Biochemical and Biophysical Research Communications **286,** 94–100 (2001)
doi:10.1006/bbrc.2001.5349, available online at http://www.idealibrary.com on IDEAL®

Transcriptional Activity of the Promoter Region of Rat Frizzled-Related Protein Gene

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Received June 27, 2001

Frizzled-related protein (Frp) is a new family of secreted proteins involved in tumorigenesis and Wnt-signaling pathway. Previous study has shown that rat Frp (rFrp) gene was found to be differentially expressed in Rat 6 fibroblast cell line overexpressing p53^{val135} (R6#13-8). The rFrp gene was otherwise silent in normal parental Rat 6 cells. To elucidate the molecular basis of the transcriptional activation of rFrp, we have isolated and analyzed a 2-kilobase pair promoter region of the rFrp gene. Mapping of transcription initiation sites of rFrp showed the existence of multiple initiation sites. Transfection studies of serial deletion constructs in both Rat 6 and CHOK1 cell lines revealed that the region from −202 to −144 contains *cis*-acting elements essential for the efficient transcription of rFrp. This work provides a transcriptional regulation basis for Frp and gives insight into its implication in tumorigenesis. © 2001 Academic Press

Key Words: **Frizzled related protein; promoter; transcriptional regulation; mutant p53.**

Frizzled-related protein (Frp) is a newly identified family of secreted proteins. Frp protein contains a secreted signal peptide at the amino terminus and is followed by an extracellular cysteine-rich domain (CRD) which is highly homologous with the CRD present in the frizzled (Fz) family proteins (1–3). Fz protein is the receptor for Wnt family proteins, and studies have implicated CRD as the putative binding site for Wnts (4). Wnt/Fz interaction triggers a series of protein–protein interactions, which lead to the accumulation of β-catenin; this accumulated β-catenin is then translocated into the nucleus where, after interacting with the DNA-binding T cell factor (Tcf) complex, it acts as a transcriptional activator (5). Since Frp shares a similar CRD with Fz family genes, it is believed that Frp might act as an antagonist, competing with Fz for Wnt proteins and therefore it might modulate Wnt signaling (6, 7). *In vitro* studies, however, have shown that Frp can directly bind to Fz receptors (8). In either modes of interaction between Frp and Wnt/Fz proteins, current data suggest that overexpression of Frp could interfere and mostly reverse the effects of Wnt-signals demonstrated in both *in vivo* and *in vitro* situations (3, 6, 7–9).

Wnt proteins and Fz receptors are best known for their connections to embryonic development and—especially—to carcinogenesis. The mechanism by which Wnt signaling leads to tumorigenesis is unclear. Recent identification of two of the downstream transcriptional target genes, c-*myc* and cyclin D1, have shed light on the oncogenesis of certain cancers due to amplification of Wnt genes or to mutations of genes along the Wnt signaling cascade (5, 10–12). The biological role of Frp genes is not known. The associations of the Frp gene family to carcinogenesis and apoptosis have only been explored recently. Two independent studies have shown that Frp homologs are either turned off or down-regulated in breast carcinomas (13, 14). Another early study indicated that a Frp gene, designated as frpAp, acts as a proapoptotic gene in rat corpus luteum (15). In cultured fibroblasts, overexpression of Frp inhibited Wnt-induced morphological transformation (8). All the above findings suggest that Frp may act as an antineoplastic gene in the course of malignant transformation. However, more recent studies have shown that ectopic expression of Frps in fact promotes growth of human malignant glioma cells by interfering with the Wnt signaling cascade (16). These contradictory roles of Frp may reflect the fact that different Frps exert different effects in different tissues and organs. To date, at least five different mammalian

Sequence information is deposited with the GenBank Accession No. AF140346, AF140347, and AF364906.

Abbreviations used: rFrp, rat frizzled-related protein; Fz, frizzled; CDR, cysteine-rich domain; RACE, rapid amplification of cDNA ends; SRY, sex-determining region Y gene product; STAT, signal transducer and activator of transcription; MZF, myeloid zinc finger; C/EBP-β, CCAAT/enhancer binding protein beta; CREB, cAMP responsive element binding protein; CBP, CREB binding protein; IK2, Ikaros 2.

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0006-291X/01 $35.00

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TABLE 1

Sequence of Oligonucleotides Used in the Study

Oligonucleotide	Nucleotide sequence (5′ to 3′)	Purpose
AP1	CCATCCTAATACGACTCACTATAGGGC	5′ RACE
rFRP GSP1	GGTCTTTTATGTATCATTCTTCTGCC	5′ RACE
6-FAM AP2	6-FAM ACTCACTATAGGGCTCGAGCGGC	GeneScan analysis
rFRP GSP2	GCTACCAGGATGGAGAGGAGCAT	GeneScan analysis
rFRP-175 F	TAGCCCTTGGCTGTGGAGT	PCR
rFRP-207F	AAAAGAGGGACTTTGGG	PCR

Frp genes have been identified (17). The precise interaction of each Frp protein in modulating Wnt signaling remains to be elucidated.

