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ARTICLES

The Rate of Folate Receptor Alpha (FRα) Synthesis in Folate Depleted CHL Cells is Regulated by a Translational Mechanism Sensitive to Media Folate Levels, While Stable Overexpression of its mRNA is Mediated by Gene Amplification and an Increase in Transcript Half-Life

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Abstract DC-3F/FA3 cells (FA3) were obtained by selection of Chinese hamster lung fibroblasts for growth in folic acid free media, supplemented with 15 pM [6S]-5-formyltetrahydrofolic acid. These cells, as a result of low level gene amplification and RNA stabilization, were found to overexpress folate receptor alpha (FRα) mRNA by more than five hundred fold. The expression level of the receptor, a 43 kDa GPI-linked plasma membrane glycoprotein, was found to be inversely related to changes in media folate concentrations while its steady state mRNA level remained unaffected. In low folate, the rate of receptor synthesis was found to increase by more than three fold, while its half-life stabilized as compared to that observed in high folate media. Although DC-3F cells were found to contain low amounts of FRα mRNA, receptor expression was undetectable, and changing media folate concentrations had no effect on the expression of either. Hence, while selection for growth in low folate leads to stable overexpression of FRα mRNA, receptor expression is regulated at the level of protein synthesis by a mechanism sensitive to media folate levels. J. Cell. Biochem. 81:205–219, 2001. © 2001 Wiley-Liss, Inc.

Key words: gene amplification; transcription; folate receptors; mRNA stability; folate depletion; translation

The folate vitamins, through their ability to mediate one carbon metabolism, play a key role in maintaining the biochemical equilibrium of many species. In mammalian cells, their primary function is to provide cofactors for a variety of synthetic reactions including the production of thymidylate, purines and certain amino acids [Blakeley and Benkovic, 1984]. Maintenance of equilibrium within the cofactor pool is complex and involves interaction between a number of biochemical pathways which taken together are often referred to as the "folate cycle" [Morrison and Allegra, 1989]. However, while the "cycle" is responsible for maintaining intracellular folate homeostasis, cells must rely upon uptake of serum folate to maintain pool equilibrium and ensure survival. Restricting serum folate, therefore, leads to imbalances in folate stasis and broadly impacts cellular metabolism. In an attempt to identify changes in gene expression that occur under such conditions, and also to define the mechanisms responsible, we have selected cells for growth in sub nanomolar levels of reduced folate. Comparison of these clones with parental cells by cDNA differential display analysis has led to

Abbreviations used: RFC, reduced folate carrier; FR, folate receptor; DHFR, dihydrofolate reductase; HEPES, 4-(2-hydroxyethyl)-l-piperazine ethanesulfonic acid; FBS, fetal bovine serum; PBS, phosphate buffered saline; DPBS, Ca^{2+}/Mg^{2+} free Dulbeccos phosphate buffered saline; 5-CHOFH4, [6S]-5-formyltetrahydrofolate; HBSS, HEPES, balanced salt solution; PMSF, phenylmethyl sulfonyl fluoride.

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the identification of several genes whose expression levels are sensitive to folate depletion [Zhu and Melera, 1999]. One of these is the gene encoding folate receptor alpha (FRα).

The up-regulation of FRα expression in cells subjected to growth in low folate has been reported by several authors [Jansen et al., 1989; Brigle et al., 1991; Hsueh and Dolnick, 1993; Brigle et al., 1994]. In the few cases in which the molecular mechanisms responsible for these observations have been studied, the results have varied, and no single mechanism identified. In response to growth in 0.4 nM folinic acid, L1210 mouse leukemia cells were reported to upregulate expression by means of a chromosomal rearrangement leading to the insertion of an Intracisternal A particle within the 5′-regulatory region of the FBP1 (FRα) gene [Brigle et al., 1992]. As a result of this rearrangement, FRα expression levels were no longer responsive to the folate status of the cell. In KB-FR cells grown in folic acid free media containing approximately 2–10 nM reduced folate, increase in the steady state level of FBP (FRα) mRNA was accompanied by an increase in mRNA stability and an associated hypomethylation of the FBP gene [Hsueh and Dolnick, 1993]. When folic acid (2.3 μM) was restored for a period of 3 months, FRα mRNA and protein returned to control levels. Although a partial remethylation of the gene was noted at that time, no further studies were conducted.

Regulation of the FRα gene at the transcriptional level has been well documented, and the tissue specific expression of different FRα mRNAs is known to occur by use of differential splicing and promotor usage [Roberts et al., 1991; Elwood et al., 1997]. From the limited number of studies reported to date, however, it appears that transcription plays a minor role in the response of the gene to the folate status of the cell. Similarly, the extent to which translation may regulate FRα expression is not clear, although preliminary results to that effect have been reported in HeLa-IU1 cells [Sun and Antony, 1996]. In the present study we show that the overexpression of FRα mRNA, which occurs in CHL cells during selection for growth in 15 pM [6S]-5-CHOFH4, is mediated by low level gene amplification coupled with an alteration in transcription and a dramatic stabilization of FRα mRNA half-life. In overexpressing cells, the steady state level of the receptor is regulated by translation and its rate of synthesis is inversely related to media folate concentrations while the steady state level of its mRNA remains unchanged.

MATERIALS AND METHODS

Reagents

The natural isomer of folinic acid, [6S]-N5-formyl-5,6,7,8-tetrahydrofolate was generously provided by Cerbios-Pharma SA, (Barbengo, Switzerland). [3′, 5′, 7′, 9-^{3}H(N)]-(6S)-Leucovorin (^{3}H-6S-5-CHOFH4), 20–30 Ci/mmole, was obtained from Moravek Biochemicals (Brea, CA). Tissue culture reagents were purchased from Gibco Biologicals (Grand Island, NY). O- and N-glycosidases and PI-PLC were purchased from Boehringer Mannheim (Indianapolis, IN). Reagents for electrophoresis were obtained from Biorad (Hercules, CA) or from Sigma Chemical Co. (St. Louis, MO). All other chemicals were obtained from Sigma.

Cell Culture and Selection

Parental Chinese hamster lung fibroblasts (DC-3F) were maintained in Minimal Essential Medium (MEM) with Ham's F12 nutrient mixture (DMEM/F12), supplemented with 5% heat inactivated FBS (HyClone, Logan, UT), and 1% penicillin/streptomycin solution. Clones selected for growth under conditions of severe folate restriction were maintained in DMEM/F12 without hypoxanthine, thymidine, or folic acid, and were supplemented with 5% dialyzed, heat inactivated FBS, and 15 pM [6S]-5-CHOFH4 (FA medium). Cells were released from tissue cultured plates for passing with Ca^{2+}/Mg^{2+} free Dulbecco's phosphate buffered saline (DPBS) at 4°C. Selection was initiated by growing DC-3F cells for 7 days in DMEM/F12 in which the FBS had been replaced by 5% dialyzed FBS. Cells were then replated, and when the culture reached 60–70% confluence, folic acid, thymidine, and hypoxanthine were removed and [6S]-5-CHOFH4 added to a concentration of 5 nM. The population was maintained for 10 days at which time the media was changed and the dead cells removed. On Day 13, after growth had resumed, the cells were split. Beginning on Day 15, and over the course of the ensuing 17 days, the [6S]-5-CHOFH4 concentration was reduced by 50% in stepwise increments to

0.15 nM. Cells were carried in this concentration for 7 days and were then plated in 0.015 nM [6S]-5-CHOFH4. The population was passed for 30 days at which time one half was replated in 0.015 nM and the other half in 0.001 nM [6S]-5-CHOFH4. The latter culture did not survive. Cells plated in 0.015 nM folate were cultured for an additional 30 days at which time they had stabilized with a doubling time of approximately 36 h. Clones from this population, DC-3F/FA3, FA7, and FA14, were isolated for further study.

