Dihydrofolate Reductase Protein Inhibits Its Own Translation by Binding to Dihydrofolate Reductase mRNA Sequences within the Coding Region†

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ABSTRACT: Previous studies suggest that dihydrofolate reductase (DHFR) regulates its own translation. Moreover, intracellular levels of DHFR protein increase following exposure to the antifolate methotrexate (MTX), suggesting that MTX may release the translational inhibition mediated by DHFR [Chu et al. (1993) *Biochemistry 32***,** 4756−4760; Ercikan et al. (1993) *Adv. Exp. Med. Biol. 338,* 537−540]. To further investigate the role of DHFR in translational autoregulation, we have considered the possibility that DHFR directly contacts its cognate mRNA. Binding studies using a series of truncated DHFR mRNAs as probes localized the DHFR/RNA interaction to a 100-base-pair region containing two putative stem−loop structures; initial studies indicated that both of these loop structures are involved in protein binding. Moreover, the binding of MTX to DHFR prevents interaction of the protein with its cognate mRNA, thereby relieving translational autoregulation.

Dihydrofolate reductase (DHFR),[1] which catalyzes the reduction of 7,8-dihydrofolate (H_2folate) to 5,6,7,8-tetrahydrofolate, is an essential enzyme for regeneration of the cellular reduced folate pools required for synthesis of purines, thymidylate, and certain amino acids. As such, it has proven to be an important drug target, particularly in cancer chemotherapy. For example, methotrexate (MTX), a tight-binding inhibitor of DHFR, is widely used in the treatment of several malignancies, including acute lymphocytic leukemia, non-Hodgkin's lymphoma, osteosarcoma, choriocarcinoma, and breast and head and neck cancer (Schweitzer et al., 1990; Blakley, 1984). MTX is also used in the treatment of nonmalignant diseases, including psoriasis and rheumatoid arthritis, and as an immunosuppressive agent.

It has been documented in leukemia patients that levels of DHFR increase upon administration of methotrexate (Bertino et al., 1963, 1965), an observation that has generated considerable interest in the mechanism(s) underlying the control of DHFR protein levels. It is now known that the regulation of DHFR synthesis is complex and is mediated at both the transcriptional and posttranscriptional levels. While the majority of research efforts have focused on the factors involved in transcriptional regulation of DHFR, particularly during the cell cycle, analyses of cellular response to MTX have suggested that DHFR levels can also be regulated at the level of translation (Bertino et al., 1965; Domin et al., 1982). However, the molecular basis of this translational regulation is largely unknown.

Exposure of cells to MTX, both *in vitro* and *in vivo*, results in a rapid increase in DHFR levels (Bertino et al., 1965; Hillcoat et al., 1967; Domin et al., 1982; Bastow et al., 1984; Cowan et al., 1986). This was first documented three decades ago, when it was observed that patients receiving MTX had increased DHFR protein levels in their blood cells, apparently due to an increase in MTX-bound DHFR (Bertino et al., 1963, 1965). Subsequent experiments showed that incubation with MTX resulted in the maintenance of DHFR levels in cycloheximide-treated cells, whereas cells treated with cycloheximide in the absence of MTX exhibited a marked decrease in DHFR protein levels. These results led to the suggestion that the binding of MTX to DHFR stabilized the enzyme, resulting in an increase in steady-state DHFR levels. However, later studies showed that the half-life of DHFR protein was similar in the presence and absence of MTX, arguing that the increase in DHFR was not due to protein stabilization by MTX (Domin et al., 1982; Bastow et al., 1984). Similarly, the increased DHFR activity was not due to a transcriptional change, since DHFR mRNA levels were similar in the presence and absence of MTX (Bastow et al., 1984; Cowan et al., 1986) and the increase in DHFR activity in the presence of MTX was also observed in cells treated with actinomycin D.

Having ruled out transcription and mRNA stability as mechanisms underlying the rapid increase in enzyme activity following MTX treatment, the most plausible explanation was an increase in translation of DHFR mRNA. Recently, our laboratory (Ercikan et al., 1993) and others (Chu et al., 1993a) have shown that the in vitro translation of DHFR mRNA in reticulocyte lysates is specifically inhibited in the presence of DHFR protein. Using UV-cross-linking and gel mobility shift assays, we now extend these studies and show that this inhibition is due to the binding of DHFR to its own

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[1] Abbreviations: DHFR, dihydrofolate reductase; wt, wild type; MTX, methotrexate; H_2folate, dihydrofolate; PCR, polymerase chain reaction; 3′UTR, 3′ untranslated region; 5′UTR, 5′ untranslated region.

