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Structure and regulation of a polymorphic gene encoding folate receptor type γ/γ′

Huiquan Wang, John F. Ross and Manohar Ratnam*

Department of Biochemistry and Molecular Biology, Medical College of Ohio, Toledo, OH 43699-0008, USA

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ABSTRACT

The human folate receptor (hFR) type γ and γ′ are constitutively secreted proteins that are expressed primarily in hematopoietic tissues and are potential serum markers for certain hematopoietic malignancies. hFR-γ′ is a variant of hFR-γ with a two base deletion in its cDNA resulting in a truncated polypeptide. The gene encoding hFR-γ′ was isolated from a placental genomic library. The gene has five exons, four introns and a 5′ flanking sequence which contains multiple putative regulatory elements. From RNase protection assay and RACE analysis, the major site of transcriptional initiation was identified at –56 nt. Systematically deleted fragments in the 5′ region of the genomic DNA of FR-γ′ were ligated into the PGL_3 Basic plasmid and the reporter luciferase activity was assayed in cell lysates from transiently transfected NIH3T3 cells. From those results, putative positive and negative regulatory regions in the 5′ flanking sequences were noted, and a TATA-less proximal promoter was located between –206 and –22 nt. Gel mobility shift and supershift analyses as well as mutagenesis experiments revealed that Sp1 and ets binding elements in the proximal promoter region confer transcriptional activity. From partial sequencing of genomic DNA, genomic Southern blots, RACE analysis and RNase protection assays, it appears that hFR-γ shares the gene organization of hFR-γ′. The results of the analysis of genomic DNA in spleen tissues from several individuals, were consistent with the interpretation that hFR-γ and hFR-γ′ are encoded by a polymorphic gene.

INTRODUCTION

The mammalian folate receptor (FR) occurs as a family of homologous glycopolypeptides which bind folate compounds and antifolates with high affinity (1). The receptor binds folic acid (K_D < 1 nM) with a 1:1 stoichiometry. The cDNAs for isoforms of FR from human (hFR-α, hFR-β and hFR-γ/γ′) and murine (mFR-α, mFR-β) sources have been isolated (2–6). Although the FR isoforms show >70% identity in their deduced amino acid sequences, they display significant differences in their relative affinities and stereospecificities for reduced folate compounds and antifolate drugs (7,8). hFR-α and hFR-β are attached to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor (2,9–11) and internalize bound folate/antifolate and folate conjugates by an endocytic mechanism (12–14). hFR-γ is a soluble folate binding protein that is constitutively secreted due to the lack of an efficient signal for GPI modification (15). The cDNA for hFR-γ′ is identical to that of hFR-γ with the exception of a two base deletion that predicts truncation of 138 C-terminal amino acids resulting in a 81 residue polypeptide (6). The hFR isoforms are also differentially tissue-specific and differentially elevated in several malignancies (6,16–19). In general, hFR-α is expressed in certain normal epithelial cells and is vastly elevated in certain carcinomas. hFR-β is absent in most normal tissues, is moderately expressed in placenta, spleen and thymus and is elevated in certain malignancies of non-epithelial origin including myeloid leukemia. The expression of hFR-γ/γ′ is generally restricted to tissues and malignancies of hematopoietic origin including lymphoid cells (15). The FR isoforms are, therefore, regarded as promising tumor-specific targets for a number of experimental cancer therapies and also as potential prognostic and diagnostic serum markers (20–30).

The genes encoding FR have been located on chromosome 11 (q13.3–q13.5) by *in situ* hybridization (31). Recently, the organization and functional analysis of the hFR-β and hFR-α genes have been reported (32–34). Although the structures of the two genes are similar in their protein coding regions, fundamental differences exist in the organization of their 5′ untranslated regions and in the regulatory elements responsible for their transcription.

In order to understand the mechanism for the tissue specificity of the folate receptor isoforms and their regulation in malignant cells, it is necessary to first characterize the genomic structure and the regulatory elements of each hFR gene. The focus of the present study is the organization and regulation of the genes encoding hFR-γ and hFR-γ′. A gene encoding hFR-γ′ was first isolated from a human placental genomic library and characterized. The transcriptional initiation site(s) in this gene were mapped by independent methods. The proximal promoter region was located by functional analysis and possible positive and negative upstream transcriptional regulatory regions were noted. Specific regulatory elements constituting the proximal promoter were identified. Finally, the experiments were extended to the gene encoding hFR-γ to understand the genetic basis for the difference between hFR-γ and hFR-γ′.

*To whom correspondence should be addressed. Tel: +1 419 381 4131; Fax: +1 419 383 6228; Email: mratnam@gemini.mco.edu

MATERIALS AND METHODS

Restriction enzymes, T4 polynucleotide kinase, T4 DNA ligase, M-MLV reverse transcriptase and Taq DNA polymerase were purchased from Life Technologies. [α-^{32}P]UTP (3000 Ci/mmol), [γ-^{32}P]ATP (6000 Ci/mmol), [α-^{32}P]dATP (3000 Ci/mmol) and [α-^{33}P]dATP (2000 Ci/mmol) were purchased from E.I. Dupont-New England Nuclear. Tissue explants from normal spleen, snap frozen in liquid nitrogen, were purchased from the Cooperative Human Tissue Network (Columbus, OH).

Screening of the genomic library and isolation of phage DNA

A human genomic library from placenta in the λFIX II vector was purchased from Stratagene (La Jolla, CA). Individual plaques (~5×10^6) were screened by probing with the cDNA for hFR-γ. The identification of the hybridizing clones and subsequent purification of phage DNA were carried out according to standard procedures (35).

DNA sequencing

Purified phage DNA (~25 fmol), purified (using glassmilk; Bio101 Inc.) PCR product (~100 fmol), or plasmid DNA (~50–100 fmol) was sequenced using a modification of the standard dideoxynucleotide chain termination method with the AmpliCycle Sequencing Kit (Perkin Elmer). Both the DNA strands of the isolated genomic DNA clone, except for the major portion of intron 2, were sequenced. The nucleotide sequence of each subcloned DNA fragment was confirmed prior to transient transfection.

Southern blots

Genomic DNA (30 µg) was completely digested with *Dde*I and separated by electrophoresis on an 8% polyacrylamide gel. The DNA was then electrophoretically transferred to a Nylon membrane at 4°C for 30 min at 10 V followed by 2 h at 40 V. Subsequent treatments of the membrane for Southern blot analysis were as described (35). A 30mer oligonucleotide probe (GTAT-GAGTGCTGTTCCCACAAACATTAACC) corresponding to the third intron was end-labeled with ^{32}P and the hybridization was carried out at 65°C overnight.

Plasmid cloning

A selected genomic clone was digested with the restriction enzymes, *Spe*I and *Sac*I; the resulting 4033 bp DNA containing the 5′ flanking region was purified from an agarose electrophoretic gel according to the procedure described in the GeneClean Kit (Bio101, Inc.), and then inserted in the pBSK vector (Stratagene) digested with *Sst*I and *Spe*I. This subclone served as the template for producing DNA fragments for the promoter analysis which were obtained either by digestion with the appropriate restriction enzymes or by PCR amplification. The DNA fragments were inserted into the *Sma*I-digested and phosphatase-treated PGL_3 Basic vector (Promega).

Isolation of genomic DNA and total RNA

Genomic DNA was purified from ~0.5 g pulverized tissue by standard procedures (35). Total RNA from 0.5–1.0 g of tissue was isolated by the guanidinium thiocyanate/phenol/chloroform single-step extraction method (36), using an RNA Isolation Kit (Stratagene). The final RNA pellet was washed with 70% ethanol, dried, and resuspended in diethylpyrocarbonate (DEPC) treated water. The RNA concentration was determined by measuring the absorbance at 260 nm. The integrity of the RNA was ensured by inspection of the ethidium bromide stained rRNA bands upon agarose gel electrophoresis.