Labeling and Isolation of Plasma Membranes

The N-hydroxysuccinimide (NHS) ester of [^{3}H]-6S-5-CHOFH4 was prepared according to the method of Henderson and Zevely (1984). Cells at 70–80% confluence were released from the surface with three rinses of DPBS at 4°C, and washed twice in HBSS buffer [Jansen et al., 1989] containing 107 mM NaCl, 20 nM HEPES, 26.2 mM $NaHCO_3$, 5.3 mM KCl, 1.9 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 7.0 mM D-glucose. They were then adjusted to pH 7.4 with NaOH, and 1×10^8 cells resuspended in the same buffer at 37°C. Derivitized folinic acid was added to a final concentration of 200 nM (50 μl/ml cell suspension) and incubated with occasional swirling for 10 min at 37°C. The reaction was stopped by the addition of 10 volumes of ice cold HBSS. Cells were pelleted and washed twice in ice-cold HBSS, resuspended in 2 ml acid saline for 2 min at 0°C, and finally washed an additional two times in 10 volumes DPBS.

After the final wash, cells were resuspended in five volumes of homogenization buffer (10 mM Tris, pH 7.6 at 0°C, 0.1 mM $MgCl_2$, 1 mM EDTA, 10 μM antipain, and 10 μM chymostatin), and were allowed to swell for 20–30 min on ice before disruption with a Dounce homogenizer. The resulting suspension was centrifuged at 600*g* for 10 min at 4°C to remove nuclei and whole cells. The supernatant was then adjusted to a final volume of 11 ml with homogenization buffer and spun in an SW41 rotor at 100,000*g* for 45 min at 4°C, in a Beckman Model 65 Ultracentrifuge. The membrane pellet was resuspended in a small volume (200–300 μl) of 1/2 × homogenization buffer containing 1 × protease inhibitors, and stored at −80°C. Protein concentrations were determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL), with bovine serum albumin as the standard.

Membrane preparations were separated by SDS-polyacrylamide gel electrophoresis, after which the gels were treated with Entensify (New England Nuclear, Boston, MA) for autoradiography.

Treatment of Cells with PI-PLC

Glycosyl phosphatidylinositol (GPI) linked proteins were removed from the cell surface as described previously [Yan and Ratnam, 1995]. Briefly, cells were plated in duplicate wells of six well plates and treated for 1 h with 1 ml of buffer (50 mM Tris-HCI, pH 7.5, and 10 mM EDTA) alone or with 1 ml of buffer containing 0.3 U of phosphatidylinositol-specific phospholipase C (PI-PLC). They were then washed with 1 ml of ice-cold acid saline buffer (10 mM sodium acetate, pH 3.5, and 150 mM NaCl) and rinsed again with PBS. One milliliter of PBS, containing 15 pmol of [^{3}H]-6S-5-CHOFH4 and 15 pmol of unlabeled [6S]-5-CHOFH4, was added to each well and the cells incubated for 1 h at 4°C at which time they were again washed twice with 1 ml of cold PBS. Bound radioactivity was released from the cell surface by washing each well with 1 ml of acid saline and quantified by scintillation counting. In a separate well, the specificity of [^{3}H]-6S-5-CHOFH4 binding was demonstrated by its inability to label the cells after they were preincubated for 1 h in the presence of 100 nM [6S]-5-CHOFH4.

Deglycosylation

Proteins were deglycosylated as described by Fan et al. (1991) with minor modification. Membrane preparations, upto 25 μl, were diluted with 25 μl of buffer containing 50 mM Tris-HCL (pH 8.8 at 24°C), 2% SDS, and 1% Nonidet P-40, and incubated for 3 h at room temperature. 0.1 M dithiothreitol was then added and the mixture boiled for 3 min. After cooling to room temperature, the denatured membrane preparation was digested by the addition of 3–10 mU/μg protein of either *N*-glycosidase or *O*-glycosidase for 1 h at 37°C. Deglycosylation controls were run simultaneously using enzymes that had been inactivated by boiling for 10 min.

FA3 Cell cDNA Library Construction and Cloning of a Hamster Folate Receptor cDNA

Poly(A+) RNA was isolated from FA3 cells using the Fast Track mRNA isolation system

(Invitrogen). An FA3 cDNA library was constructed using the λTriplEx lambda vector system and the Great Length cDNA Synthesis Kit from Clontech with directions provided by the vendor. Approximately 1.5×10^6 plaques were obtained from the unamplified library, and were screened at low stringency with a [^{32}P]-dCTP labeled human FRγ cDNA probe, kindly provided by M. Ratnam [Shen et al., 1995]. Three rounds of screening were performed and four clones were isolated. Conversion of the four λTriplEx clones to pTriplEx clones was performed according to the vendor. The identity of each was determined by DNA sequencing using an ABI automated DNA Sequencer. Computer analyses of the nucleotide and predicted amino acid sequences were performed using Omiga software from Oxford Molecular Group.

Southern, Northern and Western Blot Analysis, mRNA Turnover and Estimation of Transcription Rate

The FRα specific insert from clone pFA3-P2k was released by digestion with *Not I* and labeled to a specific activity of $>10^9$ cpm/μg with [^{32}P]-dCTP using the Random Primer Labeling System (GIBCO, BRL), and used as a probe for both Southern and Northern analysis. High molecular weight genomic DNA was obtained from cultured cells as described [Sambrook et al., 1989]. *EcoRI* digestions were carried out at 37°C, in the buffer recommended by the supplier, for 5–7 h using 4–7 units of enzyme per μg of DNA. Digestion products were analyzed by Southern blotting [Ausubel et al., 1989]. After washing, membranes were exposed to autoradiography film with intensifying screens for 12–48 h at −70°C. The relative intensities of the resulting bands were determined using an Alpha Innotech Image Analyzer.

For Northern analysis total cellular RNA was isolated with TRIzol LS reagent (GIBCO, BRL). 20 μg was fractionated on a 1% agarose gel and blotted onto GeneScreen Plus nylon membranes. These were prehybridized for 4 h at 42°C in 5 × SSPE, 5 × Denhardt's solution, 1% SDS, 50% formamide, 10% Dextran Sulfate, and 100 μg/ml denatured and fragmented salmon sperm DNA. Radiolabeled probe was added, and hybridization proceeded for 24 h at 42°C. After washing, autoradiography was performed using double intensifying screens. The membranes were then stripped and rehybridized with a labeled β-actin control cDNA probe.

Western blots were performed as described [Shen et al., 1997a] with modification. Approximately 1×10^7 cells, removed from the surface of T75 flasks by scrapping, were suspended in 300 μl of cold PBS containing 1% Triton X-100 and 2 mM PMSF. Debris was removed by centrifugation. Fifty microliter of 10% SDS and 150 μl of 2 × SDS sample buffer were added to the supernatant and the protein concentration determined. Forty microgram of protein was added to each well of a 12.5% SDS-polyacrylamide gel. Following electrophoresis for 4 h at 50 mA, fractionated proteins were electrophoretically transferred onto nitrocellulose paper, probed with a human FRα specific polyclonal antibody kindly supplied by M. Ratnam [Shen et al., 1997], and analyzed using the ECL system from Amersham (Arlington Heights, IL).

To estimate FRα mRNA half-life, pulse-chase experiments were carried out essentially as described by Yang et al. [1995]. Cells were washed twice with either pre-warmed DMEM/F12 (DC-3F cells) or FA media (FA3 cells) and then incubated for 30 min in fresh media containing 75 μCi/ml [^{3}H]-uridine (38 Ci/mmol, Amersham). They were then rinsed once with pre-warmed media and incubated in the presence of 40 mM non-radioactive uridine for various lengths of time. Total RNA was isolated from duplicate cultures at each time point and hybridized for 48 h at 42°C with an excess (1μg) of denatured FRα specific cDNA immobilized on 12 mm circular nitrocellulose filters. Filters were then washed, digested for 1 h in the presence of 20 mg/ml RNaseA, rinsed, and dried. Bound radioactivity was measured in a liquid scintillation counter.

