Tissue-specific promoters of the α human folate receptor gene yield transcripts with divergent 5' leader sequences and different translational efficiencies

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The α human folate receptor (α hFR), or KB cell folate receptor, gene contains two major promoters that produce transcripts, KB1 and KB4, varying only in the length and sequence of their 5' untranslated regions (UTRs). Using RNase protection assays specific for each isoform, we show that the level of expression of these two transcripts is tissue-specific, indicating that promoter usage is regulated, not constitutive. RNA stabilities and translational efficiencies of the KB1 and KB4 transcripts were compared to determine the functional significance of the different 5' UTRs. Analyses of RNA turnover *in vivo* with actinomycin D to block new transcription and *in vitro* with a cytoplasmic extract indicate no discernible differences in the stabilities of the two transcripts. However, the KB4 transcript is 2–3-fold more efficiently translated in wheat germ extracts *in vitro* and transfected CHO cells *in vivo*. Also, high ionic strength, which favours

the formation of RNA secondary structure, differentially affects the translational efficiencies of the two transcripts. Translation of the longer KB1 mRNA is 2–5-fold more inhibited by hypertonic conditions than translation of the KB4 mRNA. Because the 5' UTR of KB1 is approximately four times longer than the 5' UTR of KB4, 149 bp (75%) of the KB1 5' UTR were deleted to determine whether the long leader sequence inhibited translation. The resulting derivative, dKB1, has a 5' UTR similar in length, but not sequence, to the 5' UTR of KB4. dKB1 is translated at a level approaching that of KB4 in wheat germ extracts, indicating that the upstream portion of the 5' leader sequence contributes to the relative translational inefficiency of KB1. Hence, one consequence of tissue-specific promoter usage is the production of α hFR transcripts with different 5' non-coding regions that affect translational efficiency.

INTRODUCTION

The human folate receptor (hFR) is an important component in the cellular accumulation of folates and folate analogues used in chemotherapy [1–3]. In humans, three isoforms of the folate receptor have been identified [4–7]. The most abundant isoform found in a wide variety of cell types was originally isolated from the KB epidermoid carcinoma cell line and is designated KB-hFR or α hFR.

Multiple α hFR cDNA isoforms have been isolated from various cell lines [4,8,9]. These cDNA isoforms vary in the length and sequence of the 5' leader but share the same 3' untranslated region (UTR) and open reading frame encoding a 257-residue polypeptide. We have recently cloned the α hFR gene and a novel α hFR cDNA isoform designated KB4 [10,11]. The KB1 (c32) [4] and KB4 isoforms are the major transcripts expressed in the KB cell line, which constitutively overexpresses the hFR protein. On the basis of an analysis of genomic DNA, we have shown that the α hFR gene potentially encodes all reported α hFR cDNA isoforms including the 5' UTRs of KB1 and KB4 in putative exons 1 and 4 respectively. Sequences flanking putative exons 1 and 4 exhibit promoter activity and have been designated the P1 and P4 promoters [10,11]. From these results it seems that the heterogeneous 5' UTRs reported for KB1 and KB4 result from transcription initiated from the P1 and P4 promoters respectively.

The discovery of these two promoters in the α hFR gene introduced the possibility that expression is regulated not only at the level of transcription but also at the level of translation. The two 5' non-coding regions produced by differential promoter

usage could be important in determining the expression of α hFR through an effect on either RNA turnover or translational efficiency. In some instances, sequences in the 5' UTRs of mRNA species contribute secondary structure and binding of associated proteins that might regulate RNA stability [12]. However, most studies on the effects of the 5' UTR have found differences in translational efficiency. Secondary structure, decreased accessibility to the m⁷G cap, and some sequence elements in the 5' UTR all decrease the efficiency with which mRNA species produce protein [13–17]. Moderately stable hairpin structures close to the 5' end are thought to block access to the m⁷G cap, whereas structures located downstream apparently inhibit translation by decreasing the processivity of the ribosomal complex [17]. Hence it is possible that specific sequences and/or structures in the 5' non-coding regions of the α hFR transcripts could influence their translation.

Recently, transcripts originating from both the P1 and P4 promoters have been found in RNA species from several tissue types [10]. Therefore it is important to understand whether the different 5' UTRs affect receptor expression. In this study we have quantitatively analysed the tissue-specific activity of the P1 and P4 promoters with RNase protection assays (RPAs). These assays revealed a broad range in the level of expression of α hFR as well as the tissue-specific activities of the two promoters. Using three separate assays for translation, we found that the transcripts derived from the P1 and P4 promoters differed by as much as 3-fold in translational potential. However, assays of RNA degradation showed no effect of the different 5' UTRs on the rate of RNA turnover.

Abbreviations used: CHO, Chinese hamster ovary; hFR, human folate receptor; RPA, RNase protection assay; TBE, 0.089 M Tris/borate/0.089 M boric acid/0.002 M EDTA; UTR, untranslated region.