Reverse transcription and PCR analysis

The 10 µl reverse transcription reaction contained 1.0 µg total RNA, 0.05 M KCl, 5 mM $MgCl_2$, 1 mM each of dNTPs (Gibco-BRL), 1 U RNasin RNase inhibitor (Promega), 5×10^{-4} OD units of random hexamer primers (5×10^{-4} OD U/µl; United States Biochemical), and 5 U MMLV-reverse transcriptase (5 U/µl; Gibco-BRL) in 0.01 M Tris–HCl buffer, pH 8.3. The reaction mixture was first incubated at room temperature for 10 min to allow the random hexamer primers to anneal. The temperature was raised to 42°C and held for 15 min. The reaction was stopped by heating at 99°C for 6.5 min.

Following reverse transcription, the entire product was combined with additional buffer (0.05 M KCl, and 2 mM $MgCl_2$ in 0.01 M Tris–HCl, pH 8.3), 15 µM of each primer (upstream primer, GGACATGGCCTGGCAGATGATGC; downstream primer, GCCCCAGCATTCATGGCC) and 1.25 U Taq polymerase (Perkin Elmer) in a 50 µl reaction volume. The reaction mix was heated for 2 min at 95°C. The PCR cycle comprised, in sequence, 1 min at 95°C, 1 min at 60°C and 1 min at 72°C. After 25–35 cycles, the reaction was held at 72°C for 6.5 min.

For PCR amplification of genomic DNA, 2.0 µg of the DNA template was combined with Taq buffer (0.05 M KCl, 2 mM $MgCl_2$ in 0.01 M Tris–HCl, pH 8.3), 0.2 mM each of dNTPs (Gibco-BRL), 15 µM of each primer and 1.25 U Taq polymerase (Perkin Elmer) in a 50 µl reaction volume. The PCR cycles were carried out as described above for RT-PCR using Taq polymerase. The primer sequences used in experiments to distinguish hFR-γ and hFR-γ′ were: upstream primer, CACGGCCAGCACCAGCCAGG-AGCTG; downstream primer, GTGGGAACAGCACTCATACC.

Mapping of the transcription initiation sites by RACE and RNase protection assay

For the RACE analysis, a 26mer primer specific for the FR-γ/γ′ cDNA (CAGGAATCAATAATCCCACGAGACGG) was used for the first strand cDNA synthesis. The reaction was carried out at 42°C in a final volume of 25 µl in the presence of 1.0 µg of the total RNA from normal spleen tissue samples, 8 U SuperScript II RT (Life Technologies), 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 100 nM of the above primer and 400 µM dNTPs. After RNase H digestion and first strand cDNA purification, the first strand cDNA was tailed with polyC using terminal deoxynucleic acid transferase (Life Technologies). The dC-tailed cDNA was amplified by PCR using a nested primer and an anchor primer from Life Technologies Company. The PCR product was purified and digested with *Spe*I and *Pst*I and then inserted into the pBSK vector (Stratagene) for DNA sequence analysis.

For the RNase protection assay, antisense RNA fragments with the 5′ end at –206 nt and the 3′ end either at –13 nt in the first exon or at the *Bgl*II site in the first intron were obtained by *in vitro* transcription utilizing the T7 RNA polymerase promoter in the pBSK plasmid. The RPA II kit from Ambion was used for the

RNase protection assay. Forty μg of total RNA from normal spleen tissue and 40 μg of tRNA for the negative control were incubated with the labeled antisense probe (5.0×10^5 c.p.m.) at 42°C overnight. The actin RNA antisense transcript provided by Ambion was used as the positive control. The protected fragments were separated by electrophoresis on a 15% urea–polyacrylamide gel followed by autoradiography.

Transient transfection and luciferase assay

NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum in a 35 mm dish at 37ºC in 5% CO_2. At 50–70% confluence, the cells were transfected with 2 μg of each promoter–luciferase construct and 1 μg of pSV-β-gal (Promega), which served as the internal control, using lipofectamine (Life Technologies) according to the vendor's instructions. Forty-eight hours after transfection, the cells were harvested in the reporter lysis buffer provided in the luciferase assay system (Promega) and centrifuged at 14 000 *g* for 1 min at room temperature. The supernatant was assayed for luciferase and β-galactosidase activities and for the total protein concentration. The luciferase activity was determined by a chemiluminescent assay using the reagents from the luciferase assay system (Promega) in a luminometer (Lumat LB9501, Berthold) following the protocol provided by the vendor. β-Galactosidase activity was measured colorimetrically using the system purchased from Promega. The luciferase activity was corrected for protein measured by the Bradford (Bio-Rad) assay and normalized to β-galactosidase activity to correct for differences in the transfection efficiency. All of the assays were performed in duplicate. The final results are the mean of at least three independent experiments.

Gel-mobility shift assay

Nuclear extracts from NIH3T3 cells cultured in DMEM were prepared using standard methodology (37). All of the procedures were carried out at 4°C. The dialyzed nuclear extract was stored in 100 μl aliquots at –70°C. The protein concentrations in the extracts were ~9 μg/μl as determined by the Bradford assay (BioRad).

Two sense oligonucleotide sequences, CAAGAGGGGAAGTCCACAAG (–116 to –97 nt) and TCCACAAGGGGCGGTGGCTCC (–104 to –85 nt), together with their respective complementary antisense oligonucleotides were prepared for the gel-mobility shift assays; the sequences (underlined) GGAA and GGGCGG are the classical consensus binding sites of the ets and Sp1 transcription activators, respectively. Two other oligomers, CAAGAGGGCTAGTCCACAAG and TCCACAAGTTCGGTGGCTCC, with mutations (underlined) in the essential dinucleotides in the sequences for the binding of ets and Sp1 transcription activators, respectively, were used for competition studies in the gel-mobility shift assay to determine the specificity of the binding of ets or Sp1 transcription activators. Equimolar quantities of complementary oligonucleotides were denatured in TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0) by heating at 100°C for 5 min and annealed by cooling to room temperature. Nuclear extract (4.5 or 9.0 μg) or recombinant Sp1 (0.5 fpu) (Promega) was incubated with the ^{32}P end-labeled probes (10 000 c.p.m.) at room temperature for 30 min in 20 μl of binding solution [25 mM HEPES buffer, pH 8.0, containing 50 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM DTT, 2 μg of poly(di-dC)-poly(dI-dC) and 10% glycerol] with or without 50 or 100 ng of cold competitor. In order to immunologically identify the protein component of the protein–DNA complexes, nuclear extracts were first incubated with the probes for 15 min at room temperature followed by the addition of 2 μg of anti-human Sp1 antibody (Santa Cruz Biotechnology Corp.) or 2 μg of normal rabbit serum (negative control) and the incubation continued at room temperature for a further 15 min. The reaction mixture was then electrophoresed on a 4% polyacrylamide gel and subjected to autoradiography.

Site-directed mutagenesis of the proximal promoter PGL_3 constructs

A synthetic sense oligonucleotide, 5′-CAAGAGGGCTAGTCCACAAGAATTCTGGCTCCC-3′, in which the putative ets and Sp1 transcription activator binding sequences were substituted at the positions underlined, was used to generate the double mutation in the proximal promoter (–206 to –22 nt)–PGL_3 construct by PCR using the mutagenic oligonucleotide in combination with upstream and downstream primers as described (11). The proximal promoter–PGL_3 constructs with only one of the two mutations were created by further mutation using the double mutant construct as the template and an oligonucleotide that restored either the Sp1 or the ets element. The PCR products were then digested at *Kpn*I and *Hin*dIII restriction sites present in the upstream and downstream primers and subcloned into the PGL_3 Basic vector. In one construct, the sequence –81 to –60 nt was substituted with a divergent sequence by similar PCR methods. The new sequence substituted at –81 to –60 nt is 5′-AGCGCTACTAGTCCTCTTTGCA-3′ and has little homology with the original sequence (5′-CAAGGTCACAGAGCAAGCTGGT-3′).

