Characterization of a *cis*-acting regulatory element in the protein-coding region of human dihydrofolate reductase mRNA

Ningwen TAI*†, John C. SCHMITZ*†, Tian-min CHEN*† and Edward CHU*†¹

*Department of Medicine and Pharmacology, Yale Cancer Center, Yale University School of Medicine, New Haven, CT 06520, U.S.A., and †VACT Cancer Center, VA Connecticut Healthcare System, West Haven, CT 06516, U.S.A.

Previous studies have shown that human DHFR (dihydrofolate reductase), in addition to its critical role in DNA biosynthesis, functions as an RNA-binding protein. The interaction between DHFR and its own mRNA results in translational repression. In this study, we characterized the *cis*-acting elements on human DHFR mRNA that are required for the DHFR mRNA–DHFR protein interaction. Using a series of gel-shift and nitrocellulose filter-binding assays, a 164 nt RNA sequence, corresponding to nt 401–564, was identified within the coding region that binds to DHFR protein with an affinity similar to that of full-length DHFR mRNA. To document *in vivo* biological activity, various DHFR sequences contained within the coding region were cloned on to the 5' end of a luciferase reporter plasmid, and transient transfection experiments were performed using human colon cancer RKO cells. In cells transfected with p644/DHFR:401–564,

luciferase activity was decreased by 50 % when compared with cells transfected with the p644 plasmid alone. Luciferase mRNA levels were identical under each of these conditions, as determined by Northern-blot analysis. In cells transfected with p644/DHFR:401–564, luciferase activity was restored to almost 100 % of control when cells were treated with the antifolate analogue methotrexate or with a short-interfering RNA targeting DHFR mRNA. These findings provide evidence that the DHFR 401–564 sequence is a DHFR-response element. *In vitro* and *in vivo* studies further localized this *cis*-element to an 82 nt sequence corresponding to nt 401–482. This work provides new insights into critical elements that mediate RNA–protein interactions.

Key words: dihydrofolate reductase, RNA-binding protein, RNA–protein interaction, translational regulation.

INTRODUCTION

The events controlling cellular gene expression are complex. Both post-transcriptional and translational regulatory mechanisms play central roles in this process, and they are mediated, in part, by the interaction of RNA-binding proteins with target cellular RNAs to form ribonucleoprotein complexes. Most of the well-established *cis*-acting elements have been localized to the 5'- and/or 3'-UTRs (untranslated regions) [1]. The IRP (iron regulatory protein) is one of the best characterized RNA-binding proteins identified to date, and it plays a critical role in the precise control of iron homeostasis within a given cell [2–4]. A wide range of cellular proteins, including creatine kinase, testis-specific Cu/Zn superoxide dismutase, and zebra fish DAZ (deleted in azoospermia)-like protein, have also been demonstrated to function as RNA-binding proteins. Each regulates translation by targeting sequences in either the 5'- or 3'-UTRs of their target cellular mRNAs [5–7]. In addition, there is now a growing list of RNA-binding proteins that recognize the coding region of their target mRNAs. For example, the folate-dependent enzyme TS (thymidylate synthase) binds to a sequence contained within the coding region (nt 480–550) of its own mRNA [8,9], a protein of unknown function interacts with the coding region of rat protein D mRNA [10], a protein recognizes the CAG repeat elements in the coding region of Huntington mRNA [11], and a cytoplasmic protein interacts with a translational control element in the coding region of pro-opiomelanocortin mRNA [12].

Previous studies have demonstrated that human DHFR (dihydrofolate reductase), in addition to its critical role in DNA biosynthesis, functions as an RNA-binding protein [13–16]. DHFR protein forms a ribonucleoprotein complex with its own

mRNA, and this RNA–protein interaction results in translational repression. A model of DHFR translational autoregulation has been proposed, and this control mechanism would appear to have particular biological relevance in that it offers an efficient process for the levels of DHFR to be precisely controlled within a given cell. Of note, treatment with an inhibitor compound of DHFR disrupts this normal regulatory mechanism, thereby leading to the induced expression of DHFR and the acute development of cellular drug resistance.

In the present study, we characterized in further detail the molecular elements mediating the interaction between human DHFR protein and its target human DHFR mRNA. Specifically, we have further defined the *cis*-acting elements within the DHFR mRNA that are required for RNA recognition. Using a series of *in vitro* RNA-binding and filter-binding assays and *in vivo* transfection experiments, we identified an 82 nt nucleotide sequence within the coding region of human DHFR mRNA that functions as a specific DHFR-response element.