As described above, most studies on Frp have focused on the downstream effects of Frp, leaving the regulation of Frp expression largely unexplored. In our previous studies on p53^{val135}-induced cell transformation, rat Frp (rFrp) was identified as one of the differentially expressed genes activated in untransformed (R6#13-8) and transformed (R6#T2) Rat 6 fibroblast cell lines overexpressing p53^{val135} tumor suppressor gene. This rFrp gene is normally silent in the parental and vector control Rat 6 (R6) cells (18). To further our understanding of the functional role of rFrp and its regulation, we have cloned and the promoter region of rFrp and studied the transcriptional regulation of the gene in our cell systems under which the rFrp is differentially expressed. Here we report the identification of promoter regions responsible for the positive and negative transcriptional regulation of the rFrp gene. Our work forms a fundamental basis in the regulation of Frp and gives insights into a possible role of Frp in tumorigenesis.

MATERIALS AND METHODS

Rapid amplification of 5′ cDNA ends (5′ RACE). Cloning of rFrp cDNA was performed using Marathon cDNA amplification kit (Clontech) according to the instruction manual. The adaptor-ligated cDNA library was constructed using poly(A)$^+$ RNA extracted from R6#13-8 cells which highly express rFrp (18). The first PCR was performed using adaptor primer AP1 and rFrp gene specific primer GSP1 corresponding to 1690–1715 of the rFrp cDNA (GenBank Accession No. AF140346). Reamplification was performed using the same primer set and the first PCR product as template. The primer sequences are listed in Table 1. Parameters used in PCR were 25 cycles of 94°C for 45 s, 65°C for 45 s, and 72°C for 4 min. The reamplified PCR products were subcloned into pCRII vector (Invitrogen) and sequenced by BigDye Terminator kit (PE Biosystems).

GeneScan analysis. Transcription initiation sites were mapped using a RACE-based method in conjunction with the ABI GeneScan Analysis (19). The 5′ untranslated regions of rFrp cDNA were amplified from first PCR products by nested PCR using a pair of internal primers: a fluorescently labeled adaptor primer 6-FAM AP2 and rFrp GSP2 corresponding to the nucleotides 246–268 of rFrp cDNA. The nested PCR product was mixed with GeneScan 400HD [ROX] size standard (PE Biosystems), denatured and electrophoresed on a 5% polyacrylamide gel at constant 3000 V at 51°C for 2.5 h in an ABI Prism 377 DNA Sequencer. The data were analyzed by GeneScan Analysis, version 3.1 software (PE Biosystems).

Genomic library screening and DNA sequencing. Rat liver Lambda DASH genomic library (Stratagene) (1×10^6 pfu) was screened using the ^{32}P-labeled 1.7-kb rFrp cDNA as a probe (Probe I). Nine positive phage clones obtained were further hybridized with a ^{32}P-labeled 271-bp rFrp cDNA (nucleotides 141–411) (Probe II) to identify clones containing putative promoter region. A clone with 21-kb insert containing putative rFrp promoter region was obtained. DNA was extracted and was further analyzed by restriction mapping and Southern hybridization using Probe II. A positive 4.5-kb fragment was subcloned into the pBluescript II KS+ (Stratagene) and sequenced. The complete DNA sequencing was performed on both strands by primer walking, and carried out by the DNA sequencing and synthesis facilities at the Iowa State University of Science and Technology.

Construction of reporter plasmids carrying deletions. The rFrp genomic fragment of 2096 bp (nucleotides −2013 to +83) was subcloned into the reporter vector, pSEAP2-B (Clontech) through *Xho*I and *Nru*I sites. A series of 5′ unidirectional deletion plasmids was constructed by exonuclease III and mung bean nuclease digestion from the Frp (−2013/+83) plasmid. The Frp (−175/+83) and Frp (−207/−130) plasmids were constructed by PCR amplification of the promoter sequence using primers rFRP −175F and rFRP −207F flanking both ends and cloning the resulting fragment into pSEAP2-B. The end points and sequences for all plasmids were determined and confirmed by sequencing using BigDye Terminator kit (PE Biosystems). The DNA plasmid for transfection was prepared by using the QIAGEN plasmid purification kit.

Cell culture, transient transfection, and reporter assays. The Rat 6 embryo fibroblast cell line (R6) is a subclone of an immortalized F2408 rat embryo cell line (20). R6#13-8, a p53^{val135}-overexpressing cell line was described in detailed in a previous study (18). The Chinese hamster ovarian cell line (CHOK1) was purchased from ATCC. All cell lines were grown in Dulbecco's modified Eagle medium (DMEM) (Life Technologies, Inc.) supplemented with 10% calf serum, penicillin, and streptomycin at 37°C in a humidified incubator with 5% CO_2 in air. Cells were seeded into 6-well plates at a concentration of 2×10^5 cells per well 24 h prior to transfection. LipofectAMINE PLUS Reagent (Life Technologies, Inc.) was used for transfection according to the manufacturer's instruction. In each transfection, 1 μg of Frp reporter construct was cotransfected along with 0.5 μg of control luciferase plasmid, pGL2-C (Promega) for normalization of transfection efficiency. Each transfection was done in triplicate. Conditioned medium and cell lysate were collected 48 h after transfection and used for analyzing alkaline phosphatase and luciferase activities respectively. Alkaline phosphatase activity was assayed by the Great EscAPe SEAP chemiluminescence detection kit (Clontech). Control luciferase activity was assayed by the luciferase reporter gene assay detection kit (Roche). Light signals were recorded by the tube luminometer, Monolight 2010. The results are

presented as relative phosphatase activity comparing with the basic reporter, pSEAP2-B, after normalization with the luciferase activity.