RESULTS

Isolation of genomic DNA clones encoding folate receptor type γ

A total of seven clones in the human placental genomic library hybridized to the hFR-γ cDNA probe. The isolated phage DNA from each clone was directly sequenced partially and determined to correspond to hFR-γ. The phage DNA was digested with *Sac*I in order to determine the size of the insert. A clone containing a DNA insert longer than 20 kb was chosen for further sequencing using primers corresponding to the cDNA sequence. About 4 kb of DNA upstream of the 5′ end of the cDNA sequence as well as a major portion of the downstream region including the protein coding regions were sequenced.

Nucleotide sequence and organization of the gene for hFR-γ

The previously published cDNA sequence for hFR-γ was contained within the isolated genomic clone. Identification of consensus intron/exon boundaries and alignment with the coding region of the cDNA (Fig. 1) initially revealed four exons (174, 189, 136 and 304 bp) interrupted by three introns (~1800, 197 and 104 bp) spanning at least 3 kb. A two base pair (TA) deletion in the second exon indicated that the gene encoded hFR-γ (Fig. 1). Sequence analysis of the region –630 to –13 nt, using the MacVector 4.5 program (IBI), revealed several putative regulatory elements. Among these a TATA box (–593 to –588 nt), an ets element (–109 to –106 nt) and a GC box (–97 to –92 nt) were noted (Fig. 1).

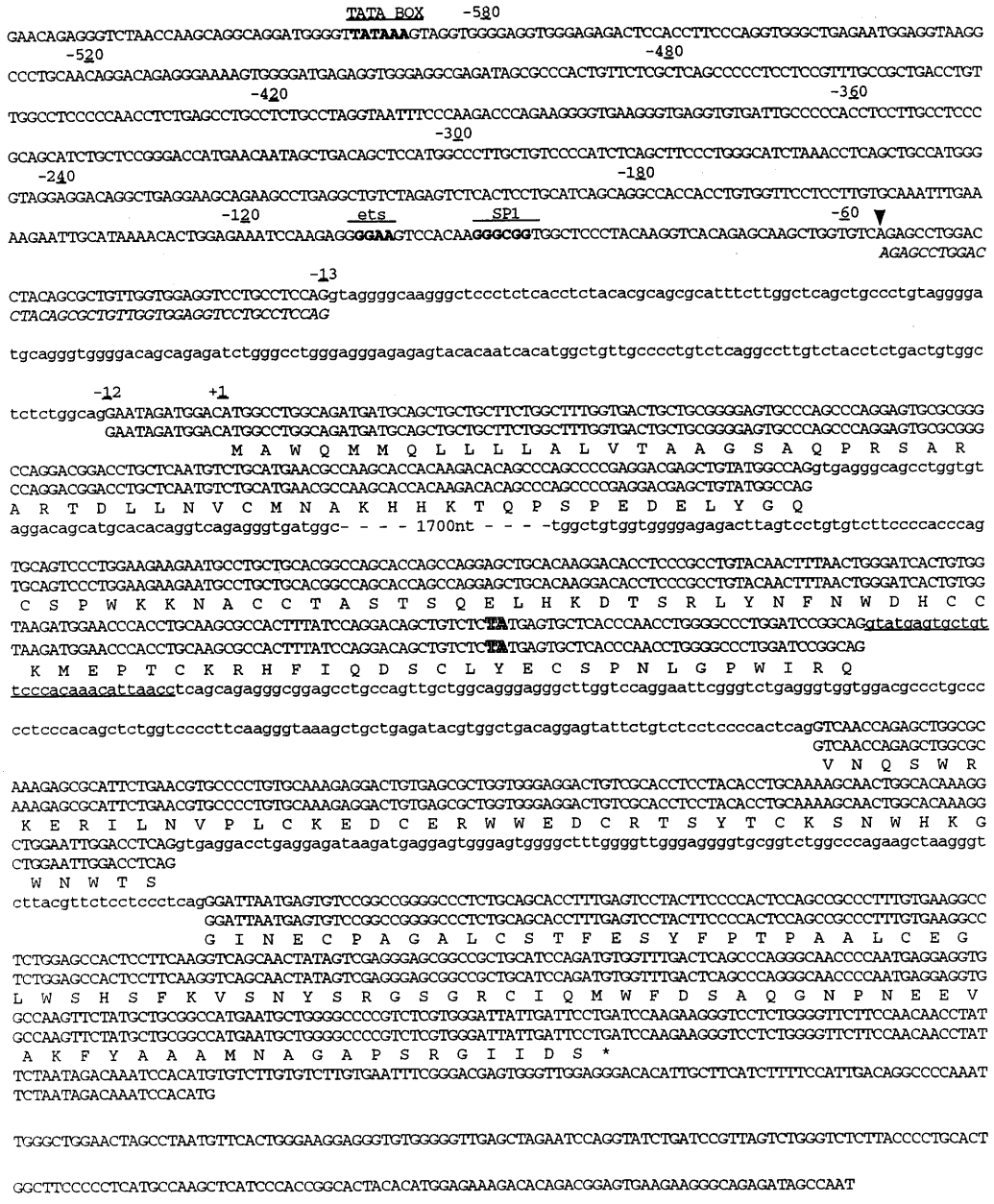

Figure 1. The nucleotide sequence of the hFR-γ/γ′ gene aligned with its cDNA and the deduced amino acid sequences of the coding regions. The genomic DNA sequence is indicated in uppercase (exons and the 5′ flanking region) or lower case (introns) letters. The cDNA sequence (6) is aligned with the genomic DNA sequence (uppercase letters) and the first exon analyzed in this study by RACE is indicated (italics). The deduced amino acid sequence is indicated in the single letter code. The numbering of the genomic nucleotide sequence begins at the first base (A) of the methionine codon for translational start and is negative in the 5′ direction. Sequences constituting a putative TATA box, GC box and ets elements are highlighted and overlined. The underlined sequence in the third intron is the 30mer probe used in the genomic Southern blot in Figure 6. The shadowed letters indicate the nucleotides (TA) present in the cDNA for hFR-γ but not hFR-γ′. The arrowhead indicates the major transcription initiation site.

Mapping the transcription initiation site(s)

To identify the 5′ end(s) of the full length cDNA, initially, the RACE strategy was used. Total RNA from normal spleen tissue, previously determined by RT-PCR analysis to express hFR-γ′ alone, was reverse transcribed using an oligonucleotide primer corresponding to the cDNA sequence encoding a C-terminal segment of hFR-γ′. The cDNA strand was subsequently tailed with polyC and amplified by PCR using a nested downstream primer and an anchor primer. After digestion with the restriction enzymes *Spe*I (contained in the anchor primer) and *Pst*I (in the coding region of the cDNA), the C-tailed PCR products were subcloned into the pBSK plasmid and sequenced. The sequence analysis of multiple cDNA clones revealed heterogenous 5′ ends, all of which occurred between –75 and –52 nt (e.g., Fig. 2A). The 5′ UTR of those clones all contained an additional exon when compared to the published cDNA sequence. The heterogeneity in the 5′ ends of the cDNA sequences suggested the occurrence of multiple transcription initiation sites.