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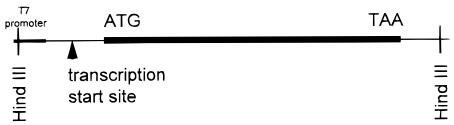

FIGURE 1: Sequence of the "full-length" human DHFR cDNA used for these studies (Chen et al., 1984). The sequence upstream from the start codon is GGGGGGGCGGGGCCTCGCCTGCACAAAT-AGGGACGAGGGGGCGGGGCGGCCacaatttcgcgccaaact-tgaccgcgcgttctgctgtaacgagcgggctcggaggtcctcccgctgctgtc. The nucleotides in capital letters indicate the region upstream from the transcription start site and the nucleotides in small letters are the entire 5′UTR. The sequence of 91 nucleotides downstream to the stop codon (i.e. 3′UTR) contained in the construct is: tatgaaggt-gttttctagtttaagttgttccccctccctctgaaaaaagtatgtatttttacattagaaaaggt-**AAGCT**Ttgactttagatc. The nucleotides in boldface type indicate the *Hin*dIII restriction enzyme recognition site.

mRNA. Furthermore, we delineate regions of the DHFR mRNA involved in this autoregulation.

EXPERIMENTAL PROCEDURES

Materials. Nucleoside monophosphates, restriction enzymes, Sephadex G-50 columns, and RNase-free DNase I were obtained from Boehringer Mannheim (Indianapolis, IN). Taq polymerase was purchased from Perkin-Elmer (Norwalk, CT). Heparin was obtained from U.S. Biochemical Corp. (Cleveland, OH). Centricon-100 and Centricon-30 columns were obtained from Amicon (Beverly, MA). All other molecular biology grade reagents were purchased from Fisher (Pittsburgh, PA).

Human recombinant DHFR was purified utilizing Affi-Gel Blue chromatography (Bio-Rad Laboratories, Hercules, CA). Affinity chromatography utilizing MTX−Sepharose with elution by folate or dihydrofolate was not used as these coenzymes interfere with binding of DHFR to its cognate mRNA. The enzyme after Affi-Gel Blue chromatography was estimated to be 20% pure. The actual amount of DHFR was determined by titrating DHFR with MTX (Fanin et al., 1993). Following purification, DHFR was stored in a buffer containing 25 mM Tris, pH 7.5, and 400 mM KCl and kept at −70 °C.

Rabbit polyclonal antibody to human DHFR was the kind gift of Dr. Bruce Dolnick (Roswell Park Memorial Institute, Buffalo, NY.)

Preparation of Plasmid Construct and in Vitro RNA Synthesis. DHFR cDNA was 807 nucleotides containing the 5′UTR, the coding region, and the 3′UTR [referred to as "full-length" DHFR (Figure 1)]. The nucleotides upstream from the start codon contained the entire 5′UTR plus 51 bases that are upstream to the transcription start site and T7 promoter with the *Hin*dIII site (30 bases). The sequence downstream from the stop codon (i.e., 3′UTR) contained 91 nucleotides. This cDNA was generated by PCR primers containing a T7 promoter at the 5′ end and a primer at the 3′ end, each of which contained a *Hin*dIII restriction site. The cDNA generated was then ligated into pCR-Script (SK)+ vector (Stratagene, La Jolla, CA) using blunt-end ligation, according to the manufacturer's instructions.

Full-length DHFR mRNA and smaller fragments of DHFR mRNA were generated by the following procedure. Full-length DHFR cDNA was obtained by digesting the vector with *Hin*dIII. Smaller fragments of DHFR cDNA were obtained by PCR amplification using a full-length DHFR cDNA as the template. By using primers at the 5′ end that contained the T7 promoter, there was no need to remove the template when transcribing the PCR product, since the template lacked the T7 promoter. The products of PCR reactions were passed through either Centricon-100 or Centricon-30 columns, depending on their size, to remove the primers and the unincorporated nucleotides. I*n vitro* transcription was performed using T7 RNA polymerase according to the manufacturer's instructions (Promega, Madison, WI). Radioactively labeled RNA transcripts were generated using [α-^{32}P]CTP (specific activity 200 Ci/mmol; NEN, Boston, MA).

