Characterization of the Human Forkhead Gene FREAC-4

EVIDENCE FOR REGULATION BY WILMS’ TUMOR SUPPRESSOR GENE (WT-1) AND p53*

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We describe the cloning and sequence analysis of a nearly full-length cDNA as well as a corresponding 5.2-kilobase pair genomic fragment encoding FREAC-4, a member of the forkhead family of transcription factors. The cDNA is collinear with respect to the coding region of the intronless genomic clone. The conceptual translation product predicts a protein of 465 amino acids with a hyperacidic amino-terminal end, a DNA binding forkhead domain and a carboxy-terminal part that is rich in homopolymeric runs of prolines and alanines. The transcription start is identified using an RNase protection assay. A 2.7-kilobase pair genomic DNA fragment, located immediately upstream of the translation start, was fused to a luciferase reporter gene. Significant levels of luciferase activity were detected when this construct was transfected into two kidney-derived cell lines, 293 and COS-7 cells, whereas only background reporter gene expression was observed in a cell line of nonkidney origin. Cotransfections with plasmids expressing WT-1, WTAR (a mutated form of WT-1), p53, and a mutated form of p53 revealed a complex pattern of regulation with a 3-fold induction with WT-1, a 7-fold induction with mutated p53, and a 4-fold repression with wild-type p53. A 5′-promoter deletion series delimited a DNA fragment necessary for WT-1 inducibility in cotransfection experiments. This fragment is shown to contain at least one cis-element that is capable of interacting with recombinant WT-1.

The forkhead gene family of transcription factors belongs to the “winged helix” class of DNA-binding proteins (1, 2), a name derived from the x-ray crystallography data on HNF-3γ (3). When the homeotic Drosophila gene fork head (4), the original founding member of this gene family, was compared with HNF-3 (5) a stretch of sequence homology revealed a common DNA binding motif, the forkhead domain (6, 7). This motif, contained within some 100 amino acids, has since been identified in over 40 genes isolated from a wide range of organisms such as Saccharomyces cerevisiae (8, 9), Caenorhabditis elegans (10), zebrafish (11), Xenopus laevis (12), mouse (13), rat (14), and humans (15). Forkhead genes have been shown to be instrumental in embryogenesis of higher mammals, in particular during development of the nervous system (16–20) as well as directly involved in tumorigenesis (21–25) and tissue-specific gene expression in liver (26, 27), lung (14, 28), and adipose tissue (29). A mutation in the nude locus of mice and rats, disrupting the forkhead domain of the whn gene, has been linked to the abnormal hair and thymus development, causing these animals to be immune deficient (30). The majority of forkhead genes have yet not been ascribed any biological function, which is not surprising since only small fragments of sequence, typically the DNA binding domain, exist for several of these genes (13–15, 31).

We have previously published sequences corresponding to the DNA binding domains of seven forkhead genes, which we named FREAC-1 to FREAC-7 (forkhead-related activators), together with data on DNA binding properties and tissue distribution of expression (15). In this paper we focus on one of these genes, FREAC-4. Genomic and cDNA sequence of FREAC-4 is presented as well as determination of the transcription start. Two kidney-derived cell lines, both expressing FREAC-4 mRNA, were identified and used in transfection experiments. These experiments demonstrated that FREAC-4 is regulated by two tumor suppressor genes, WT-1 and p53. We also identify a cis-element in the promoter of FREAC-4 that binds recombinant WT-1.

EXPERIMENTAL PROCEDURES

Isolation and Sequencing of cDNA and Genomic Clones—Isolation of original cDNA clones for FREAC-4 has been described previously (15). A human λ DASH genomic library was screened with probes derived from FREAC-4 cDNA clones. Inserts from positive phages were subcloned and sequenced bidirectionally. In some instances, exonuclease III was used to generate a set of nested deletions that served as templates for sequence reactions (32). A Pharmacia ALF sequencer using T7 polymerase (Pharmacia Biotech Inc.) or Thermosequenase (Amersham Corp.) and fluorescent-labeled primer or fluorescent-dATP was used. Some regions were also sequenced with [α-35S]dATP and Sequenase (U.S. Biochemical Corp.).