Table 1. Exon–intron splice junctions of the FRγ/γ′ gene

	5′-Splice donor	Intron size (bp)	3′-Splice acceptor	Intron type
Intron 1	AGgtaggggcaagg	138	gctctctggcagGA	
Intron 2	AGgtgagggcagcc	>1800	ttccccacccagTG	0
Intron 3	AGgtatgagtctgttc	197	ctccccactcagGT	0
Intron 4	AGgtgaggacctga	104	ctcctccctcagGG	1
Consensus sequence	AGgtaagt		YYYYYYncagGN	
Frequency (%)	100 100 100 100 33 67 100 0		100 100 100 100 75 75 100 100 100 75	

While multiple putative transcription initiation sites were observed by RACE, the method does not allow determination of their relative frequencies. To identify the major population among the polymorphic mRNAs, the complementary RNase protection assay was employed using an antisense RNA probe beginning at –206 nt and reaching the first intron, i.e., between *Xba*I and *Bgl*II restriction sites (Fig. 3A). The RNase protection assay revealed a major transcription initiation site for hFR-γ′ at –56 nt, corresponding to one of the RACE products (Fig. 2B). An identical result was obtained using another antisense RNA probe (–206 to –13 nt) which does not contain any portion of the first intron (results not shown).

From the preceding results, it is clear that the complete hFR-γ′ gene contains five exons and four introns whose exon/intron splice junctions are summarized in Table 1. The exon/intron junctions are in good agreement with the consensus sequences at both the donor and acceptor splice sites. Introns 2 and 3 are of type 0, whereas intron 4 is of type 1, with the intron splice site after the first G of the glycine codon.

Identification of putative transcriptional regulatory regions

To localize the *cis*-elements responsible for regulating transcription of the hFR-γ′ gene, a fragment of ~3.5 kb from the 5′ region of the gene, containing the major portion of the 5′ UTR exon, from –3530 to –22 nt (Fig. 3A), was inserted in the PGL_3 Basic vector in which transcriptional activity can be monitored by measuring the expression of a luciferase reporter gene. A series of subclones in the same vector was generated by systematic deletion of the DNA insert as indicated in Figure 3B. Measurement of luciferase activity in the transfected NIH3T3 cell lysates (Fig. 3C) revealed that compared with the fragment from –3530 to –206 nt, the relative reporter activities of the fragments from –3530 to –22 nt and from –206 to –22 nt were ~8 and 29 times higher, respectively.

While a proximal promoter obviously resides within the –206 to –22 nt region, the deletion analysis suggested that the region upstream of the proximal promoter may be dissected into at least three possible regulatory regions, from –3530 to –1350 nt, from –1349 to –1105 nt and from –1104 to –207 nt (Fig. 3B and C). Thus, the promoter activity of the fragment –1104 to –22 nt was relatively low, indicating a negative regulatory effect of the sequence between –104 and –207 nt on the proximal promoter. However, in this construct, inclusion of the sequence from –1349 to –1105 nt (i.e., in fragment –1349 to –22 nt) restored the promoter activity to a level comparable to that of the proximal promoter suggesting the presence of one or more positive regulatory elements in the region –1349 to –1105 nt. Further, the lower promoter activity of the fragment –3530 to –22 nt may be ascribed to a negative regulatory effect of the sequence from –3530 to –1350 nt. The fragment –1349 to –1105 nt by itself displayed a relatively weak promoter activity that was inhibited by both upstream (–3530 to –1350 nt) and downstream (–1104 to –207 nt) sequences. Taken together, the above results demonstrate a strong proximal promoter and, in addition, an undetermined

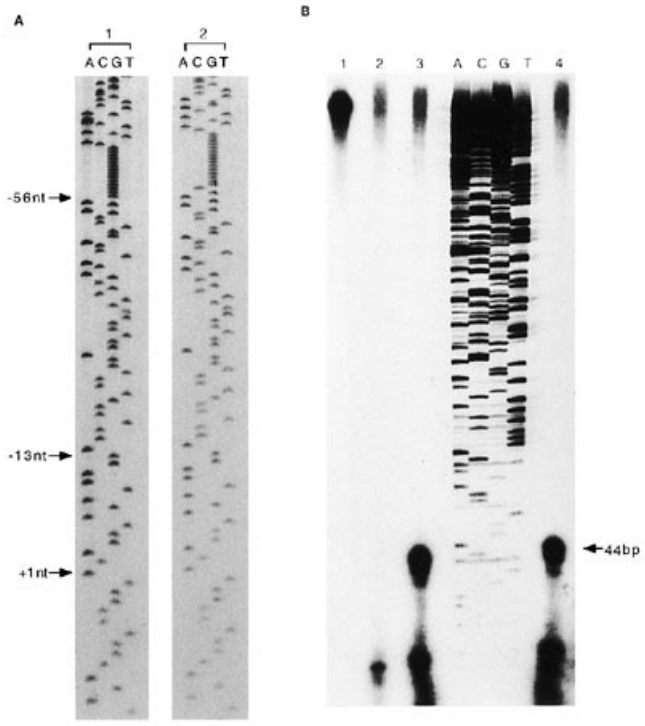

Figure 2. Mapping the transcription initiation site(s) by RACE and RNase protection assay. (**A**) RACE analysis: DNA sequence data for a subcloned RACE product from a normal spleen tissue sample expressing the mRNA for hFR-γ′ but not hFR-γ (panel 1) or a normal spleen tissue sample expressing the mRNA for hFR-γ but not hFR-γ′ (panel 2). The nucleotide positions in the genomic DNA sequence are indicated by arrows; +1 nt is the translation initiation site; –13 nt is the last nucleotide of the first exon; –56 nt is the transcription initiation site. (**B**) RNase protection assay: a 282 base ^{32}P labeled transcript was incubated with 40 µg of either total RNA from normal spleen or yeast tRNA (negative control) for the RNase protection assay carried out as described in Materials and Methods. The autoradiograph shows the results of RNase protection experiments using the labeled probe alone (lane 1) or the labeled probe plus yeast tRNA (lane 2) or total RNA from spleen tissues (lanes 3 and 4). In lane 3, the total RNA was obtained from tissue expressing the mRNA for hFR-γ′ but not hFR-γ and in lane 4 the total RNA sample contained mRNA for hFR-γ but not hFR-γ′. The DNA sequencing ladder serves as the molecular size maker. The size of the major protected fragment is indicated (arrow). A similar result was obtained using the RNA probe from –206 nt to –13 nt (result not shown).

number of negative and positive putative regulatory regions upstream of the proximal promoter. In NIH3T3 cells, the entire 5′ flanking DNA (–3530 to –207 nt) exerts a net negative regulatory effect on the proximal promoter (Fig. 3B and C). When HeLa cells and human 293 fibroblasts were transfected with the luciferase constructs in Figure 3B, the patterns of relative promoter activities of the DNA fragments were similar to that obtained in NIH3T3 cells (results not shown).