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MATERIALS AND METHODS

RPAs

The relative abundance of α hFR transcripts originating from the P1 and P4 promoters of the α hFR gene were determined with RPAs. Total RNA species from normal human tissues were purchased from Clontech. The 5' *EcoRI*–*HincII* restriction fragments from the KB4 cDNA [11] and the KB1 cDNA (c32 cDNA clone) [4] were subcloned into pGEM-4Z for use as KB4 α hFR and KB1 α hFR riboprobes respectively (Figure 1A). The KB1-32 construct, containing the 32 bp of the 5' UTR proximal to the ATG start site, was synthesized by PCR with the KB1 *EcoRI*–*HincII* fragment in pGEM-4Z as a template; the sense primer was –32 bp to –8 bp of KB1 and the anti-sense primer was derived from the T7 promoter. The PCR product was subcloned into PCRII (Invitrogen) and verified by DNA sequencing. This short construct was used to quantify KB1 transcripts more accurately as described in the Results section. The recombinant α hFR vectors were linearized with *EcoRI*, and riboprobes (specific radioactivity more than 0.5×10^9 c.p.m./ μ g) corresponding to the anti-sense strand were synthesized with the reagents and protocol from the Promega *in vitro* transcription kit and 5' [α - 32 P]CTP (specific radioactivity 800 Ci/mmol) purchased from Amersham. A riboprobe synthesized from the β -actin anti-sense control template (Ambion) was radiolabelled at one-fortieth of the specific radioactivity (127 bp protected fragment) and included in the RPAs to facilitate the comparison between experiments and control for the integrity of the RNA. A truncated α hFR internal control, consisting of a sense strand of the c10 [3] construct synthesized *in vitro* and linearized with *AvaI* (156 bp protected fragment; see Figure 1A) was included to standardize between samples and experiments.

RPAs were performed as described [18]. Briefly, total tissue RNA (25 μ g), internal control RNA (approx. 2 pg), α hFR riboprobe (100 000 c.p.m.) and β -actin riboprobe (10 000 c.p.m.) in 30 μ l of hybridization buffer [40 mM Pipes (pH 6.4)/80% (v/v) formamide/0.4 M NaCl/1 mM EDTA] were denatured at 85 °C for 8 min and then reannealed at 45 °C for 18 h. The single-stranded RNA was digested by the addition of 350 μ l of RNase buffer [10 mM Tris/HCl (pH 7.5)/200 mM NaCl/100 mM LiCl/2 mM EDTA] containing RNase A (14 μ g) and RNase T₁ (0.7 μ g) followed by incubation at 25 °C for 15 min. The digestion was terminated by the addition of 20 μ l of 10% (w/v) SDS and 100 μ g of proteinase K and incubation at 37 °C for 30 min. After extraction in an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, by vol.), 10 μ g of carrier (wheat germ) tRNA was added and the RNA species were precipitated with ethanol. Precipitated RNA was resuspended in 6 μ l of RNA loading buffer [0.5 $\times$ TBE (where TBE is 0.089 M Tris/borate/0.089 M boric acid/0.002 M EDTA)/80% formamide/0.2% Bromophenol Blue/0.2% Xylene Cyanol], denatured for 5 min at 95 °C and resolved on a 6% (w/v) sequencing wedge gel run in 0.5 $\times$ TBE at 60 W constant power. Sequencing ladders were used to estimate the fragment sizes. The gels were dried and autoradiographed at –70 °C with intensifying screens. The abundance of the protected fragments was determined with a Molecular Dynamics Phosphorimager. Values for specific transcripts were standardized to the internal control. The relative abundances of transcripts from the P1 or P4 promoter were quantified by determining the intensity of protected fragments that were probe-specific. Total transcript abundance was determined by summing the value of all the fragments protected by the α hFR probes; the values were normalized to the expression in placenta. Promoter utilization was calculated as a percentage of total transcript abundance [e.g. $100 \times (\text{abundance}$

of specific KB1 or KB4 α hFR transcripts/total α hFR transcript abundance)]. The relative expression of β -actin in the various tissues was also determined by phosphorimager analysis and normalized to β -actin expression in placenta.

Construction of KB-hFR plasmids

KB1 (c32 cDNA) was subcloned into pGEM-4Z as previously described [4]. The KB1 cDNA was also subcloned by blunt-end ligation into the *HindIII* site of pRC/CMV eukaryotic expression vector (Invitrogen, San Diego, CA, U.S.A.). dKB1 was generated by PCR from KB1 with the Amplitaq reagents and protocol (Perkin-Elmer). The 5' sense primer corresponded to bp 150–164 of KB1 and the anti-sense primer was the T7 universal primer (Promega). Both primers contained *EcoRI* sites that were subsequently used for subcloning the PCR product into the *EcoRI* site of pGEM-4Z.

A 3' truncated KB4 cDNA was amplified from human kidney mRNA with reverse transcriptase-PCR as described previously [11]. The PCR product was subcloned into pGEM-4Z and pRC/CMV as described above. The full-length KB4 cDNA was constructed by exchanging the 3' *NcoI*–*XbaI* fragment of KB1 with the *NcoI*–*XbaI* fragment of KB4 in pGEM-4Z. All constructs were confirmed by restriction enzyme digestion and DNA sequencing.

Analysis of RNA stability in KB cells

KB cells were grown to 90% confluency in 10 cm dishes in minimal essential medium with 10% (v/v) fetal calf serum (MEM/10%FCS). Transcription was inhibited by bathing the cells in MEM/10%FCS with actinomycin D (5 μ g/ml) as described [19,20]. Total cellular RNA was isolated from cells at 2 h time points with the Qiagen RNeasy kit. RPAs were used to quantify the relative concentrations of the KB1 and KB4 transcripts at each timepoint. The KB1-32 riboprobe was used and transcript levels of the specific (KB1) and splice site (KB4) fragments were quantified by phosphorimager analysis as described above for RPAs. The assays were performed in duplicate.