RESULTS AND DISCUSSION

Isolation of the 5′ flanking region of the rFrp gene. A genomic clone of 4.5-kb was obtained by genomic library screening using the 1715-bp rFrp cDNA (GenBank Accession No. AF140346) as a probe. The DNA sequence of the rFrp genomic clone (4483-bp) has been submitted to GenBank data bank (Accession No. AF#140347) (Fig. 1). Sequence alignment between the rFRP cDNA and genomic DNA revealed that the genomic clone consisted of a 2013-bp promoter region (−2013 to −1), a 692-bp exon 1 (+1 to +692), and 81-bp exon 2 (+1893 to +1973), a 66-bp exon 3 (+2059 to +2124) and the remaining intron sequences. These three exons contain the same nucleotide sequence as the rFrp cDNA. All intron–exon boundaries contained the consensus GT-AG splice sequence.

Analysis of the promoter region of the rFrp gene by the TFSEARCH version 1.3 software showed that a potential TATA box located at −28 to −34. The promoter region also contains a number of consensus motifs for hematopoietic specific factors STAT3 (−1577 to −1569), GATA (−1755 to −1748, −1306 to −1299), IK2 (−1345 to −1335, −805 to −794, −717 to −707), Lyf-1 and MZF1 (−295 to −288). Another group of putative sex-determining transcriptional factor-binding motifs were found within −1737 to −1728, −566 to −557 for Sox-5, and within −1786 to −1780, −410 to −404 for SRY. These motifs may play a role in regulating Frp expression in a tissue-specific manner. Other putative regulatory elements found were GC box (−535 to −528), and a stretch of CT repeat at nucleotides −1551 to −1508. No p53 binding motif was found in the sequenced region.

Determination of transcription initiation sites. A RACE-procedure was used to map the transcription initiation site of the rFrp gene. One round of PCR using a gene specific primer (GSP) and an adaptor primer (AP) was followed by second round of nested PCR with a fluorescently labeled adaptor primer (6-FAM AP2) and a second gene specific primer in the reaction (Fig. 2A). The labeled PCR products were sequenced and read using the GeneScan analyzer as described under Materials and Methods. The size of the PCR products minus the size of the adaptor sequence (36 bp) corresponds to the location of the transcription initiation site upstream from the GSP2 primer. Sizes of the PCR

