Specific Binding of Human Dihydrofolate Reductase Protein to Dihydrofolate Reductase Messenger RNA in Vitro

Edward Chu,* Chris H. Takimoto, Donna Voeller, Jean L. Grem, and Carmen J. Allegra

NCI-Navy Medical Oncology Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland 20892

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ABSTRACT: Dihydrofolate reductase (DHFR) is a critical enzyme in de novo purine and thymidylate biosynthesis. An RNA gel mobility shift assay was used to demonstrate a specific interaction between human recombinant DHFR protein and its corresponding DHFR mRNA. Incubation of DHFR protein with either its substrates, dihydrofolate or NADPH, or with an inhibitor, methotrexate, repressed its ability to interact with DHFR mRNA. An in vitro rabbit reticulocyte lysate translation system was used to show that the addition of exogenous human recombinant DHFR protein to in vitro translation reactions specifically inhibited DHFR mRNA translation. These studies suggest that the direct interaction between DHFR protein and its mRNA may be a mechanism for regulation of DHFR synthesis.

The enzyme dihydrofolate reductase (DHFR) (E.C. 1.5.1.3) catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate, a key intermediate in one-carbon transfer reactions. For this reason, DHFR is required for the de novo synthesis of purines, thymidylate, and certain amino acids (Blakley, 1969; Allegra, 1990). Because of its critical role in maintaining the cellular pool of reduced folates, DHFR represents an important target enzyme in cancer chemotherapy.

Intracellular levels of DHFR are regulated by various factors, including the concentration of serum factors, changes in cyclic AMP levels, viral infection, and exposure to antimetabolites such as methotrexate (MTX) and the fluoropyrimidines (Frearson et al., 1967; Kellems et al., 1979; Hendrickson et al., 1980; Gudewicz et al., 1981; Yoder et al., 1983; Schuetz et al., 1988). Alterations in DHFR expression result from gene amplification (Alt et al., 1976; Nunberg et al., 1978; Kaufman et al., 1979) as well as from both transcriptional (Wiedemann et al., 1979; Wu & Johnson, 1982; Santiago et al., 1984) and posttranscriptional (Bertino et al., 1965; Hillcoat et al., 1967; Cowan et al., 1986) regulatory mechanisms. Recent studies have demonstrated that exposure of cultured human cancer cell lines to the antifol MTX results in an acute increase in DHFR protein that is independent of the levels of DHFR mRNA and DHFR gene copy number (Domin et al., 1982; Bastow et al., 1984). It has been proposed that the induction of DHFR protein in response to MTX exposure may result from a translational control mechanism (Bastow et al., 1984). To date, however, there have been no studies to address directly the role of either RNA/protein interactions or other forms of translational control in regulating DHFR expression. In the present study, we demonstrate that human recombinant DHFR protein binds specifically to its own mRNA in vitro. Our findings suggest that this interaction is associated with inhibition of DHFR mRNA translation.

MATERIALS AND METHODS

Isolation of Total RNA. Isolation of total cellular RNA from human breast cancer MCF-7 cells was based on the method of Chomczynski and Sacchi (1987) and was performed as previously described (Chu et al., 1991a). The RNA concentration was determined by measuring UV absorbance at 260 nm, and the integrity of RNA was verified by electrophoresis of an aliquot on a 1% formaldehyde–agarose gel.

Reverse Transcription of RNA. Synthesis of DHFR cDNA from total cellular RNA was performed by a modification of the method of Gubler and Hoffman (1983) as described by Dicker et al. (1989). Using the Strategene first-strand cDNA synthesis protocol (Stratagene, San Diego, CA), 10 μg of total cellular RNA was incubated with 3 μL of random primers for 5 min at 65 °C. The mixture was allowed to cool at room temperature for 10 min, to which 5 μL of transcription buffer, 5 μL of 0.1 mM dithiothreitol, 1 μL of RNase block, 3 μL of a 25 mM deoxynucleotide triphosphate solution, and 1 μL of M-MMLV reverse transcriptase (20 units/μL) were then added. The reaction was incubated for 1 h at 37 °C, and the solution was then stored at −20 °C.