After *in vitro* transcription the cDNA was digested with RNase-free DNase I at a concentration of 1 unit/μg of template DNA for 15 min at 37 °C. The transcribed RNA was extracted with 1 volume of phenol−chloroform, vortexed for 1 min, centrifuged at 15 000 rpm for 2 min, and reextracted with 1 volume of chloroform−isoamyl alcohol. The upper phase was loaded on a Sephadex G50 column (Boehringer Mannheim, Indianapolis, IN) and was centrifuged at 1100*g* for 4 min to separate the RNA sample from unincorporated nucleotides.

The integrity and the size of the RNA were determined by denaturing agarose gel electrophoresis according to Sambrook et al. (1989).

RNA−Protein Binding Assays. Electrophoretic gel mobility shift assays were performed according to Chung et al. (1995) with minor modifications. Briefly, [^{32}P]CTP-labeled DHFR mRNA (0.25 pmol) was incubated with DHFR protein (2.3 pmol) in binding buffer [20 mM Tris-HCl, pH 7.2, 50 mM KCl, 10% (v/v) glycerol, and 0.1 mM DTT] in a total volume of 30 μL for 15 min at 37 °C. Following the addition of heparin (375 μg; U.S. Biochemical Corp., Cleveland, OH) to eliminate protein that was bound non-specifically to mRNA (15 min, 37 °C), an aliquot of this mixture was directly loaded on a 1% agarose gel in TAE buffer (40 mM Tris acetate, pH ∼8.5, and 1 mM EDTA). The gel was run for 2 h at 50 V, dried, and autoradiographed for visualization.

UV-cross-linking assays were performed according to Leibold and Munro (1988) as modified by Chu et al. (1993b). [^{32}P]-labeled DHFR mRNA was incubated with DHFR protein in binding buffer for 15 min at 37 °C. Following the addition of heparin, this mixture was UV-cross-linked using a Stratalinker (Stratagene, La Jolla, CA) for 20 min. After UV-cross-linking, RNase A/T1 (5 units of each; Boehringer Mannheim, Indianapolis, IN) was added and incubated for 15 min to degrade unprotected mRNA. The reaction mixture was then directly loaded on an SDS−7.5% polyacrylamide gel and run at a constant current of 25 mA. The gel was dried and autoradiographed for visualization.

RESULTS

Binding of DHFR Protein to Its Cognate mRNA. Previous work indicated that exogenously added DHFR protein specifically inhibited translation of this protein in a reticulocyte assay using DHFR mRNA (Ercikan et al., 1993; Chu et al., 1993a). In order to show specific binding of DHFR protein to its mRNA, radiolabeled full-length DHFR mRNA was incubated with purified DHFR protein, and after UV-cross-linking, the mixture was separated on SDS−PAGE. When radiolabeled DHFR mRNA was irradiated in the absence of DHFR protein (Figure 2, lanes 1) or when UV