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  nt.                                                                      a.a.
-2013 GTCGACTGGAAAGTGAAGAGTGAGGTGAGATCTGGGGTCAAAATCTTGAGAGCAGTCTGC
-1953 ATATAGCAGGGGAGAGAAAGAAGGACAAGGAGACTAACCCCAGAGAAGCACTCAGCTAGA
-1893 GGACAATGGCTTTAGTGCTAAAGCTGGAAGCCTTTTTGGAGGGCTGAAAGAGGAAGGACA
                                                     SRY
-1833 ATCGACAAGAATTTCCTGAGTCCCTCAGCTCTGTGTAACAGTCTATGAAACACAGAGCAG
                      GATA               Sox-5
-1773 ACAGACTGTCTGTGGGCCTGATAGGTAGGGTTACAGACAACAATGAAGACAACTCAGGCT
-1713 AAGCATGAACTTGGGGTGAGCTCATCAATGGGCTCTACTCCTCTGTTCCAAATGCCTGTC
-1653 TTAACATTTTCCTTCTTTTAGTTTTTTCTTGATTTCTTTTAGTGCCGGGATGACCTAGGG
                     STAT3
-1593 TCTCATCTACTTTAGCTTGAAGGAAAGTGGTGGGAAGTTTTGCTCTCTCTCTCTCTCTCT
-1533 CTCTCTCTCTCTCTCTCTCTCTCTTTCTTCTCATTAGAAAGTTAATTTGATTTTTTTT
-1473 CTGAGTGGGGGGATTTAGATTACACTTGGTATTTCACTACTGGAGAAGGCATGAGTTGTC
-1413 TTTTGGGCCACCTATTCTGCTTAATGATTTTTTACCATGGTTTGGCATCTTTGGACTTAA
             IK2                                     GATA
-1353 GACTTATTCTGTGGGAAAGCAGAATGCCCTTTTTGATTGGTCTGGATAGATAGGTACTCT
-1293 AGGCAGATGCTAAATAGTATCAGACCTCTTATGAGCGTTAACATTTTCATAGTGTTTGTC
-1233 TCTACACCTTATGCAATAAAGACAATAAAAACAGTTGTGTTTAGTTTTGTTTTGTTCCAT
-1173 TTTGAGCAGCTGCTTTAATCCTCAGTCAGATTCATGCCTAAGCTAAATTGTATGTCTCAG
-1113 TTTTAGAGAGGGAAAAGAATTTCCTTAAACCCAGGTTTCAAACTCAACCATACCTTTAAA
-1053 GATTAACCATTCAGAGAACTTGTTTTCTTATTGTCAATGATGACAGCAAATTTATACCAG
 -993 AAGTCTTGCTGTCTGATTGGCAGACATGTGGTGTAAATTTGCAACAGTAACAACTCTTTT
 -933 TGTGAATCAGCCTTGATTTTCCTGTCTCTTTCTAATGCAAAGAGGAGGAAAGTAAACTTG
 -873 CTTTCCAAGGCTGGTGAATTCTTAGTCAAGTAAAAATAATCTGCAATTATTCAACGATCG
              IK2
 -813 CGATGTTGGACTGGGAAAAATCTTTCAGCATGGGGCGTCTCAAGTTACCTTTGTTTTCTT
                                            IK2
 -753 CAGTTTTCCAGTCATATTGGGCTCAGAAATTAGTATTTTAGGGAAAGCAGAGAGTAGAGA
 -693 AACTTCTCCAACACAAAGATGCTCTTGCAACCCTGGGCAGTTCCAAGTTCTGGAGCAGGC
 -633 ACAAACCAAATGCCTGGTAAAAACTTGAGCCAGAACTAAGTCCAGTGGCCACAAGACAAC
            Sox-5                            GC box
 -573 ATCAGTCGCAACAACAATAACAAACTCTGCACAACTCAGGGCGGGCTGGGAGAGGCTTAT
 -513 ATAAATAGCCTGTGCTAGTTCCAACACCTACAGTGCTAGGCTTTCCCAGCTCCACACCAC
                                                    SRY
 -453 CCTCACTACTCAGGCGTCTCTCTCGCCCCCTACAGGAAAGGAAAAACAAATGTTTCAAAA
 -393 TTAAAGGGCAACAACAGGGTCGTATTGCTCTAACTCTGTACTTAGAGAAACTTTAGGCTG
 -333 ACTTAGCAAAGAGATTCCTCAAAGTCTCCCCTGCACCGGCTTATATGCAGCGTCCCAAAT
 -273 GACTGAAAGCAAGGCGCTCCTTTTCTTGATCTGAAGAAAAAAGACTAGCAGACTGAGGGG
                STAT3/Lyf-1/MZF1
 -213 GAAAAAAAAAGAGGGACTTTGGGGAAATGCAGAACTATAGCCCTTGGCTGTGGAGTGGC
      C/EBP-β/GATA-1/CREB
 -153 GGAGATGATGTAATCGCTTCTGCAGGAGAGGCTGGGGGTGGAGCAGCGGAGCAGGAGCCG
                                                          TATA
  -93 GCTCTGCTTTCGCCTCTGCCAAGGCTGCTGAGCCCACGTCAGGGGACGCGTCTGGATAAA
                                  +1 ┌──>                  ▼
  -33 TAGGGTCCCACAATGGCCGTGGCCCGCTGCGCTCCGAGCTGCGGGGCGGGAGACTGGAGC
  +28 TTCCTGCGCGGGTCTGTGCTCTGGTGGGTGAGTGCTGTCCCTGCCAGTGTCCGGGCGCCC
  +88 AGGGCTGATCGACGGCCAGAACCCCAGAACTCCCGTTCAGTGCGGACTGGAGCTCCAGCA
 +148 GAAACGCTCACTTCGCTGCCCCTAGGTAAAGACATAAGGAGATCAGAGGGACTTGAAGGG
 +208 AAGGTCCCCAGTGGGGACCGCAGCCCAGGAGGACAGTGCGatgctcctctccatcctggt
                                                M  L  L  S  I  L  V   7
 +268 agcGTTATGCCTGTGGCTGCGCCTGGCTCTGGGAGTGCGCGGAGCGCCCTGCGAGGCTGT
        A  L  C  L  W  L  R  L  A  L  G  V  R  G  A  P  C  E  A  V   27
 +328 GCGCATCCCCATGTGTAGGCACATGCCCTGGAACATCACCCGGATGCCCAACCACCTGCA
        R  I  P  M  C  R  H  M  P  W  N  I  T  R  M  P  N  H  L  H   47