Polymerase Chain Reaction Conditions. DNA from the first-strand synthesis reaction was used as template for PCR amplification. The reaction conditions were those specified by the Perkin-Elmer Cetus protocol (Perkin-Elmer Cetus, Emeryville, CA); amplification was performed in a total volume of 100 μL. Primer DHFR 1 (ATCGGATCC-CCGCCAAACTTGACCGCGCGT) corresponds to a sequence that starts 10 bases downstream from the presumptive DHFR transcriptional start site (Chen et al., 1984) and represents the 5′ primer. The *Bam*HI restriction site, as underlined, was included at the 5′-end of primer DHFR 1. Primer DHFR 2 (ATCAAGCTTACTTTTCTAATGTA-AAAAT) corresponds to a sequence 77 bases downstream from the termination codon and represents the 3′ primer. The *Hin*dIII restriction site, as underlined, was included at the 5′-end of primer DHFR 2. These two primers define a sequence that includes the entire coding region of human DHFR. PCR conditions employed were according to Dicker et al. (1989).

Preparation of Plasmid Constructs and in Vitro mRNA Transcription. PCR products were analyzed by nondenaturing gel electrophoresis. DNA corresponding to DHFR was isolated as previously described (Dicker et al., 1989). Purified DHFR DNA was digested with *Bam*HI and *Hin*dIII and cloned into the *Bam*HI and *Hin*dIII sites of pGEM-4Z (Promega, Madison, WI). The sequence was confirmed by the dideoxynucleotide method of Sanger et al. (1977). DHFR

mRNA was synthesized with SP6 RNA polymerase after linearization with *Hin*dIII. Human c-*myc* cDNA was a generous gift from Shoshana Segal (NCI-Navy Medical Oncology Branch, National Cancer Institute), and it was transcribed into its corresponding mRNA by using SP6 RNA polymerase. Human chromogranin A cDNA was a gift from Lee Helman (Pediatric Branch, National Cancer Institute), and its corresponding chromogranin A RNA was synthesized as previously described (Chu et al., 1991b). Yeast and human preplacental lactogen RNAs were supplied in the protein-processing translation system (New England Nuclear–Dupont), and brome mosaic virus (BMV) was obtained from Promega (Madison, WI). All mRNA transcripts were evaluated on a 1% formaldehyde–agarose gel to verify their integrity and size. The concentration of unlabeled RNA was determined by spectrophotometry. Labeled transcripts were made by inclusion of [α-^{32}P]CTP at 200 Ci/mmol, and the concentration of radioactively labeled RNA was calculated from the specific activity of ^{32}P incorporation.

RNA–Protein Binding Assay. Electrophoretic gel mobility shift assays were performed as previously described (Leibold & Munro, 1988; Chu et al., 1991b). Labeled RNAs (0.04 pmol) were incubated with human recombinant DHFR protein (30 pmol) (Huang et al., 1989), a generous gift from James Freisheim (Department of Biochemistry, Medical College of Ohio, Toledo, OH), for 15 min at room temperature. RNase T1 (3 units; Boehringer Mannheim) was then added for 10 min, followed by incubation with heparin (5 mg/mL; Sigma) for an additional 10 min at room temperature. The entire reaction sample (total volume, 30 μL) was resolved on a 4% polyacrylamide (acrylamide/methylenebis[acrylamide] 100/1) gel (gel dimensions, 15 × 17 cm) for approximately 2 h at 450 V. Gels were dried and then visualized by autoradiography.

Competition experiments were performed with human recombinant DHFR protein (30 pmol) and radiolabeled DHFR mRNA (0.04 pmol; 50 000 cpm). These conditions were selected on the basis of experiments using a fixed amount of DHFR mRNA with varying concentrations of DHFR protein to determine linearity of binding. Unlabeled competitor RNAs were mixed with labeled probes prior to the addition of human recombinant DHFR protein. In competition experiments examining the effect of either dihydrofolate, NADPH, MTX, 5-methyltetrahydrofolate, or 5-fluorouracil (5-FU) on DHFR protein binding, each of these compounds was preincubated with DHFR protein for 15 min prior to incubation with radiolabeled DHFR mRNA.

