31, 32). In this assay, cellular RNA or synthetic DHFR pseudo-mRNA standards are hybridized with radiolabeled RNA probes complementary to specific regions of the DHFR message. After RNase digestion, protected probe regions are separated from low-molecular-weight digestion products by gel electrophoresis or by precipitation with trichloroacetic acid. Comparison of cellular RNA signals with standards allows precise quantitation of the absolute number of DHFR molecules per cell.

Figure 4 shows an RNase protection assay of DHFR mRNA levels in H⁻αR300T cells. Proliferative H⁻αR300T cells (lane 7) exhibited 1,500 DHFR mRNA molecules per cell. (In contrast, proliferative nonamplified cells contain four DHFR messages [31].) Upon induction, levels of DHFR mRNA decreased, which indicated that the mechanisms regulating DHFR mRNA levels were not inactivated during selection of cell line H⁻αR300T. Comparison of the 20-h signal with the standard curve indicated that committed cells contained 156 DHFR mRNA molecules per cell (lane 10), a 9.6-fold decrease.

To test whether the translational efficiency of DHFR mRNA decreased during commitment, parallel measurements of DHFR synthesis rates and DHFR mRNA levels

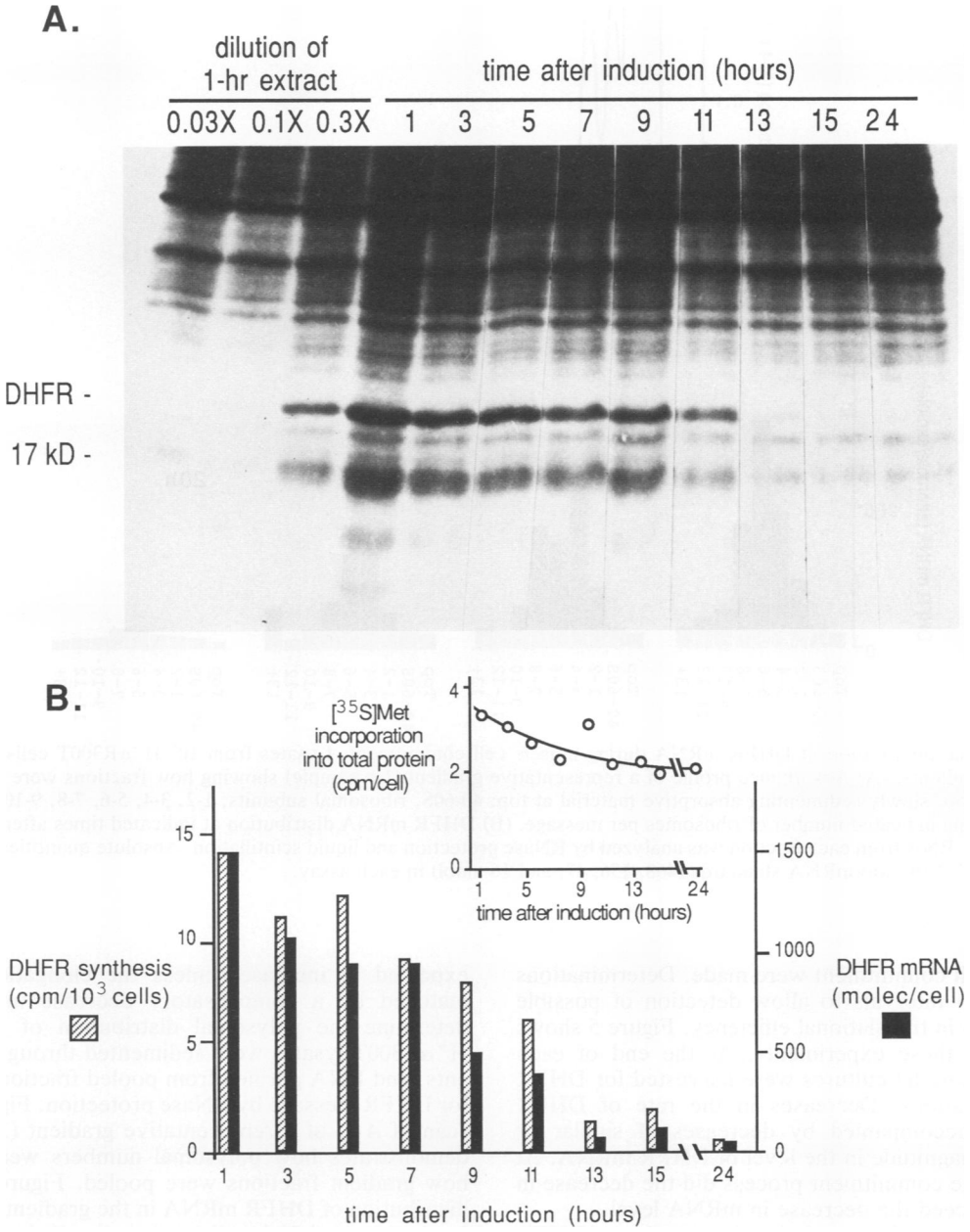

FIG. 5. DHFR synthesis rates and DHFR mRNA levels during muscle cell commitment. Parallel cultures of H$^-$αR300T cells were harvested for DHFR synthesis rate and DHFR mRNA level determinations. (A) DHFR synthesis rates. Cultures were incubated for 1 h in 100 μCi of [^{35}S]Met per ml. Proteins in clarified lysates (10^5 cell equivalents per lane) were separated by electrophoresis through 12% polyacrylamide-SDS gels and