EXPERIMENTAL

Synthesis of double-stranded siRNAs (short interfering RNAs)

Double-stranded siRNAs were designed to target a sequence corresponding to nt 35–54 on human DHFR mRNA. The target sequence was as follows: 5'-CCCAGAACATGGGCATCGGC-3'. The selected sequences were screened against all known human genes using a BLAST search to confirm that only human DHFR mRNA would be targeted. The respective sense and antisense RNA sequences were synthesized by the Yale Keck Molecular

Abbreviations used: UTR, untranslated region; TS, thymidylate synthase; DHFR, dihydrofolate reductase; siRNA, short interfering RNA; MTX, methotrexate.

¹ To whom correspondence should be addressed, at VACT Cancer Center, VA Connecticut Healthcare System (e-mail chueyale@yahoo.com).

Core Facility (Yale University, New Haven, CT, U.S.A.). Each RNA contained an additional two 2'-deoxythymidine nucleotides on the 3'-end. All RNAs were deprotected by incubating at 25 °C for 30 h in 1 M tetrabutylammonium fluoride (Sigma, St Louis, MO, U.S.A.). An equivalent volume of RNase-free water was added to quench the reaction. Equal amounts of complementary RNAs in tetrabutylammonium fluoride/water solution were combined, heated to 95 °C for 5 min, and allowed to cool slowly to room temperature. siRNAs were gel-purified by resolving on a 15 % acrylamide gel. Scrambled siRNA whose target is not present in mammalian cells was purchased from Dharmacon Research (Lafayette, CO, U.S.A.).

Cell culture

Human colon cancer RKO cells were maintained in 25 cm² plastic tissue-culture flasks (Becton Dickinson Labware, Franklin Lakes, NJ, U.S.A.) in growth medium containing RPMI 1640 medium (Invitrogen, Carlsbad, CA, U.S.A.) with 10 % dialysed fetal bovine serum (Invitrogen). This cell line has been well-characterized previously [17].

Synthesis of plasmid constructs

The luciferase reporter plasmid p644 was constructed by cloning an early growth response gene promoter at the *Bgl*III restriction site of plasmid pGL2-basic (Promega, Madison, WI, U.S.A.). Plasmid pGEM-7Z:DHFR containing the full-length human DHFR cDNA was used as template for PCR amplification of various DHFR fragments. Sequences corresponding to DHFR:5'-UTR, DHFR:3'-UTR, DHFR:1–200, DHFR:220–370, DHFR:401–564, DHFR:401–482, DHFR:441–522, DHFR:483–564 and TS:3'-UTR were PCR-amplified, digested with *Hind*III, and cloned into the *Hind*III restriction site of plasmid p644 to generate heterologous luciferase reporter constructs (Figure 1). The sequence and orientation of the cloned DHFR and TS inserts were confirmed by sequencing analysis, which was performed by the Yale Keck Molecular Core Facility.

The oligonucleotide sequences used in the PCR reactions are listed as follows, and the underlined nucleotides represent *Hind*III or *Bgl*III restriction sites. DHFR:5'-UTR, sense primer, 5'-ATCGAAGCTTCAAACTTGACCGCGCTTCTG-3'; DHFR:5'-UTR, antisense primer, 5'-ATCGAAGCTTAGATCTGACAGCAGCGGAGGACCTCC-3'; DHFR:3'-UTR, sense primer, 5'-ATCGAAGCTTTATGAAGGTGTTTCTAGTTT-3'; DHFR:3'-UTR, antisense primer, 5'-ATCGAAGCTTAGATCTACCTTTCTAATGTAAAATACAT-3'; DHFR:1–200, sense primer, 5'-ATCGAAGCTTATGGTTGGTTCGCTAAACTGC-3'; DHFR:1–200, antisense primer, 5'-ATCGAAGCTTAGATCTGGTTCGATCTTCTCAGGAATG-3'; DHFR:220–370, sense primer, 5'-ATCGAAGCTTTTATGTTCTCAGCAGAGAACTC-3'; DHFR:220–370, antisense primer, 5'-ATCGAAGCTTAGATCTCCTTATAACAGAACTGCCAC-3'; DHFR:401–564, sense primer, 5'-ATCGAAGCTTCTATTTGTGACAAGGATCATG-3'; DHFR:401–564, antisense primer, 5'-ATCGAAGCTTAGATCTTTAATCATCTTCTCATATAC-3'; DHFR:401–482, sense primer, same as DHFR:401–564 sense primer; DHFR:401–482, antisense primer, 5'-ATCGAAGCTTAGATCTGGCAGAAAGTTTATTTCTCC-3'; DHFR:441–522, sense primer, 5'-ATCGAAGCTTTTTTTTC-CAGAAATTGATTTG-3'; DHFR:441–522, antisense primer, 5'-ATCGAAGCTTAGATCTTTTCTCCTCCTGGACATCAGA-3'; DHFR:483–564, sense primer, 5'-ATCGAAGCTTAGAATACCCAGGTGTTCTCTC-3'; DHFR:483–564, antisense primer, same as DHFR:401–564 antisense primer; TS:3'-UTR, sense primer, 5'-ATCGAAGCTTTTCAACGGAGCTTGAAGGAT-3';

