Redundant Intronic Repressors Function to Inhibit Fibroblast Growth Factor Receptor-1 α-Exon Recognition in Glioblastoma Cells*

Wei Jin, Eileen S.-C. Huang, Weiqi Bi, and Gilbert J. Cote‡

From the Section of Endocrine Neoplasia and Hormonal Disorders, Department of Medical Specialties, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

The human fibroblast growth factor receptor-1 primary transcript is alternatively processed to produce receptor forms that vary in their affinity for fibroblast growth factor. The inclusion of a single exon ( α ) in normal brain glial cells produces a low affinity form of the receptor. Recognition of the α -exon is dysregulated during neoplastic transformation of glial cells to produce a high affinity receptor form. In this study, we have identified a second intronic repressor of RNA splicing located approximately 250 nucleotides upstream of the α -exon. Deletion or mutation of this sequence resulted in a significant increase in exon recognition in glioblastoma cells. This intronic repressor was found to share significant sequence homology with an intronic repressor element located downstream of the α -exon. The two repressor elements are functionally redundant in that they are capable of inhibiting α -exon recognition when positioned upstream or downstream of the exon. Finally, the elements were found to mediate enhanced exclusion of an unrelated exon, but only the repressors were placed flanking the exon. However, under these conditions, the cell-specific exon exclusion was no longer maintained. These results suggest that although the α -exon inclusion is actively repressed in glioblastomas, the absence of trans-activators appears to be key to the production of the high affinity form of fibroblast growth factor receptor-1 in glioblastomas.

The underlying mechanisms involved in the regulated recognition of exons during RNA processing remain largely unknown ( 1–4 ). This problem is compounded by the continued discovery of genes that utilize alternative RNA processing as a mechanism for the creation of new gene products or the regulation of gene expression. The FGFR\(^1\) gene family provides but one example of several hundred. FGFR-1 is a member of this complex gene family encoding membrane-associated tyrosine kinase receptors ( 5–7 ). These receptors are widely expressed and have diverse functions that are mediated through the action of one or more of at least 14 FGF peptide ligands ( 5–7 ). FGFR-1 gene transcripts undergo regulated RNA processing that affects no fewer than 6 of the 20 coding exons ( 5–7 ). The decision to include or exclude a specific exon greatly affects the functionality of FGFR-1. Alternative RNA splicing controls changes that occur in both receptor affinity and ligand specificity. Because FGFR plays a primary role in neuronal cell growth and differentiation pathways, precise maintenance of splicing regulation is critical. Dysregulation of FGFR-1 alternative splicing occurs in the malignant transformation of glial cells. In normal human glial cells, the predominantly expressed form of FGFR-1 (FGFR-1α) has three extracellular Ig-like domains involved in ligand binding. However, in glial cell tumors, the exclusion of a single exon (the α -exon) results in the production of a form of FGFR-1 (FGFR-1b) that has two Ig-like domains ( 8 ). The absence of the third domain has been shown to give FGFR-1b an affinity for acidic and basic FGFs 10-fold greater than that of the FGFR-1a ( 9, 10 ). Predominant expression of this high affinity form of FGFR-1 in glial cells is believed to provide a cell growth advantage and to possibly contribute to glial cell malignancy. A reduction of FGFR-1 expression mediated by antisense oligonucleotides significantly reduces the growth of glioblastoma cells in culture ( 11, 12 ).

Our laboratory has focused on clarifying the underlying mechanisms involved in the regulated recognition of the α -exon in glial cells. In previous studies, we established that cell-specific recognition of the α -exon is maintained in chimeric RNA transcripts (containing only the α -exon and flanking sequence) and that an exonic enhancer sequence is required for α -exon inclusion ( 13, 14 ). However, in glioblastoma cells, intronic sequences are required to ensure α -exon skipping ( 15 ). In this study, we describe the identification of a second regulatory intron sequence that is required to prevent α -exon inclusion. The element shares extensive sequence homology and was found to be functionally redundant with the previously identified downstream repressor ( 15 ).

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The human astrocytoma cell line SNB-19 and the human choriocarcinoma cell line JEG-3 were maintained as described previously ( 13, 14 ).