visualized by autoradiography. DHFR bands were excised and assayed for associated radioactivity by liquid scintillation. The positions of the DHFR band and the 17-kDa (17-kD) amplified band are indicated. All other bands represent rates of synthesis of other major cellular proteins and serve as controls indicating that overall rates of protein synthesis were maintained during withdrawal from the cell cycle. The three leftmost lanes contain dilutions of the 1-h H$^-$αR300T sample (supplemented with proliferative cell extracts from identically labeled nonamplified cells, such that 10^5 cell equivalents were loaded). Background radioactivity was determined by measuring the DHFR-associated radioactivity in dilutions of the 1-h sample and extrapolating to zero-amplified cell equivalents. DHFR mRNA levels were quantitated by RNase protection by using 5×10^4 cell equivalents (0.5 μg) of RNA. After RNase digestion, samples were precipitated with trichloroacetic acid and assayed by liquid scintillation. Standard curve signals were used to determine the number of attomoles of DHFR mRNA in each sample, which in turn were converted to DHFR mRNA molecules per cell on the basis of the number of cell equivalents in each sample. (B) Summary of the data from three polyacrylamide-SDS gel analyses of DHFR synthesis rates (hatched bars) and four RNase protection analyses of DHFR mRNA levels (solid bars). The inset shows total incorporation of [^{35}S]Met at each time point. To correct for possible variations in the specific activity of the [^{35}S]Met pool, DHFR-specific radioactivity was normalized to total incorporated radioactivity for each time point. molec, molecules.