Northern Blot and RNA Preparation—Total RNA was prepared as described by Chomczynski and Sacchi (33). Twenty micrograms of total RNA was electrophoresed in a 1% agarose-formaldehyde gel and blotted onto Hybond-C extra filters (Amersham; 34); hybridized at 44 °C in 50% formamide, 5 × SSC and washed at high stringency (0.1 × SSC, 0.1% SDS, 62 °C). Probe was made from a DNA fragment outside of the conserved DNA binding region (nucleotides 3896–4216 in Fig. 1).

RNase Mapping of Transcription Start—A genomic DNA fragment spanning from nucleotide 2122 to 2485 (Fig. 1) was subcloned into plBluescript SK+ (Strategene). T7 RNA polymerase and [α-32P]UTP were used to label a cDNA antisense probe. Various restriction enzymes were used to produce templates for T3 and T7 RNA polymerases in order to create run-off transcripts of defined lengths, to be used as size markers. Labeled antisense probe, approximately 170,000 cpm Cerkon for each reaction, was added to 50 μg of total RNA in a hybridization buffer (80% formamide, 100 mM sodium citrate, pH 6.4, 300 mM sodium acetate, pH 6.4, 1 mM EDTA) at 52 °C overnight. After digestion with RNase A and RNase T1, the protected fragment was electrophore-
Cell Culture and Transfections—All cells were obtained through the American Tissue Culture Collection (ATCC): 3T3 (mouse embryonic fibroblasts; ATCC CL-173), HeLa (human cervix cancer; ATCC CRL-7923), 293 (human embryonic kidney; ATCC CRL-1573), and COS-7 (monkey-transformed kidney; ATCC CRL-165). All cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.). 

A typical transfection contained 100 ng of luciferase reporter plasmid and 100 ng of cotransfected plasmid, expression constructs encoding various transcription factors, or an expression vector void of insert as a control. These plasmids were diluted into 560 μl of OptiMEM together with 2 μg of LipofectAMINE (Life Technologies, Inc.) and added to a subconfluent monolayer of cells cultured in a gelatin-coated (Sigma) 16-mm tissue culture well. Cell harvest and luciferase assay were performed according to Promega Corp. (Technical Bulletin 101). To compensate for differences in transfection efficiency, 10 ng of a β-galactosidase-expressing plasmid, pCMV β gal (Clontech), was added to each transfection. β-galactosidase activity was measured using a Lumi β-galactosidase assay (Clontech). Luciferase values were normalized against β-galactosidase activities. For transfections, a FREAC-4 luciferase (FREAC-4-luc) construct was used. To make this reporter gene construct, a DNA fragment corresponding to nucleotides 1–2654 (Fig. 1) was cloned into the XhoI and HindIII sites, upstream of the luciferase gene, in pGL2-basic (Promega). Exonuclease III was used to create various 5' deletions of FREAC-4-luc (32).

RESULTS

Sequence and Structure of FREAC-4—A cDNA extending from nucleotide 2521 to 4792 in Fig. 1 was isolated from two cDNA libraries as three overlapping clones. An open reading frame encoding the characteristic forkhead domain was identified. The conceptual translation product of this reading frame predicts a protein of 465 amino acids. The initiation codon was assigned to the first in-frame ATG within a Kozak consensus sequence (37). A polyadenylation signal was identified at positions 4755–4760, 32 nucleotides downstream of which a poly(A) tail is added. 

Sequenced derived from genomic clone is in lowercase and uppercase; the cDNA sequence is in uppercase. ATATAA motif (nucleotides 2243–2247), the predicted transcription start (nucleotide 2274), and a polyadenylation signal (nucleotides 4755–4760) are underlined. A poly(A) tail is added to the cDNA immediately 3' to nucleotide 4792. The reporter construct FREAC-4-luc was made by fusing nucleotide 2654 to a luciferase reporter gene; the fusion point is marked with a plus sign (+).