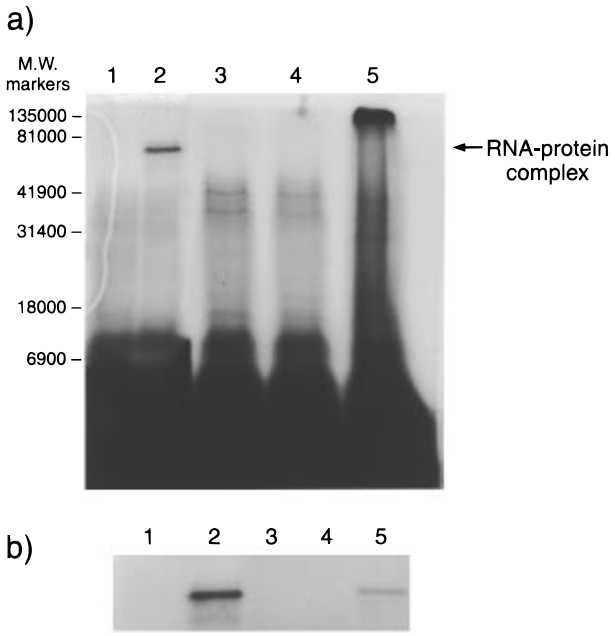

FIGURE 2: Specific binding of DHFR protein to DHFR mRNA using a UV-cross-linking assay. (a) Lane 1, labeled DHFR mRNA alone. Labeled DHFR mRNA was incubated with recombinant DHFR protein followed by UV irradiation (lane 2). The DHFR protein/DHFR mRNA was treated with proteinase K before (lane 3) and after irradiation (lane 4). Labeled DHFR mRNA was incubated with recombinant DHFR protein without UV irradiation (lane 5). (b) Lanes 1 and 2, same as above. Bovine serum albumin (lane 3) and plasma γ globulin (lane 4) were incubated with labeled DHFR mRNA. Labeled DHFR was competed with unlabeled DHFR mRNA (lane 5). The arrow shows the RNA−protein complex.

irradiation was omitted (Figure 2a, lane 5), no RNase A/T1-resistant complex was generated, indicating that the binding of DHFR protein to its mRNA was not covalent in nature. However, when radiolabeled DHFR mRNA was incubated with DHFR protein, a major band of ca. 80 kDa was formed (Figure 2, lanes 2). When the RNA−protein complex was treated with proteinase K either before (Figure 2a, lane 3) or after UV irradiation (Figure 2a, lane 4), no RNA−protein complex was detected, suggesting that the labeled complex was DHFR mRNA−DHFR protein adduct, since no complex was noted with unrelated proteins (bovine serum albumin and plasma γ globulin; Figure 2b, lanes 3 and 4, respectively). The addition of unlabeled DHFR mRNA inhibited complex formation (Figure 2b, lane 5), while the addition of a nonspecific mRNA (insulin) only slightly competed for DHFR protein binding to its mRNA (data not shown).

As the reticulocyte translation assays had shown that MTX could relieve the inhibition of DHFR translation by its mRNA, increasing concentrations of this antifolate were tested in the UV-cross-linking assay. MTX, a specific and tight-binding inhibitor of DHFR, decreased complex formation of the ca. 80 kDa complex in a concentration-dependent manner (Figure 3a). MTX added to DHFR protein first or to DHFR mRNA first or added following incubation of DHFR and its mRNA did not change the level of competition observed (Figure 3b), indicating that MTX could still relieve the translational inhibition produced by DHFR even after the protein had bound its mRNA. The MTX concentration required to inhibit binding of DHFR protein to its cognate mRNA is relatively high. A possible explanation for this observation is that the ratio of dissociation constant for

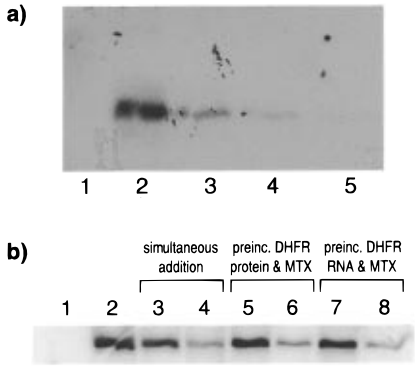

FIGURE 3: Inhibition of binding of labeled "full-length" DHFR mRNA to DHFR protein by MTX demonstrated by a UV-cross-linking assay. (a) DHFR mRNA was incubated in the absence (lane 1) or in the presence of DHFR protein (lane 2−5). In lanes 3−5, radiolabeled DHFR mRNA was preincubated with 0.66, 1.33, and 2.66 mM MTX for 15 min at room temperature before the addition of DHFR protein. (b) Radiolabeled DHFR mRNA was incubated in the absence (lane 1) or presence of DHFR protein (lane 2). In lanes 3 and 4, DHFR mRNA, DHFR protein, and MTX (0.3 and 3.3 mM, respectively) were incubated simultaneously. In lanes 5 and 6, DHFR protein was preincubated with the same concentrations of MTX as in lanes 3 and 4 for 15 min at room temperature before the addition of DHFR mRNA. In lanes 7 and 8, DHFR mRNA was preincubated with MTX (0.3 and 3.3 mM, respectively) to which DHFR mRNA was then added.