Analysis of RNA stability in cell extracts

RNA stability in a cell extract was measured as described by Pei and Calame [21]. Briefly, cytosol was prepared from KB cells cultured in MEM. Adherent KB cells were washed twice with PBS, scraped off the culture plate and collected by centrifugation. Pellets were resuspended in 2 vol. of 0.25 M sucrose containing 0.5 M dithiothreitol and a cocktail of protease inhibitors (2.5 μ g/ml leupeptin, 2.5 μ g/ml pepstatin, 2.5 μ g/ml aprotinin and 150 μ g/ml PMSF), vortex-mixed for 10 s and then incubated on ice for 10 min. Lysis of the cells was confirmed by the Trypan Blue exclusion test. The KB cell extract was clarified by centrifugation for 1 min at 13 000 g and the supernatant was recentrifuged for 30 min at 13 000 g. The second supernatant, hereafter referred to as cytosol, was stored at –40 °C.

SP6 RNA transcripts capped with 7-methyl-GpppG were synthesized with the Ambion Message Machine kit reagents and protocols with a 4:1 ratio of cap analogue to GTP. The pGEM-4Z hFR plasmid DNAs were purified by Qiagen maxiprep and linearized with *XbaI*. Template DNA (1 μ g) was used in each reaction and 25 μ Ci of either [32 P]UTP or [32 P]CTP were used to radiolabel the RNA. The radiolabelled RNA was resuspended in 100 μ l of water. There was approx. 12–15% incorporation of the 32 P-labelled ribonucleotide under these conditions.

Turnover of each radiolabelled transcript was assayed at 37 °C in a 50 μ l reaction consisting of 25 μ l of reaction buffer [20 mM

Tris/HCl (pH 8)/5 mM KCl/150 mM NaCl/5 mM MgCl₂, 10 ng of RNA (approx. 300 000 c.p.m.) and 25 μ l of cytosol. At 0.5, 1, 2 and 4 h, 10 μ l of the reaction were removed and added to 100 μ l of stop buffer [2 mM EDTA/20 mM Tris/HCl (pH 8)/0.5 % SDS]. All samples were then extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, by vol.), and the aqueous phase containing the RNA was analysed by agarose gel electrophoresis and phosphorimager quantification (Molecular Dynamics).

Stable transfection of Chinese hamster ovary (CHO) cells

CHO cells were stably transfected with α hFR pRC/CMV constructs by using the calcium phosphate protocol as described [22]. DNA for transfection was purified with the Qiagen maxiprep system. Stable transfectants were selected in 500 μ g/ml G418 (Gibco) and maintained in 250 μ g/ml G418. Cells were grown in monolayers at 37 °C under air/CO₂ (19:1) in folate-deficient minimal essential medium with 200 nM folic acid and 10 % (v/v) fetal calf serum.

Immunoprecipitation of hFR from transfected CHO cells

Stable transfectants were plated in 10 cm dishes (Falcon) and cultured to 75 % confluency. Cells were washed twice with PBS and then incubated in 5 ml of methionine- and cysteine-free minimal essential medium (Gibco) supplemented with 1 % (v/v) fetal calf serum for 1 h at 37 °C. Cells were metabolically labelled in 2.5 ml of the above medium with 0.1 μ Ci/ml [³⁵S]methionine/cysteine translabel (ICN) for 4 h at 37 °C. For high-ionic-strength experiments, the labelling medium was modified by increasing the NaCl concentration by 0.12 M (final NaCl concentration 0.245 M). At the end of the labelling period, the plates were placed on ice, the medium was removed, cells were washed with ice-cold PBS and harvested with 20 mM EDTA in PBS. hFR was immunoprecipitated as described [3]. Equal

Figure 1 Determination of the abundance of α hFR transcripts in human tissues

(A) Derivation of probes used in the RPAs. At the top is a schematic representation of the α hFR gene showing the intron/exon organization and the splice sites identified for the cDNAs from which the RPA probes are derived. Exons are numbered and represented by boxes, introns are represented by a line and an arrowhead marks the splice site in exon 4. Notation: A, *Aval* restriction site; H, *HincII* restriction site; P, *cis* elements with promoter activity [10,11]. Panels 1, 2 and 3 are diagrams of the 5' *HincII* restriction fragments from the α hFR cDNA species used for synthesis of the riboprobes; panel 4 represents the *Aval* restriction fragment used for the α hFR internal control. Arrowheads mark the splice site that is 8 bp upstream of the open reading frame (ORF), which is illustrated by the open box. The divergent 5' UTRs are represented by shaded boxes corresponding to the potential 5' exons. (B, C) Autoradiographs from the RPAs. (B) The KB1 (lane KB) and KB1-32 (remaining lanes) anti-sense riboprobes as well as the actin anti-sense riboprobe were hybridized with total RNA from the indicated normal human tissues (25 μ g) or the KB cell line (5 μ g), with a constant amount of the internal control RNA added to each sample. (C) The KB4 and actin anti-sense riboprobes were hybridized with the same tissue and KB RNA species including the internal control RNA. RNase digestions and electrophoresis of the protected fragments were performed as described in the Materials and methods section. Labels: Act, the actin protected fragment (128 bp); int C, the internal control protected fragment (156 bp); SS, fragments protected through the splice site. In (B) the bracket labelled KB1 delineates the protected fragments produced by the P1 promoter's multiple start sites within exon 1, and the label KB1-32 identifies the protected KB1-32 probe (204 bp) used to quantify the multiple KB1 fragments. In (C) the label KB4 refers to the full-length protected KB4 probe (217 bp). Wheat germ tRNA (25 μ g) with internal control sense RNA (tRNA) served as the negative control in both assays. Sequencing ladders were run in parallel for size delineation (in bp) as indicated at the right. Cell and tissue sources are labelled in lanes as follows: KB, KB cell; tRNA, tRNA negative control; 1, thymus; 2, spleen; 3, lung; 4, kidney; 5, liver; 6, uterus; 7, heart; 8, brain; 9, pancreas; 10, skeletal muscle; 11, salivary gland; 12, placenta; 13, small intestine; 14, testis; 15, cerebellum; 16, prostate; 17, stomach; 18, mammary gland.

amounts of total cell protein per sample, determined by the Micro bicinchoninic acid protein assay (Pierce), were used for immunoprecipitation. Immunoprecipitates were resolved by SDS/PAGE [12.5% (w/v) gel]; gels were prepared for fluorography by incubation for 1 h in Enlightening (NEN). Radio-labelled bands of immunoprecipitated hFR were quantified with a Molecular Dynamics Phosphorimager.