Gel Mobility Shift Assay—Double-stranded oligonucleotides with 5'-overhangs were used. Oligonucleotide A (nucleotides 2253–2277, Fig. 1) and oligonucleotide B (nucleotides 2345–2389, Fig. 1) were labeled with [α-32P]dCTP and [α-32P]dATP (Amersham) and Klenow fragment. For competition experiments a 50-fold molar excess of a double-stranded oligonucleotide containing a WT-1 binding site from the TGF-β1 promoter known to bind WT-1 (GGGGGCGCCCGCGCTCCCGCCGCCGTG, from Dey et al. (35)) was used. Binding reactions were carried out in a volume of 20 μl containing 10 mM Tris-HCl, pH 7.8, 70 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 10% glycerol, and 1 μg of poly(dI-dC). Reaction were conducted at room temperature for 20 min, and the samples were then resolved on a 5% polyacrylamide gel (29:1) in Tris-glycine buffer (25 mM Tris, 190 mM glycine, 1 mM EDTA) with 5% glycerol at 14°C and 15 V/cm for 90 min. Human recombinant WT-1 protein was produced as described by Morris et al. (36). In brief, a plasmid with a flanking T7 RNA polymerase promoter site harboring the full-length amino acid coding sequence of human WT-1 and a plasmid void of insert (a control) were transcribed in vitro with T7 RNA polymerase. The RNAs were translated in a reticulocyte lysate in the presence of [35S]methionine. An aliquot from the translation mixtures was analyzed on a 10% SDS-polyacrylamide gel electrophoresis, and the molecular mass of the WT-1 translation product (a single band) was estimated to be 53 kDa (not shown), in agreement with the calculated molecular mass of human WT-1. The translation mixture with the control plasmid rendered no detectable band.
HeLa cells, probed for FREAC-4 and FREAC-4 transcript has been estimated to be 2.5 kilobase pairs. A genomic fragment of 5181 nucleotides from the upstream FREAC-4 fragment (nucleotides 1–2654; Fig. 1) has been used to estimate the size of the protected fragment to 212 nucleotides. Size marker 364 is undigested cRNA antisense probe, and 145 and 210 are cRNA run-off transcripts. RNA from 293 cells protects a fragment of 212 nucleotides, whereas RNA derived from yeast, HeLa cells, and 3T3 cells does not protect any fragment. This designates nucleotide 2274 as the transcription start.

Apart from the DNA binding forkhead domain (amino acids 123–222, Fig. 1), FREAC-4 has a hyperacidic NH2-terminal domain (amino acids 1–122, Fig. 1) and a COOH-terminal domain (amino acids 223–465, Fig. 1) with several stretches of homopolymeric runs of alanines and prolines, often seen in transcription factors. The NH2-terminal domain is extraordinary in its high density of charged amino acids; 40 out of the 100 most NH2-terminal amino acids carry a charged side chain. Of the 80 most NH2-terminal amino acids, 33 are either aspartic or glutamic acid; they are grouped in two “hyperacidic clusters.” This situation is very similar to what has been described for the COOH terminus of the nuclear factor UBF. In UBF this region is essential for transactivation and is phosphorylated by casein kinase II (38). As in UBF, there are potential phosphorylation sites for casein kinase II in the corresponding region of FREAC-4 (39). Within the COOH-terminal domain of FREAC-4 we have identified a region (amino acids 241–312), by means of a computer homology search, as having similarities with the NH2-terminal region of WT-1. The main feature of this region is two homopolymeric runs of prolines separated by 45 amino acids. This region is located in the NH2-terminal part of WT-1, and deletions of these proline-rich regions partially inactivate the repressor function of WT-1 (40).

A genomic fragment of 5181 nucleotides from the FREAC-4 gene was also sequenced, and the cDNA was found to be colinear with the genomic sequence. Since the FREAC-4 transcript is estimated to be approximately 2.5 kilobase pairs (see Fig. 4) and the cDNA sequence spans a region of 2272 nucleotides, starting in the 3′-end at the first nucleotide that is not part of the poly(A) tail (Fig. 1), this gene is most likely intronless.