Identification of putative *cis*-elements in the proximal promoter region by gel-mobility shift assay

Two putative *cis*-elements for transcriptional regulation occur in the proximal promoter region identified above. They are, an Sp1 site (GGGCGG) (–97 to –92 nt) and an ets element containing the consenses GGAA sequence (–109 to –106 nt) (Fig. 1). The binding of nuclear proteins to putative *cis*-elements in the proximal promoter region was determined by gel-mobility shift assays. When a ^{32}P-labeled synthetic oligonucleotide, corresponding to the fragment –104 to –85 nt in the hFR-γ′ gene and containing the Sp1 element (but not the ets element) was incubated with nuclear extract from NIH3T3 cells, the gel mobility shift resulted in two major bands whose intensity was dependent upon the amount of the extract. The mobility shift of the upper band could be competed by the unlabeled probe (Fig. 4A). When the unlabeled probe was altered to disrupt the putative Sp1 element by substitution with the sequence GTTCGG, it could no longer compete in the gel mobility shift assay (Fig. 4A) indicating the specific binding of a protein(s) in the NIH3T3 nuclear extract to the Sp1 element. Incubation with antibody specific for the human Sp1 transcription activator resulted in a supershift of the upper band (Fig. 4A); the supershift was not observed with normal rabbit immunoglobulin (negative control). This result indicates the binding of the Sp1 transactivator in NIH3T3 cells to the corresponding *cis*-element in the gene encoding hFR-γ′. The lower band in Figure 4A was not competed off by the unlabeled probe in the concentration range tested, suggesting the binding of an unidentified protein(s) that occurs abundantly, i.e., at a concentration in excess of the competing probe, possibly with a low affinity.

Similar experiments were carried out with a partially overlapping synthetic oligonucleotide probe corresponding to the sequence –116 to –97 nt in the hFR-γ′ gene (Fig. 4B). This fragment contains an ets element but not the canonical Sp1 element discussed above. Incubation of the ^{32}P-labeled probe with nuclear extract from NIH3T3 cells produced gel mobility shift resulting in four major bands whose intensities were dependent on the concentration of the nuclear extract (Fig. 4B). The mobility shifts of the upper three bands were competed by the unlabeled probe (Fig. 4B). When the putative ets binding site in the competing unlabeled probe was disrupted by changing the sequence GGAA to GCTA, the lower of the three bands was unaltered in the gel mobility shift assay (Fig. 4B), indicating the binding of nuclear protein(s) to the ets motif in the NIH3T3 cells. On the other hand, the upper two bands were diminished in intensity suggesting the binding of non-ets protein(s) to the probe. In the presence of antibody to Sp1, the mobility of one of the upper two bands clearly showed antibody-specific supershift (Fig. 4B), suggesting the association of Sp1 (or Sp1-like protein); the second band, that did not show the supershift, could indicate a non-ets and non-Sp1 protein that is bound to the probe. To further investigate the nature of the protein(s) that gave a supershift with anti-Sp1 antibody, recombinant Sp1 protein was used. This protein produced a gel mobility shift of the sequence –116 to –97 nt containting CT mutation similar to that produced by nuclear extract (Fig. 4C); these results strongly suggest the presence of a non-canonical Sp1 binding element in this region. The lowest band in Figure 4B, which could not be competed off by excess unlabeled probe, suggests the binding to the probe of an abundant protein in the nuclear extract possibly with a low affinity similar to the lower band in Figure 4A.

The presence of additional functionally important *cis*-elements within the proximal promoter (–206 to –22 nt) was examined by gel mobility shift assay. The sequence –206 to –112 nt (represented by two overlapping 50mer probes) did not give a distinct gel mobility shift. The sequence –84 to –22 nt showed the binding of proteins by gel mobility shift (data not shown); however, deletion of the sequence from –51 to –22 nt or substitution of the sequence from –81 to –60 nt did not have a major effect on the promoter activity (see below).

Functional analysis of the proximal promoter by mutagenesis and deletion

The Sp1 and ets elements were mutated both individually and in combination in order to test their contribution to the proximal promoter activity in the hFR-γ′ gene. Accordingly, the plasmid PGL_3 hFR-γ′ (–206, –22) shown in Figure 5, as well as the constructs, containing disruptive mutations in the Sp1 element and/or in the ets element (described in Materials and Methods) were transfected into NIH3T3 cells. Measurements of the reporter luciferase activity indicated that both the Sp1 and the ets elements are functional in contributing to promoter activity (Fig. 5). While mutation at both the sites virtually abrogated the promoter activity, mutations at the individual sites produced ~65% (ets) and 83% (Sp1) reduction in promoter activity (Fig. 5). The results show a cooperative effect of the Sp1 and ets elements in contributing to the proximal promoter activity.

Since gel mobility shift analysis suggested the binding of nuclear proteins to the sequence –84 to –22 nt (results not shown), it was of interest to mutate this region in order to determine the possible functional significance of such interactions in the proximal promoter activity. Deletion of the sequence –51 to –22 nt or substitution of the sequence –81 to –62 nt caused only a small reduction in the promoter activity (Fig. 5). Deletion of the region –81 to –22 nt abolished the promoter activity (Fig. 5) presumably due to the elimination of the major transcription initiation site (–56 nt) mapped in this study. Possible functional significance of sequences in the proximal promoter that did not appear to bind nuclear proteins were not investigated in this study.

Relationship between hFR-γ and hFR-γ′

To test the possibility that separate genes encode hFR-γ and hFR-γ′, samples of spleen tissue from several normal individuals were examined for the presence of each gene. The putative genes were identified by PCR amplification of a portion of the third exon which includes the TA deletion in hFR-γ′ followed by sequence analysis of the amplified products. As a complementary approach, some of the genomic DNA samples were also analyzed by Southern blot after digesting the DNA with *Dde*I. The latter method takes advantage of the fact that the TA deletion in the hFR-γ′ gene results in the formation of an additional *Dde*I restriction site that would be absent in a putative hFR-γ gene. It