Northern analysis

Total RNA was purified from CHO stable transfectants with the Qiagen RNeasy kit. RNA (15 µg) from each CHO transfectant was denatured in 50% formamide and resolved by electrophoresis in 1.4% agarose in TBE buffer. RNA transfer and hybridization were performed as described in the Schleicher and Schuell manual. The RNA was transferred to Nytran (Schleicher and Schuell) and the blot was hybridized simultaneously with cytoplasmic actin and hFR probes radiolabelled by random priming (Promega Prime-a-Gene kit) with ³²P dCTP (10⁶ c.p.m./ml of hybridization solution for each probe). After washing for 1 h at 65 °C in 1 × SSPE/0.1% SDS, the blots were autoradiographed and quantified by phosphorimager analysis.

Translation *in vitro*

Both reticulocyte lysate and wheat germ extract (Promega) were used for translation assays *in vitro*. The protocol in the Promega technical manual was followed with these reaction conditions: 40 µg/ml RNA transcribed *in vitro*, 0.8 µCi/µl [³⁵S]cysteine, 80 or 150 mM potassium acetate as indicated and either 0.5 mM magnesium acetate (reticulocyte lysate) or 2 mM magnesium acetate (wheat germ extract) in a 25 µl reaction volume. Reticulocyte lysate reactions were incubated at 30 °C, whereas wheat germ extract reactions were incubated at 25 °C. After 1 h, 25 µl of 2 × SDS sample buffer were added to the samples, which were then boiled for 3 min to terminate the reactions.

The translation reactions were analysed by SDS/PAGE [15% (w/v) gel] with 2.5 or 5 µl of the reaction mix. Gels were fixed and fluorographed, and αhFR bands were quantified by phosphorimager as described above. An aliquot of each reaction was resolved on at least two gels and the results were averaged.

RESULTS

Transcript origin and relative abundance

We have previously isolated two cDNA isoforms of αhFR (KB1 and KB4) that differ in their 5' leader sequences and seem to originate from different promoter regions (designated P1 and P4; Figure 1A) in the αhFR gene [10,11]. To determine the levels of activity of the P1 and P4 promoters in normal human tissues we performed RPAs with isoform-specific riboprobes. The riboprobes corresponded to the anti-sense strand of the 5' 204 bp of KB1-32 and the 5' 217 bp of KB4. These riboprobes, diagrammed in Figure 1A, spanned the splice site located -8 bp upstream of the start of translation such that the 3' terminal 180 bp sequence was common to all isoforms, whereas the 5' terminal sequences were isoform-specific. Hence each riboprobe would protect not only the isoform-specific mRNA (fragments greater than 180 bp) but also other αhFR mRNA species sharing the 180 bp sequence downstream of the splice site.

With a full-length KB1 riboprobe (383 bp) to identify transcripts originating from the P1 promoter, total RNA from KB cells (Figure 1B, lane KB) protected multiple fragments of the KB1 riboprobe ranging in size from approx. 190 bp to 380 bp. Similar results were obtained previously in assays containing

Table 1 Relative expression and promoter utilization of αhFR in normal human tissues

Values were determined from two independent RPAs with the KB1-32 (RPA 1) and KB4 (RPA 2) probes as shown in Figure 1. All values were normalized to the internal control. Expression is denoted by the total level of αhFR mRNA (all fragments protected by αhFR probe) in each tissue relative to the level in placenta. Promoter utilization is presented as the percentage of KB1- or KB4-specific transcripts relative to the total αhFR mRNA in the given tissue with either the KB1-32 (P1, RPA 1) or KB4 (P4, RPA 2) probes. The relative expression of actin refers to cytoplasmic actin in each tissue standardized to the level in placenta. + indicates a detectable signal less than 2% of the level in placenta. Abbreviation: ND, no detectable signal under these experimental conditions.

Tissue	RPA ...	αhFR expression (%)		αhFR promoter utilization (%)		Relative expression of actin	
		1	2	P1	P4	1	2
Placenta		100	100	32	63	1.0	1.0
Lung		110	98	38	46	2.3	2.2
Kidney		99	91	52	37	1.3	1.3
Liver		+	6	+	+	0.5	0.6
Salivary gland		130	120	25	53	0.7	0.6
Small intestine		n.d.	4	n.d.	+	1.4	1.2
Pancreas		+	4	+	+	0.1	0.2
Brain		6	9	99	n.d.	1.7	1.6
Cerebellum		+	3	+	+	0.9	0.9
Uterus		5	4	37	62	2.0	2.3
Mammary		86	92	29	60	1.5	1.6
Prostate		+	6	+	+	1.6	1.7
Testis		7	7	49	32	1.5	1.5
Heart		+	+	+	+	0.4	0.5
Skeletal muscle		n.d.	+	n.d.	+	0.3	0.3
Thymus		+	+	n.d.	+	2.9	2.8
Spleen		n.d.	+	n.d.	+	1.7	1.8
Stomach		14	21	28	58	0.6	0.6

RNA from human kidney, lung and brain [10]. The protection of multiple fragments suggests that in these tissues transcripts originate from multiple start sites downstream of the P1 promoter but within exon 1. To facilitate quantification of the transcripts originating from the P1 promoter, a 5' truncated KB1 probe (KB1-32; Figure 1A) was used to 'sum' the longer transcripts into a single 204 bp fragment, as shown in Figure 1(B). The KB1-32 probe yielded three protected fragments, two corresponding to the KB1 isoform-specific transcripts at 204 bp and 190 bp, and a protected fragment at 180 bp (SS) representing other αhFR mRNA species, predominantly KB4.