 +388 CCACAGCACTCAGGAGAACGCCATCCTGGCCATCGAGCAGTACGAGGAGCTGGTAGACGT
        H  S  T  Q  E  N  A  I  L  A  I  G  Q  Y  E  E  L  V  D  V   67
 +448 GAACTGCAGCTCTGTACTGAGCTTCTTTCTCTGTGCCATGTACGCACCCATCTGTACCCT
        N  C  S  S  V  L  S  F  F  L  C  A  M  Y  A  P  I  C  T  L   87
 +508 GGAGTTCCTGCACGATCCCATCAAACCCTGCAAATCTGTGTCCCAGCGTGCACGCGACGA
        E  F  L  H  D  P  I  K  P  C  K  S  V  C  Q  R  A  R  D  D  107
 +568 CTGCGAGCCCCTCATGAAGATGTATAACCACAGCTGGCCAGAGAGCCTGGCTTGCGATGA
        C  E  P  L  M  K  M  Y  N  H  S  W  P  E  S  L  A  C  D  E  127
 +628 GCTGCCTGTCTATGACCGTGGAGTGTGCATCTCTCCGGAGGCGATAGTCACTGACCTTCC
        L  P  V  Y  D  R  G  V  C  I  S  P  E  A  I  V  T  D  L  P  147
 +688 GGAAGGTAAGAGAAGGTATCAGGAAGGCCAAGACACTCTAGGGACTAACACATTCAGAGA
        E                                                           148
 +748 GAAGCAGAGGGTTTCTGCTTAGAGCCCACCAGTTCCTGTCCCAGAATAGTTCACTGAGCC
 +808 TTGTGGGAGGTGAAGGGCAGGGATTTCTCAAGGGAGTGAAACATTCTAGAGCTCCTTGAA
 +868 GGACATTGGGAATAAGTTTGCTTGAGTATAACCCATTTAAGCGTTTTTATTTAAGGTAAC
 +928 TAGCAGAAACAATAGCCTAGGCAAAGAAATACCATGTACATGGGTCCGTGTAAGCTTCCA
 +988 CGGTTTACCTTCTCTTGCTCTTGAAACAGTCCCCCACGTGGTGGTGGCTAAGAACGCTCT
+1048 CATTTAGTGCTGGTTGGGAGTTCTTTCTCAAAGAGCAATAATAATTTCCACTCCCCACCA
+1108 GGATAAAGTTTGTAAATGTAGTCACCCTGGGTAAACTCCGGTCTGTGTCTATGTTGTGGT
+1168 CACTGAGCAGAGCACTGTGGCATGTTGGTTAATCTGATCCTGTGTCAGTTCCTCCGTTTT
+1228 GTTTTTGTTTTTTTCTTTTATGCTGCTTTAGTGTTTTTGTTGGAAGGAACACTATGAATT
+1288 CATTTCTTGAAATGAAACCTGAGAACATTATTGCATTATTTTATGGAGGCTTCCTCTAAA
+1348 TCAGATCATCCTTTATTTAAAGAGACTGGGTCTAGGATGTGAGTCAGGGGGATGGCGTGA
+1408 GTTATGAACCTTGCTTTAAACTCTTGCCTTATTCAGAAGACTAGAAAAATAGTTTAAATT
+1468 AAACAATTGCTTCGACTTCATTAAGCCTGGAAGTTATGCTTAGTACTGTTGATAATGCGT
+1528 CTGTGGACTATTTCTGCACCACAGTAGGAGATAGCAATGTGGAAACAGTCTGGTTGTTCT
+1588 AATAGAAGACCTGTTAACTTGTTGACTTGGAACAGCAGTATCAGGGTATCTGAGGGAGGG
+1648 AGGGCTGAGACTCATCCAGGCTGGTGTCATGGATGTTCATTTGCATCAAGTTCTGTTTGA
+1708 AAAAGGAAATTCTTGACTCACAGACTTCTTTCTTAGGCCTGAGGTTTACTGAGAAAGGTA
+1768 CTTTTATTATGGCGATATTGTGTTGGATGTTTGTGTTGGCCTGCAGCCTCCAATGGTTCC
+1828 TTATTCCATTTGAATGAGGGGACCCAGTCATTGACTACATCCCCATGGTTCTGTTCATGT
+1888 TTCAGATGTGAAGTGGATAGACATCACACCAGATATGATGGTTCAAGAAAGGTCCTTTGA
            D  V  K  W  I  D  I  T  P  D  M  M  V  Q  E  R  S  F  D  167
+1948 TGCTGACTGTAAACATCTGAGCCCTGGTGAGTTTTGTCCTCAGGCTTCCTTTGCTTTGTG
        A  D  C  K  H  L  S  P                                      175
+2008 CTCACGGTGATGTCCCATCCCCACTGTCCATGTATTTTATGTCTCTTTCAGATCGGTGCA
                                                      D  R  C       178
+2068 AGTGCAAAAAGGTGAAGCCAACTTTGGCAACGTACCTGAGCAAAAACTACAGCTATGGTA
      K  C  K  K  V  K  P  T  L  A  T  Y  L  S  K  N  Y  S  Y       197
+2128 AGTCTTCTGATAACAGCATTTGCTCTGGGACTTGGCACACCAAGGCACCGAAATCACAGG
+2188 GACTAGATGTAGTAGTCAAGGAAAAGGGGATGTTGGATGGGTAATCAAATGGTTTCTGGA
+2248 CCGTTCAACAAACATAAAGTTTCTTTTCTGGGTAAATGAAAGCAAGCACAAGATAAATAA
+2308 CACTTAAAAATGGTCTCGGTGAAAACTGGTAAGCACAGAATGGCCGAGGCTGCTGGTCTC
+2368 CTTCTCAGGTTGTCCTTCCCTGTAGGAAGGACAGGGCTTTGGACCAGGATTTGTTTATCT
+2428 GAAGATCCGAATTCGTTCCCTTTAGTGAGGGTTAATTGTCGAC
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FIG. 1. Nucleotide sequence of the 5′ flanking region of rFrp gene. The rFrp genomic sequence (−2013 to +2470) is shown. The radiolabeled rFrp cDNA probe used for genomic library screening is underlined. Putative regulatory elements and the potential TATA box are boxed. The GSP2 primer used to determine the start site is shown in lowercase. The determined major transcriptional initiation site is designated as +1 and marked by an arrow. Minor initiation site is indicated with solid triangle.