Figure 1 Structure of human DHFR cDNA and heterologous luciferase report plasmids

(A) Map of the human DHFR cDNA. The 700 nt human DHFR cDNA includes 59 nt of 5'-UTR, the coding region (564 nt), and 77 nt of the 3'-UTR. The shaded box represents the DHFR RNA response element corresponding to nt 401–564. (B) Structure of heterologous plasmids. Details of construction of the luciferase heterologous plasmids are included in the Experimental section. The solid black box indicates the EGR-1 promoter and the dark grey box represents the protein-coding region of firefly luciferase gene. The light grey box represents the DHFR RNA sequence cloned into the *Hind*III restriction site of the p644 recombinant plasmid.

TS:3'-UTR, antisense primer, 5'-ATCGAAGCTTAGATCTGCC-TTTTATTATGGAAAGAAC-3'.

In vitro RNA transcription

A ³²P-radiolabelled human DHFR mRNA probe (DHFR:1–564) was synthesized *in vitro* with T7 RNA polymerase (Promega) after linearization of pET28a(+):DHFR with *Hind*III. Sequences corresponding to DHFR:1–200, DHFR:220–370, DHFR:301–564, DHFR:401–564, DHFR:401–482, DHFR:441–522 and DHFR:483–564 were synthesized *in vitro* using PCR-generated DNAs and T7-MEGAscript™ kit or T7-MEGAscript™ kit (Ambion, Austin, TX, U.S.A.). The primers used in the PCR reaction are listed as follows, and the underlined nucleotides represent the T7 promoter/primer sequence. DHFR:1–200, sense primer, 5'-TAATACGACTCACTATAGGGATGGTTGGTTCGCTAAACTGC-3'; DHFR:1–200, antisense primer, 5'-GGTCGATTCTTCTCAGGAATG-3'; DHFR:220–370, sense primer, 5'-TAATACGACTCACTATAGGGTATGTTCTCAGCAGAGAACTC-3'; DHFR:220–370, antisense primer, 5'-CCTTATAACAGAACTGCCAC-3'; DHFR:301–564, sense primer, 5'-TAATACGACTCACTATAGGGTACTGAACAACCAGAAATTA-GCA-3'; DHFR:301–564, antisense primer, 5'-TTAATCATCTTCTCATATAC-3'; DHFR:401–564, sense primer, 5'-TAATACGACTCACTATAGGGCTATTTGTGACAAGGATCATG-3'; DHFR:401–564, antisense primer, same as DHFR:301–564 antisense primer; DHFR:401–482, sense primer, same as DHFR:401–564 sense primer; DHFR:401–482, antisense primer, 5'-GGCAGAAGTTTATTTTCTCC-3'.

All RNA transcripts were resolved on a 15 % polyacrylamide/8 M urea gel to confirm their integrity and size, and they were subsequently gel-purified. The concentration of unlabelled RNA was determined by UV spectrophotometry. Labelled transcripts

were made by inclusion of [α - 32 P]CTP at 800 Ci/mmol (NEN Dupont, Boston, MA, U.S.A.), and the concentration of radioactively labelled RNA was determined from the specific activity of 32 P incorporation.

Purification of human recombinant DHFR protein

Human His-tagged DHFR protein was purified according to methods described previously [16]. The integrity and purity of the recombinant His-tagged DHFR protein were analysed by resolving by SDS/PAGE followed by Coomassie Blue and silver staining, respectively.

RNA gel-mobility shift assay

RNA gel-mobility shift assays were performed using a modification of methods described previously [13,16,18]. Each reaction (total volume, 20 μ l) contained 18 mM Hepes (pH 7.5), 0.9 mM MgCl₂, 18 mM KCl, 100 mM 2-mercaptoethanol (Sigma), 3% glycerol, 40 units of Prime RNase Inhibitor (Eppendorf-5 Prime, Boulder, CO, U.S.A.), 20 μ g/ml BSA, 25 μ g/ml yeast tRNA (BD Sciences, San Diego, CA, U.S.A.), 32 P-radiolabelled DHFR mRNA (100 000 c.p.m.; 3.8 fmol) and DHFR protein (42.6 pmol). The initial incubation was for 15 min at room temperature. RNase T1 (15 units; Ambion) 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 was resolved on a 4% non-denaturing polyacrylamide gel (acrylamide/methylenebisacrylamide, 60:1; gel dimensions, 15 cm $\times$ 17 cm) for approx. 60 min at 500 V. Gels were dried and then exposed to Kodak XMR film (Eastman Kodak Company, Rochester, NY, U.S.A.) and visualized by autoradiography.

