Isolation and Characterization of a Folate Receptor mRNA-binding trans-Factor from Human Placenta

EVIDENCE FAVORING IDENTITY WITH HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN E1

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The interaction of an 18-base cis-element in the 5'-untranslated region of human folate receptor (FR)-α mRNA with a cytosolic trans-factor protein is critical for the translation of FR mRNA with a cytosolic trans-factor protein is critical for the translation of FR mRNA. In vitro studies using antibodies against human heterogeneous nuclear ribonucleoprotein E1 (hnRNP E1) revealed that this interaction is mediated by a 18-base cis-element in the 5'-untranslated region of the FR mRNA. The functional role of this interaction is suggested by the observation that antibodies against hnRNP E1 specifically inhibited translation of FR mRNA in a dose-dependent manner, and the antibody effect could be reversed in a dose-dependent manner by either purified trans-factor or the complete trans-factor. Collectively, the data favor identity of the FR mRNA-binding trans-factor and hnRNP E1, confirm its critical role in the translation of FR, and highlight yet another role of multifunctional hnRNP E1 in eukaryotic mRNA regulation.

Folate receptors (FR)1 mediate the cellular uptake of physiologic folates and some antifolates in normal and malignant cells (reviewed in Refs. 1 and 2). Human placenta is rich in FR and was the source from which FR were first isolated to apparent homogeneity as a truncated species (3) through the action of an endogenous metalloprotease (4, 5). The full-length FR species that are anchored to the membrane by glycosyl phosphatidylinositol anchoring (6) play a crucial role in transplacental maternal-to-fetal transport of folate (7). Among the three FR isoforms (designated α, β, and γ), the most ubiquitous is the α-isomorph (referred hereafter as FR-α).

In studies designed to identify the basis for the inverse relationship between the extracellular folate concentration and FR expression (8), we identified that the up-regulation of FR in cervical carcinoma cells is primarily at the translational level (9). Further studies identified a 46-kDa cytosolic protein in human cervical carcinoma cells, which specifically interacted with a CU-rich 18-base cis-element in the 5'-untranslated region (5'-UTR) of human FR-α mRNA, and that this interaction was critical for the translation of FR in vitro (10). Moreover, because this FR mRNA-binding trans-factor was widely distributed in cells expressing little to no FR-α, we predicted that this RNA-binding protein probably has additional functions that are unrelated to FR translation (10).

RNA-binding proteins are known to play a pivotal role in the metabolism, function, and fate of RNA in the post-transcriptional regulation of gene expression (11–14). In the past decade, several groups of RNA-binding proteins have been identified (15, 16) that are variously involved in mRNA stability, storage, splicing, transportation, and translational regulation. Accordingly, to characterize the FR mRNA-binding trans-factor (10), and further understand the role of this trans-factor in regulation of FR expression at the translational level, we isolated and characterized this protein from human placenta, which is a rich source. In this report, we present evidence that favors identity of the FR mRNA-binding trans-factor with heterogeneous nuclear ribonucleoprotein E1 (hnRNP E1). In addition, functional analysis of this protein using antibodies against hnRNP E1 confirms its critical role in the biosynthesis of FR at the translational level.

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‡ The abbreviations used are: FR, folate receptor; hnRNP E1, heterogeneous nuclear ribonucleoprotein E1; FPLC, fast flow liquid chromatography; GST, glutathione S-transferase; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; UTR, untranslated region; RT, reverse transcription; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid.

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Materials—Restriction endonucleases, T4 DNA ligase, RNase A, RNase T1, proteinase K, RNase inhibitor, mRNA isolation kit, and the in vitro transcription kit (containing pSP78-19/19 plasmids) were from Roche Molecular Biochemicals. Unless noted otherwise, all other chemicals of the highest analytical grade were from Sigma. Full-length FR-α cDNA was from Dr. P. C. Elwood. Sense and antisense 18-base DNA oligonucleotides corresponding to the FR-α mRNA cis-element (10) and sense and antisense oligonucleotides corresponding to the 19-base cis-element in the 3′-UTR of 15-lipoxygenase RNA (25) were synthesized by Life Technologies, Inc. The DNA sequencing kit (Sequenase version 2.0), [α-32P]UTP, [α-32P]CTP (specific activity >3000 Ci/mmol), [α-32P]ATP, Redivue Pre-Mix [32P]Smethionine, all FPLC HiTrap chromatography columns, fast flow liquid chromatography (FPLC) equipment, and GST fusion protein expression vector pGEX-T-1 were from Amersham Pharmacia Biotech (Uppsala, Sweden). Mini-SDS-PAGE electrophoresis cell, mini-trans blot transfer cell, and SDS-PAGE ready gels were from Bio-Rad. Access RT-PCR introductory system and the 5′ end labeling kit were from Promega (Madison, WI). Plasmid preparation kits were from Qiagen (Valencia, CA).