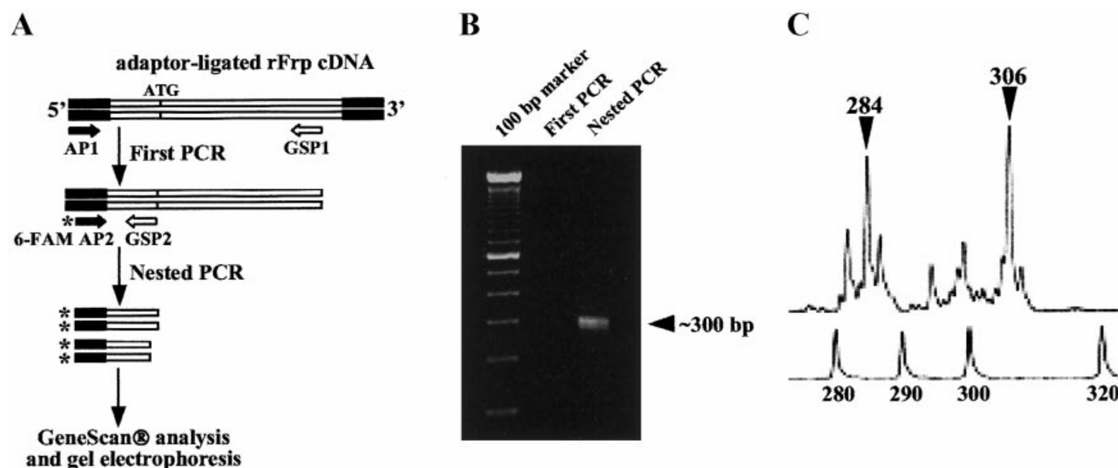

FIG. 2. Mapping of transcriptional start site of rFrp gene by RACE and GeneScan analysis. (A) The scheme of the RACE-based technique. Amplification of rFrp cDNA was done using AP1 and GSP1 in the first PCR. The nested PCR was performed with 6-FAM AP2 and GSP2 (located at the start codon) using the first PCR product as template. The 6-FAM-labeled PCR products are indicated by asterisks. The size of the nested PCR products was analyzed by GeneScan software. (B) The PCR products of rFrp cDNA electrophoresed on an ethidium bromide stained 2% agarose gel. No observable PCR product was shown in first PCR and a 300-bp PCR product was generated in nested PCR. (C) Determination of the nested PCR product size by GeneScan analysis. Dominant peaks at 284 and 306 indicate that PCR products of different sizes were produced in the nested PCR. The GeneScan 400HD [ROX] size standard is shown below. The major peak (306) corresponds to the nucleotide "C" which is the first nucleotide of our previously cloned rFrp cDNA (GenBank Accession No. AF140346).

products are indicated as peaks where the locations are precisely aligned with an internal fluorescent size ladder, while the number of peaks reflects the number of initiation sites. Figure 2B shows that DNA fragment about 300 bp was amplified in two rounds of PCR. GeneScan analysis shows that major products of 284 and 306 bp in length were detected (Fig. 2C). The nucleotide corresponds to the major 306-bp peak exactly matched with the first nucleotide of our cloned rFrp cDNA. Therefore, we defined this nucleotide to be the major transcription initiation site. The presence of PCR products of rFrp with various sizes suggests the existence of multiple transcription initiation sites.

Promoter domain of rFrp residing at −202 to +83. To identify potential cis-acting elements important for rFrp transcription, a series of unidirectional 5′ deletions of the rFrp promoter region flanking from −2013 to +83 was fused to the pSEAP2-B plasmid carrying a promoterless alkaline phosphatase gene (Fig. 3A). The resulting constructs were transiently transfected into R6#13-8 cells expressing high level of endogenous rFrp (18). Compared with the promoterless vector, pSEAP2-B, the Frp (−2013/+83) construct exhibited a 5.3-fold increase in phosphatase activity. Sequential deletion of promoter sequence from −2013 to −202 resulted in a gradual increase in phosphatase activity from 5.3-fold to 9.6-fold. These data suggest the release of suppressive effect of negative *cis*-acting elements resulted in a gradual increase in promoter activity. Maximum promoter activity was exhibited by the Frp (−202/+83) construct. Further deletion from the region −202 to −144 as shown by Frp (−175/+83) and Frp (−144/+83) constructs resulted in drastic drops in phosphatase activity (Fig. 3B) from 10-fold to 3.5- and 0.6-fold, respectively. These results suggest that the region −202 to −144 is critical for the promoter activity of rFrp in R6#13-8 cells. However, when the Frp (−207/−130) construct containing the region −202 to −144 was tested for promoter activity, it showed only minimal phosphatase activity (Fig. 3B). This result suggests that the region −202 to −144 alone cannot confer full promoter activity, and that the downstream sequence (−143 to +83), which possesses a TATA box and transcription start site, is required for basic promoter activity. Sequence analysis of the region −202 to +83 showed that two nucleotide stretches from −196 to −185 and −149 to −141 contain putative *cis*-acting elements: i.e., STAT3, Lyf-1, and MZF1 in the region −196 to −185 and C/EBP-β, GATA-1 and CREB in the region −149 to −141 (Fig. 1). Sequence comparison reveals that these two domains are well conserved in rat, mouse (GenBank Accession No. AF364906), and human (Fig. 4), further supporting the significance of these regions in the regulation of rFrp.