Competition experiments were performed with human recombinant DHFR protein (42.6 pmol) and 32 P-radiolabelled DHFR mRNA (3.8 fmol; 100 000 c.p.m.). These conditions were selected based on control experiments using a fixed amount of radiolabelled DHFR mRNA with varying concentrations of DHFR protein to document the linearity of binding.

Nitrocellulose filter-binding assay

Nitrocellulose filter-binding assays were performed using a modification of methods described previously [16,19]. Each reaction (total volume, 20 μ l) contained 18 mM Hepes (pH 7.5), 0.9 mM MgCl₂, 18 mM KCl, 100 mM 2-mercaptoethanol, 40 units of Prime RNase Inhibitor, 20 μ g/ml BSA, 25 μ g/ml yeast tRNA, 32 P-radiolabelled human DHFR mRNA (3.8 fmol; 100 000 c.p.m.) and DHFR protein (42.6 pmol). The initial reaction was incubated for 15 min at room temperature, after which 15 units of RNase T1 were added for 15 min, followed by incubation with heparin (5 mg/ml) for an additional 10 min at room temperature. The samples were filtered through a 0.22 μ m nitrocellulose filter (Millipore, Bedford, MA, U.S.A.) supported in a Millipore glass-fritted 25 mm filter apparatus. Nitrocellulose filters were washed twice with 30 mM Hepes (pH 7.5), and the bound radiolabelled RNA retained on the filter was counted in a scintillation counter in 10 ml of Bio-Safe IITM scintillant (Research Products International Corp., Mount Prospect, IL, U.S.A.).

Transient transfection and luciferase assay

Transient transfection experiments were performed as described previously to determine the effect of DHFR RNA sequences on

expression of the heterologous luciferase construct [9,20]. In brief, cells were plated at 2×10^5 cells/25 cm² flask and grown to approx. 50% confluence. In each transfection experiment, cells were incubated with 1 μ g of plasmid DNA and 5 μ g of Lipofectamine 2000 (Invitrogen). Cells were also co-incubated with 20 ng of pRL-SV40 plasmid DNA (Promega), which encodes *Renilla* (sea pansy) luciferase. The expression of *Renilla* luciferase served as an internal control for transfection efficiency. After incubation at 37 °C for 48 h, cells were harvested using trypsin/EDTA (Invitrogen) and washed twice with ice-cold 1 $\times$ PBS. Luciferase activity was measured with the Dual-Luciferase Reporter 1000 Assay System (Promega).

Isolation of total cellular RNA and Northern-blot hybridization analysis

RKO cells were washed three times with ice-cold 1 $\times$ PBS buffer and harvested from 25 cm² tissue-culture flasks. Total RNA was isolated from cell pellets using the RNeasy kit (Qiagen, Valencia, CA, U.S.A.). RNAs were resolved on a 1% agarose/formaldehyde gel to verify integrity and size. The concentration of RNA was determined by UV spectrophotometry.

For Northern-blot analysis, total cellular RNA (10 μ g) was resolved on a 1% agarose/formaldehyde gel and then transferred to a positively charged BrightStar-Plus nylon membrane (Ambion) by capillary transfer. Antisense RNA probe was synthesized *in vitro* using a PCR-generated template complementary to nt 926–1226 of the luciferase cDNA. A 28 S antisense probe was generated from a template purchased from Ambion. RNA probes were gel-purified and biotin-labelled using the BrightStar PsoralenBiotin non-isotopic labelling kit (Ambion). The filter membranes were pre-hybridized for 2 h at 65 °C, followed by probe hybridization overnight at 65 °C. The BrightStar BioDetect kit (Ambion) was then used for RNA detection.

Western immunoblot analysis

Cells were harvested and processed as described previously [21]. Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, U.S.A.), and equal amounts of total cellular protein (50 μ g/lane) were resolved by SDS/PAGE (12.5% gels) according to the method of Laemmli [22]. The resolved proteins were then transferred to a nitrocellulose filter membrane (Bio-Rad). The membrane was incubated in 1 $\times$ PBS/0.1% Tween 20, containing 5% non-fat dried milk, for 2 h at room temperature. The blots were then incubated with mouse monoclonal anti-DHFR antibody (BD Biosciences) at a 1:1000 dilution, followed by incubation with a horseradish peroxidase-conjugated secondary antibody (Bio-Rad) at a 1:1,000 dilution. Proteins were visualized with a chemiluminescence detection system using the Pierce Super Signal substrate (Pierce, Rockford, IL, U.S.A.).