Plasmid Construction—The 1.1-kilobase pair full-length human FR-α cDNA, which was subcloned into the pSP18 vector downstream of the SP6 RNA polymerase promoter, was designated pSP18-FR-α (10). To construct a plasmid containing the 18-base FR-α cis-element, sense and antisense oligonucleotides containing two 18-base oligonucleotide repeats with PstI and BamHI restriction sites at the ends were synthesized. After annealing the sense and antisense oligonucleotides and digestion, the purified DNA was subcloned into the pSP18 vector between restriction sites PstI and BamHI. The new plasmid was designated as pXL37A. The 18-base sequence (see Fig. 4A) was determined by DNA sequencing.

Preparation of radiolabeled RNA (cis-Element) and DNA Oligonucleotides from FR mRNA/cDNA—To prepare the 18-base cis-element RNA fragment, pXL37A was linearized by PstI. To prepare radio-labeled RNA probe, [α-32P]UTP was included in the transcription reaction, and RNA transcripts were purified using NucTrap push-through Sephadex G-50 columns (Stratagene, La Jolla, CA). 5′ end labeling was used to prepare γ-32P-labeled 18-base DNA oligonucleotides probes. Double-stranded 18-base pair DNA was generated by annealing equimolar amounts of single-stranded sense and antisense 18-base oligonucleotides by heating at 95 °C for 5 min followed by cooling to 22 °C.

RNA-Protein Binding Assays and Analysis of Complexes—RNA-protein binding reactions and electrophoretic mobility gel-shift assay (18) were performed as described (10). In some experiments, reducing or oxidizing reagents were added to the reactions to detect effects on the RNA-protein interaction. Briefly, [32P]cis-labeled cis-element (10,000 cpm) was reanimated with 20 μg of partially purified placental cytosolic extract (that had been dialyzed to remove dithiothreitol before Northwestern blot analysis using 32P-labeled 18-base pair DNA probes. Double-stranded 18-base pair DNA probes were electrophoresed before Northwestern blot analysis using 32P-labeled 18-base cis-element RNA probes (10). For Southwestern blot analysis, following SDS-PAGE and transfer to nitrocellulose, the separated proteins were probed with 32P-labeled 18-base single-stranded or double-stranded DNA probes.

Preparation of crude protein extracts—Cytosolic protein extracts (5-100 fraction) from 5 × 107 Hela-IU1 cells were prepared as described (10). Placental cytosolic extracts were collected as follows. Full-term placentas were placed in an ice bath shortly after normal vaginal delivery and processed within 4 h of delivery. After removal of the umbilical cord, membranes, blood clots, and necrotic tissue, each placenta was diced into 2 × 2 cm pieces and then washed twice with 1000 volumes of 10 mM potassium phosphate (pH 7.5) (Buffer A). After centrifugation at 30,000 × g for 30 min, the supernatant was supplemented with 1 mM DTT and 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and filtered through a Minutian ultrafiltration system (Millipore Corp., Bedford, MA) using a 300,000 M, cut-off. The sample was then collected into cut-off membranes, and 0.5 ml of 32P-labeled RNA probe. After the addition of 10% glycerol (v/v), the sample was frozen at −80 °C until further use. The protein concentration of the cytosolic extracts from human placenta (hereafter referred to as "crude sample") was determined using the BCA method. Nuclear extracts from human placenta were also prepared as described (19).