To test whether the transcriptional regulation of the rFrp promoter would only apply to R6#13-8 cells, the rFrp promoter activity was studied in the CHOK1 ovarian epithelial cell line, which is commonly used for transient report assays and found to express the endogenous rFrp in our hand (data not shown). The rFrp promoter displayed a nearly identical activity pattern for the serial 5′ deletion constructs in CHOK1 cells as in R6#13-8 cells (Fig. 3C vs 3A). This result indicates that the control mechanism is not a phenomenon

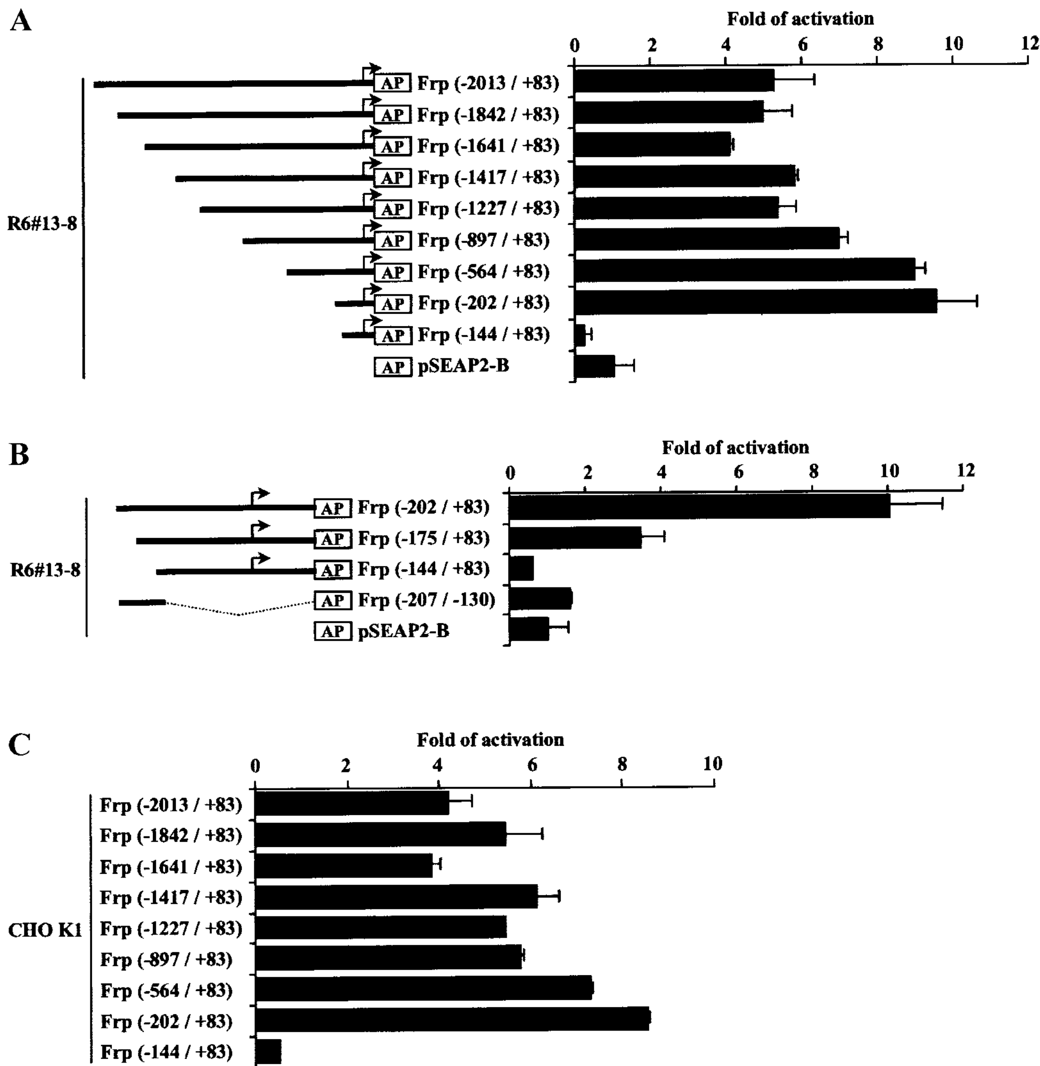

FIG. 3. Deletion analysis of the promoter region of rFrp gene. (A) 5′ unidirectional deletion analysis of rFrp promoter region from −2013 to +83 in R6#13-8 cells. (B) Proximal promoter (−202 to +83) activity of rFrp in R6#13-8 cells. One microgram of serial 5′ deletion constructs containing portions of rFrp promoter driving the expression of secreted alkaline phosphatase (AP) was transfected into R6#13-8 cells along with 0.5 μg of the control luciferase vector, pGL2-C. The numbers indicate the endpoint of deletion constructs with respect to the transcription start site of the rFrp gene. Values are normalized with the luciferase activity. The phosphatase activity is shown relative to the activity of the promoterless basic vector, pSEAP2-B taken as 1. Each data point represents the mean ± SE for three transfections from a representative experiment. (C) Serial 5′ deletion constructs were assayed for activity in CHO K1 cells.

unique to R6#13-8 cells which show aberrant expression of rFrp.