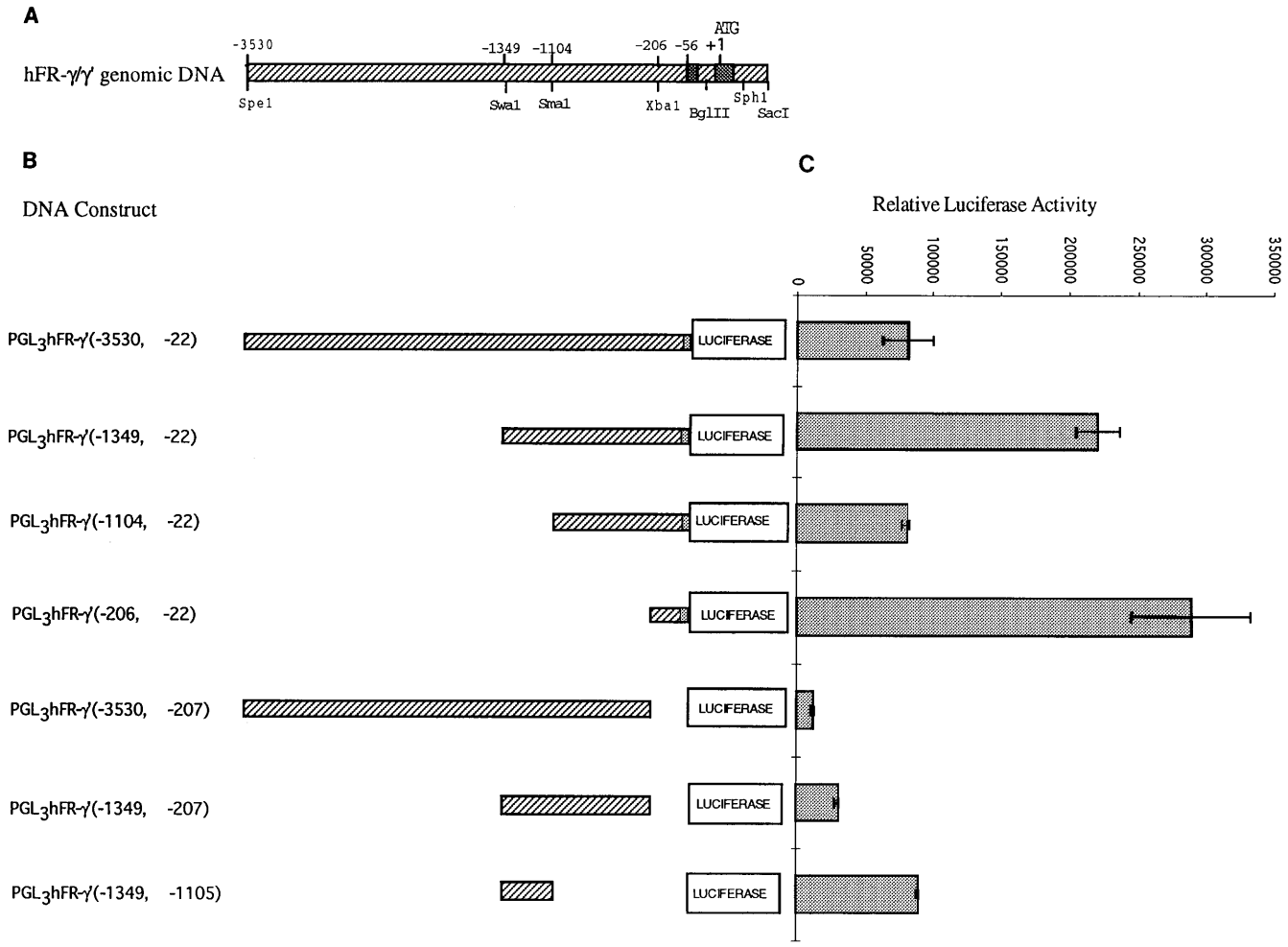

Figure 3. Functional analysis of the 5′ flanking region of the hFR-γ/γ′ gene. (**A**) Diagram of the 5′ flanking region of the hFR-γ/γ′ gene. The map coordinates are relative to the translation start site at +1 as indicated. The relative locations of restriction sites and exons (shaded areas) are indicated. (**B**) Systematically deleted 5′ flanking DNA fragments are schematically represented by the bars adjacent to the promoterless luciferase reporter gene (PGL3 Basic), depicted by open boxes, and are aligned with the schematic in (A). (**C**) Analysis of the promoter activity of the PGL3 constructs. Each construct together with the pSV40-β gal plasmid were cotransfected into NIH3T3 cells as described in Materials and Methods. Luciferase and β-galactosidase activities and the protein concentrations of the cell lysates were measured as described in Materials and Methods. The results are normalized in relative light units for the same protein concentration and transcription efficiency. The bar graph shows the mean of three independent duplicate transfections.

would be predicted from the sequence of the FRγ′ gene that the *Dde*I digestion should result in two fragments (176 and 66 bp) flanking this additional *Dde*I site, while in the absence of the TA deletion, the two DNA segments should remain associated as a single 244 bp fragment. Of 10 normal spleen samples tested by PCR, six yielded product corresponding to the hFR-γ′ gene alone and one corresponding to the putative hFR-γ gene alone while the remaining samples yielded product corresponding to both hFR-γ and hFR-γ′ (Table 2). Southern blot analysis of five representative samples tested (Fig. 6) confirmed the results of the PCR analysis.

The spleen sample that was homozygous for the putative hFR-γ gene was used to further characterize the gene encoding hFR-γ. Both RACE analysis and RNase protection assay (Fig. 2) revealed that the transcription initiation site for the putative hFR-γ gene corresponds to that of the hFR-γ′ gene. Further, except for the TA deletion, the sequences of the exons including the 5′ UTR, the sequences flanking the splice junctions as well as the sizes of the first, third and fourth introns of the putative hFR-γ gene were identical to those of the hFR-γ′ gene by PCR analysis (data not shown). Taken together, the preceding results strongly suggest that hFR-γ and hFR-γ′ are allelic genes.

Table 2. Genomic DNA analysis of spleen tissue by PCR and sequencing

Number of samples	Genotype of DNA
1	hFR-γ
6	hFR-γ′
3	hFR-γ + hFR-γ′

DISCUSSION

The elucidation of the gene structure of hFR-γ′ reported here, together with corresponding previously reported data for hFR-α (33,34) and hFR-β (32), is an essential step toward the understanding of the transcriptional mechanisms underlying the

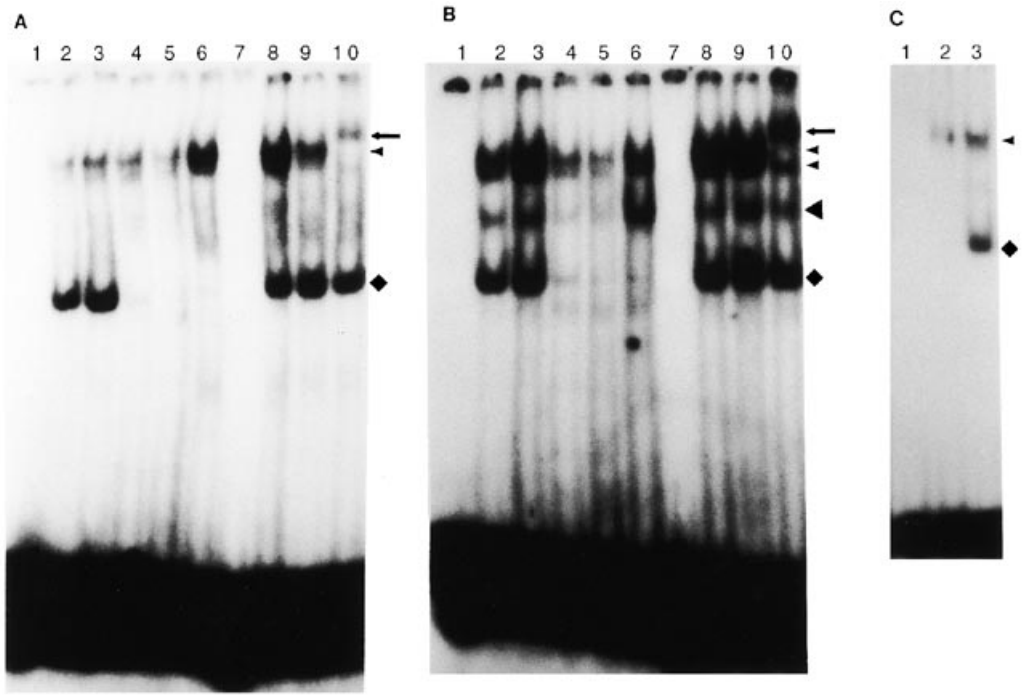

Figure 4. Gel-mobility shift assays and supershift assays to identify the binding of nuclear proteins to oligonucleotides corresponding to the proximal promoter sequence in the hFR-γ′ gene. The assays were performed using: (**A**) ^{32}P labeled probe from –104 to –85 nt; (**B**) ^{32}P labeled probe from –116 to –97 nt. Lanes 1 and 7, probe alone; lane 2, probe plus 4.5 μg of nuclear extract; lanes 3 and 8, probe plus 9.0 μg of nuclear extract; lane 4, same as lane 3 plus 50 ng of unlabeled oligonucleotide; lane 5, same as lane 3 plus 100 ng of unlabeled oligonucleotide; lane 6, same as lane 3 plus 100 ng of unlabeled mutated oligonucleotide; lane 9, same as lane 3 plus 2.0 μg of normal rabbit serum; lane 10, same as lane 3 plus 2.0 μg of polyclonal rabbit anti-human Sp1 antibody. (**C**) ^{32}P-labeled probe from –116 to –97 nt containing CT mutation. Lane 1, probe alone; lane 2, probe plus 0.5 p.f.u. recombinant human Sp1 protein (Promega); lane 3, probe plus 4.5 μg of nuclear extract. The supershifted bands are denoted by arrows. The positions of the other bands are marked by arrowheads, triangles and diamonds.

differential tissue specific regulation of the hFR isoforms and their differential elevation in various malignancies. From a comparison of cDNA sequences (2,4,6), hFR-β and -γ display a closer similarity than either protein does with hFR-α suggesting that the gene encoding hFR-α diverged earlier in evolution than those encoding hFRs-β and -γ. Consistent with this view, the hFR-β and hFR-γ genes have a similar intron–exon organization, consisting of five exons and four introns with the protein coding sequence beginning in exon 2; on the other hand, the hFR-α gene has seven exons and six introns with multiple transcripts resulting from the use of alternative promoters as well as alternative splicing involving exons 1–4 (33,34) (Fig. 7).