Purification of the Placental trans-Factor—Unless otherwise stated, all purification steps (Table I) were carried out at 4 °C and RNA binding activity was detected by Northwestern blot analysis. The crude sample was thawed to 4 °C and trans-factor was precipitated by 40% saturated ammonium sulfate. The pellet was rinsed with 40% saturated ammonium sulfate, following which the trans-factor was dissolved in 10 ml of Buffer B (10 mM potassium phosphate, pH 7.0, containing 0.6 M ammonium sulfate) containing 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 0.1% SDS, and 10 mM DTT. The sample was then loaded onto a 5-ml Hitrap phenyl-Sepharose CL-4B column that was equilibrated with Buffer B using a Pharmacia FPLC system. After elution, the sample and washing with 10 column volumes of 50% Buffer B plus 50% Buffer C (10 mM potassium phosphate, pH 7.0, containing 0.5% CHAPS, 1 mM DTT, 0.5 mM PMSF, 10% (v/v) glycerol), the trans-factor was eluted with Buffer C and fractions of 0.5 ml were collected.

The eluted fractions containing RNA binding activity (fractions 5–9) were pooled and directly loaded onto a HiTrap heparin column (consisting of 5-ml columns connected in series; Amersham Pharmacia Biotech) that was equilibrated and washed with Buffer C until the absorbance at an optical density of 280 was at base line. The proteins that remained bound to the column were eluted with 50 ml of Buffer B with KCl in a linear gradient from 0 to 1000 mM, and trans-factor-positive fractions (eluting between 0.3 and 0.4 M KCl) were pooled and diluted with an equal volume of Buffer C. After adding tRNA (35 μg/ml), tRNA (20 μg/ml), and RNase inhibitor (40 units/ml), the mixture was applied to a 5-ml HiTrap poly(U)-Sepharose 4B column, which was equilibrated with Buffer C. After washing the column with Buffer C, the protein fractions containing RNA-binding activity were collected. The sample was then eluted with 50 ml of Buffer C with KCl in a linear gradient of 0–1000 mM. Fractions of 0.5 ml were collected, and trans-factor-positive fractions (eluting between 0.38 and 0.6 M KCl) were dialyzed against Buffer C using Slide-a-lyzer dialysis cassettes (Pierce). Finally, the sample was filtered (0.2 μm) and stored at −80 °C.

Electrophoretic Elution and Renaturation of the trans-Factor from SDS-PAGE—Purified protein fractions from the (U-Sephrose step) were electrophoresed on 10% SDS-PAGE. Pre-stained protein markers (Bio-Rad) were loaded on either sides of the gel. Following electrophoresis, the band corresponding to the trans-factor was excised and the proteins were electro-eluted in 2 ml dialysis bags containing 25 mM Tris-glycine and 0.1% SDS. After filtration through 0.2-μm filters, GST fusion protein expression vector pGEX-T-1 were loaded onto 5-ml HiTrap phenyl-Sepharose CL-4B column that was equilibrated with Buffer C containing 10 ml of Buffer B with KCl in a linear gradient from 0 to 1000 mM, and trans-factor-positive fractions (eluting between 0.38 and 0.6 M KCl) were dialyzed against Buffer C using Slide-a-lyzer dialysis cassettes (Pierce). Finally, the sample was filtered (0.2 μm) and stored at −80 °C.

Semiquantitative Assay for Recovery of the trans-Factor during Purification—To semiquantitatively estimate the relative recovery of FR mRNA-binding trans-factor during each purification step, aliquots from each step were analyzed using a single purification of [32P]labeled 18-base cis-element probe of known specific activity. The binding of the fixed amount of excess RNA probe with trans-factor present in each purification step was determined by a gel-shift assay. After drying the gel on Whatman paper, the areas of RNA-protein complexes (verified by autoradiography) were excised and immersed in 10 ml of BioSafe II counting mixture (Research Products International), and radioactivity was determined by liquid scintillation counting.

Northwestern and Southwestern Blot Analysis—Functionally active trans-factor was monitored during each purification step by Northwestern blots. Briefly, various aliquots of trans-factor protein were first separated by 10% gradient SDS-PAGE gels by electrophoresis before Northwestern blot analysis using [32P]labeled 18-base cis-element RNA probes (10). For Southwestern blot analysis, following SDS-PAGE and transfer to nitrocellulose, the separated proteins were probed with [32P]labeled 18-base single-stranded or double-stranded DNA probes.