The KB4 riboprobe was used (Figure 1C) to identify transcripts originating from the P4 promoter. In every tissue except brain, a protected fragment corresponding to the KB4 mRNA (217 bp) was detectable, with high expression in placenta, lung, kidney, salivary gland and mammary tissues. In these tissues and in brain there was also a band (SS) corresponding to transcripts that matched the 3' 180 bp sequence, indicative of RNA species transcribed from promoter(s) upstream of the P4 promoter and spliced into the -8 bp acceptor site in exon 4. The presence of protected fragments at approx. 190 bp in some tissues suggests that there are transcription start sites downstream of the P4 promoter or that another splice site is used upstream of the -8 bp splice site in exon 4.

The expression of the αhFR KB1 and KB4 transcripts in tissues relative to the expression in placenta, as determined from the RPAs, is shown in Table 1. Most of the tissues studied expressed αhFR mRNA species, but at variable levels ranging

Figure 2 Decay rates of KB1 and KB4 mRNA species in actinomycin D-treated KB cells

(A) RPA. KB cells were exposed to actinomycin D for the indicated durations. The RPA was performed with 2 μ g of total RNA as described in the legend to Figure 1 by using the KB1-32 riboprobe. At $t = 8$ h, both KB1 and KB4 transcripts were approx. 50% degraded, as determined after each lane had been normalized to the internal control. Actin is shown in the lower panel for comparison. (B) Relative decay of KB1 and KB4 transcripts. The KB1/KB4 ratios for each time point were calculated from the intensity of protected fragments specific to each isoform as shown in the RPA in (A).

from less than 2% to approx. 130% of that observed in placental tissue. Interestingly, P1 and P4 promoters are utilized simultaneously in tissues and the relative activities of the promoters are tissue-specific. For example, the α hFR transcripts in RNA from human brain, kidney and testis originate predominantly in the P1 promoter region. In contrast, most α hFR transcripts from placenta, lung, salivary gland, uterus, mammary and stomach tissues originate in the P4 promoter. Transcripts originating from the P1 and P4 promoters accounted for most (more than 80%) of the α hFR transcripts in the tissues analysed (Table 1).

RNA turnover

To test whether the different 5' UTRs affect RNA stability we used KB cells, a cell line that expresses transcripts from both the P1 and P4 promoters, to measure differences in the rate of turnover of the KB1 and KB4 transcripts. First, actinomycin D was used to inhibit new transcription in intact cells to allow us to monitor the decay of the endogenous α hFR mRNA species *in vivo*. RPAs with the KB1-32 probe were used to distinguish between the KB1 and KB4 transcripts (full-length KB1 transcripts protect a 204 bp fragment, whereas KB4 transcripts protect a 180 bp fragment downstream of the splice site). Approx. 50% of both the KB1 and KB4 transcripts were degraded over the 8 h time course (Figure 2A). To make a precise comparison

Figure 3 Stability of KB1 and KB4 mRNA species in KB cell extracts

(A) Degradation of KB1 and KB4 transcripts *in vitro*. Radiolabelled transcripts of KB1 and KB4 were incubated at 37 °C in a reaction mix containing a cytosolic extract of KB cells. At the indicated times, an aliquot of the reaction mix was removed, RNA extracted, resolved by agarose gel electrophoresis and autoradiographed. (B) Time course of KB1 and KB4 degradation. Intact transcripts were detected and quantitated by phosphorimager analysis (Molecular Dynamics) and expressed as a percentage of the transcript level at zero time. Symbols: $\square$, KB1 transcripts; $\blacksquare$, KB4 transcripts. Each point is the average of two independent experiments. Error bars represent the ranges of the two values.

of the relative decay rates of the KB1 and KB4 transcripts, the ratio of KB1 to KB4 in each lane of the RPA (equals one timepoint) was calculated. As shown in Figure 2(B), the KB1/KB4 ratio did not change after actinomycin D treatment; each transcript was degraded at the same relative rate. Therefore the different 5' UTRs of the KB1 and KB4 transcripts do not seem to affect the rate of degradation in intact KB cells.

Secondly, because the expression of the KB4 transcript is approx. 15-fold higher than that of KB1 in KB cells, we used an *in vitro* model of RNA stability [21] to compare the absolute rates of degradation. Equal quantities of KB1 mRNA and KB4 mRNA were added to the assay *in vitro* so that the rates of degradation could be measured at the same RNA concentration. Stabilities of radiolabelled transcripts of KB1 and KB4 synthesized *in vitro* were assayed in a KB cell cytoplasmic extract supplemented with 5 mM $MgCl_2$ to enhance the rate of degradation. Both the KB1 and KB4 transcripts were degraded approx. 80% over a 4 h incubation, with no significant differences in the rates of degradation (Figures 3A and 3B). Consequently, in both *in vivo* and *in vitro* models for assessing RNA turnover, the KB1 and KB4 mRNA species have similar stabilities.