The present study also undertook to investigate the genetic basis for the expression of hFR-γ versus hFR-γ′. The only difference in the cDNAs for the two proteins is the presence (hFR-γ) or deletion (hFR-γ′) of two adjacent bases in the coding region. The isolated gene encoding hFR-γ′ was virtually indistinguishable from a putative gene encoding hFR-γ characterized in the genomic DNA of a sample of spleen tissue that expressed hFR-γ but not hFR-γ′. The major transcriptional initiation site identified in the hFR-γ′ gene by the complementary approaches of RACE and RNase protection assays corresponded to that of the putative hFR-γ gene (Fig. 2). The exon sequences of the putative hFR-γ gene were identical to those of the hFR-γ′ gene except that exon 3 contained the divergence observed in its cDNA sequence. Further, the sizes of the introns tested by PCR in the putative hFR-γ gene as well as the intron sequences flanking the splice junctions (Table 1) were identical. These results strongly suggest that hFR-γ and hFR-γ′ are not encoded by distinct members of the hFR gene family or generated from a single transcript by post-transcriptional mechanisms, but rather, that the two proteins result from gene polymorphism. The allelic nature of the gene encoding hFR-γ or hFR-γ′ is further evidenced by the analysis of the genomic DNA from spleen tissues obtained from several individuals which showed the presence of genomic DNA encoding either protein alone or both proteins (Table 2).

An (A+T) rich sequence, i.e., a TATA box element, occurs in the hFR-γ/γ′ gene 532 bp upstream of transcription initiation site (Fig. 1). Functional TATA elements generally occur 30–70 bp upstream of the transcriptional start (38). The distance of the TATA element in the hFR-γ/γ′ gene from the transcriptional start, the ability of the proximal promoter region to function in the absence of a TATA element (Fig. 3) and the absence of significant promoter activity in the segment –1349 to –207 nt, which contains the TATA element (Fig. 3), argue against the involvement of a TATA element in regulating the expression of hFR-γ/γ′. From the results of this study and the previous reports on hFR-α and hFR-β genes (32–34), it appears that TATA-less promoters are characteristic of the hFR gene family.

The basal promoter activity in the hFR-γ′ gene was assigned in this study to a proximal promoter region between –206 and –22 nt (Fig. 3). From a computer assisted search, the only candidate regulatory elements identified in this region were a single consensus sequence for the binding of Sp1 (–97 to –92 nt) and a

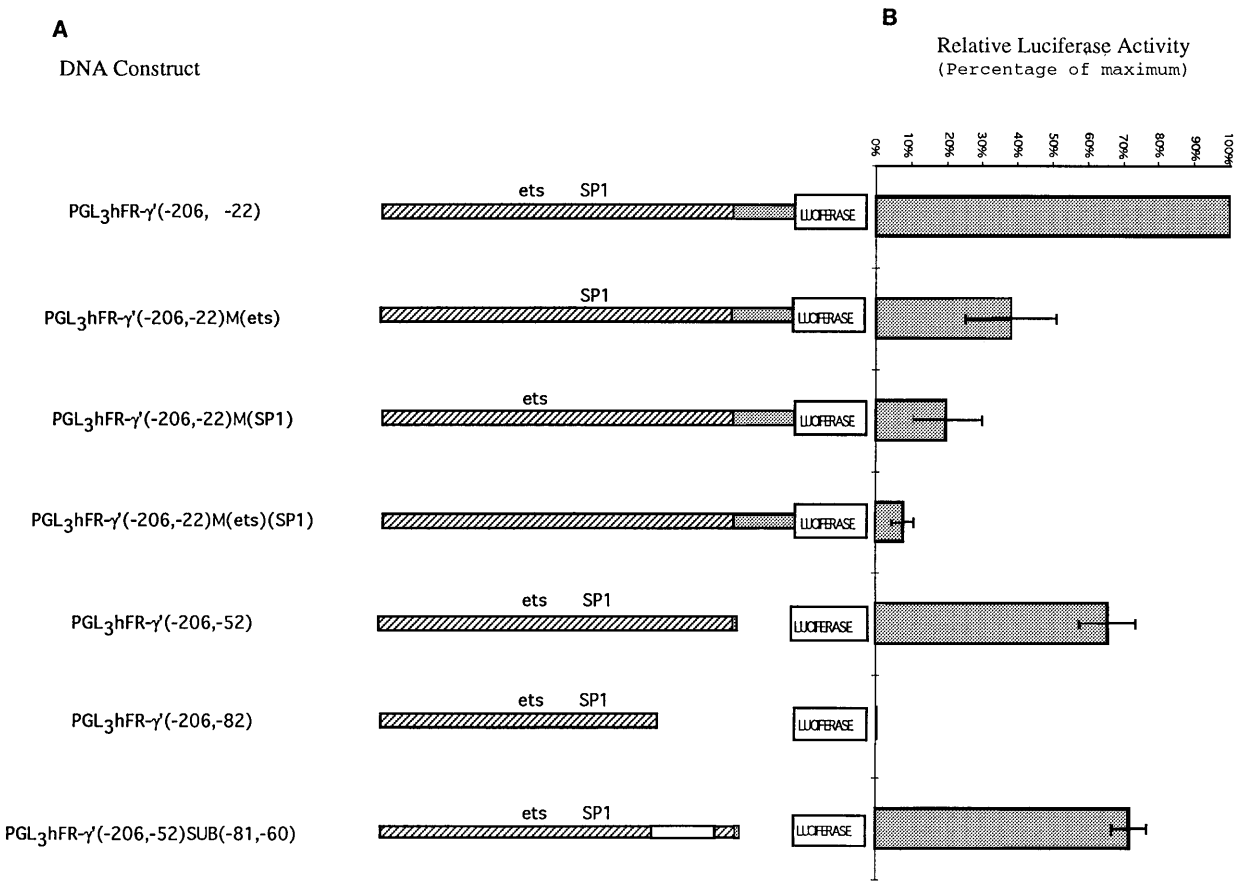

Figure 5. Functional analysis of the proximal promoter by mutagenesis and deletion. (**A**) The wild-type proximal promoter DNA segment and the segment containing either single or double mutations are schematically represented by the bars adjacent to the promoterless luciferase reporter gene (PGL3 Basic), depicted by open boxes. The open bar represents the substituted sequence (see Materials and Methods). The arrowhead indicates the major transcription initiation site mapped in this study. The wild-type Sp1 or ets elements are labeled above the bars. (**B**) Analysis of the promoter activity of the PGL3 constructs. Each construct together with the pSV-β-gal plasmid were cotransfected into NIH3T3 cells as described in Materials and Methods. Luciferase and β-galactosidase activities and the protein concentrations of the cell lysates were measured as described in Materials and Methods. The results are normalized in relative light units for the same protein concentration and transcription efficiency. The bar graph shows the mean of three independent duplicate transfections.

single ets element (–109 to –106 nt) (Fig. 1). Mutagenesis and functional analysis of the proximal promoter transfected into NIH3T3 cells revealed that both the elements are required for optimal promoter activity (Fig. 5). Further, gel-mobility shift assays demonstrated the specific recognition of the Sp1 and ets elements by nuclear proteins in NIH3T3 cells (Fig. 4). Nuclear protein interactions with other regions in the proximal promoter fragment did not appear to have a major effect on the promoter activity as suggested by either gel mobility shift or by mutagenesis. However, the inability to observe nuclear protein binding to the other sequences in the proximal promoter does not necessarily preclude their functional importance in promoter activity and they will need to be investigated further.