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FIG. 1. A, evidence that the FR mRNA-binding trans-factor is present in human placental cytosolic protein extracts. For gel-shift assay, increasing amounts of human placental cytosolic crude extracts (lanes 2–5) were reacted with 32P-labeled 18-base RNA cis-element followed by electrophoresis in 6% native gels. Lane 1 contained no protein extract, and lanes 2–5 contained 10, 20, 40, and 80 μg of protein plus proteinase K. B, Northwestern blot analysis. The experiments in A and B were repeated three times with less than 10% variation between results. C, analysis of the trans-factor by SDS-PAGE during various purification steps. Samples containing cis-element binding activity following each purification step were analyzed on 4–20% SDS-PAGE and stained with Coomassie Blue. Lane 1, after ultrafiltration, 20 μg; lane 2, after ammonium sulfate precipitation, 20 μg; lane 3, after hydrophobic interaction (phenyl-Sepharose) chromatography, 20 μg; lane 4, after heparin chromatography, 10 μg; lane 5, after poly(U) affinity chromatography, 1 μg; lane 6, M, standards, 20 μg (rabbit muscle phosphorylase = 97.4 kDa; bovine serum albumin = 66.2 kDa; hen egg white ovalbumin = 45 kDa; bovine carbonic anhydrase = 31 kDa; soybean trypsin inhibitor = 21.5 kDa; lysozyme = 14.5 kDa). D, gel-shift assay of the electro-eluted trans-factor following attempts at renaturation with various buffers. Following SDS-PAGE of partially purified trans-factor (5 μg) the 43-kDa trans-factor was electro-eluted, denatured with guanidine-HCl, and then diluted 50-fold with various renaturing buffers (lanes 3–9). After concentration and reaction with 32P-labeled cis-element RNA, RNA-protein complexes were analyzed by gel-shift assays. Lane 1, positive control to mark position of RNA-protein complex; lane 2, negative control (addition of SDS-PAGE loading buffer and boiling of sample used in lane 1) prior to gel-shift assay; lane 3, gel electro-solution buffer; lane 4, phosphate-buffered saline; lane 5, 10 mM Hepes buffer; lane 6, Northwestern binding buffer (without Denhardt’s solution); lane 7, Denhardt’s solution (1×); lane 8, 10 mM Hepes plus Denhardt’s solution (1×); lane 9, Northwestern binding buffer plus Denhardt’s solution (1×). The experiments in C and D have been repeated over five times with similar results.

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**Purification of the trans-Factor from Human Placenta**

Progressive purification of the trans-factor was assessed by Northwestern blots and SDS-PAGE. Various properties of the trans-factor were exploited in its purification from human placental cytosolic extracts (Table I; Fig. 1A). Although the predicted molecular mass of the trans-factor was 43 kDa, trans-factor proteins were retained by a 100-kDa cut-off membrane; this suggested its association in a complex with other proteins. Accordingly, following ultrafiltration using a 300-kDa cut-off membrane, the sample was concentrated over a 30-kDa cut-off membrane.

Fig. 1C shows the progressive purification of the trans-factor using the purification scheme summarized in Table I. Following ammonium sulfate precipitation, the trans-factor, which bound to a hydrophobic interaction column (phenyl-Sepharose), was further purified based on its resistance to elution by up to 80% of Buffer C. Therefore, after eluting other proteins, the trans-factor was eluted by Buffer C, which was supplemented with 0.5% CHAPS (CHAPS was selected because several other ionic, nonionic, or zwitterionic detergents (Nonidet P-40, SDS, Triton X-100) affected the RNA-protein interaction). The trans-factor interacted with the heparin column at pH 7.0, following which it was eluted by a linear gradient of KCl.