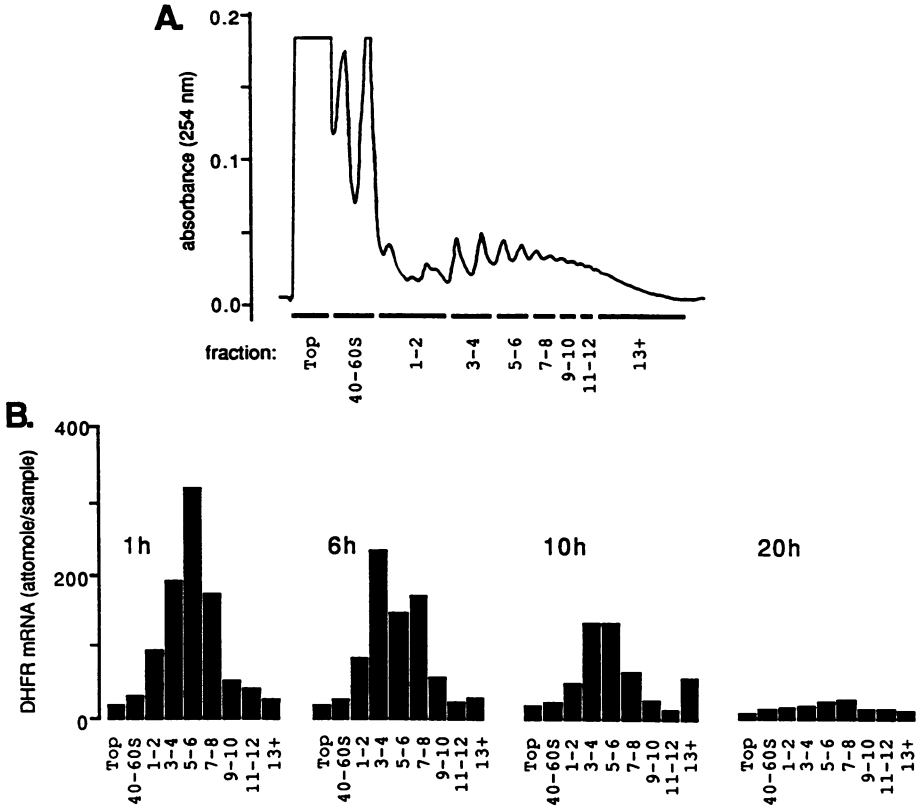

FIG. 6. Polysomal distribution of DHFR mRNA during muscle cell commitment. Lysates from 10^6 $H^-\alpha R300T$ cells were sedimented through sucrose gradients. (A) Absorbance profile of a representative gradient (1-h sample) showing how fractions were pooled for DHFR mRNA analysis. Top, slowly sedimenting absorptive material at top; 40-60S, ribosomal subunits; 1-2, 3-4, 5-6, 7-8, 9-10, 11-12, and 13+, polysomes containing indicated number of ribosomes per message. (B) DHFR mRNA distribution at indicated times after commitment was induced. Half of the RNA from each fraction was analyzed by RNase protection and liquid scintillation. Absolute quantities were determined by inclusion of DHFR pseudo-mRNA standards (468, 156, 47, and 16 amol) in each assay.

during muscle cell commitment were made. Determinations were done at 2-h intervals to allow detection of possible transient changes in translational efficiency. Figure 5 shows the results from these experiments. At the end of each labeling period, parallel cultures were harvested for DHFR mRNA determinations. Decreases in the rate of DHFR synthesis were accompanied by decreases of similar or slightly greater magnitude in the level of DHFR mRNA. At no time during the commitment process did the decrease in synthesis rate exceed the decrease in mRNA level.

As an additional test for translational repression of DHFR synthesis, the polysomal distribution of DHFR mRNA was determined at various times during commitment. Usually, in reports of translational regulation, rates of translational initiation are reduced (6, 15), often via sequestration of mRNA in nonpolysomal ribonucleoprotein (2, 14). Regulation of translational initiation would cause characteristic changes in the polysomal distribution of DHFR message. If the rate of translational initiation on a particular message was reduced, the mean number of ribosomes engaged on each mRNA would decrease, leading to a measurable shift in the message distribution to smaller polysomes. If initiation was blocked completely, such as when message is sequestered as ribonucleoprotein, the message would disappear from the polysomal fractions and appear in the more slowly sedimenting nonpolysomal fractions. Conversely, if translational elongation was inhibited, polysome size would be expected to increase (unless the elongational block was matched by a compensatory decrease in initiation). To determine the polysomal distribution of DHFR mRNA, $H^-\alpha R300T$ lysates were sedimented through sucrose gradients, and RNA purified from pooled fractions was analyzed for DHFR message by RNase protection. Figure 6A shows a scan of A_{254} of a representative gradient (1-h sample) and demonstrates how polysomal numbers were assigned and how gradient fractions were pooled. Figure 6B shows the distribution of DHFR mRNA in the gradients prepared from 1-, 6-, 10-, and 20-h cell lysates. At all times, only a small fraction of the DHFR message was in the nonpolysomal material at the top of the gradient. Also, no significant shift in DHFR polysome size during commitment was observed. At all time points, an average of five to six ribosomes were engaged on each DHFR mRNA.