RESULTS

Mapping of the binding site on DHFR mRNA

Prior studies from this laboratory had shown that human DHFR interacted with its cognate mRNA with high specificity. In contrast, RNA sequences corresponding to the 5'- and 3'-UTRs did not interact with human His-tagged recombinant DHFR protein, while the RNA sequence corresponding to the coding region bound to DHFR protein with a relatively high affinity similar to that of the full-length DHFR mRNA [13,16]. These initial

Table 1 Relative binding affinity of DHFR RNA sequences

Binding of human recombinant His-tagged DHFR protein to DHFR mRNA sequences was determined by comparing the concentration of unlabelled RNA that inhibited specific binding of 32 P-radiolabelled human DHFR:1–564 mRNA by 50 %. RNA competition experiments were performed using the filter-binding assay. Each value represents the mean $\pm$ S.E.M. from at least three experiments.

RNA sequence	IC ₅₀ (nM)
DHFR:1–564	3.71 $\pm$ 0.37
DHFR:1–200	6.90 $\pm$ 0.85
DHFR:220–370	13.97 $\pm$ 0.48
DHFR:301–564	4.26 $\pm$ 0.48
DHFR:401–564	4.57 $\pm$ 0.23
DHFR:1–64	> 500
DHFR:165–214	> 500
DHFR:515–564	> 100
TS:200–219	> 500

experiments suggested that the main binding site was contained within the coding region of DHFR mRNA.

To begin to map this site more precisely, we initially synthesized *in vitro* RNA sequences contained within the coding region corresponding to DHFR:1–200, DHFR:220–370 and DHFR:401–564. A series of nitrocellulose filter-binding assays were then performed to determine their relative binding affinity to DHFR protein. As seen in Table 1, the 164 nt fragment corresponding to DHFR:401–564 interacted with DHFR protein with nearly the same affinity (IC₅₀, 4.57 nM) as the sequence corresponding to the entire coding region (IC₅₀, 3.71 nM). The DHFR:401–564 sequence displayed the highest affinity to DHFR protein when compared with the other RNA sequences tested. Two other sequences within the coding region, DHFR:1–200 and DHFR:220–370, also interacted with DHFR protein, albeit at a relative lower affinity, 1.8- and 3.8-fold, respectively, when compared with DHFR:401–564. In contrast, several RNA sequences corresponding to DHFR:1–64, DHFR:165–214 and DHFR:515–564 displayed extremely poor binding to DHFR protein. This initial series of experiments demonstrated that the DHFR:401–564 sequence bound with the highest affinity to DHFR protein and that this element was important for RNA recognition. However, these findings suggested that binding sites may also be present within the DHFR:1–200 and DHFR:220–370 sequences. In addition to the filter-binding assay, we performed a series of experiments using the RNA gel-shift assay, and nearly identical results were obtained (results not shown).

***In vivo* biological effect of DHFR RNA sequences**

We next characterized the potential *in vivo* biological significance of the DHFR:401–564 sequence by investigating whether this 164 nt sequence could confer the property of DHFR-dependent translational regulation on to an unrelated mRNA. For these studies, we made use of a heterologous luciferase p644 indicator plasmid that had been placed under control of an EGR-1 promoter element [9]. The heterologous luciferase constructs are outlined in schematic form in Figure 1. Various DHFR sequences were inserted immediately upstream of the sequence encoding the luciferase gene to yield several heterologous constructs. Both the parent p644 and the heterologous p644/DHFR:401–564 plasmids were transiently expressed in human colon cancer RKO cells. As seen in Figure 2, the greatest reduction of luciferase activity was observed in cells transfected with p644/DHFR:3'-UTR. However, there was also a significantly decreased level (approx. 50 %) of luciferase activity in cells transfected with p644/DHFR:401–564

Figure 2 Effect of DHFR sequence on luciferase expression *in vivo*

Luciferase heterologous constructs were transiently expressed in human colon cancer RKO cells. Luciferase activity was measured 48 h post-transfection using the dual-luciferase reporter assay system as described in the Experimental section. The activity in cells transfected with parent p644 plasmid was defined as 100 %. Each value represents the mean $\pm$ S.E.M. from at least three experiments. *Statistical significance as determined by the paired Student's *t* test, $P < 0.02$, compared with the luciferase activity of cells transfected with parent p644.

when compared with cells transfected with the parent plasmid. A significant decrease in luciferase activity (approx. 25 %) was also observed in cells transfected with p644/DHFR:1–200 or p644/DHFR:220–370, although the reduction was not as significant as observed when either DHFR:3'-UTR or DHFR:401–564 was placed upstream of the luciferase reporter construct. In contrast, the presence of DHFR:5'-UTR or TS:3'-UTR sequences upstream of the luciferase reporter had no impact on luciferase activity. These initial transfection experiments suggested that the presence of the DHFR:3'-UTR, DHFR:401–564, DHFR:1–200 and DHFR:220–370 sequences could significantly down-regulate the expression of luciferase.