The 18-base cis-element is a C/U-rich sequence (containing 11 cytosine and 6 uridine residues). Although the trans-factor interaction with the cis-element could be competed by poly(C) (but not by poly(U)) (10), the very strong interaction of the trans-factor with poly(C)-Sepharose precluded elution from this column with conventional buffers. Surprisingly, in the presence of CHAPS, we found that the trans-factor could bind poly(U)-Sepharose and was eluted by a linear gradient of KCl. This was a key step in the purification scheme (Fig. 1C, lane 5). To eliminate minor protein staining bands (that were noted only on silver-stained SDS-PAGE) that co-eluted with the trans-factor following the poly(U)-Sepharose step, that portion of the gel containing the 43-kDa trans-factor was electro-eluted. An aliquot of this electro-eluted protein exhibited a single 43-kDa protein on repeat SDS-PAGE followed by silver staining. To prove that this protein was the trans-factor, which another aliquot was denatured with guanidine and renatured and staining. To prove that this protein was the trans-factor, which another aliquot was denatured with guanidine and renatured and then assessed for the capacity to interact with the cis-element with gel-shift assays. As shown in Fig. 1D, although there were no RNA-protein complexes when electro-eluted (denatured) 43-kDa trans-factor protein was assessed for RNA interaction, such RNA-protein complexes were best observed after the electro-eluted and denatured trans-factor was renatured with Northwestern binding buffer containing 1X Denhardt’s solution (lane 9). Thus, the purified protein doublet that bound the 18-base RNA cis-element on Northwestern blots was the same protein that gave rise to the RNA-protein complexes on gel-shift assays. Accordingly, the electro-eluted, apparently homogeneous trans-factor was subjected to amino acid sequence analysis.

**Characterization of the Purified trans-Factor from Human Placenta**—After confirmation of apparent homogeneity based on SDS-PAGE, the trans-factor was characterized as follows.

**Molecular Weight Determination**—Earlier, we identified that the cervical carcinoma (HeLa-IU1) cell trans-factor had a Mr of 46,000; this was based on the use of prestained standards used in Northwestern blots (10). However, using unstained Mr standards, the same 46-kDa protein migrated as a 43-kDa species on SDS-PAGE (data not shown). Similar findings were noted with the human placental trans-factor. Thus, the corrected Mr of the trans-factor is 43,000.

Northwestern blots of the crude human placental trans-factor revealed a single protein band. However, the purified trans-factor migrated as a doublet on SDS-PAGE (in addition to the 43-kDa species, there was another 38-kDa species). Both these species were recognized by specific hnRNP E1 antibodies, and amino acid sequences of a peptide fragment from both proteins were identical (discussed below). Together, these results suggest that the 38-kDa species is either a degradation product of the 43-kDa trans-factor that was generated in vitro during protein purification or a closely related isoform.

**Binding of trans-Factor to Single-stranded DNA**—Three probes were used to determine whether the trans-factor could bind to single-stranded 18-base sense or antisense DNA oligonucleotides, or 18-base pair double-stranded DNA oligonucleotides. The results of gel-shift assays (Fig. 2A) and Northwestern blots (Fig. 2B) indicated that the trans-factor could also bind to single-stranded sense 18-base DNA oligonucleotides but not single-stranded antisense or double-stranded DNA oligonucleotides.

**Effects of Reducing and Oxidizing Agents on RNA-Protein Interactions**—Some RNA-protein interactions can be affected by reducing or oxidizing agents (22). As shown in Fig. 2C, with progressively increasing concentrations of DTT, D,L-homocysteine, and L-cysteine, there were dose-dependent effects in generation of RNA-protein complexes. Conversely, with the addition of increasing concentrations of oxidizing agents, N-ethylmaleimide, and trans-4,5-dihydroxy-1,2-dithiane (oxidized DTT), the RNA-protein complex formation was progressively quenched (Fig. 2D). Thus, the interaction of the cis-element and trans-factor was increased by reducing agents and inhibited by oxidizing agents.

**Evidence Favoring Common Identity of the FR mRNA-binding trans-Factor and hnRNP E1**

**Amino Acid Sequencing Studies**—To further characterize the purified trans-factor, both 43- and 38-kDa proteins were subjected to amino acid sequence analysis. Because the N-terminal amino acid was blocked, internal amino acid sequence analysis of peptide fragments following cleavage with cyanogen bromide or further treatment with O-phthalaldehyde was carried out. Two peptides from the 43-kDa trans-factor and one peptide fragment from the 38-kDa species were analyzed. The amino

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<td>1.2</td>
<td>181.5</td>
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a One unit of activity corresponds to 1 pmol of bound RNA/mg of protein in the standard gel-shift assay.

b The lysate was derived from six placentas.