The rate of FRα mRNA synthesis was estimated by nuclear run off transcription assays. Preparation of nuclei, labeling with [^{32}P] UTP, isolation of nuclear RNA, and hybridization to slot blots were as described in detail [Ausubel et al., 1989]. Slot blots were prepared using nylon membranes and purified FRα and GAPDH cDNA fragments that had been denatured by incubation for 30 min in 0.2 M NaOH and neutralized with 6 × SSC before loading. After hybridization, the membranes were washed and incubated at 37°C for 30 min with 10 μg/ml RNaseA to remove nonspecifically bound RNA.

They were then washed for 1 h in 2 × SSC at 37°C and analyzed by autoradiography.

Metabolic Labeling, Measurement of Folate Receptor Synthesis and Turnover

In order to estimate the time required to saturate the [^{35}S]-methionine pool, cells were pre-incubated in DMEM/F12 without methionine or FBS for 15 min. Fresh media, containing 5% FBS and 100 μCi/ml [^{35}S]-methionine, were then added. At appropriate times, cells were rinsed with PBS, removed by scrapping and disrupted by sonication in 0.01 M potassium phosphate buffer (pH 7.4) containing 1 mM PMSF and 1% Triton. The sonicate was cleared by low speed centrifugation. Twenty microliter aliquots from the resulting supernatants were either counted directly in a liquid scintillation spectrometer, or mixed with an equal volume of ice-cold 10% TCA. After 10 min on ice, the TCA precipitates were collected by filtration in GF-C glass-fiber filters, and washed with ice-cold 5% TCA, 95% ethanol and diethyl ether. After drying, they were counted and the amount of [^{35}S]-methionine in the TCA soluble fraction, i.e., the methionine pool, was estimated by subtraction of the filter-bound radioactivity from the total radioactivity present in the initial cleared sonicate.

To measure receptor turnover, cells were labeled for 40 min with [^{35}S]-methionine under the conditions described above. Labeling medium was removed and, after rinsing with PBS, replaced by DMEM containing 100 μg/ml cycloheximide. At various time points cells were rinsed twice with ice-cold PBS, scrapped from the surface in 0.01 M K_2HPO_3 (pH 7.4) containing 1 mM PMSF and 1% Triton X-100, and disrupted by sonication. Receptors were then isolated as described previously [da Costa and Rothenberg, 1996]. In brief, receptor bound folate was released by adjustment of the sonicate to pH 3.5 with the addition of citric acid. Free folate was then removed by the addition of 50 mg/ml charcoal, followed by low speed centrifugation. The resulting supernatant was adjusted to pH 7.4 and mixed with epoxy-activated agarose crosslinked with folic acid (Sigma). The mixture was slowly rotated for 48 h at 4°C, pelleted by centrifugation at 3000 rpm for 5 min, and rinsed first with a large volume of 0.01 M K_2HPO_3 containing 1 M NaCl and then with 0.01 M K_2HPO_3 alone. Receptor was released from the resin by incubating at pH 3.0 as described [da Costa and Rothenberg, 1996] and the amount recovered was determined by scintillation counting.

The rate of receptor synthesis was measured after a similar 40 min labeling period by determining the amount of [^{35}S]-methionine incorporation into purified receptor as a function of time.

RESULTS

Selection and Preliminary Characterization of Clones

The population of DC-3F cells selected for growth in FA media was maintained for 30 days at which time three clones, DC-3F/FA3 (FA3), DC-3F/FA7 (FA7), and DC-3F/FA14 (FA14), were isolated by serial dilution. The stepwise selection strategy used here has been shown in a number of cases to result in the amplification of genes whose overexpression provides survival advantage under different growth conditions [Melera, 1991]. Hence, the three clones were analyzed for cytogenetic abnormalities consistent with the presence of amplified genomic sequences. Preliminary results obtained from Giemsa banded metaphase chromosome spreads of FA3 cells indicated the presence of abnormally banded regions (ABRs) [Lewis et al., 1982; Biedler et al., 1988] located on an X chromosome (Biedler, Spengler, and Melera unpublished). Similar, but not identical regions were present in all three clones, but not in DC-3F, suggesting that amplification of genomic sequences had occurred during the selection procedure.

Identification of a Folate Binding Protein in FA3 Cells

The NHS ester of [6S]-5-CHOFH4 is an affinity probe that specifically interacts with folate binding proteins and becomes covalently linked via its ester group [Fan et al., 1991]. As shown in figure 1A, reaction with the NHS ester of [^{3}H]-6S-5-CHOFH4 labels a predominant but somewhat diffuse 43 kDa molecule on FA3 (lane 2) that is not detectable on DC-3F (lane 1). A slightly larger but weaker band was also noted. These were both reduced to a single discreet band of approximately 30 kDa by digestion of the membrane preparations with N-glyconase (Fig. 1B), indicating that the different sized bands were due to varying levels of glycosylation. A similar doublet with a more

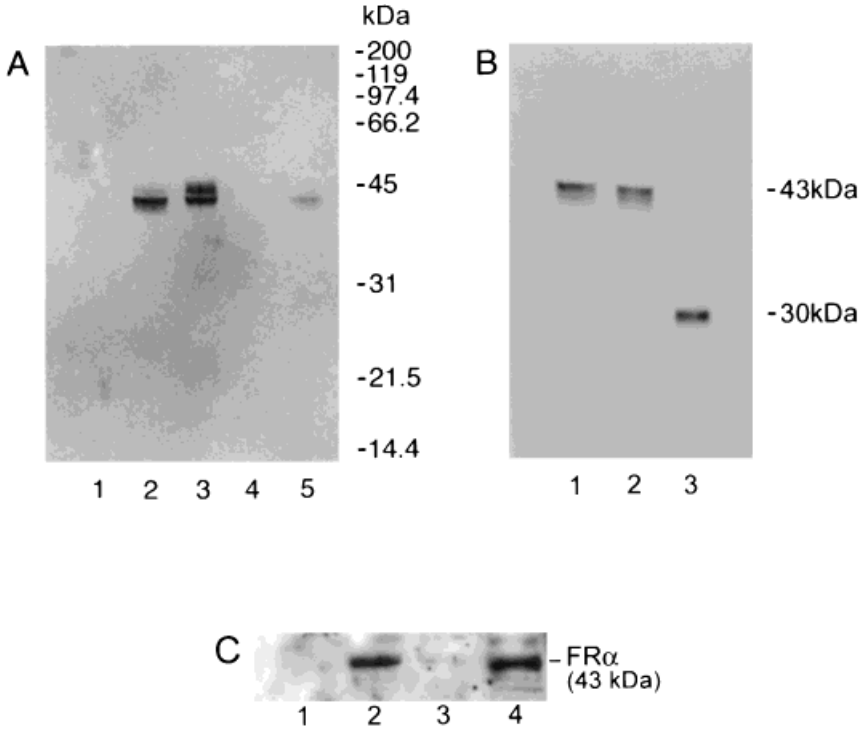

Fig. 1. FA clones express folate binding proteins. (**A**) Labeling with the NHS ester of [^{3}H]-6S-5-CHOFH4. Plasma membranes were prepared from labeled cells as described in Materials and Methods and 30 μg of protein from each sample added to the wells of a 12.5% SDS-polyacrylamide gel. **Lane 1:** DC-3F; **Lane 2:** FA3; **Lane 3:** FA14; **Lane 4:** FA3 cells removed from FA medium and cultured in MEM/F12 for 21 days; **Lane 5:** FA3 cells removed from FA medium, cultured in MEM/F12 for 21 days, and then replated in FA medium for another 21 days. Coomassie blue stained controls (not shown) indicated that approximately 50% less protein had been loaded into Lane 5. (**B**) Analysis of labeled membranes digested with *N*-glyconase. Preparation and analysis of membranes was as described in (A). **Lane 1:** [^{3}H]-6S-5-CHOFH4 labeled FA3 membrane; **Lane 2:** Labeled membranes treated with N-glyconase that had been inactivated by boiling for 10 min prior to use; **Lane 3:** labeled membranes treated with active *N*-glyconase. Similar treatment with *O*-glyconase had no effect (Alliegro and Melera, unpublished observations). (**C**) Western blot analysis of total cell protein with a FRα-specific polyclonal antibody. Forty microgram of total cell protein from the various cell lines was prepared and analyzed as described in Materials and Methods. **Lane 1:** DC-3F; **Lane 2:** FA3; **Lane 3:** FA3 cells removed from FA medium and cultured in MEM/F12 for 21 days; **Lane 4:** FA3 cells removed from FA medium, cultured in MEM/F12 for 21 days, and then replated in FA medium for another 21 days.

pronounced larger band was found on FA14 cells (lane 3) and FA7 (not shown). The doublet in FA14 was also reduced to a single band of 30 kDa by *N*-glyconase digestion (not shown). FA7 was not further analyzed. These results are very similar to those reported by others and show that folate binding proteins expressed by hamster cells are N-glycosylated to an extent similar to those found in mouse and human [Elwood, 1989; Luhrs, 1991; Brigle et al., 1994; Shen et al., 1997].