In Vitro Translation. In vitro translation reactions (total volume, 30 μL) were performed using the New England Nuclear rabbit reticulocyte lysate system as previously described (Chu et al., 1991b). Translation products were analyzed by SDS–PAGE (12.5% acrylamide) according to the method of Laemmli (1970).

RESULTS

The interaction between DHFR protein and its corresponding DHFR mRNA was examined using an electrophoretic gel mobility shift assay. For these experiments, we used a 700-nt human DHFR mRNA that includes the entire protein-coding region, 59 nt of the 5′-untranslated region (UTR), and 77 nt of the 3′-untranslated region (Figure 1). This DHFR mRNA transcript includes an additional 37 nucleotides in its 5′-UTR when compared to those previously reported (Masters & Attardi, 1983; Masters et al., 1983; Chen et al., 1984). A number of studies have now identified the

FIGURE 1: Map of the human DHFR cDNA. The 700-nt DHFR cDNA includes 59 nt of 5′-untranslated region, the entire coding region, and 77 nt of 3′-untranslated region. The sequence of the complete 5′-UTR is CAAACTTGACCGCGCGTTCTGCTG-TAACGAGCGGGCTCGGAGGTCCTCCCGCTGCTGTC. Sequence coordinates are according to Masters and Attardi (1983).

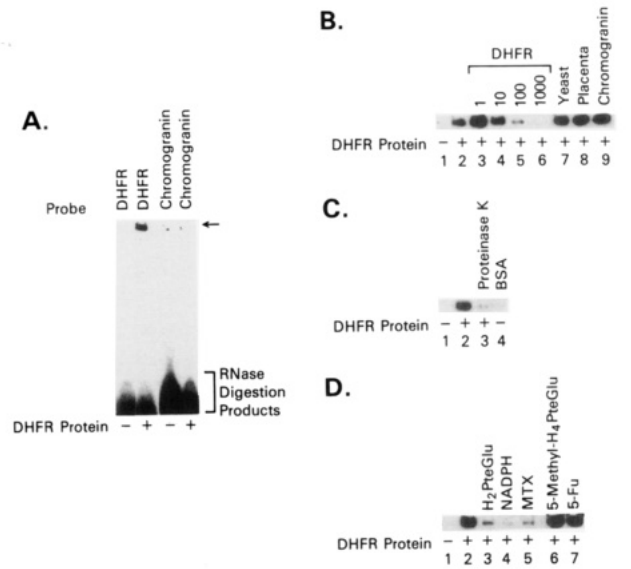

FIGURE 2: Specific binding of DHFR protein to DHFR mRNA. (A) Labeled human DHFR RNA (50 000 cpm, 0.04 pmol; lanes DHFR) and human chromogranin RNA (50 000 cpm, 0.04 pmol; lanes chromogranin) were incubated in the absence or presence of homogeneously pure human recombinant DHFR protein (30 pmol). The specific complex is indicated by the arrow. (B) Labeled DHFR RNA (50 000 cpm, 0.04 pmol) was incubated in the absence (lane 1) or presence (lane 2) of pure DHFR protein (30 pmol). This same amount of DHFR protein was included in reactions represented in lanes 3–9. Competition studies were performed with 1-fold (lane 3), 10-fold (lane 4), 100-fold (lane 5), and 1000-fold (lane 6) excess unlabeled DHFR RNA and 1000-fold molar excess of either yeast (lane 7), human preplacental lactogen (lane 8), or human chromogranin mRNA (lane 9). (C) DHFR protein was preincubated with proteinase K (1 μg/μL) for 15 min and then included in a reaction containing radiolabeled DHFR RNA (lane 3). Bovine serum albumin (30 pmol) was incubated with radiolabeled DHFR RNA (lane 4). (D) Labeled DHFR RNA (50 000 cpm, 0.04 pmol) was incubated in the absence (lane 1) or presence, where indicated (lanes 2–7), of DHFR protein (30 pmol). In lanes 3–7, DHFR protein was first incubated with either dihydrofolate (300 pmol) (lane 3), NADPH (300 pmol) (lane 4), MTX (30 pmol) (lane 5), 5-methyltetrahydrofolate (300 pmol) (lane 6), or 5-FU (300 pmol) (lane 7) for 15 min at room temperature, to which the radiolabeled DHFR mRNA was then added.