To provide evidence for the specific molecular event(s) regulating the changes in luciferase enzyme activity in RKO cells, we next performed a series of experiments in which an siRNA was designed to target a sequence on human DHFR mRNA corresponding to nt 35–54. Control experiments had been performed to show that this DHFR-targeted siRNA effectively inhibited the expression of DHFR protein in RKO cells at concentrations in the range of 1–10 nM (results not shown). The respective luciferase heterologous constructs were transfected into control RKO cells or RKO cells pre-treated with the DHFR-targeted siRNA at a concentration of 5 nM for 24 h. To document the molecular level at which luciferase expression was controlled, we determined the levels of luciferase mRNA by means of Northern-blot analysis. As seen in Figure 3(A), luciferase mRNA levels were identical in RKO cells treated with or without siRNA and following transfection with the respective luciferase constructs. In RKO cells treated with the DHFR-targeted siRNA, DHFR protein was undetectable by Western immunoblot analysis (Figure 3B). As an important control, the expression of an unrelated housekeeping protein, α -tubulin, remained unaffected by siRNA treatment and/or upon transfection with the respective luciferase recombinant plasmids.

Figure 3 Effect of DHFR RNA sequence on luciferase expression in RKO cells with or without treatment with DHFR-targeted siRNA

(A) Northern analysis of luciferase mRNA in RKO cells. Human colon cancer RKO cells were treated with 5 nM DHFR-targeted siRNA or scrambled siRNA for 24 h before transfection with the respective luciferase heterologous plasmid. Total cellular RNA was isolated and then subjected to Northern-blotting analysis. Control refers to the cells without transfection with siRNA and plasmids. (B) Western-blotting analysis. Human colon cancer RKO cells were treated with 5 nM of DHFR siRNA or scrambled duplex siRNA for 24 h and then transfected with the respective luciferase heterologous plasmid. Total cellular protein was harvested and then subjected to Western-blotting analysis. (C) Effect of DHFR RNA sequences on luciferase activities in control and DHFR-siRNA-treated RKO cells. Human colon cancer RKO cells were treated with 5 nM of DHFR siRNA or scrambled siRNA for 24 h and then transfected with the respective luciferase heterologous plasmid. Luciferase activity was measured 48 h post-transfection using the dual luciferase reporter assay. The activity in cells transfected with p644 was defined as 100%. Each value represents the mean $\pm$ S.E.M. from at least three experiments. *Signifies statistical significance as determined by the paired Student's *t* test, $P < 0.02$, compared with the luciferase activity of cells transfected with p644/DHFR:401–564 alone.

Luciferase activity remained unchanged following transfection with p644, p644/DHFR:3'-UTR and p644/DHFR:220–370 regardless of siRNA treatment (Figure 3C). In contrast, luciferase

Figure 4 Effect of MTX treatment on luciferase expression *in vivo*

Plasmids p644, p644/DHFR:3'-UTR, p644/DHFR:1–200, p644/DHFR:220–370 and p644/DHFR:401–564 were each transiently expressed in human colon cancer RKO cells in the absence or presence of 5 nM MTX. Luciferase activity was measured after 48 h post-transfection using the dual-luciferase reporter assay system. Luciferase activity in cells transfected with p644 in the absence of MTX was defined as 100%. Each value represents the mean $\pm$ S.E.M. from at least three experiments. *Signifies statistical significance as determined by the paired Student's *t* test, $P < 0.005$, compared with the luciferase activity of cells transfected with p644/DHFR:401–564 alone.

activity in cells transfected with p644/DHFR:401–564 was reduced by 50%, but was restored to nearly 100% control levels in cells treated with the DHFR-targeted siRNA. Moreover, treatment of RKO cells with scrambled siRNA did not restore luciferase activity to control levels following transfection with p644/DHFR:401–564.

To provide further evidence that the DHFR:401–564 sequence was responsive to alterations in intracellular levels of DHFR protein, we incubated transfected RKO cells with the antifolate analogue MTX (methotrexate). Previous studies from this laboratory and others have shown that treatment of a wide range of human cancer cell lines with MTX and other antifolate analogues resulted in the acute induction of DHFR protein [23–26], which was controlled, at least in part, at the translational level. As seen in Figure 4, treatment with MTX resulted in a significant 2-fold increase in luciferase activity in RKO cells transfected with p644/DHFR:401–564. In contrast, no such increase in luciferase activity was observed in response to MTX when RKO cells were transfected with either the parent p644 plasmid or the p644/DHFR:3'-UTR, p644/DHFR:1–200 or p644/DHFR:220–370 constructs.