Ets and other oncogene promoters typically lack TATA and CCAAT box sequences (39) and ets binding sites (EBS) occur in promoter/enhancer sequences of a variety of genes. Promoter analysis has revealed that the regulatory function of ets proteins may require digitization or interaction with other transactivators such as Sp1 (40–44) and AP1 (39). Recently, the mechanism for the synergistic effect of ets and Sp1 was proposed to involve enhancement of the affinity of Sp1 to a non-canonical Sp1 binding sequence due to the binding of ets protein(s) at an upstream site (45). The mutagenesis experiments in this study suggested cooperation between the Sp1 and ets binding sites in producing the optimal activity of the basal promoter in hFR-γ′ (Fig. 5). Curiously, the gel-mobility shift and supershift assays revealed the specific binding of a nuclear protein(s) to a sequence in the basal promoter outside of both the Sp1 and ets elements as well as the binding of Sp1 or an Sp1 like protein possibly to a second non-canonical Sp1 binding site (–113 nt -GAGGGGAAG- –106 nt) overlapping the EBS (Fig. 4). The DNA sequence specificity of the unidentified non-Sp1 and non-ets protein(s) and its possible functional significance in a transactivating complex as well as possible competition between Sp1 and ets for binding to overlapping sequences are the subject of further investigations.

Although the proximal promoter of the hFR-γ/γ′ gene is functional in NIH3T3 fibroblasts (Figs 3 and 5) as well as in human 293 fibroblast and HeLa cells (data not shown), which do not express hFR-γ/γ′, the occurrence of a functional ets element within this sequence may be significant in determining the specificity of hFR-γ/γ′ for hematopoietic cells based on the following reports. Many lymphoid specific genes contain EBS in their promoters (39) and activation of ets is observed in certain types of leukemia. Several members of the ets oncogene family

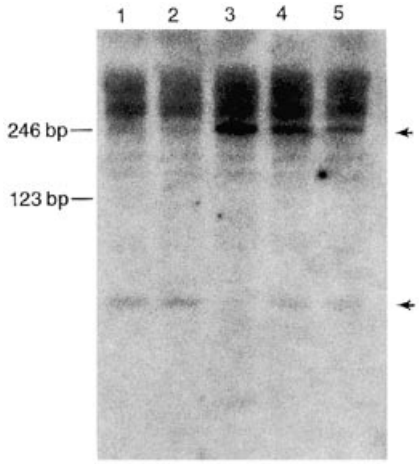

Figure 6. Genomic Southern blot to identify the genes for hFR-γ′ or hFR-γ. Human genomic DNA (30 mg) from normal spleen tissue samples from five individuals was digested with *Dde*I and electrophoresed in an 8% polyacrylamide gel as described in Materials and Methods. The experimental procedure for the Southern blot is described in Materials and Methods. The probe used is indicated in Figure 1. The genotypes of the DNA samples were previously determined (Table 2) by PCR amplification and sequence analysis as: hFR-γ′ (lanes 1 and 2); hFR-γ (lane 3) and hFR-γ plus hFR-γ′ (lanes 4 and 5). The positions of DNA size markers (the 123 bp ladder) are indicated on the left. The arrows indicate the DNA fragments discussed in the text.

are expressed in hematopoietic tissue including Ets-1, Ets-2, GABP, Fli-1 and Pu.1 (46,47). In the absence of a TATA element, it appears that regulatory proteins binding upstream may interact with transactivators associated with the basal promoter resulting in hematopoietic cell type specificity of gene expression (47). It has been proposed that interaction between proteins associated with ets and Sp1 elements and a GATA element several hundred bases upstream may be required for enhanced and cell type specific promoter activity in megakaryocytes (45). Possible additional mechanisms of ets mediated cell type specificity include the differential (activation versus repression) effects of different ets proteins (e.g., ets-1 and ets-2) upon binding to the same site as well as modification by cofactors or by phosphorylation (39).

Alternate promoters have been identified in the hFR-α gene that result in transcripts having different lengths of the 5′ UTR (34). One of the basal promoters of that gene appears to be primarily directed by a cluster of GC-rich sequences that are non-canonical Sp1 binding sites, each of which contributes to promoter activity. The second promoter has not yet been characterized, but appears to lack a functional TATA box (34). The hFR-α gene lacks ets elements in its regulatory sequences. The basal promoter in the hFR-β gene is regulated by a single non-canonical Sp1 binding sequence and tandem repeats of EBS (32). Thus, there are certain differences among the hFR isoforms in the organization of their proximal promoter regions, which may contribute at least in part to their narrow cell type specificities. A role for as yet unidentified upstream *cis*-elements in determining the tissue specificity of hFR-γ/γ′ in possible conjunction with EBS is suggested by the following observations: (i) the presence of both positive and negative regulatory regions in the ~3.2 kb DNA fragment upstream of the proximal promoter in the hFRγ/γ′ gene (Fig. 4); (ii) the net negative regulation by the sequence upstream of the proximal promoter in the non-hematopoietic cells used in this study (Fig. 4); (iii) the absence of EBS in the gene encoding the epithelial cell specific hFR-α (33,34); and (iv) the expression of hFR-β in hematopoietic tissue but with a different cell type specificity from that of hFR-γ/γ′ together with the fact that the gene encoding hFR-β also contains both ets and Sp1 elements in a TATA-less promoter.

The present study highlights both similarities and differences in the organization and regulation of the human folate receptor genes. The results warrant a detailed and comparative analysis of the 5′ flanking basal promoters together with upstream sequences of the three genes in order to understand the mechanisms underlying the narrow tissue specificities and malignancy associated modulation of this clinically important family of proteins.

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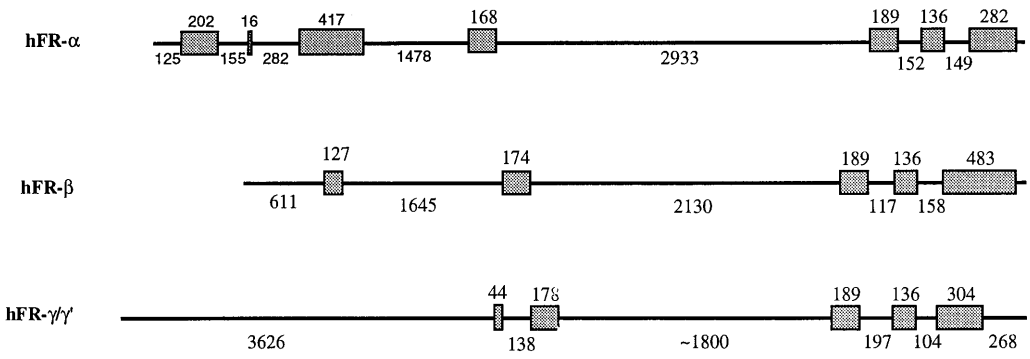

Figure 7. Schematic diagram depicting the organization of the genes encoding the human folate receptor isoforms. The boxes indicate exons. The lines indicate introns and the 5′ flanking region. The numbers indicate the size of each segment (in base pairs). The data for hFR-α and hFR-β is derived from previous publications (32–34).

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