Treatment of FA3 cells with phosphatidylinositol-specific phospholipase C, prior to labeling with the NHS ester of [^{3}H]-5-CHOFH4, eliminated binding of the label (Table I). This indicated that the 43 kDa glycosylated peptide is GPI linked to the plasma membrane, a characteristic consistent with its being a member of the folate receptor family [Lacey et al., 1989; Luhrs and Slomiany, 1989]. Western blot analysis (Fig. 1B) of total cell lysates using a human folate receptor alpha specific polyclonal antibody confirmed that FA3 cells express folate receptors and DC-3F cells do not. Hence

TABLE I. Sensitivity of [^{3}H] 5-CHOFH4 Binding to the Action of PI-PLC

	Amount of [^{3}H] 5-CHOFH4 bound (pmol/10^7 cells)	
Cells	(−) PI-PLC	(+) PI-PLC
FA3	2.30 ± 0.21	0.53 ± 0.03
DC-3F	0.10 ± 0.01	0.07 ± 0.01

Prior to labeling with [^{3}H] 5-CHOFH4, cells were treated for 1 h in the presence, (+), or absence, (−) of 0.3 U of PI-PLC, as described in Materials and Methods. The average of three experiments ± SEM are shown.

the inability to surface label DC-3F cells with the NHS ester of folinic acid or to bind [^{3}H]-folic acid (Zhu and Melera, unpublished) can be attributed to their lack of receptor expression rather than failure to correctly sort the receptors to the plasma membrane. That DC-3F cells can express functional receptors has been confirmed by transfection with a FRα cDNA expression construct [Zhu et al., 1998].

FA3 Cells Overexpress FRα mRNA

A cDNA for the receptor was obtained by screening an FA3 cDNA library at low stringency, with a [^{32}P]-labeled human FRα cDNA probe. 1×10^6 plaques from the unamplified library were analyzed and four clones, pFA3-F1e, pFA3-P2k, pFA3-P1a, and pFA3-Sli, were isolated, sequenced, and found to be identical. Each contained the same inferred open reading frame, the same 3′- untranslated region (UTR) terminating with a poly (A) tail, and the same 96 base 5′-UTR. The nucleotide sequence of the pFA3-P2k insert may be found under Genbank accession number AF061256. It contains 1136 base pairs and a single open reading frame of 261 amino acids that displays 89% and 79% identity with the coding regions of mouse and human FRα cDNAs respectively. A poly (A) tail is present in 24 nucleotides downstream from a consensus polyadenylation signal, AAUAAA, located at position 988. The predicted molecular mass of the encoded protein is 28,710 Da. Three consensus sites for N-linked glycosylation occur at amino acid residues 67, 159, and 199, which, when aligned for maximal homology correspond directly to those found in mouse and human FRγ [Shen et al., 1997]. The overall amino acid sequence homology with mouse and human FRα is 92% and 79% respectively.

Recent structure-function analysis [Shen et al., 1997; Maziarz et al., 1999] has identified the amino acid residues that define the differences in substrate specificity for both human and mouse α and β receptors. When aligned for maximal amino acid sequence homology, and compared at each point of divergence, (Fig. 2A) the hamster sequence shares 100% homology with mouse FRα, and only 42% homology with either human or mouse FRβ. Moreover, comparison at the carboxy terminus, across the region containing the GPI cleavage sites, shows that the hamster receptor shares 84% homology with mouse FRα and only 25% homology with FRβ (not shown). Based upon these results we conclude that the sequence cloned in plasmid pFA3-P2k represents the hamster homologue of FRα.

Evidence that FRα transcripts account for the large majority of the receptor mRNA expressed in FA3 cells is provided by the PCR experiment in Figure 2B. A set of primers homologous with mouse and human FRα and β as well as hamster FRα was identified. Because of the high degree of sequence conservation between FRα and β displayed in mouse and human, this set of primers is also expected to be homologous with hamster FRβ. These primers span regions of non-homology that contain different restriction sites, therefore, the composition of the folate receptor mRNA pool can be estimated by using restriction enzymes to digest the amplified DNA product. This approach assumes that the restriction site differences found to exist within the regions of non-homology between mouse FRα and β, and human FRα and β will also occur between hamster FRα and β. As shown in the figure, the amplified PCR product obtained from FA3 cells was completely digested by four different restriction enzymes whose sites are known to be present in the hamster FRα sequence. If a significant amount of FRβ mRNA was present in FA3 cells, either undigested DNA would have remained after digestion or an unexpected restriction pattern would have resulted. Since neither was observed, and it is extremely unlikely that hamster FRα and β share extensive nucleotide sequence homology across the regions analyzed, we conclude that the predominant form of folate receptor mRNA expressed in FA3 cells is alpha. Similar experiments using RNA prepared from DC-3F cells provided the same results (data not shown). These data do not, however, rule out the possibility that other forms of FR mRNA may be expressed in these cells.

Overexpression of the FRα Gene is Mediated by Gene Amplification and mRNA Stabilization

Comparison of northern blots of total RNA (Fig. 3A), indicated that FRα mRNA transcripts of approximately 1.2 kb in size are overexpressed by more than 500 fold in each of the FA clones. Long-term exposure of these blots shows the presence of similar sized transcripts in DC-3F cells. Image analysis of the southern blot presented in Figure 3B however,