RNase Mapping of the Transcriptional Start Site—A cRNA antisense probe of 364 nucleotides, corresponding to nucleotides 2122–2485 (Fig. 1), was synthesized together with size markers (see “Experimental Procedures”). The radiolabeled cRNA probe was hybridized with total RNA derived from the following cell types: yeast, 3T3, HeLa, and 293 cells. In total RNA derived from 293 cells, a fragment slightly larger than a size marker of 210 nucleotides was protected. The size of the protected fragment was estimated to be 212 nucleotides (Fig. 2). Thus, the transcription start is located 212 nucleotides upstream of nucleotide 2485, i.e. at nucleotide 2274 (Fig. 1). The 5′-end of the transcript is located at nucleotide 2274 and the 3′-nucleotide at 4792. This predicts a transcript size of 2519 nucleotides, which agrees well with an estimated transcript size on Northern blots of 2.5 kilobase pairs (Fig. 3). The transcription start is located some 30 nucleotides downstream of a canonical TATAA-motif. Notably, only total RNA from the kidney-derived cell line 293 contained the FREAC-4 transcript.

A FREAC-4 Reporter Gene Construct Is Active in Two Kidney-derived Cell Lines but Not in HeLa Cells—In order to study transcriptional activation of the FREAC-4 gene we fused a genomic fragment (nucleotides 1–2654; Fig. 1) upstream of a luciferase reporter construct to make FREAC-4-luc. Using a Northern blot assay we could detect FREAC-4 mRNA in COS-7 and 293 cells, both of kidney origin, whereas a non-kidney-derived cell line such as HeLa cells did not express FREAC-4 (Fig. 3). When the FREAC-4-luc construct was transfected into COS-7 and 293 cells, we observed significant reporter gene activity. In HeLa cells no activity above that of background could be detected (Fig. 4), as was the case with a reporter gene construct lacking the FREAC-4 sequence (not shown). This demonstrates a cell type-specific promoter activity derived from the genomic upstream FREAC-4 fragment (nucleotides 1–2654; Fig. 1).

The Tumor Suppressor Genes WT-1 and p53 Have Opposite
Effects on FREAC-4-luc Reporter Gene Activity in Cotransfections—When the FREAC-4-luc construct was cotransfected with a WT-1 expression plasmid, approximately a 3-fold induction was observed (Fig. 5). A dominant negative mutation of WT-1 has been identified in a patient with Wilms’ tumor. In this case, the third of four zinc fingers in the COOH terminus of WT-1 has a deletion (41). This mutation, WTAR, can suppress the activity of wild-type WT-1 in a trans-dominant fashion (42).

When FREAC-4-luc was cotransfected with a WTAR expression plasmid, no significant difference in reporter gene activity was observed as compared with that of a cotransfection with an expression plasmid void of insert (Fig. 5). Another tumor suppressor gene, p53, has recently been implicated as a modulator of WT-1. It has been suggested that WT-1 acts as a repressor of transcription in the presence of wild-type p53 and as an activator in the absence of wild-type p53 (43). To test whether p53 is involved in the regulation of FREAC-4, we transfected a p53 expression plasmid together with FREAC-4-luc; this represses reporter gene activity approximately 4-fold (Fig. 5). In contrast, cotransfections with a mutated form of p53 (Arg175 → His) increases reporter gene activity by a factor of 7. This p53 mutation has been shown to inhibit the activity of wild-type p53 (44). Thus, the induction can be seen as the release of a “p53 break” that in the presence of wild-type p53 reduces FREAC-4 reporter gene activity. Since high levels of p53 (45) and WT-1 (46) are expressed in the kidney during embryogenesis, it is conceivable that these genes take part in the regulation of FREAC-4.

Transfection of a 5’-Deletion Series of FREAC-4-luc and Gel Shift Assay Using Recombinant WT-1 Identifies a WT-1 Binding Site within the FREAC-4 Promoter—Two derivatives of FREAC-4-luc, -527 and -152 and expression plasmids encoding mock (expression vector void of insert), WT-1 (Wilms’ tumor suppressor gene-1), and mWT-1 (a dominant negative WT-1 mutation named WTAR (42)). A luciferase reporter plasmid void of any FREAC-4 promoter sequence was used as a control (vector). Reporter gene activity is expressed as relative activity with the activity of FREAC-4-luc cotransfected with mock set to 1.0. Values are the means of at least three independent transfections ± S.D.