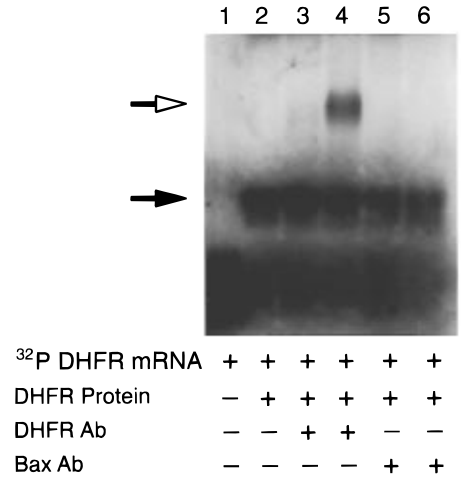

FIGURE 4: Demonstration of a DHFR mRNA−DHFR protein complex using a gel mobility shift assay. Lane 1, labeled DHFR mRNA alone. Labeled DHFR mRNA was incubated with recombinant DHFR protein (lane 2). Increasing amounts (1 and 10 μL) of either DHFR antibody (lanes 3 and 4) or Bax antibody (lanes 5 and 6) were incubated with labeled DHFR mRNA and DHFR protein. See Experimental Procedures for details.

complex formation of MTX and DHFR protein in the presence and absence of NADPH is 4800 (Nakano et al., 1994). In the UV-cross-linking assay NADPH is not added since NADPH can compete for the binding of DHFR protein to its own mRNA (Chu et al., 1993). In addition high levels of MTX may be required for inhibition of binding of DHFR to DHFR mRNA as the pH of the binding buffer is pH 7.2, a pH at which the binding of DHFR protein to MTX is less tight than at pH 6.0, where binding may be stoichiometric (Bertino et al., 1964).

The presence of a complex between DHFR protein and its cognate mRNA could also be shown using a gel retardation assay (Figure 4). In the presence of polyclonal antibody to DHFR, the DHFR mRNA was supershifted (Figure 4, lanes 3 and 4). A nonspecific antibody (to Bax

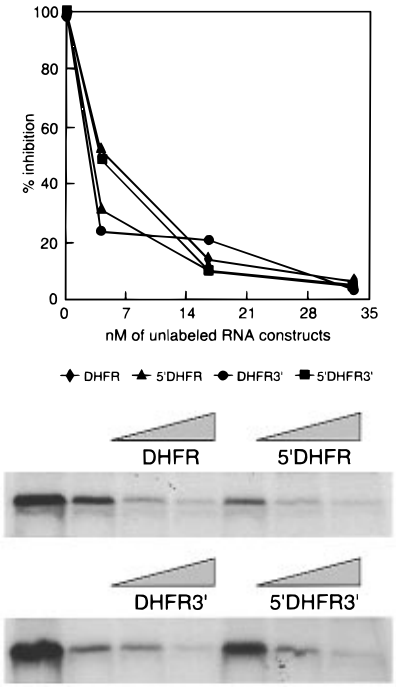

FIGURE 5: Inhibition of binding of labeled "full-length" DHFR mRNA to DHFR protein by unlabeled transcribed DHFR RNAs. Unlabeled transcripts were designated as follows: full-length transcript containing both the 5′UTR, the 3′UTR, and the coding region, 5′DHFR3′ (■); transcript containing the 5′UTR plus the coding region, 5′DHFR (▲); transcript containing the 3′UTR plus the coding region, DHFR3′ (●); and the transcript lacking both the 5′UTR and the 3′UTR, DHFR (♦). These unlabeled mRNA transcripts (4.3, 16.6, and 33.3 nM) were competed with ^{32}P-labeled full-length DHFR mRNA for DHFR protein binding. (Top panel) Graphic representation: percent inhibition vs concentration of unlabeled DHFR RNA constructs. The graph was constructed by quantifying the intensity of the bands in panel b using a densitometer (LKB 2222-020 Ultra Scan XL laser densitometer). (Bottom two panels) Competition studies using UV-cross-linking assay with unlabeled DHFR RNA constructs against ^{32}P-labeled 5′DHFR3′ (full-length DHFR). Lane 1 in each of the gel panels is a control (i.e., radiolabeled DHFR mRNA was incubated in the presence of DHFR protein without unlabeled DHFR mRNA as competitor).

protein) did not supershift the DHFR protein−mRNA band. This experiment provided additional evidence that the protein that bound to DHFR mRNA was indeed DHFR protein.