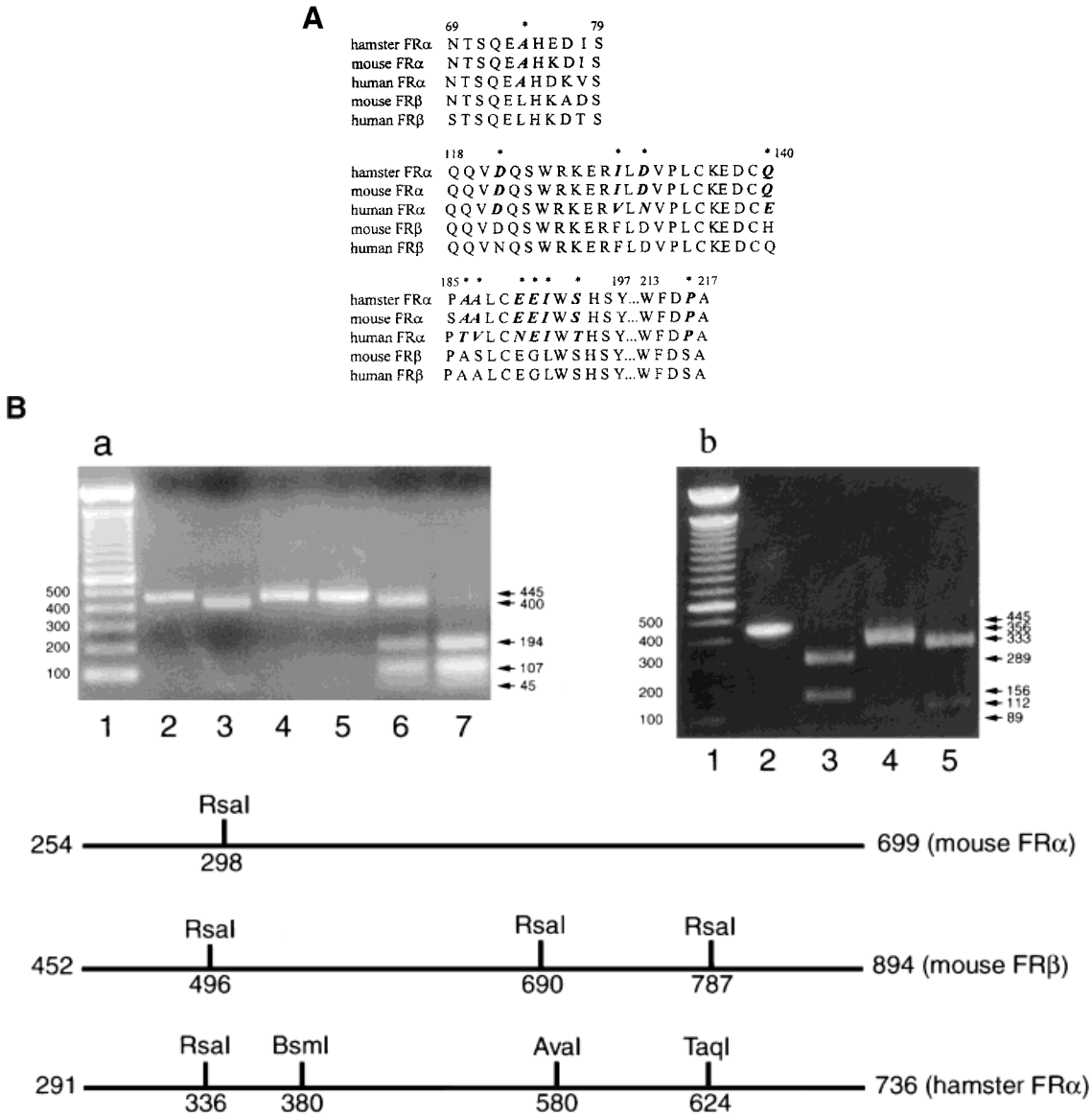

Fig. 2. Comparison of the hamster FRα amino acid sequence with those of human and mouse, and evidence that FRα is the predominant folate receptor present in FA3 cells. (**A**) Comparison of the amino acid residues in hamster with those in both mouse and human have been shown to discriminate between FRα and β. The hamster sequence is homologous with mouse in each of the 12 amino acid residues (designated with an asterisk) known to discriminate mouse FRα from mouse FRβ. It matches human FRα and β in five of those positions, and mouse FRα in only four. (**B**) FRα mRNA is the predominant form present in FA3 cells. A set of PCR primers (5′-CACCAACACAAGCCAGGA-3′, 5′-GGTCGAACCACATCTGGA-3′), were identified that are homologous between mouse and human FRα and β mRNA, and hamster FRα mRNA, but in the former two species, border on regions of non-homology. Given the high degree of sequence conservation between mammalian FR mRNAs, similar regions of non-homology are expected to be present in hamster as well. Restriction maps, based upon Genbank sequences with Accession Numbers NM_008034, NM_008035, and AF061256 for mouse FRα, FRβ, and hamster FRα, respectively, across this region are shown in the diagram. Mouse L1210 cells that express both FR α and β, and the L1210 subline Fa, reported to overexpress β, [Brigle et al., 1994], were kindly provided by ID Goldman. The primers were used to RT-PCR amplify a 445 bp cDNA fragment from the various cell lines. As indicated, digestion with RsaI generates the patterns predicted from the maps. A small amount of FRα mRNA is present in the Fa cell line as expected, but a large majority is FRβ. The pattern from FA3 cells suggests the presence of FRα alone. (**A**) **Lane 1:** 100 bp ladder; **Lane 2:** undigested DNA from FA3 (445 bp); **Lane 3:** cDNA from FA3, digested with RsaI (44 bp and 401 bp); **Lane 4:** undigested cDNA from mouse L1210 (445 bp); **Lane 5:** undigested cDNA from mouse Fa cells (445 bp); **Lane 6:** cDNA from L1210 digested with RsaI (45, 400, 194, 97 and 107 bp); **Lane 7:** cDNA from F2 digested with RsaI (45, 194, 97 and 107 bp). Note that the 107 and 97 bp fragments cannot be distinguished on these gels. (**B**) The 445 bp fragment from FA3 was digested with the three additional enzymes shown in the diagram. Only the expected restriction patterns were observed. **Lane 1:** 100 bp ladder; **Lane 2**: undigested FA3 cDNA; **Lane 3:** FA3 cDNA digested with AvaI (156 and 289 bp); **Lane 4:** digested with BsmI (89 and 356 bp); **Lane 5:** digested with TaqI (112 and 333 bp).

indicates that the gene is amplified only by four to five fold in FA3 cells and in each of the other FA clones as well. Although this modest increase in gene number is consistent with the low level of amplification that generally accompanies the presence of abnormally banded regions, [Biedler et al., 1988], it cannot explain the large increase in the steady state level of FRα mRNA found in the FA clones. Similarly, nuclear run off transcription assays indicated that only a four- to five-fold increase in the rate of FRα mRNA synthesis had taken place (Fig. 3C). While consistent with the level of gene amplification, this modest increase was also unable to explain the large amount of mRNA overexpression. Hence, the half-lives of these transcripts were measured. As shown in Figure 4, selection for growth in low folate stabilizes FRα mRNA in FA3 to the point where it's half-life is not directly measurable during the time course of these experiments. Assuming linear decay kinetics and extending the curve shown, it can be estimated that the half-life in FA3 is approximately 125 h as compared to 3 h in DC-3F.

The Expression Level of FRα in FA3, but not DC-3F Cells, is Sensitive to Media Folate Levels and is Independent of the Steady State Level of its mRNA

FA3 cells were removed from FA media and plated in standard DMEM/FI2. After 21 days they were collected and labeled with the NHS ester of [^{3}H]-6S-5-CHOFH4. Figure 1A, (lane 4) shows that FRα is not detectable on the surface of these cells. The population was then returned to FA media, and after 21 days they were once again collected and labeled with the same ligand. Figure 1A (lane 5) shows that they express receptor on their surface. Western blots of total protein preparations (Fig. 1C, lanes 3 and 4) show similar results, indicating that it is the cellular amount of FRα that has been altered rather than its presence on the cell surface alone. Media folate concentrations therefore directly affect the steady state level of FRα in DC-3F/FA3 cells. Interestingly, northern blot analyses of total RNA isolated from each of these FA3 cell populations showed that the steady state level of receptor mRNA did not change in response to media folate concentrations (Fig. 3A, lanes 5 and 6). Hence, FRα expression in FA3 is regulated at the post transcriptional level. Similar experiments with