5′-UTR as a critical determinant in regulating various RNA/protein interactions (Hentze et al., 1987; Aziz & Munro, 1987; Parkin et al., 1988; Papadopoulou et al., 1990). For this reason, it was important to use a DHFR mRNA transcript with a more complete 5′-UTR.

When ^{32}P-radiolabeled DHFR mRNA (0.04 pmol) was incubated with homogeneously pure human recombinant DHFR protein (30 pmol), a complex that was not digested by RNase T1 was formed, resulting in the retarded migration of the protein-protected fragment of the radiolabeled RNA probe (Figure 2A, second DHFR lane). Similar results were obtained when a radiolabeled DHFR mRNA transcript contining only a 22-nt 5′-UTR was used (data not shown). In contrast, incubation of DHFR protein with an unrelated radiolabeled RNA probe such as chromogranin A mRNA,

which encodes for a neuroendocrine-related protein product that is not associated with DNA synthesis, did not result in such a complex (Figure 2A, second chromogranin lane). The bottom portion of this figure represents free, digested radiolabeled RNA probe (Figure 2A). To further support the specific nature of the DHFR protein/mRNA complex, competition studies were performed. Complex formation was completely abolished by a 100-fold molar excess of unlabeled DHFR mRNA (Figure 2B, lane 5). In contrast, concentrations of up to 1000-fold molar excess of unrelated cold, competitor RNAs such as yeast mRNA (Figure 2B, lane 7), human preplacental lactogen RNA (Figure 2B, lane 8), or human chromogranin mRNA (Figure 2B, lane 9) did not compete with radiolabeled DHFR mRNA probe for DHFR protein binding. Preincubation of DHFR protein with proteinase K (1 μg/μL) (Figure 2C, lane 3) completely eliminated formation of the retarded complex, a finding that emphasizes the importance of intact protein in this interaction. There was no protection of DHFR mRNA from RNase T1 digestion when it was incubated in the presence of an unrelated protein, bovine serum albumin (30 pmol) (Figure 2C, lane 4). Moreover, the addition of exogenous human recombinant thymidylate synthase (TS), a protein similar to DHFR in terms of its critical role in one-carbon metabolism and DNA biosynthesis, did not protect DHFR mRNA from ribonuclease digestion (data not shown), a finding that further supports the specificity of the DHFR protein-inhibitory effect.

The normal substrates for the DHFR-catalyzed reaction are β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), and the oxidized folate dihydrofolate. The effect of each of these ligands and of MTX on the ability of DHFR protein to interact with its corresponding mRNA was determined. Incubation of DHFR protein with either dihydrofolate (300 pmol), NADPH (300 pmol), or MTX (30 pmol) markedly diminished binding to radiolabeled DHFR mRNA (Figure 2D). In contrast, incubation of DHFR protein with substrate compounds not directly related to the DHFR-catalyzed reaction such as 5-methyltetrahydrofolate (300 pmol) or 5-FU (300 pmol) did not inhibit subsequent formation of the RNA/protein complex (Figure 2D). The concentration of each of the substrates used in this set of experiments was at least 10-fold higher than their respective binding affinities (K_m) for human DHFR. This was done to maximize the ability of each of these compounds to interact with the enzyme. The finding that inclusion of ligands capable of binding DHFR abolishes the protein/mRNA interaction suggests either that a certain conformational state of the protein is required or that the active site of the enzyme is essential for its interaction with DHFR mRNA.