Localization of the *cis*-acting regulatory element within nt 401–564 of the coding region of DHFR mRNA

To characterize further the *cis*-acting regulatory element contained within nt 401–564, three different fragments were designed and placed upstream of the luciferase reporter gene. These sequences corresponded to nt 401–482, 441–522 and 483–564. Using a series of transient-transfection experiments, we observed that the RNA sequence corresponding to nt 401–482 was able to retain translational regulatory effects on luciferase expression similar

Figure 5 Effect of DHFR sequences on luciferase expression *in vivo*

Plasmids p644, p644/DHFR:401-564, p644/DHFR:401-482, p644/DHFR:441-522 and p644/DHFR:483-564 were each transiently expressed in human colon cancer RKO cells. Luciferase activity was measured after 48 h post-transfection using the dual-luciferase reporter assay system. The activity in cells transfected with p644 was defined as 100 %. Each value represents the mean $\pm$ S.E.M. from at least three experiments. *Signifies statistical significance as determined by the paired Student's *t* test, $P < 0.001$, compared with the luciferase activity of cells transfected with parent p644.

Figure 6 Effect of MTX and siRNA treatment on luciferase expression *in vivo*

RKO cells were treated with DHFR-targeted siRNA (5 nM) or scrambled siRNA (5 nM) for 24 h. The sequence on human DHFR mRNA that is targeted by the siRNA corresponds to nt 36–54. The respective recombinant plasmids were then transfected into RKO cells in the absence or presence of MTX (5 nM). Luciferase activity was measured after 48 h post-transfection using the dual luciferase reporter assay system. Luciferase activity in cells transfected with p644 in the absence of MTX was defined as 100 %. Each value represents the mean $\pm$ S.E.M. from at least three experiments. *Signifies statistical significance as determined by the paired Student's *t* test, $P < 0.005$, compared with the luciferase activity of cells transfected with p644/DHFR:401-482 alone.

to the intact 164 nt sequence. In particular, luciferase activity was reduced significantly by 50% when the DHFR:401-482 sequence was placed upstream of the luciferase gene. In contrast, the two other sequences were unable to alter luciferase activity significantly (Figure 5).

We next tested the effect of MTX treatment and/or DHFR-targeted siRNA on luciferase activity (Figure 6). A significant 2-fold increase in luciferase activity was observed when RKO cells were first treated with MTX and/or the siRNA directed against DHFR mRNA and then transfected with p644/DHFR:401-482. In contrast, no such increase in luciferase activity was observed in response to MTX when RKO cells were transfected with the

Table 2 Relative binding affinity of DHFR RNA sequences contained within nt 401-564

Binding of human recombinant His-tagged DHFR protein to DHFR RNA sequences contained within nt 401-564 was determined by comparing the concentration of unlabelled RNA that inhibited specific binding of 32 P-radiolabelled human DHFR:1-564 by 50 %. RNA competition experiments were performed using the filter-binding assay. Each value represents the mean $\pm$ S.E.M. from at least three experiments.

RNA sequence	IC ₅₀ (nM)
DHFR:1-564	3.71 $\pm$ 0.37
DHFR:401-564	4.57 $\pm$ 0.23
DHFR:401-482	5.35 $\pm$ 0.37
DHFR:441-522	> 100
DHFR:483-564	> 100

Figure 7 Binding of DHFR RNAs to wild-type and mutant DHFR proteins

32 P-Radiolabelled human DHFR:1-564, DHFR:401-564 and DHFR:401-482 RNAs were incubated in the absence or presence of wild-type human His-tagged DHFR protein (WT; 42.6 pmol) or mutant C6A (42.6 pmol) as described in the Experimental section.

parent p644 plasmid, nor was an increase in luciferase activity observed when RKO cells were treated with the scrambled siRNA.

We then synthesized *in vitro* each of these RNA sequences and determined their relative binding affinities using the nitrocellulose filter-binding assay. As seen in Table 2, DHFR:401-482 (IC₅₀, 5.35 nM) bound to human His-tagged DHFR protein with a relative binding affinity similar to that of DHFR:401-564 (IC₅₀, 4.57 nM). However, the sequences corresponding to DHFR:441-522 and DHFR:483-564 interacted with DHFR protein, albeit with extremely low affinity (IC₅₀ > 100 nM).

We also synthesized *in vitro* 32 P-radiolabelled RNAs corresponding to DHFR:1-564, DHFR:401-564 and DHFR:401-482, and then performed a series of RNA-binding experiments. Each of these RNA sequences was able to form a ribonucleo-protein complex with wild-type human His-tagged DHFR protein (Figure 7). Further binding studies confirmed that each of these RNA sequences bound with nearly the same affinity to human DHFR protein (results not shown). In contrast, a mutant DHFR protein with a point mutation at the cysteine residue located at the amino acid 6 position was unable to bind to any of these RNA sequences (Figure 7).