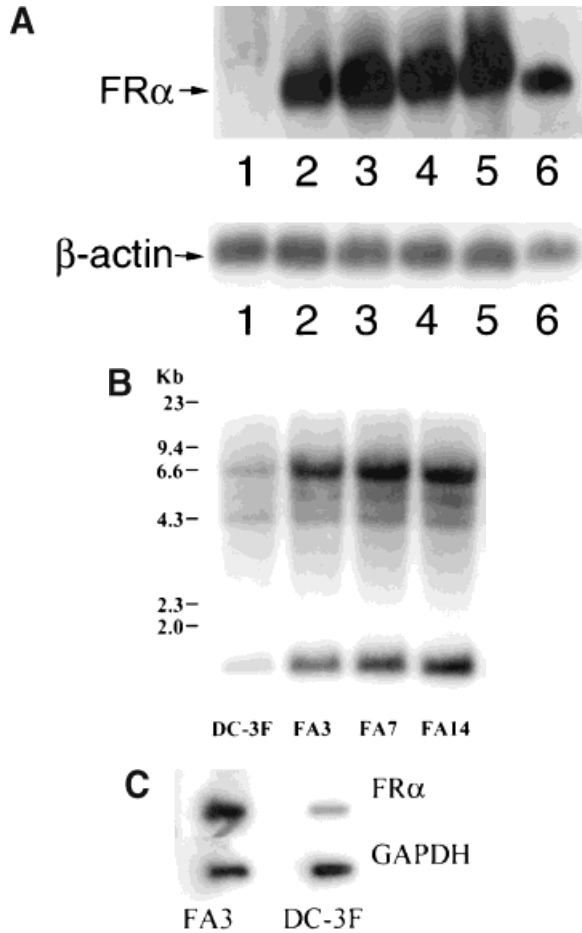

Fig. 3. Overexpression of the FRα gene is partially mediated by gene amplification. (**A**) Northern blot analysis. **Top:** 20 μg of total RNA was denatured in formaldehyde/formamide, electrophoresed through a 1% agarose gel, and blotted onto nylon membranes. Hybridization with a [^{32}P] labeled FRα cDNA probe, and subsequent analysis was as described in Materials and Methods. **Lane 1:** DC-3F; **Lane 2:** FA3; **Lane 3:** FA7; **Lane 4:** FA14; **Lane 5:** FA3 cultured in DMEM/F-12 with 5% FBS for 21 days; **Lane 6:** FA3 cells cultured in DMEM/F-12 with 5% FBS for 21 days and then replated in FA medium for 21 days. **Bottom:** (β-actin hybridization control showing that Lane 6 is under loaded. (**B**) Southern blot analysis. Ten microgram of genomic DNA was digested with EcoRI and loaded into the wells of a 0.8% nondenaturing agarose gel. After electrophoresis, the gel was blotted onto a nylon membrane, hybridized with a [^{32}P]-labeled FRα probe, and analyzed as described in Materials and Methods. **Lane 1:** DC-3F; **Lane 2:** FA3; **Lane 3:** FA7; **Lane 4:** FA14. (**C**) Nuclear runoff assay. Nuclei were isolated from 5×10^7 cells and elongating RNA transcripts labeled by incubation with [^{32}P]-UTP as described in Materials and Methods. Slot blots containing Hamster FRα cDNA and, as a control, GAPDH cDNA were prepared and hybridized with 2×10^6 CPM of the ^{32}P-labeled nuclear RNA. The blots were then washed under high stringency conditions and analyzed by autoradiography. The experiment was carried out with three independently labeled nuclear RNA preparations from FA3 and DC-3F cells with similar results.

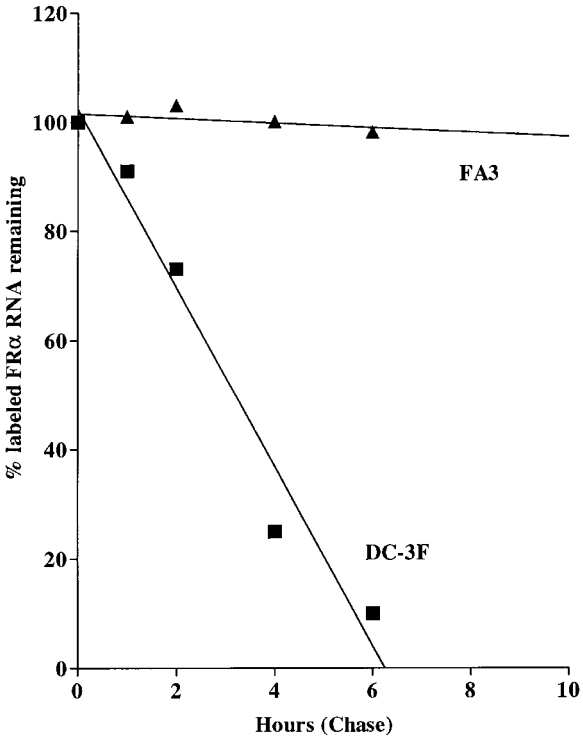

Fig. 4. FRα mRNA turnover studies. Pulse-chase labeling experiments were carried out as described in Materials and Methods. After incubation with 75 μCi/ml [5,6-^{3}H] Uridine in the appropriate medium at 37°C for 30 min (pulse), cells were washed twice and plated in fresh medium containing 10 mM Uridine. Preliminary experiments showed that under these conditions it required 2 h as opposed to 3 h of incubation following the pulse for the RNA isolated from FA3 versus DC-3F to reach maximal specific activity i.e., CPM [^{3}H]-Uridine/mg total RNA. Accordingly, RNA samples isolated from FA3 and DC-3F after 2 h and 3 h post pulse, respectively, were taken as Time 0, and four chase times of 1, 2, 4, and 6 h were analyzed. RNA turnover rates were determined by hybridization of labeled total RNA to an excess amount (1 μg) of hamster FRα cDNA immobilized on nitrocellulose filters. The experiment was repeated three times and the data shown is a representative example of the results obtained.

DC-3F cells taken from MEM/F12 and plated directly into FA media for 7 days, at which time the folate pools have been depleted by 30 fold (Zhu, Bunni, Priest, and Melera, unpublished), failed to detect expression of the receptor or changes in the steady state level of its mRNA.

FRα Expression in FA3 Cells is Regulated by Translation

The rate of FRα synthesis and turnover was determined by measuring radiolabeled amino acid incorporation into purified receptor in the presence or absence of cycloheximide (Fig. 5). To determine the rate of synthesis, [^{35}S]-methionine uptake experiments were performed to estimate the time required for saturation of the methionine pool. FA3 cells were incubated with 100 μCi/ml of the radiolabel, and the radioactivity in the TCA soluble fraction determined at various time points. As indicated in Figure 6A, the time required to saturate the pool was 40 min. Cells were therefore incubated in media containing 100 μCi/ml [^{35}S]-methionine for 40 min at which time they were harvested. Additional samples were collected at 45, 50, 55, and 70 min. The receptor was isolated from each sample by affinity chromatography and the amount of radioactivity recovered at 70 min compared to that recovered at the earlier time points taken as a measure of the synthetic rate (Fig. 6B). In FA media, 1×10^6 FA3 cells were found to incorporate 11.25 pmol of methionine into FRα in 30 min. After 15 days in DMEM/F12, 1×10^6 FA3 cells incorporated 3.5 pmol in 30 min. Hence, the rate of receptor synthesis in FA media was 3.2 fold greater than DMEM/F12.

To measure the FRα turnover-rate, cells were again labeled for 40 min at which time cycloheximide was added to the media at a final concentration of 100 μg/ml. Samples were collected and analyzed as before. The amount of radioactivity recovered from 1×10^6 FA3 cells by the affinity matrix did not change over the 30 min time course (Fig. 6C). When plated in DMEM/F12 for 15 days, a decay rate of 0.25 pmol/30 min/10^6 cells was observed. Taken together, these data indicated that under conditions of folate depletion, the rate of FRα synthesis increases and its half-life stabilizes while under conditions of folate excess the synthetic rate decreases while the decay rate increases.

DISCUSSION

We have initiated a series of studies to identify genes whose expression levels are sensitive to changes in exogenous folate levels. To accomplish this, a step wise selection procedure was used to isolate clones that survive in 15 pM [6S]-5-CHOFH4. These clones were then analyzed by cDNA differential display to identify mRNA transcripts whose steady state levels were altered by a decrease in media folate levels [Zhu and Melera, 1999]. By returning these clones to high folate media it has been possible to determine if the effects on gene expression caused by folate depletion are stable or are