**Plasmid Constructs**—The plasmid constructs pFGFR-17 and pFGFR-22 have been described previously ( 13 ). All of the constructs used, with the exception of pPPT-106 through pPPT-110, are derived from pFGFR-17 and maintain the same promoter (Rous sarcoma virus), flanking exons (human metallothionein 2α gene), and vector backbone (pGEM 4) (see Fig. 1A). All deletion constructs were created using a multistep strategy involving the introduction of two HindIII sites by mutagenesis followed by deletion and self-ligation, except for constructs pFGFR-102 and pFGFR-104, where mutagenesis followed by deletion and self-ligation, except for constructs pFGFR-102 and pFGFR-104, where mutagenesis followed by deletion and self-ligation, except for constructs pFGFR-102 and pFGFR-104, where mutagenesis followed by deletion and self-ligation, except for constructs pFGFR-102 and pFGFR-104, where mutagenesis followed by deletion and self-ligation, except for constructs pFGFR-102 and pFGFR-104, where mutagenesis followed by deletion and self-ligation, except for constructs pFGFR-102 and pFGFR-104, where mutagenesis followed by deletion and self-ligation, except for constructs pFGFR-102 and pFGFR-104, where mutagenesis followed by deletion and self-ligation, except for constructs pFGFR-102 and pFGFR-104, where mutagenesis followed by deletion and self-ligation, except for constructs pFGFR-102 and pFGFR-104, where mutagenesis followed by deletion and self-ligation, except for constructs pFGFR-102 and pFGFR-104, where mutagenesis followed by deletion and self-ligation, except for constructs pFGFR-102 and pFGFR-104, where mutagenesis followed by deletion and self-ligation, except for constructs pFGFR-102 and pFGFR-104, where mutagenesis followed by deletion and self-ligation, except for constructs pFGFR-102 and pFGFR-104, where mutagenesis followed by deletion and self-ligation, except for constructs pFGFR-102 and pFGFR-104, where mutagenesis followed by deletion and self-ligation, except for constructs pFGFR-102 and pFGFR-104, where mutagenesis followed by deletion and self-ligation, except for constructs pFGFR-102 and pFGFR-104, where mutagenesis followed by deletion and self-ligation, except for constructs pFGFR-102 and pFGFR-104, where mutagenesis followed by deletion and self-ligation, except for constructs pFGFR-102 and pFGFR-104, where mutagenesis followed by deletion and self-ligation, except for constructs pFGFR-102 and pFGFR-104, where mutagenesis followed by deletion and self-ligation, except for constructs pFGFR-102 and pFGFR-104, where mutagenesis followed by deletion and self-ligation, except for constructs pFGFR-102 and pFGFR-104, where mutagenesis followed by deletion and self-ligation, except for constructs pFGFR-102 and pFGFR-104, where mutagenesis followed by deletion and self-ligation, except for constructs pFGFR-102 and pFGFR-104, where mutagenesis followed by deletion and self-ligation, except for constructs pFGFR-102 and pFGFR-104, where mutagenesis followed by deletion and self-ligation, except for constructs pFGFR-102 and pFGFR-104, where mutagenesis followed by deletion and self-ligation, except for constructs pFGFR-102 and pFGFR-104, where mutagenesis followed by deletion and self-ligation, except for constructs pFGFR-102 and pFGFR-104, where mutagenesis followed by deletion and self-ligation, except for constructs pFGFR-102 and pFGFR-104, where mutagenesis followed by deletion and self-ligation, except for constructs
structures pFGFR-101 and pFGFR-105 contain the ISS-2 element inserted 99 bp upstream of the a-exon. The ISS-2 insert was generated by PCR amplification of pFGFR-17 using primers FP99 and FP101. For pFGFR-101, this fragment was inserted by blunt-end ligation into the HindIII site of pCR-100. For pFGFR-105, the PCR fragment was inserted into pCR-100 following mutagenic steps with different primer combinations to create an unstatistically sampled and a deletion analogous to pFGFR-100. The construct pFGFR-103 contains a downstream ISS-1 that was inserted into the EcoRV site of pFGFR-102. The fragment containing the ISS-1 element was generated by amplification of pFGFR-17 using primers FP52 and FP39. The pPPT constructs all contain rat preprotachykinin gene exons 3–5 and use the cytomegalovirus promoter and bovine growth hormone polyadenylation signal (see Fig. 6). The parent construct pPPT-106 was created by subcloning a HindIII/EcoRI fragment of RP 23 (generously provided by Paula Grabowski, University of Pittsburgh (16)) into pCR 3.1 (Invitrogen, San Diego, CA). The HindIII site was destroyed by blunt-end ligation prior to subsequent cloning steps. The insertion of the upstream ISS-1 (pPPT-107) was accomplished in multiple cloning steps. Intronic HindIII and KpnI restriction sites were introduced into pPPT-106 by mutagenesis. The ISS-1 element was inserted as a primer dimer of FP153 and FP154. A fragment containing the ISS-2 element was inserted in a similar manner. Downstream intronic HindIII and KpnI restriction sites were introduced by mutagenesis. The insert was created as a larger PCR product using primers FP151 and FP152, which were ligated into the downstream intron and finally introduced into a HindIII site by an internal deletion to create pPPT-108. Construct pPPT-109 followed an identical cloning strategy; however, pPPT-107 was used as the starting vector. The final construct, pPPT-110, contains inactivating mutations of the ISS-1 and ISS-2 elements. Mutation of the ISS-1 was performed using primers FP162 and FP163. This mutation is analogous to pFGFR-M2. The ISS-2 mutagenesis used primers FP134 and FP161 and is analogous to pFGFR-91 (15). All plasmid constructs were sequenced through ligation sites to confirm the identity of each clone.