Identification of an RNA Binding Site. To identify the putative site on the mRNA responsible for interaction with DHFR, a series of truncated DHFR mRNA transcripts were synthesized. Each of these constructs was then used as unlabeled mRNA competitors. As shown in Figure 5, these unlabeled mRNA transcripts (4.3, 16.6, and 33.3 nM) competed with ^{32}P-labeled full-length DHFR mRNA for DHFR protein binding as effectively as full-length unlabeled DHFR mRNA with similar IC_{50} values (∼2 nM; Figure 5). These results indicated that the DHFR protein binding site was within the coding region of DHFR mRNA.

To define the mRNA binding site(s) more precisely, smaller DHFR mRNA transcripts were prepared (see Experimental Procedures) and used in competition experiments (Figure 6). The RNA transcripts 1DHFR210, 200DHFR400, and 400DHFR500 did not compete for DHFR protein binding. In contrast, the RNA fragments 400DHFR807 and 500DHFR807 inhibited the binding of full-length labeled DHFR mRNA to DHFR protein, suggesting that the DHFR protein binding site is between nucleotides 500 and 807.

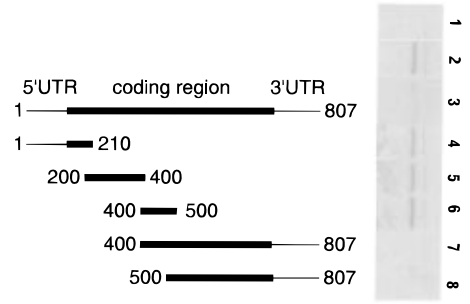

FIGURE 6: Competition of labeled "full-length" DHFR mRNA with unlabeled in vitro transcribed DHFR RNAs (33.3 nM) for binding to DHFR protein using UV-cross-linking assay. The fragments were designated as follows: the number on the left side of DHFR indicates the first nucleotide and the number on the right side indicates the last nucleotide position with respect to the full-length DHFR mRNA transcript. Labeled DHFR mRNA was incubated either in the absence (lane 1) or in the presence (lane 2) of partially purified DHFR protein. Full-length DHFR mRNA (1DHFR807, lane 3), and mRNA fragments 1DHFR210 (lane 4), 200DHFR400 (lane 5), 400DHFR500 (lane 6), 400DHFR807 (lane 7), and 500DHFR807 (lane 8) were competed with ^{32}P-labeled full-length DHFR mRNA for DHFR protein binding.

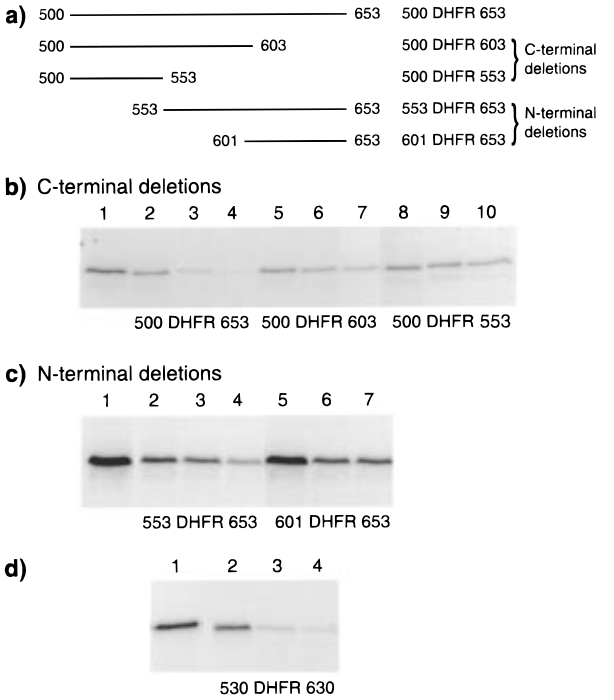

FIGURE 7: Competition experiments to define the minimal required nucleotide sequences of DHFR mRNA for binding to DHFR protein. (a) The designation of the DHFR transcripts used in panels b and c. These transcripts (5, 25, and 50 nM) were used to compete for binding to DHFR protein with ^{32}P-labeled full-length DHFR mRNA. (b) From the 3′end of 500DHFR653, 50 and 100 nucleotides was removed to create 500DHFR603 and 500DHFR553. (c) From the 5′end of the DHFR mRNA transcript (500DHFR653), 53 and 101 nucleotides were removed to prepare 553DHFR653 and 601DHFR653. (d) Fragment 530DHFR630 was used as competitor against the radiolabeled full-length DHFR mRNA transcript.