To determine the functional significance of this specific RNA/protein interaction, the effect of exogenous DHFR protein on DHFR mRNA translation was examined by means of an in vitro rabbit reticulocyte lysate translation system. Translation of rabbit lysate in the absence of exogenous mRNA transcripts yielded two protein products with one resolving at approximately 47 kDa and the other at 27 kDa (Figure 3, lane 1). When human DHFR mRNA (1.7 pmol) was included in the rabbit lysate, a band that resolved at approximately 21 kDa was observed, corresponding to DHFR protein (Figure 3, lane 2). DHFR mRNA translation was almost completely inhibited with the addition of exogenous human recombinant DHFR protein (54 pmol) (Figure 3, lane 3). In contrast, addition of exogenous bovine serum albumin (54 pmol) did not inhibit DHFR mRNA translation (Figure 3, lane 4). In addition, incubation with exogenous human recombinant TS

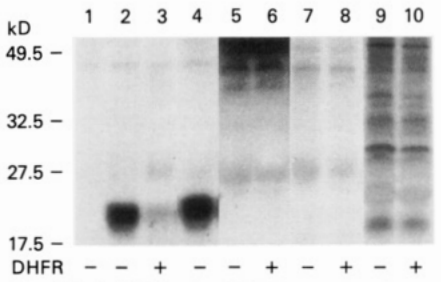

FIGURE 3: Specific inhibition by DHFR protein of human DHFR mRNA translation in vitro. Translation reactions (total volume, 30 μL) were incubated with no exogenous mRNA (lane 1) or DHFR mRNA (1.7 pmol) in the absence (lane 2) or presence (lane 3) of human recombinant DHFR protein (54 pmol). Bovine serum albumin (54 pmol) (lane 4) was included in a reaction mixture containing DHFR mRNA (1.7 pmol). Human c-*myc* mRNA (1.7 pmol) (lanes 5 and 6), yeast mRNA (1.7 pmol) (lanes 7 and 8), and brome mosaic virus mRNA (350 ng) (lanes 9 and 10) were incubated in rabbit lysates, and DHFR protein (54 pmol), where indicated, was included. Translation reactions were incubated for 60 min at 37 °C, and protein products were analyzed by SDS–PAGE (12.5% acrylamide).

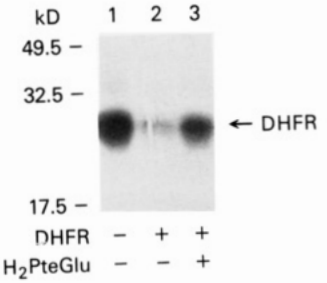

FIGURE 4: Reversal of DHFR mRNA translational inhibition by dihydrofolate (H_2PteGlu). DHFR mRNA transcript (1.7 pmol) alone (lane 1) or in the presence of human recombinant DHFR protein (54 pmol) (lanes 2 and 3), where indicated, along with H_2PteGlu (900 pmol) (lane 3) was included in reaction mixtures that were incubated at 37 °C for 60 min. Translation products were analyzed by SDS–PAGE (12.5% acrylamide), and autoradiography was as described in Materials and Methods.

protein did not inhibit the translational efficiency of human DHFR mRNA, providing additional evidence that the inhibitory effect of DHFR protein was protein-specific (data not shown). The translation of unrelated mRNA transcripts in the absence or presence of DHFR protein was next examined. Translation of an unrelated RNA such as human c-*myc* mRNA (1.7 pmol), yielding a protein product at 45 kDa, remained unaffected by the addition of exogenous DHFR protein (Figure 3, lanes 5 and 6). The specificity of the inhibitory effect of DHFR protein was further demonstrated by experiments in which the addition of exogenous DHFR protein to reaction mixtures containing other unrelated mRNA transcripts, such as yeast (Figure 3, lanes 7 and 8) or brome mosaic virus (BMV) (Figure 3, lanes 9 and 10) did not alter their respective translational efficiencies.

The effect of occupancy of the folate-binding site of DHFR enzyme on its ability to inhibit DHFR mRNA translation was also determined. When DHFR mRNA was incubated in the absence (Figure 4, lane 1) or presence (Figure 4, lane 2) of exogenous human recombinant DHFR protein, almost complete inhibition of DHFR mRNA translation by exogenous DHFR protein was again observed. Addition of the folate substrate dihydrofolate repressed the inhibition of translation by DHFR protein, restoring synthesis to nearly control levels (Figure 4, lane 3). DHFR synthesis was not altered when dihydrofolate was added to reactions containing only DHFR mRNA (data not shown).