In conclusion, changes in the level of DHFR mRNA were sufficient to account entirely for changes in the rate of DHFR protein synthesis. Our results are consistent with a model wherein DHFR synthesis rates are limited solely by levels of DHFR mRNA.

DISCUSSION

Previous studies have established that rates of DHFR synthesis (1, 17, 19, 20, 23, 26, 31, 36, 37) and levels of DHFR mRNA (8, 13, 19, 20, 23, 29, 31, 32, 37) are replica-

tion dependent. However, only two of the above studies measured DHFR mRNA levels and DHFR synthesis rates quantitatively and in parallel (23, 37) and thereby could have detected changes in the translational efficiency of DHFR mRNA. Both studies investigated activation of the DHFR gene during induction of growth-arrested cells and found no evidence for translational regulation of the DHFR gene. However, it was possible that general metabolic recovery after growth induction masked significant changes in the translational efficiency of DHFR mRNA.

In the present study, to determine whether a translational mechanism regulated DHFR expression when cells ceased replication but remained otherwise metabolically active, DHFR mRNA levels and DHFR synthesis rates were quantitatively compared during muscle cell differentiation. Whereas rates of DHFR synthesis (and to a lesser extent the 17-kDa protein) decreased dramatically during commitment, rates of synthesis of all other detectable cellular proteins remained constant (Fig. 5A), demonstrating that the committed cells were metabolically active. At all times during the commitment process, changes in the rates of DHFR synthesis were matched quantitatively by changes in the levels of DHFR mRNA. Furthermore, the polysomal distribution of DHFR message showed that shifts characteristic of translational regulation did not occur during commitment. It is possible to repress translation of an mRNA without causing a shift in the polysomal distribution of the message. For example, the rates of both translational initiation and elongation may change to the same degree. Although rare, there have been reports of translational repression without a corresponding shift in the polysomal mRNA distribution (3, 4, 11, 34, 35). Interestingly, one example of translational repression without a shift in polysome size is the translational regulation of TK (11). On the basis of both the polysomal data and direct measurements of DHFR synthesis rates and mRNA levels, we concluded that, in contrast to TK mRNA, DHFR mRNA was translated with the same efficiency in committed cells as in proliferative cells.

Our investigations to date on DHFR regulation are consistent with the following model. As muscle cells complete replication, transcription of the DHFR gene is repressed. As a result, DHFR pre-mRNA levels decrease, leading to a decrease in DHFR mRNA levels. The decrease in mRNA levels exceeds the decrease in pre-mRNA levels (32), implying that a change in message stability contributes to the overall reduction in DHFR mRNA. DHFR mRNA is translated at the same rate in proliferative and committed cells; however, because mRNA levels are much lower in committed cells, DHFR synthesis rates are greatly reduced. Because DHFR is a long-lived protein (half-life = 50 to 100 h), the decrease in DHFR synthesis does not lead to a reduction in cellular DHFR levels. Rather, it prevents overaccumulation of DHFR protein in nonreplicating cells. Because we have found that changes in DHFR synthesis rates in muscle cells are determined primarily by changes in DHFR mRNA levels, future investigations will be directed at resolving the mechanisms by which transcription of the DHFR gene is repressed during commitment. The highly amplified myogenic cell line described in this article should prove valuable for these pursuits.

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