DISCUSSION

Previous studies from our laboratory and others had suggested that the main RNA-binding site for DHFR protein was located within the coding region of human DHFR mRNA [13–16]. In the present study, we focused our efforts on further characterizing the *cis*-acting regulatory element within this region. As our initial strategy, we performed a series of *in vitro* RNA gel-mobility shift and nitrocellulose filter-binding assays and *in vivo* transfection experiments. Taken together, these studies revealed that a 164 nt sequence corresponding to nt 401-564 within the coding region is sufficient to confer the property of translational regulation on to a heterologous luciferase reporter gene. We have further localized this element to an 82 nt sequence corresponding to nt 401-482. In

addition, our *in vitro* studies suggested that human DHFR might also be able to interact with two other sequences contained within the coding region, namely those corresponding to DHFR:1–200 and DHFR:220–370. Preliminary analysis of these three elements, however, have failed to identify a consensus nucleotide sequence and/or a consensus secondary structure. However, more precise RNA sequencing and/or structural studies are required to characterize each of these binding sites more completely.

To provide further evidence that the sequence corresponding to nt 401–482 on the human DHFR mRNA was a true DHFR-response element, we investigated the expression of the luciferase reporter in human RKO cells that had been treated with a DHFR-targeted siRNA. RNA interference is a novel regulatory mechanism in which the presence of double-stranded siRNA results in gene silencing [27–29]. It has been established that the transfection of siRNAs, in the order of 20–25 nt in size, into mammalian cells can effectively and specifically down-regulate the expression of a given cellular gene [30–32]. We designed an siRNA targeting DHFR mRNA to inhibit the expression of DHFR in RKO cells. In the presence of the DHFR-targeted siRNA, luciferase activity was restored to control levels following transfection with p644/DHFR:401–564 and p644/DHFR:401–482. We observed a similar increase in luciferase activity upon transfection with p644/DHFR:401–564 and p644/DHFR:401–482 following treatment with the antifolate analogue MTX. Our earlier work has shown that treatment with MTX effectively abrogates the interaction between DHFR protein and its target *cis*-acting sequence, thereby negating the translational inhibitory effects of DHFR on luciferase activity. While the precise mechanism by which MTX abrogates the DHFR protein–DHFR mRNA interaction remains to be defined, one possibility is that a common binding domain on the DHFR protein exists for MTX ligand binding as well as RNA recognition. An alternative mechanism would be an MTX-induced alteration in the conformation of the DHFR protein that then makes the actual RNA-binding domain no longer accessible for RNA binding. Taken together, our findings provide strong evidence that the DHFR:401–482 sequence is a true DHFR-response element and that an intact DHFR protein is required for its translational regulatory effects.

Our *in vivo* transfection experiments have shown that luciferase expression was significantly reduced when the DHFR:3'-UTR sequence was placed upstream of the luciferase reporter gene. Reductions in luciferase activity were also observed when sequences corresponding to DHFR:1–200 and DHFR:220–370 were transfected into RKO cells. These findings reveal that these three sequences function as repressor-type elements. However, none of these putative repressor elements appeared to be significantly responsive to changes in the intracellular levels of DHFR protein following treatment with the DHFR-targeted siRNA and/or the antifolate MTX. These findings suggest that binding of DHFR protein to any one of these elements is not directly responsible for their biological effects, as their effects on luciferase activity were not affected by changes in the intracellular expression of DHFR protein. Our *in vitro* RNA-binding studies demonstrated, however, that human recombinant DHFR protein bound with relatively high affinity to both the DHFR:1–200 and DHFR:220–370 RNA sequences, albeit at a slightly lower affinity than the DHFR:401–482 element. Therefore, while the *in vivo* studies strongly suggest that other cellular proteins interact with these elements on DHFR mRNA, our findings do not exclude the possibility that DHFR may also play a role as a *trans*-acting element. Of note, given the absence of any change in luciferase mRNA levels, these cellular protein(s) would be expected to exert their regulatory effects at the translational level in a manner similar

to the DHFR protein. Studies are now underway to begin to identify these putative RNA-binding proteins within RKO cells.

Further studies are required to elucidate more precisely the critical molecular elements involved in the translational regulation of DHFR and the interaction between DHFR protein and its target DHFR mRNA. We have shown that Cys-6 as well as Ile-7, Arg-28 and Phe-34 are critical for RNA binding. Preliminary investigations from our laboratory suggest that none of the mutant DHFR proteins with point mutations at any of these amino acids are able to form ribonucleoprotein complexes with the DHFR:401–482 response element, providing further support to their role in RNA binding. It remains unclear as to whether these amino acids form direct contact points with the DHFR mRNA and/or whether they maintain the protein in a proper conformational state that then allows for the actual binding domain on the protein to interact with its cognate mRNA. Studies are in progress to resolve the crystal structure of the DHFR protein–DHFR mRNA complex in order to address this issue directly. Finally, this work contributes to our growing understanding of the important role of translational control in the regulation of cellular gene expression and provides new insights into the critical elements that mediate RNA–protein interactions.

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