DISCUSSION

The results of the electrophoretic gel shift binding and in vitro translation experiments, taken together, suggest that there is a specific interaction between DHFR protein and its mRNA. Moreover, it appears that formation of this protein/RNA complex is associated with inhibition of DHFR mRNA translation. These findings provide evidence of a translational autoregulatory mechanism underlying the control of DHFR expression. Recent studies by Dolnick et al. (1992) demonstrated that the ratio of TS protein to TS mRNA within cultured cell lines is 200:1. When similar calculations are performed for DHFR protein and mRNA, there is an approximately 35-fold greater level of DHFR protein relative to DHFR mRNA expression in K562 human leukemic cells (Dolnick et al., 1992; Koizumi & Allegra, 1992). With regard to the in vitro translation experiments presented in this study, a 31-fold excess of DHFR protein relative to DHFR mRNA was used. Moreover, complex formation was observed in binding studies at ratios of DHFR protein to mRNA down to 50:1 (data not shown). Thus, the relative ratios of DHFR mRNA and DHFR protein used in both the rabbit reticulocyte lysate and the gel mobility shift system appear to closely approximate those found within a cell. However, further studies are required to determine the true in vivo biological relevance of the DHFR protein/DHFR mRNA interaction observed in the present studies.

Such a translational autoregulatory model may be used to explain the experimental findings of various in vitro studies that have described acute increases in DHFR protein levels in neoplastic cells following exposure to the antifol MTX (Domin et al., 1982; Bastow et al., 1984; Cowan et al., 1986). Treatment with MTX results in the intracellular accumulation of the oxidized folate substrate dihydrofolate. As determined by our in vitro studies, dihydrofolate and MTX can each interact with DHFR protein to decrease the intracellular level of ligand-free DHFR protein required for interaction with DHFR mRNA, resulting in an enhanced translational efficiency of DHFR mRNA. Recent molecular modeling studies have revealed a detailed view of ligand-induced conformational changes for both *Escherichia coli* and human DHFR (Oefner et al., 1988; Davies et al., 1990; Bystroff & Kraut, 1991). The pteridine moiety of dihydrofolate and MTX has been shown to induce different conformational changes as a result of the inverse orientation of MTX in the enzyme-binding domain relative to that of dihydrofolate. These findings support the possibility that MTX and dihydrofolate might prevent DHFR from exerting its autoregulatory function either by altering enzyme conformation or by directly occupying the protein/mRNA binding site (Bastow et al., 1984).

Translational autoregulatory control was first characterized in a prokaryotic system in which the coat protein of bacteriophage R17 was shown to bind to the translational start site of its corresponding R17 replicase mRNA and, in so doing, repressed translation (Bernardi & Spahr, 1972; Carey & Uhlenbeck, 1983; Carey et al., 1983; Romaniuk et al., 1987). A similar mechanism has recently been described in a eukaryotic system where it was demonstrated that human thymidylate synthase gene expression is under the control of a negative, translational autoregulatory process (Chu et al., 1991b). Using electrophoretic gel mobility shift assays, human recombinant TS protein was shown to bind to a region (or regions) on its own TS mRNA, resulting in translational inhibition (Chu et al., 1992). The interaction between DHFR protein and RNA, as described in the present study, represents another example of the ability of a gene product to directly regulate its own expression. These studies, taken together, suggest that this form of translational control may represent a more general mechanism of regulation of gene expression than has been previously appreciated.

DHFR and TS each catalyze key reactions in the DNA biosynthetic pathway. Thus, the ability to regulate the expression of each of these proteins at a translational level may represent an efficient mechanism to rapidly and precisely control the intracellular level of these proteins as well as to maintain normal cellular synthetic function in the setting of an acute cellular stress. Further work is required to more completely define the molecular basis underlying the interaction between DHFR and its corresponding mRNA. However, the results of these studies provide new insights into the regulation of DHFR gene expression and suggest that this mechanism of translational autoregulation may be applicable to other critical housekeeping genes.

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