Translational efficiency *in vivo*

Another possible function of the different 5' UTRs is in determining the translational efficiency of the mRNA species. To test

Figure 4 Comparison of the expression of α hFR in CHO cells transfected with either KB1 or KB4

(A) Immunoprecipitation of α hFR from stable CHO clones containing either the KB1 or KB4 construct. CHO cells were metabolically labelled with [35 S]methionine/cysteine, and α hFR protein was immunoprecipitated as described in the Materials and methods section. Labelling medium was supplemented with 120 mM NaCl as indicated. α hFR immunoprecipitates were resolved by SDS/PAGE [12.5% (w/v) gel] and fluorographed. The positions of molecular mass standard markers [given in kDa (kD)] are indicated at the left. These results are representative of three independent labelling experiments as presented in Table 2. **(B)** Northern analysis of stable CHO clones. Northern blots were probed for α hFR and actin simultaneously. The left lane contained KB4 total RNA and the right lane contained KB1 total RNA. The ratio of α hFR to actin was used to determine the relative expression of α hFR mRNA in both clones.

Table 2 Relative translation of folate receptor mRNA isoforms

The amounts of radiolabelled protein synthesized from the KB4 and dKB1 mRNA species were normalized to the amount synthesized from the KB1 mRNA. Where $n \geq 2$, values are given as averages $\pm$ deviation for two independent experiments. Where $n \geq 3$, values are given as the means $\pm$ S.D. for independent experiments. Assays were performed and quantified as described in the Materials and methods section. Abbreviations: RL, reticulocyte lysate; WG, wheat germ extract; CHO, stably transfected CHO cell clones.

Assay	Transcript ...	Relative translation of mRNA		
		KB1	dKB1	KB4
RL		1.0	0.9 $\pm$ 0.2 ($n = 3$)	1.2 $\pm$ 0.1 ($n = 3$)
WG		1.0	2.2 $\pm$ 0.1 ($n = 2$)	2.3 $\pm$ 0.5 ($n = 4$)
CHO		1.0	—	2.8 $\pm$ 1 ($n = 3$)

whether the 5' UTR affected translational efficiency *in vivo*, we examined the translation of α hFR mRNA species with different 5' leader sequences in stably transfected CHO cells. Cells were labelled in isotonic medium or in medium containing 120 mM excess NaCl, as indicated. The rationale for adding excess NaCl is discussed below in the section on the effect of hypertonic stress.

KB1 and KB4 transcripts were transfected into CHO cells, which do not express detectable levels of endogenous folate receptor [3]. To obtain clones for each transcript that expressed similar amounts of α hFR mRNA, we screened stable clones by Northern analysis. Clones with mRNA levels that varied by less than 2-fold were used for subsequent analysis of protein expression (Figure 4B; KB4 left lane, KB1 right lane). Approx. 3-

Figure 5 α hFR mRNA transcripts

Diagrams of the mRNA transcripts used for *in vitro* assays of RNA translation are represented as follows: the unfilled region represents the α hFR open reading frame (ORF) common to all transcripts, the hatched (KB1) or shaded (KB4) regions represent the different 5' UTR sequences upstream of the ORF, and the filled region represents the common 3' UTR sequence downstream of the ORF.

Figure 6 Transcripts with the full-length 5' UTR of KB1 show lower translational efficiency *in vitro*

Capped α hFR transcripts were translated for 1 h in a 25 μ l reaction containing [35 S]cysteine and either wheat germ extract (left panels) or reticulocyte lysate (right panels). The newly synthesized radiolabelled proteins were resolved by SDS/PAGE and fluorographed. **(A)** KB1, dKB1 and KB4 mRNA translation products synthesized under standard reaction conditions (80 mM potassium acetate). **(B)** KB1, dKB1 and KB4 mRNA translation products synthesized under high-ionic-strength reaction conditions (150 mM potassium acetate). All of the lanes in **(A)** and **(B)** are taken from a fluorograph of a single gel. These results are representative of four independent experiments with the wheat germ extract and no fewer than two experiments with the reticulocyte lysate as summarized in Table 2.

fold more protein was immunoprecipitated from the KB4 clone than from the KB1 clone (Figure 4A, lanes labelled KB4 and KB1 at the left). Results from three separate labelling experiments showed that the KB1 clone consistently produced less α hFR protein than the KB4 clone (Table 2; CHO values corrected for the small differences in mRNA expression).

Translational efficiency *in vitro*

To facilitate the analysis of the effect of the 5' leader sequence on α hFR translational potential, we compared translation assays *in vitro* with the results from the experiments *in vivo* using the KB1 and KB4 mRNA species and a 5' deleted construct of KB1 (dKB1) (Figure 5). As described in detail in the Materials and methods section, the concentration of the mRNA species transcribed *in vitro* were determined by ethidium bromide staining of RNA bands resolved by agarose gel electrophoresis. Translation assays with reticulocyte lysates showed comparable amounts of

protein synthesized from the KB1 and KB4 transcripts (Figure 6A, right panel). However, when wheat germ extracts were used to assay translation, KB4 protein expression was more than 2-fold higher than KB1 (Figure 6A, left panel), a difference comparable to that observed in the stably transfected CHO cells. Results from several independent assays were quantified by phosphorimager analysis and showed consistently higher translation of the KB4 transcript in wheat germ extracts but only negligible differences in reticulocyte lysates (Table 2), suggesting that the effect of the 5' UTR on translation is dependent on the type of assay used. However, in assays both *in vivo* (CHO transfectants) and *in vitro* (wheat germ extract) the 5' UTR of KB1 seemed to depress the translation of α hFR message relative to KB4.