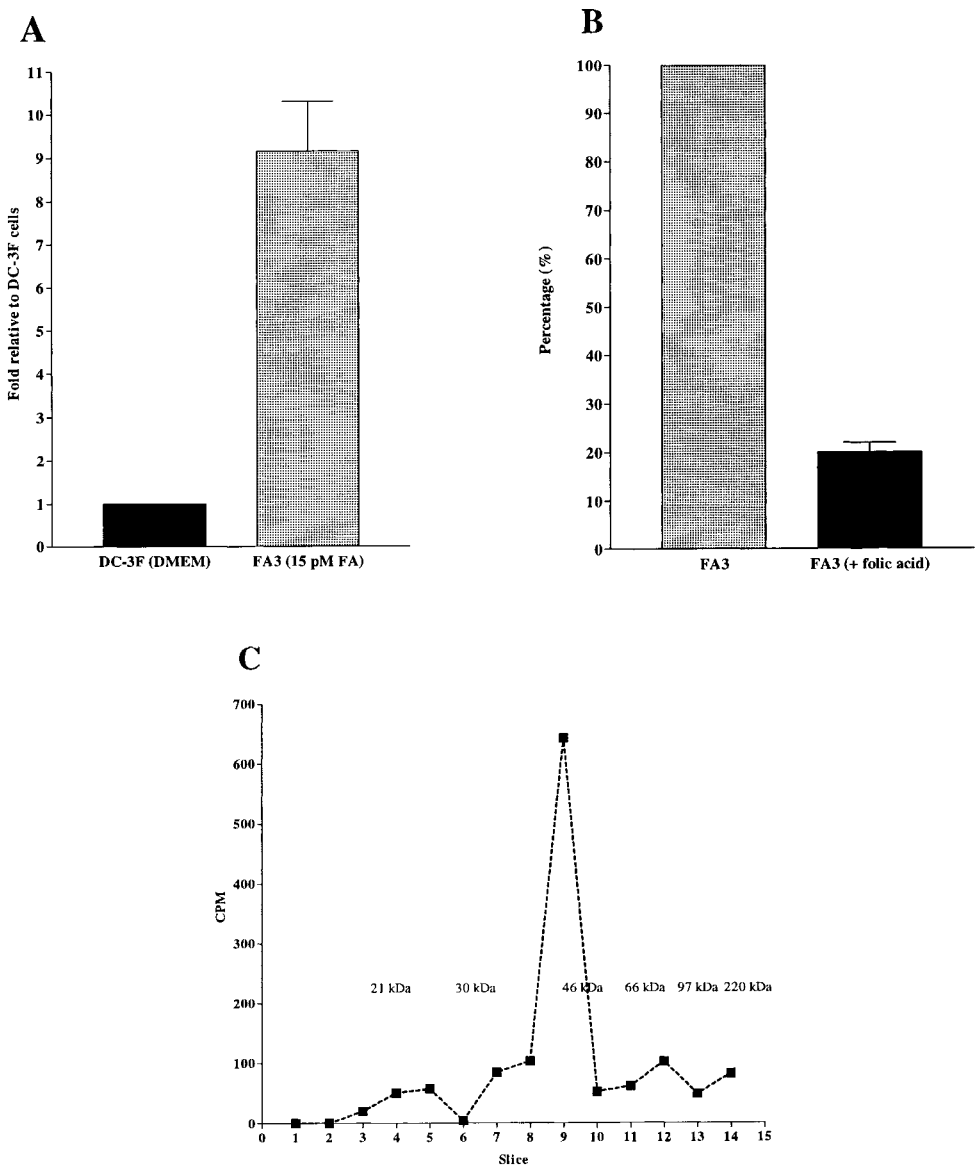

Fig. 5. Purification of hamster FRα by folic acid affinity chromatography. DC-3F and FA3 cells were labeled with [^{35}S]- methionine for 40 min. Total cell lysates were collected and prepared as described in Materials and Methods. 1×10^6 CPM from each sample were mixed with 200 µl of folic acid affinity agarose. After extensive washing, bound radioactivity was released from the matrix and the amount determined by scintillation counting. As indicated in (**A**), approximately seven times more radioactivity was recovered from FA3 as opposed to DC-3F. This is consistent with FRα overexpression in the former. The radioactivity recovered from the DC-3F lysates likely includes non-specific material as well as other folate binding proteins such as DHFR. (**B**). To show that the affinity resin specifically bound the radiolabeled material recovered from FA3, a competition experiment was performed. Radiolabeled lysate was treated with citric acid (pH 3.0) to remove bound folates from both external and internal receptors and the released folates were then removed by incubation with charcoal. After raising the pH to 7.4, an aliquot was added directly to the affinity matrix, and another incubated in 1 mM folic acid for 1 h prior to chromatography. As indicated in the figure, pretreatment of the FA3 lysate with folic acid prevents recovery of approximately 80% of the radioactivity. Hence binding to the affinity matrix is folic acid specific. (**C**). SDS-PAGE was used to determine the size of the polypeptide(s) retained by the affinity matrix. 1×10^6 CPM of radioactive lysate from FA3 were chromatographed, and thereafter elution and dialysis was analyzed on a 10% SDS-polyacrylamide gel. Each lane was cut into 1 mm slices and the slices dissolved in 100 µl H_2O_2. The radioactivity in each piece was determined by liquid scintillation counting. As shown, the majority of recovered radioactivity migrated as a single prominent peak with a molecular weight of approximately 43 kDa, the size expected for the glycosylated form of FRα (see Fig. 1). Insufficient amounts of radioactivity were recovered from DC-3F lysates to permit a similar analysis.

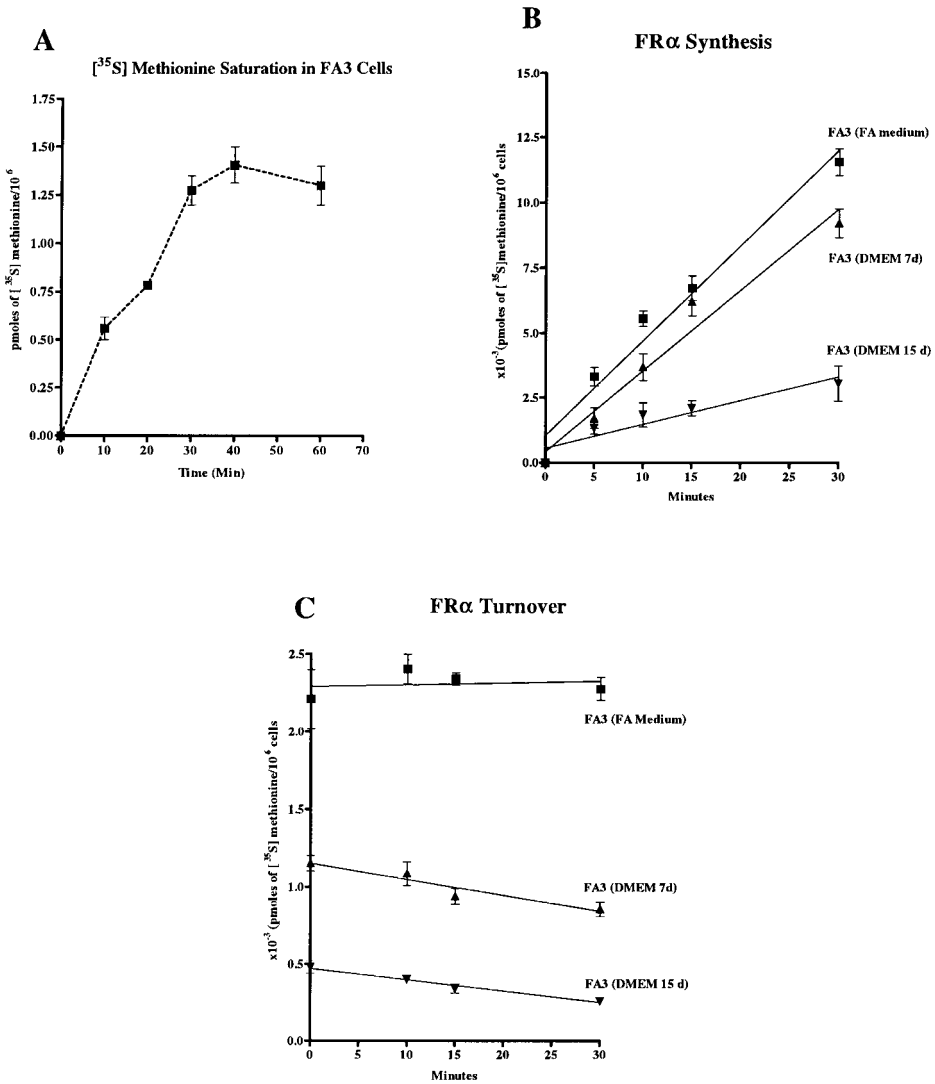

Fig. 6. FRα expression in FA3 cells is regulated by translation. (**A**) Accumulation of [^{35}S]-methionine by FA3 cells. (**B**). Determination of the FRα synthetic rates in FA3 cells plated in medium containing different folate levels. As shown in (A), incubation of FA3 cells for 40 min in 100 μCi/ml [^{35}S]-methionine saturates the methionine pool, and this was considered as Time 0 for these experiments. Cell lysates, each containing 1×10^6 CPM, and prepared from the time points indicted, were subjected to folic acid affinity agarose chromatography. Receptor purification was carried out as described in Materials and Methods and the radioactivity bound at each time point determined. Rate estimations were determined by linear regression analysis of the data. (**C**) To estimate FRα half-life, cells were again labeled with [^{35}S]-methionine for 40 min, at which time the labeling medium was removed and the cells chased in non-radioactive medium containing 100 μg/ml cycloheximide for the time periods indicated. Cell lysates containing 1×10^6 CPM were chromatographed on folic acid affinity agarose and the receptor isolated. Each of the experiments in this figure were done twice, three data points were collected at each time point. The values shown represent the mean and standard deviation of the mean for one of those experiments. The results were analyzed by linear regression.

reversed when folate is restored and to begin to define the mechanisms responsible.