**TABLE I**

Purification of the FR mRNA-binding trans-factor

**Folate Receptor mRNA-binding trans-Factor**
Binding of Recombinant hnRNP E1 to 18-base FR mRNA cis-Element—To explore the relationship between trans-factor and hnRNP E1, we first expressed and purified recombinant human hnRNP E1. As shown in Fig. 3A, the affinity-purified GST-hnRNP E1 fusion protein was 69 kDa (lane 2). After treatment with thrombin, the dominant protein was 43 kDa (lane 3). By Western analysis (Fig. 3B), both the 69-kDa fusion protein (lane 2), the cleaved 43-kDa protein (lane 3), and purified trans-factor (lane 5) bound to the 32P-labeled 18-base cis-element (Fig. 3B, lanes 1–3 and 5), but there was no signal with GST alone (lane 4). (The additional smaller signal in lane 3 was probably a proteolytic fragment generated by thrombin).

Immunological Studies to Assess Cross-reactivity—Next, we generated rabbit-specific anti-hnRNP E1 (oligopeptide) antibodies to a synthetic 19-amino acid peptide fragment of hnRNP E1 that was neither in the conserved K homology (KH) domain of hnRNP (15, 16) nor in the microsequenced peptide of the 43- and 38-kDa trans-factors. The capacity for the recognition of the full-length hnRNP E1 as well as 43- and 38-kDa trans-factors by this purified antibody was then determined by Western blot analysis. As demonstrated in Fig. 3C, the specific rabbit anti-hnRNP E1 (oligopeptide) antibodies clearly recognized the 69-kDa recombinant fusion protein within the bacterial lysate, the purified 43-kDa hnRNP E1 proteins, as well as the two purified 43- and 38-kDa trans-factors.

Interaction of the Human 18-base cis-Element with the Murine Homolog of hnRNP E1—The tissue distribution of the human 18-base cis-element also interacted with the murine protein in a distribution similar to that described for αCP-1. We observed positive signals for murine placenta, bone marrow, liver, heart, kidney, brain, and lung on both gel-shift assays and Western blots, respectively (data not shown), further suggesting that the related murine protein(s) interacted with the cis-element in a manner similar to that for human placent al trans-factor and hnRNP E1.
FIG. 4. Analysis of the interaction of the 19-base cis-element in the 3'-UTR of FR-α RNA/DNA and the 19-base cis-element in the 3'-UTR of 15-lipoxygenase RNA/DNA with human placental trans-factor and recombinant hnRNP E1 by gel-shift assays, Northwestern blots, and Southwestern blots. A and B, the sequence of the 19-base cis-element in FR-α mRNA 5'-UTR and the 19-base cis-element in the 3'-UTR of 15-lipoxygenase. The numbers indicate positions in the FR-α mRNA 5'-UTR and 15-lipoxygenase cDNA from the first nucleotide of the translation initiation site. C-F, α-32P-labeled 18-base FR RNA and 15-lipoxygenase RNA or γ-32P-labeled 18-base single-stranded sense DNA oligonucleotides of FR and 15-lipoxygenase were reacted with 40 μg of human placental extract enriched for 43-kDa trans-factor and 1 μg of recombinant hnRNP E1 in the indicated studies. 15-LOX, 15-lipoxygenase; HP, human placenta; E1, hnRNP E1; asterisk-marked lane contained only free probe.