To examine the effect of the length of the 5' UTR of KB1 on translational efficiency, we compared the translation of the full-length KB1 with a 5' deleted construct of KB1 (dKB1). The dKB1 construct was identical with KB1 except that the first 149 bp of the 5' UTR were deleted as described in the Materials and methods section (see Figure 5). When assayed in the wheat germ extract, the truncation of the KB1 5' UTR increased the translational efficiency of the mRNA to a level comparable to translation of KB4 (Figure 6A, left panel, and Table 2). These results suggest that the 5' end of the leader sequence is responsible for the low translational efficiency of KB1 in wheat germ extracts.

Effect of hypertonic stress

Previous studies have shown that high concentrations of K⁺ or other cations decrease the translational efficiency of mRNA species with 5' non-coding regions that contain moderate and/or stable stem-loop structures [23,24]. To test whether there would be greater differences in the relative translation of the α hFR transcripts under these conditions, the tonicity of either the wheat germ extract or the reticulocyte lysate was varied by increasing the potassium acetate concentration from 80 to 150 mM.

In the wheat germ extract, translation of all three transcripts was inhibited by the increase in potassium acetate concentration (Figure 6B, left panel). However, translation of the KB1 transcript was more sensitive to the high-tonicity medium [factor of decrease, 4.5 ± 1 (S.D.), $n = 4$] than dKB1 or KB4 which showed similar sensitivities (factor of decrease, dKB1 = $2.6\text{--}3.4$, $n = 2$; KB4 = 2.5 ± 1 , $n = 4$). In contrast, the higher potassium acetate concentration did not reproducibly affect the translation of any of the transcripts in the reticulocyte lysate assay (Figure 6B, right panel).

Kozak [23] has shown that differences in translational efficiency dependent on moderately stable secondary structure in the 5' UTR are enhanced under conditions of hypertonic stress *in vivo*. Hence we also tested the effect of high tonicity on translation in the CHO stable transfectants. For this experiment we compared the amount of α hFR protein immunoprecipitated from cells radiolabelled in either isotonic medium or hypertonic medium (supplemented with 0.12 M NaCl) as described by Kozak [23]. As shown above (Figure 4), the higher-tonicity medium disproportionately inhibited the synthesis of KB1. Results from three separate experiments showed that inhibition of KB1 translation at high salt concentrations averaged 50% compared with the 0–15% inhibition seen for translation of KB4. These results are comparable to those previously described [23], where the introduction of a moderately stable hairpin structure (-126 kJ/mol) in the 5' UTR of proinsulin made this transcript sensitive to high tonicity. It is possible that secondary structure in the 5' leader is responsible for the relatively low translational

efficiency of the KB1 transcript because high tonicity inhibits translation of KB1, whereas truncation of the KB1 5' UTR ameliorates this inhibition in the wheat germ assay.

DISCUSSION

We have identified α hFR transcripts in normal tissues that code for the same protein but contain unique 5' leader sequences. Previous studies demonstrated that these mRNA species, designated KB1 and KB4, are transcribed from different promoters flanking exons 1 and 4 of a single-copy gene [10,11]. Differential promoter usage results in the production of transcripts with different 5' UTRs but identical coding sequences and 3' UTRs [11]. By using RPAs that were designed to distinguish between the two isoforms we show that these mRNA species are frequently co-expressed. However, the transcription of KB1 relative to KB4 is tissue-specific; in brain, testis and kidney KB1 is the predominant isoform, whereas in salivary gland, placenta, uterus, lung, stomach and mammary tissues KB4 is the predominant isoform.

The RPAs also indicated that transcription from the P1 promoter initiated from multiple sites within putative exon 1. It is possible that P1 contains several promoter elements or that the promoter 'stutters', as has been described for the TATA-less promoters [25]. Multiple initiation sites in P1 result in the production of KB1 mRNA species with 5' UTRs of different lengths. These results are strikingly similar to those described for superoxide dismutase (SOD)-1 expression in mouse testis [26]. Like α hFR, alternative promoters in the SOD-1 gene produce distinct mRNA species from a single gene. The resulting SOD-1 RNA species have 5' leaders of different lengths and are differentially expressed in somatic and germ cells. Interestingly, the SOD-1 upstream promoter has multiple transcription start sites, analogous to what we describe for KB1-hFR. Also, those authors found that the longer transcripts from the upstream promoter were half as efficiently translated in reticulocyte lysates.

Because the α hFR mRNA species are expressed in a tissue-specific pattern, we were interested in determining the consequences of differential promoter usage leading to the transcription of mRNA species with different 5' UTRs. Heterogeneity in promoter usage and 5' sequence might contribute to gene expression through transcriptional regulation or through post-transcriptional mechanisms such as RNA turnover or translational efficiency. Evidence for the regulated use of multiple promoters has been described in a mouse cell line in which acquisition of methotrexate resistance was associated with the transcription of folate receptor message with an alternative 5' UTR [27]. Also, promoter-dependent translational regulation has been described for other genes, including the regulation of *c-myc* in *Xenopus* development, where the 5' UTR from one promoter inhibits translation but the 5' UTR from another promoter does not [16].

Initially we tested whether the 5' UTR has an effect on RNA turnover. RNA turnover rates have been shown to regulate the expression of some proteins [12] and might be relevant to the variable pattern of tissue expression of the α hFR mRNA isoforms. In addition, an increase in stability of α hFR mRNA has been shown to be a factor in the increased level of folate receptor protein expressed by KB cells grown in folate-depleted medium [20]. To test whether differences in the 5' UTR could affect the stability of α hFR mRNA species, we used actinomycin D to inhibit new transcription and measured the decay of the KB1 and KB4 RNA species. By using RPAs to separate and quantify the two isoforms, we found no detectable differences in the relative decay rates. Similarly, by using an assay *in vitro* of

RNA stability, we found no differences in the rates of degradation of the two mRNA species. On the basis of these results, the 5' UTR does not seem to be a major determinant of α hFR mRNA stability.