Gene Amplification and Stabilization of Transcript Half-life Mediate Overexpression of FRα mRNA in FA3 Cells

Overexpression of FRα mRNA in FA3, FA7 and FA14 is the result of a positive selection for growth in 15 pM [6S]-5-CHOFH4 and is partially mediated by low-level amplification of the FRα gene. Since folate depletion is known to enhance uracil misincorporation into DNA and to result in DNA strand breaks that would be expected to promote gene amplification [Blount et al., 1997; Kim et al., 1997], it is somewhat surprising that gene amplification has not

been reported to mediate FRα overexpression in other systems. These include mouse L1210 and MEL cells and human KB cells, in which growth in low folate (0.4, 0.5, and <10 nM respectively) was used to elevate FRα levels [Brigle et al., 1991; Brigle et al., 1994; Hsueh and Dolnick, 1993]. An increase in the gene copy number of the receptor has been observed in a human squamous cell carcinoma cell line [Orr and Kamen, 1994]. However, no increase in receptor expression occurred and the receptor expressed was nonfunctional. Many explanations including the intrinsic ability of a given cell line to successfully amplify autosomal genes as indicated, perhaps, by its p53 status [Livingstone et al., 1992; Yin et al., 1992], could prevent amplification of the receptor gene. The use here, however, of a stepwise selection procedure using a cell line, DC-3F, known to readily produce gene amplification mutants in response to drug selection [Biedler et al., 1988], coupled with the very high selection pressure imposed is likely to have played a major role in establishing such mutants. Indeed, 15 pM [6S]-5-CHOFH4 may actually define the lower limit of extracellular reduced folate required for viability of CHL cells in culture since lowering the selective concentration below that concentration prevents survival.

While the four-fold amplification of the FRα gene contributes to its overexpression in FA3 cells, the rate of transcription per gene does not differ (Fig. 3C). Hence it is the apparent 40-fold increase in transcript half-life which occurs during selection that accounts for the several 100-fold increase in the steady state level of FRα mRNA (Fig. 5). Stabilization of FBP mRNA, albeit to a much lesser extent, has also been found to contribute to the increase in FBP mRNA observed when KB cells are cultured in 10 nM folate [Hsueh and Dolnick, 1993]. In that case, the mRNA half-life increased 2.5 fold from 5.7 h in folate replete cells to approximately 14 h under conditions of folate depletion. Hence, in folate replete medium, the half-lives of human and hamster FRα mRNAs appear to be similar; i.e. 5.7 h versus 3 h, respectively.

The stability of a number of mRNA transcripts encoded by different genes has been reported to be regulated by interaction of the RNA binding protein, AUF-1 to adenine/uridine rich cis elements (ARE sequences) located in the 3′-UTR [Chen and Shyu, 1995; DeMaria et al., 1997]. Examination of this region in hamster FRα mRNA expressed in both FA3 and DC-3F cells shows the presence of two such sequences, 5′-AAUUAUUU-3′, and 5′-AAUUAUUU-3′ located 18 nucleotides apart between positions 973 and 1008 of the sequence posted in Genbank. These share extensive homology with known AREs such as 5′-AUUA-3′ and 5′-UUAUUUA(U/A)(U/A)-3′ [Chen and Shyu, 1995] and their presence in the 3′- UTR of FRα mRNA is consistent with regulation at the level of transcript stability. Interestingly, the 3′- UTR of human FRα contains two similar sequences, 5′-UUUUA-3′ and 5′-UAUUU-3′ located 50 nucleotides apart between positions 779 and 840 of the sequence reported in Elwood [1989]. An identical sequence, 5′-UUUUA-3′ is also located in the 3′-UTR of mouse FRα at position 776 [Brigle et al., 1991]. The presence of these sequences suggest that mRNA stability may play a role in regulating the steady state level of FRα mRNA in mammalian cells. However, the extent to which this mechanism is actually used is unclear since it is only under conditions of long-term selection that the stability of FRα mRNA has been reported to be altered [Hsueh and Dolnick, 1993; and the present work]. Neither DC-3F nor FA3 cells responded to fluctuating media folate concentrations by altering their FRα mRNA level in the short-term i.e., within 7 and 21 days respectively. Hence, the half-lives of FRα transcripts in hamster fibroblasts do not appear to be sensitive to folate pool size. Whether this mode of regulation is active during development and differentiation or in various disease states remains unknown.

FRα Expression in FA3 Cells is Regulated by Translation

The Northern blot data shown in Figure 3A, and the protein synthesis data in Figure 6 establish that the steady state expression level of FRα in FA3 cells is regulated at the level of protein synthesis and is sensitive to media folate concentrations. This may also be the case in DC-3F cells where FRα mRNA, but not the protein is expressed. Interestingly, 5′ RACE (Rapid Amplification of CDNA Ends) experiments have indicated that the population of FRα transcripts expressed in FA3 cells contains four different 5-UTRs, only one of which is present in DC-3F (Zhu and Melera, unpublished). Since it has been reported that transcription of the FRα gene in human KB cells is

complex and generates multiple transcript forms containing different 5-UTRs that vary in their translational efficiency [Roberts et al., 1991; Elwood et al., 1997], it is reasonable to consider the possibility that the transcript found in DC-3F cells is inefficiently translated, thus explaining the lack of receptor expression. Experiments to clarify this issue are in progress.

Culturing FA3 cells in DMEM/F12, in which the folic acid concentration is >2 μM, decreases the rate of FRα synthesis by more than three fold in 15 days. It is important to point out that the effect is initially seen after 7 days (Fig. 6B). Given a doubling time of approximately 36 h (Zhu and Melera, unpublished), only five cell doublings occur in 7 days, making it extremely unlikely that the effect observed is due to reselection of the cells. Rather it is a response of the receptor synthetic rate to changes in media folate levels, which are directly reflected by alterations in the intracellular folate pool size (Zhu, Bunni, Priest, and Melera, unpublished). The same argument applies to the changes noted in the half-life of the receptor (Fig. 6C).

The observed changes in its rate of synthesis and turnover provide a likely explanation as to why the receptor cannot be detected in FA3 after 21 days in DMEM/F12 (Fig. 1). But it also raises the question as to why receptor expression should be down regulated in the presence of high folic acid concentrations when it is known that the receptor can mediate folic acid uptake [Spinella et al., 1995]. One scenario involves the reduced folate carrier (RFC), which we have shown is expressed in DC-3F and FA3 cells [Melera et al., 1993], and other transport systems postulated to be involved in folic acid uptake [Spinella et al., 1995; Assaraf et al., 1998]. When the external folic acid concentration is high, these may provide a more efficient transport route for folic acid than that mediated by receptors whose affinity for folic acid is very high and as a result, upon internalization do not readily release the ligand [Kamen et al., 1988]. In any event, we have shown that in FA3 cells, the rate of FRα receptor synthesis is inversely proportional to media folate levels thus providing an opportunity to understand the mechanisms that control receptor expression and to clarify how they respond to the ability of the RFC and other transport systems to sustain internal folate levels.

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