Shorter fragments of DHFR RNA transcripts of this region were prepared and used in competition experiments (Figure 7a). RNA fragment 500DHFR653 inhibited the binding of full-length labeled DHFR mRNA to DHFR protein as well as the DHFR mRNA fragment 500DHFR807 (Figure 7b, lanes 1−4), narrowing the DHFR binding site to within the coding region between nucleotides 500 and 653. A radio-

labeled DHFR mRNA (nucleotides 500−653) was used to confirm that this fragment was able to form a complex with DHFR protein. When this mRNA fragment was incubated with human DHFR protein, a complex of ca. 80 kDa was formed, which was the same size obtained with radiolabeled full-length DHFR (data not shown). To attempt to further narrow down the RNA binding site for DHFR protein, transcripts with further deletions from both the 5′ end and the 3′end of the 500DHFR653 mRNA transcript were prepared (Figure 7a). Removal of 50 nucleotides from either the C-terminal (500DHFR603, Figure 7a) or N-terminal end (553DHFR653, Figure 7a) partially inhibited the binding of full-length labeled DHFR mRNA to DHFR protein (Figure 7b,c). However, deletion of 100 nucleotides from either the N-terminal (601DHFR653, Figure 7a) or C-terminal end (500DHFR553, Figure 7a) resulted in RNA fragments that competed only marginally against the full-length labeled DHFR mRNA (Figure 7b,c). These results suggested that the sequences between nucleotides 553 and 601 may be of importance since both the transcripts 553DHFR653 and 500DHFR603 that partially competed against the full-length DHFR mRNA contained this nucleotide region. Although either of these latter transcripts completely inhibited the binding of full-length labeled DHFR mRNA to DHFR protein, an unlabeled mRNA transcript (530DHFR630, Figure 7d) competed almost as effectively as did 500DHFR653, indicating that nucleotides 500−530 and 630−653 may not be essential for binding to DHFR.

DISCUSSION

Previous studies showed that DHFR mRNA translation is inhibited in the presence of DHFR protein in an *in vitro* reticulocyte translation assay; other unrelated proteins did not influence the translational efficiency of DHFR mRNA (Ercikan et al., 1993; Chu et al., 1993a). The addition of either dihydrofolate, a natural substrate of DHFR, or MTX, an antifolate, along with exogenous DHFR protein relieves this inhibition. Furthermore, addition of MTX, a specific inhibitor of DHFR, increased translation of DHFR, confirming that the DHFR protein specifically inhibits translation of the DHFR mRNA. In the present study, we have demonstrated a specific interaction between DHFR and its mRNA, which likely underlies this autoregulation. Moreover, the inhibition of this DHFR protein/DHFR mRNA complex by MTX provides an explanation for the earlier observation that DHFR protein levels can increase following MTX treatment (Bertino et al., 1965; Hillcoat et al., 1967; Domin et al., 1982; Bastow et al., 1984; Cowan et al., 1986).

UV-cross-linking experiments defined a protein binding site(s) between nucleotides 500 and 630 within the protein coding region of DHFR RNA. While the exact role of this site in RNA/protein complex formation has yet to be determined, studies in other systems have indicated that target elements for RNA-binding proteins are generally found in loops, bulges, or interior loops, which offer single-stranded regions for binding (McCarthy & Kollmus, 1995). For example, stem and loop structures have been shown to be essential for the regulation of ferritin and transferrin (Kozak, 1992), two proteins that regulate the uptake and detoxification of iron in mammalian cells. Regulation of both proteins involves a similar stem and loop structure, called the iron response element (IRE). The IRE is located within the 5′UTR in ferritin mRNA, and ferritin translation is inhibited by the binding of iron-responsive binding protein (IRE-BP) to the IRE. On the other hand, transferrin levels are regulated by the stabilization of transferrin mRNA upon binding of IRE-BP to five IRE motifs in the 3′UTR (Kozak, 1992).

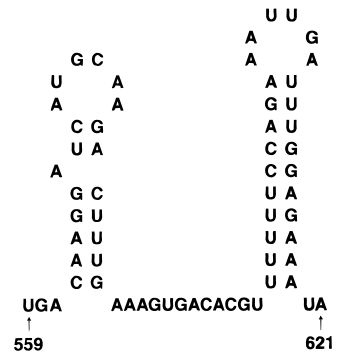

FIGURE 8: Computer-generated secondary structures between nucleotides 559 and 621 of DHFR mRNA. See text for details.