**Fig. 5.** Cotransfections, using COS-7 cells, with FREAC-4-luc and expression plasmids encoding mock (expression vector void of insert), WT-1 (Wilms’ tumor suppressor gene-1), mWT-1 (a dominant negative WT-1 mutation named WTAR (42)), p53, and mp53 (a mutated form of p53 with an Arg to His mutation at amino acid 175 known to suppress the activity of wild type p53 (44)). Reporter gene activity is expressed as fold induction as compared with a FREAC-4-luc cotransfected with an expression vector void of insert (mock). Values are the means of at least three independent transfections ± S.D.

Effects on FREAC-4-luc Reporter Gene Activity in Cotransfections—When the FREAC-4-luc construct was cotransfected with a WT-1 expression plasmid, approximately a 3-fold induction was observed (Fig. 5). A dominant negative mutation of WT-1 has been identified in a patient with Wilms’ tumor. In this case, the third of four zinc fingers in the COOH terminus of WT-1 has a deletion (41). This mutation, WTAR, can suppress the activity of wild-type WT-1 in a trans-dominant fashion (42). When FREAC-4-luc was cotransfected with a WTAR expression plasmid, no significant difference in reporter gene activity was observed as compared with that of a cotransfection with an expression plasmid void of insert (Fig. 5).

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Transfection of a 5’-Deletion Series of FREAC-4-luc and Gel Shift Assay Using Recombinant WT-1 Identifies a WT-1 Binding Site within the FREAC-4 Promoter—Two derivatives of FREAC-4-luc, -527-luc and -152-luc extending 527 and 152 nucleotides upstream of the transcription start, were used to study the inducibility conferred by WT-1 in cotransfection experiments. As can be seen in Fig. 6, both -527-luc and -152-luc are induced approximately 3-4-fold as compared with mock cotransfections. Since the luciferase vector void of any FREAC-4 promoter sequence is not induced by WT-1 cotransfections, we concluded that the FREAC-4 promoter sequence in -152-luc
be seen in Fig. 7. Oligonucleotide A renders a retardation band that is specific for the WT-1-containing extract. This points out the retardation product specific for the WT-1-containing extract.

DISCUSSION

We have sequenced cDNA and genomic clones corresponding to FREAC-4. A hyperacidic motif in the NH₂-terminal part and a proline-rich region in the COOH-terminal domain of FREAC-4 are, based on sequence similarities with the transcription factors UBF and WT-1, identified as possible effector sites. Computer homology searches have identified BF-2 (48), a recently cloned mouse cDNA with a high degree of sequence similarity to that of FREAC-4, as a good candidate for the mouse homolog of FREAC-4. This notion is underscored by matching patterns of expression, i.e. predominantly brain and kidney cells (48). In a previous publication we have shown that mRNA derived from kidney, of adult as well as fetal origin, together with testis are the only tissues out of 21 different sources of RNA that express FREAC-4 mRNA (15). In situ hybridization data from Hatini et al. (48) as well as our unpublished observations demonstrate a similar pattern of expression to that of WT-1, i.e. the area around the IV ventricle, medulla spinalis, kidney, and testis (46, 49, 50). It is interesting to note that (i) WT-1 and FREAC-4 both are transcription factors, (ii) WT-1 transactivates a FREAC-4 reporter gene construct in a cotransfection assay, and (iii) they have similar patterns of expression. Cotransfection experiments indicate the tumor suppressor genes p53 and WT-1 as potential regulators of FREAC-4. A WT-1 binding site within the FREAC-4 promoter is identified. Thus, there is evidence of circumstantial nature of several overlapping characteristics between FREAC-4 and WT-1. With this in mind, it is interesting to note that about 10% of Wilms’ tumors have mutations in the coding region of the WT-1 gene (51–53). This suggests that mutations are either present in regions other than the coding parts of the WT-1 gene (e.g. introns and cis-regulatory regions) or that mutations in other genes contribute to tumor formation. In this context we would like to speculate that FREAC-4 could play a role. In a recent publication we have assigned FREAC-4 to the chromosomal position of 5q12–13 (54). Patients with chromosomal duplications in this region exhibit common clinical features such as dysplastic kidneys and mental disorders (55, 56). The anatomical sites for these clinical findings seem to match the tissue distribution of FREAC-4 mRNA. Hence, an increased gene dosage of FREAC-4 could not be ruled as a possible cause of these abnormalities.

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