Differential activity of the rFrp promoter in R6 and R6#13-8 cells. The gene rFrp was initially identified through differential mRNA display of normal and transformed p53^{val135}-overexpressing R6 cells (18). The transcript was found to be highly expressed in R6#13-8 cells, but not detectable in R6 cells using Northern blotting analysis. To investigate the promoter activity of rFrp in relation to the differential expression of rFrp previously reported, we conducted reporter assays using Frp (−2013/+83), Frp (−202/+83) and Frp (−144/+83) constructs in these two cell lines (Fig. 5). Luciferase construct was cotransfected with each Frp construct and the phosphatase activity was normalized by the luciferase activity. The relative phosphatase activities of the test constructs in each cell line were similar, but the Frp constructs exhibited significant higher phosphatase activity in R6#13-8 than in R6 cells. This result correlates well with the lack or extremely low level of expression of the endogenous rFrp in R6 cells and high level of expression of rFrp in R6#13-8 cells. In this regard, we can conclude that the up-regulation of rFrp observed in R6#13-8 cells is very likely to be controlled at the level of transcription. According to the current sequence analysis, no known

p53 consensus binding motif was identified from the promoter region to the third intron of the rFrp. Therefore, the upregulation of the rFrp as seen in R6#13-8 cells did not result from the direct interference of the transcriptional activity of p53 present in the cell lines. One of the potential regulatory elements, CREB, found in the promoter domain interacts specifically with the nuclear transcriptional coactivator CREB binding protein (CBP) (21–23). The transactivation domain of CREB directly complexes with the CBP through the KIX domain (24), which is also a newly discovered contact point for the p53 tumor suppressor protein (25). The KIX binding domain of p53 is crucial for p53 transactivation function. CBP and p53 have been shown to act synergistically in transcriptional regulation (26). The intimate association between the CREB, CBP and p53 proteins could possibly explain the aberrant expression of rFrp observed in R6#13-8 cells overexpressing mutant p53. It is possible that overexpression of mutant p53 might interfere with the function of wild-type p53 as a coactivator in the CREB/CBP protein complex. However, further investigation is required for verification of this hypothesis.

To our knowledge, this is the first report to characterize the transcriptional regulation of rFrp. We have shown that the proximal end of the promoter region (−202 to −144) contains critical elements for rFrp gene expression. Our results presented here suggest a possible mechanism by which the rFrp is differentially regulated in the normal and mutant $p53^{val135}$-transformed R6 cells. Our work also provides valuable information toward the understanding of the regulation of rFrp and gives insights into its possible role in both tumorigenesis and development.

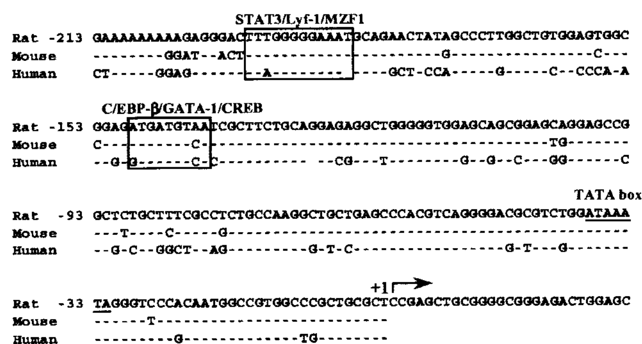

FIG. 4. Nucleotide sequence alignment of the rat Frp promoter region −213 to +1 with mouse and human homologs. Identical nucleotides are indicated by dashes. Two nucleotide stretches −196 to −185 and −149 to −141 which contain the putative *cis*-acting elements are boxed. Putative *cis*-acting elements STAT3, Lyf-1, and MZF1 are located in the region −196 to −185 and C/EBP-β, GATA-1, and CREB are located in the region −149 to −141.

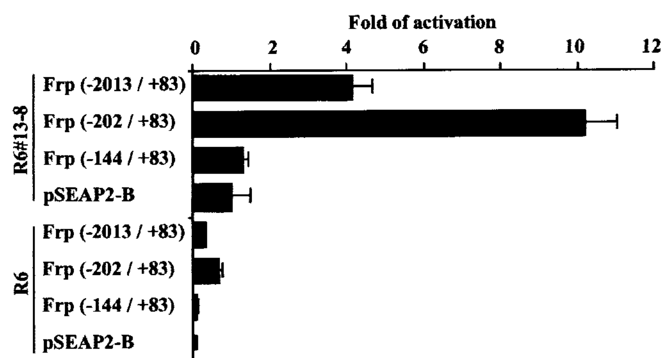

FIG. 5. Comparative rFrp transcriptional activity assayed in R6, R6#13-8 cells. Each Frp construct, Frp (−2013/+83), Frp (−202/+83), and Frp (−144/+83) was cotransfected with control luciferase vector, pGL2-C into R6 and R6#13-8 cells. Values are normalized with the luciferase activity and expressed relative to the activity of the promoterless basic vector, pSEAP2-B. Each data point represents the mean ± SE for three transfections from a representative experiment.

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