In contrast, differences in the translational efficiency of the KB1 and KB4 messages were found in assay systems both *in vivo* and *in vitro*. In stably transfected CHO cells there was 3-fold higher expression of α hFR protein in the KB4 clone relative to the KB1 clone, even though both contained comparable amounts of α hFR mRNA. Because the proteins produced are identical there should be no differences in protein turnover; instead, the differences in the pulse-chase labelling of α hFR in these transfected cells most probably reflects differences in translational efficiency. We also measured translational efficiency with translation systems *in vitro* from both reticulocyte lysates and wheat germ extracts. In the wheat germ extracts, translation of KB4 mRNA was more than twice as efficient as KB1 mRNA, whereas in reticulocyte lysates the translational efficiencies of the two transcripts were comparable.

Several groups have reported that the 5' UTR might influence the translational efficiency of eukaryotic mRNA transcripts through any of the following structural features: (1) AUG codon sequence context, (2) occurrence of upstream AUG codon(s), (3) length of the 5' leader, and (4) secondary structure in the 5' leader [13–17,28]. For the α hFR mRNA species, analysis of the sequence in the upstream untranslated region showed that both KB1 and KB4 transcripts have the same sequence surrounding the initiation codon (ACAGACAUGG) and neither transcript has additional, upstream AUG codons; hence the primary sequence of the 5' leader does not predict differences in translational efficiency. However, the 5' leader sequence of KB1 is 166 bp longer than the 5' leader of KB4. One possibility is that the 5' UTR of KB1 contains elements of secondary structure that could inhibit translation. Such effects have been described both *in vitro* and *in vivo* for the decreased translational efficiency of other mRNA species with 5' secondary structure [14,15,17,23,28–30]. The following experiments support the hypothesis that the long 5' leader sequence of KB1 affects the translational potential of the KB1 transcript through an increased potential to form secondary structure. First, in both the wheat germ extract and the stably transfected CHO cells the KB1 mRNA was more sensitive to high tonicity than the KB4 mRNA. High concentrations of potassium acetate in assays *in vitro* or NaCl in the culture medium of transfected cells have been shown to decrease the translational efficiency of transcripts with secondary structure in the leader sequence because a high salt concentration enhances the stability of RNA duplexes [23,24,28]. Secondly, the translational potential of the truncated message, dKB1 (in which 75 % of the KB1 5' UTR has been deleted), was increased relative to the native KB1 and showed a lower sensitivity to inhibition by high K^+ levels in the wheat germ extract assay. Because long, unstructured 5' UTRs do not inhibit translation [28,29] it is unlikely that the increased efficiency of the dKB1 transcript is due simply to the decrease in length of the 5' UTR. Instead, the loss of inhibition in the 5' deleted KB1 transcript might be due to the loss of potential secondary structure or the loss of a specific inhibitory sequence. Future experiments with targeted deletions will help to determine the critical feature(s) of the KB1 5' UTR.

Variation in the relative rates of translation that we observed in the reticulocyte and wheat germ assays *in vitro* could be a reflection of the translational capacity of these systems. At high translational capacity, moderately stable secondary structures might not be inhibitory, whereas under less optimal conditions such structures could slow the rate of translation [29–32].

Reticulocytes have the abundant translational machinery required for the key role that these cells have in haemopoiesis; hence this system is preferred for the translation of cloned genes. Although wheat germ extracts might initially be expected to be equivalent to reticulocyte lysates, it has been shown that efficient translation of some mRNA species requires supplementation of the extract with the initiation factors eIF-4A, 4F, 4G and eIF-3 [32]. Previous studies have indicated that mRNA species with 5' secondary structure require higher levels of eIF-4F than transcripts with less structure [24,29,31,32,34,35]. Hence, when the activity of the eIFs is rate-limiting, transcripts with more secondary structure might be less efficiently translated. Similarly, the translational machinery of CHO cells, like wheat germ extract, might be sufficient for sustaining a constitutive level of protein synthesis, but might not be optimal for the translation of some messages, such as KB1.

Nevertheless the significance of alternative promoter usage remains. Why would some cell types preferentially express a transcript that is less efficiently translated? The answer might be related to the regulation of cell growth. Cell proliferation requires folates for DNA synthesis, and increased expression of the folate receptor would potentially increase folate uptake. Hershey [36] showed that rapidly growing cells have higher rates of protein synthesis, apparently due to an increase in the activity of initiation factors. If dividing cells are poised for efficient translation, the inhibitory effect of the 5' UTR of KB1 could be relieved, thus allowing for an increased expression of folate receptor when the cells have a higher demand for folates. This would be equivalent to the efficient translation of KB1 that we observed in reticulocyte lysates. A similar model has been proposed for another protein required for cell proliferation, ornithine decarboxylase (ODC). ODC is poorly translated and has a 5' UTR rich in secondary structure. However, stimulation of cells with insulin results in increased phosphorylation of eIF-4B and eIF-4E and a corresponding increase in translation of ODC mRNA [17,29]. Therefore secondary structure in the 5' UTR could function as a mechanism for regulation of the expression of proteins required during periods of rapid cell growth.

In summary, we have quantified the tissue-specific expression of two mRNA isoforms of α hFR that result from regulated promoter usage. The two isoforms, KB1 and KB4, differ only in the length and sequence of the 5' UTR. Relative to KB4, the 5' leader of KB1 depressed translation in assays both *in vivo* and *in vitro*, whereas the turnover rates of the transcripts were equivalent. Future experiments will be directed towards determining the physiological significance of the tissue-specific expression of these α hFR mRNA isoforms.

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