Analysis of DHFR mRNA between nucleotides 500 and 653 revealed two potential stem−loop structures, (Figure 8), one lying between positions 559 and 585 and the other including sequences from 590 to 621, which could form stable secondary structures suitable for DHFR binding [analysis of mRNA folding was performed using PCGene software according to Zuker and Stiegler (1981)]. The stability of these stem−loop structures measured as free energy of formation (ΔG) is calculated to be about −8.2 kcal/mol. The free energy of formation refers to the equilibrium constant for the for the stem−loop structure versus unfolded structure. Free energy of the first loop between nucleotides 559 and 585 is −2.0 kcal and that of the second loop is −6.2 kcal. These secondary structures may be further stabilized by interaction with DHFR protein, resulting in inhibition of translation. Deletion analysis of this area suggested that both of the stem and loop structures were involved in protein binding. Interestingly, the mouse DHFR RNA contains only one of the potential stem−loop structures while the hamster homologue includes neither. This is particularly intriguing in light of earlier studies (Ercikan et al., 1993; Cowan et al., 1986) that showed barely significant increases in DHFR levels following MTX treatment of rodent cell lines compared to the 6−12-fold increase observed in human cells, consistent with a requirement for these structures in translational autoregulation.

Another mammalian gene that has been shown to be autoregulated at the translational level by RNA/protein interactions is thymidylate synthase (TS); this regulation involves two RNA domains, one of which also lies within the protein coding region (Chu et al., 1991, 1993b). Similar to DHFR, TS mRNA translation is inhibited by its own protein and this inhibition is relieved by either its substrate (dUMP), its cofactor (methylene tetrahydrofolate), or its specific inhibitor (FdUMP). Moreover, TS protein and mRNA were shown to form a complex in cultured human colon cancer cells (Chu et al., 1994), supporting the physiological relevance of the *in vitro* studies.

It is intriguing to speculate on the potential biological significance of the translational autoregulation of TS and DHFR. Both are encoded by genes whose expression is highly regulated during the cell cycle. Both are essential enzymes for the de novo synthesis of dTMP, and therefore for proper replication of DNA. The expression of these two

proteins increases as the cell goes from G1 to S phase. Although this increase is primarily regulated at the level of transcription by the nuclear protein E2F (De Gregori et al., 1995; Slansky & Farnham, 1996), translational autoregulation may provide an additional mechanism for tightly controlling expression levels of these gene products in order for cells to rapidly increase the synthesis of DHFR and TS proteins in response to low levels of substrates or cofactors or to inhibition by antimetabolites. In tumor cells, where the function of E2F can be affected by mutations or deletions in pRb (Weinberg, 1995), which sequesters the E2F/DP complex, translational regulation may play an even more critical role than it does in normal cells.

A question that remains to be addressed is the nature of the protein domain(s) involved in RNA binding. Visual inspection of the amino acid sequence of DHFR reveals no known protein/nucleic acid binding domains. However, Hentze (1994) proposed that a common motif for enzymes that catalyze reactions with mono- or dinucleotides as substrates or cofactors is an overlapping RNA−(di)nucleotide-binding domain. Many dehydrogenases, such as lactate dehydrogenase, glutamate dehyrogenase, and glyceraldehyde dehydogenase, bind to their cognate mRNAs. Moreover, RNA binding enzymes such as catalase and acotinase, which do not use NAD(P)H as a substrate, were found to have NAD(P)H binding sites (Hentze, 1994). Both DHFR and TS enzymes utilize nucleotide cofactors (NADPH for DHFR and dUMP for TS). Therefore it is possible that the nucleotide binding site regions are also the sites for their mRNA binding.

Taken together, our results suggest that translation of DHFR mRNA is negatively regulated by its own protein product, which likely explains the previous observation that MTX exposure induces an increase in the DHFR protein levels in cells. MTX, H_2folate, and NADPH, by altering DHFR conformation (Appleman et al., 1988; Bystroff & Kraut, 1991), prevent DHFR protein from binding to its mRNA and autoregulating its synthesis. Since increases in DHFR protein may result in clinical resistance to antifolate treatment, these data encourage the search for novel DHFR inhibitors that do not relieve DHFR translation. Alternatively, compounds that compete with DHFR protein for mRNA binding, e.g., peptides, may be useful as specific inhibitors of DHFR translation.

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