Interaction of the 19-base RNA in the 3'-UTR of 15-Lipoxygenase mRNA with Human Placental trans-Factor—hnRNP E1 is known to bind to a poly(C)-rich 19-base element in the 3'-UTR of the 15-lipoxygenase RNA (25). Therefore, we determined if this 19-base RNA and/or single-stranded sense DNA could interact with the single 43-kDa placental trans-factor (Fig. 4). Both the 18-base FR cis-element and the 19-base 15-lipoxygenase sequences, although not identical, are poly(C)-rich (Fig. 4, A and B). The single human placental 43-kDa trans-factor and recombinant hnRNP E1 interacted in a similar manner with 18-base FR RNA and 19-base 15-lipoxygenase RNA on gel-shift assays and Northwestern blots (Fig. 4, C and D). A similar interaction was also documented when placental trans-factor and hnRNP E1 were reacted with single-stranded sense 18-base DNA oligonucleotides of FR and 19-base DNA oligonucleotides of 15-lipoxygenase and analyzed by gel-shift assays and Southwestern blots (Fig. 4, E and F). These data lend further support to the common identity of the 43-kDa human placental trans-factor and hnRNP E1.

Functional Analysis of the FR mRNA-binding trans-Factor

Because interaction of the trans-factor with the FR mRNA cis-element is critical for the translation of FR (10), we determined if antibodies to the hnRNP E1/trans-factor could inhibit the translation of FR in vitro. Although reticulocytes are rich in hnRNP E1 and were the source for purification of this protein (24), it was critical in this experiment to first demonstrate that the exogenous addition of hnRNP E1 did not further increase expression of FR during in vitro translation. There was no change in the efficiency of translation of FR when either increasing concentrations of recombinant hnRNP E1 (Fig. 5A) or purified trans-factor (data not shown) was added to the in vitro translation reaction mixture. However, with the addition of increasing concentrations of purified specific anti-hnRNP E1 (oligopeptide) antibodies, there was a dose-dependent inhibition of FR mRNA translation (Fig. 5, A and B, lanes 2–4). Addition of nonimmune (control) IgG (lane 8) did not have significant effect in reducing translation. Furthermore, despite the presence of a concentration of anti-hnRNP E1 (oligopeptide) antibody known to completely inhibit FR translation, the addition of increasing concentrations of purified trans-factor to the reaction mixture led to a dose-dependent restoration of FR synthesis in vitro (Fig. 5, B and C, lanes 5–7). These results lent further support to the concept that the protein that we had purified (the trans-factor/hnRNP E1) was indeed functional and played a critical role in the translation of FR in vitro. Experiments using recombinant hnRNP E1 in place of trans-factor also gave similar results, further supporting the identity of these proteins.

DISCUSSION

The evidence supporting our isolation of functionally active placental trans-factor to apparent homogeneity were the findings of a 43- and 38-kDa doublet on silver-stained SDS-PAGE with no other contaminating proteins, and demonstration of binding of this doublet to 18-base FR cis-element on Northwestern blots and gel-shift assays. Although a single 43-kDa protein was observed in the crude placental cytosolic extract, purified trans-factor(s) resolved into the 43- and 38-kDa doublet in the absence and presence of reducing agents. A trivial (and likely) explanation is that, with progressive purification, the smaller protein was generated by an endogenous placental protease. In support of this possibility, partial microsequencing data of an internal peptide fragment revealed identity with one another, and functional assays revealed that both protein bands interacted with the 18-base mRNA cis-element. Immunological studies also indicated that each was recognized by the same anti-hnRNP E1 antibody. However, such data do not rule out...
the possibility that the 43- and 38-kDa trans-factor species are isofoms. By analogy, the 43-kDa hnRNP E1 has 80% homology with hnRNP E2 (a smaller isofom); likewise, there are also two closely related mouse poly(C)-binding proteins (αCP-1 and αCP-2) (23).

The trans-factor was localized by cell fractionation studies predominantly in the cytoplasm with some activity in the nucleus in human placenta. This is consistent with our results that the trans-factor binds RNA and single-stranded DNA (but not double-stranded DNA) in vitro. This is also similar to previous reports with other hnRNP proteins (25–28). Such data raise the possibility that this hnRNP E1/trans-factor may function during gene transcription of FR or during transport of mRNA from nucleus to cytoplasm. In the latter context, the in vitro translation data clearly establish a cytosolic function for this heterogeneous nuclear RNP.

Several lines of evidence favored identity between the 43-kDa human placental FR mRNA-binding trans-factor and hnRNP E1. These include structural (similar $M_r$, shared internal peptide sequences), common binding characteristics of both proteins to the 18-base cis-elements of FR (RNA and DNA), binding of the 18-base FR RNA to murine tissues known to contain a hnRNP E1-related protein, and immunological (shared epitopes) studies. In addition, the same poly(C)-rich 19-base element in the 3′-UTR of the 15-lipoxygenase mRNA that binds hnRNP E1 (24) also interacted with the placental FR-mRNA-binding trans-factor. Finally, the capacity of specific anti-hnRNP E1 (oligopeptide) antibodies to quench the translation of FR in vitro, and the dose-dependent reversal of the inhibitory effects of antibody by purified FR mRNA-binding trans-factor or purified recombinant hnRNP E1 was additional evidence for identity. When taken together, these data allow for the following conclusions. (a) The trans-factor we isolated and characterized was functional and played a critical role in the translation of FR, further supporting our early report (10). (b) Because of significant structural similarities between the trans-factor and hnRNP E1, these data indicate yet another function of hnRNP E1 in translational synthesis of FR. This has important implications for red cell physiology because FR are also expressed in proliferating erythroid progenitors and precursors (burst-forming unit-derived cells as well as colony-forming unit-derived cells) (29, 30). Thus, it is likely that in addition to its functional role in erythroid differentiation through its interaction with cis-elements in α-globin mRNA (31) and 15-lipoxygenase mRNA (24), hnRNP E1 will also be involved in erythroid cell proliferation by interacting with the cis-element of FR mRNA. (c) Although hnRNP E1 is known to interact with the 3′-UTR of mRNAs of 15-lipoxygenase (24), α-globin (31), and erythropoietin (32), until our report, no previous studies identified binding of hnRNP E1 to the 5′-UTR of mammalian mRNA. This observation is nevertheless similar to the interaction of hnRNP E1 interaction with the cloverleaf structure in the 5′-UTR of poliomyelitis virus RNA (33).

The requirement of thiol reagents to optimize the 18-base FR mRNA cis-element and trans-factor interaction suggests that redox states may be important in the regulation of expression of FR under physiological conditions. The identification (9)2 that the thiol amino acid homocysteine increases the binding of trans-factor to the 18-base FR mRNA cis-element is very relevant to the physiology of FR expression as it relates to the extracellular availability of folate. For example, homocysteine is increased in folate deficiency (34), and, in cultured cervical carcinoma cells, this also leads to an up-regulation of FR at the translational level (9).2 We have recently accumulated substantial evidence to support a model where substrate buildup of homocysteine (in folate deficiency) leads to an increased interaction of the cis-element and trans-factor/hnRNP E1, which, in turn, facilitates increased synthesis of FR at the translational level and eventually results in up-regulation of FR (9).2 If similar mechanisms are operative during erythroid development, (folate deficiency-induced) homocysteine-mediated augmentation of binding of hnRNP E1 to C/U-rich cis-elements in mRNAs of FR, 15-lipoxygenase, and α-globin should result in coordinated up-regulation of FR and α-globin but down-regulation of 15-lipoxygenase in megaloblastic erythroid precursors. This merits further study.

The FR mRNA-binding trans-factor/hnRNP E1 may also play a critical role in the integrity of neural crest cell and neural tube function during development. Recent “knockout” (35) and “knock-down” (36) studies have demonstrated the importance of FR in the prevention of neural tube defects in murine models. However, comprehensive studies in humans have failed to identify a role for polymorphisms of FR in the etiology of neural tube defects (37, 38). Recently, we determined the consequences of perturbation of the integrity of the translational regulatory domain of the murine FR in day 8–10 embryos (39) by using antisense oligonucleotides designed to quench the interaction of murine trans-factor/αCP-1 and a murine FR mRNA cis-element. The resulting embryos developed neural tube defects, as well as other defects, including malrotation and abnormal cardiac and eye development. Thus, these data predict that congenital or acquired alterations in the FR mRNA-binding trans-factor/hnRNP E1 expression could be the basis for some neural tube defects and neurocristopathies in humans (40).

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Isolation and Characterization of a Folate Receptor mRNA-binding trans-Factor from Human Placenta: EVIDENCE FAVORING IDENTITY WITH HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN E1

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