**Mutagenesis**—All of the mutations were introduced using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. In some cases, the procedure was modified to employ three primers in the reaction as described previously (14, 15). The majority of the constructs required at least two independent mutagenic steps with different primer combinations to obtain the final plasmid. Detailed information on the creation of specific plasmids will be provided upon request. All of the constructs have been sequenced to confirm the specific nucleotide changes.

**Transfections**—All of the transfections were performed using DOSPER liposomal reagent (Roche Molecular Biochemicals) according to the manufacturer’s suggested protocol. Briefly, the day before transfection, 800,000 JEG-3 cells or 1 million SNB-19 cells were plated into a 35-mm dish in a 6-well plate. On the day of a transfection, a DNA/DOSPER mixture (1:6 ratio) was incubated at room temperature for 45 min. The mixture was then diluted by adding 4 ml of serum-free medium and added to each well. Ten to 15 μg of DOSPER was used for the transfection, which was allowed to proceed for 6 h prior to changing to serum-containing medium. All of the transfection reagents were prepared in the JetStar Isolation Kit (Genomed, Research Triangle Park, NC) according to the manufacturer’s protocol, and the purity and integrity of each preparation was confirmed by agarose gel electrophoresis.

**RNA Isolation and Reverse Transcription-PCR—**mRNA was isolated 72 h after transfection using the mRNA Capture kit (Roche Molecular Biochemicals), and reverse transcription-PCR reactions were performed as described previously (14). All PCRs were performed in a 50-μl solution containing 20 μl Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 0.5 mM dNTPs, 5 units of Taq polymerase, 0.2 μl H2233 downstream primer, 0.25 μl unlabeled DSS primer, and 0.03 μl 32P-end-labeled DSS forward primer. Primers used to analyze the RNA derived from the pPPT clones were T7 and FP82. PCR products were then analyzed by polyacrylamide gel electrophoresis. Quantification and statistical analysis of exon inclusion were performed as described previously (14). Individual values for the percentage of exon inclusion were derived, following subtraction of background counts and then averaged to obtain the mean value ± S.D. for a minimum of three independent transfections.

**Rat FGFR-1 Gene Analysis**—A rat genomic sequence was derived from the genomic P1 clone DMPC-HFF 12894, which was obtained from Genome Systems, Inc. (St. Louis, MO), by providing the oligonucleotide primers that map to the a-specific exon for the FGFR-1 gene (FP52 and FP53). DMPC-HFF 12894 was subjected to EcoRI digestion, and the resultant fragments were subcloned into pBluescript using standard methodology. Clones containing the a-exon were identified by colony hybridization using labeled FP52 as a probe. The genomic sequence was obtained from a single positive clone using a combination of standard and automated sequencing.

**Oligonucleotide Primers**—The DNA oligonucleotide primers used were as follows: FP11, 5′-GGGGAGCTTGCCAGAAGACGTCCTGAC-3′; FP12, 5′-GGGGAGCTTGCCAGAAGACGTCCTGAC-3′; FP26, 5′-GGGGAGCTTGCCAGAAGACGTCCTGAC-3′; FP27, 5′-GAATTCCTCGAGTTGTT-CAATCTGGGACAAAGCTCTCCCTTAG-3′; FP38, 5′-CTAATTGCTACTCTGATGAAATTTA-3′; FP39, 5′-CAATTGCTACTCTGATGAAATTTA-3′; FP40, 5′-CTAATTGCTACTCTGATGAAATTTA-3′; FP53, 5′-CTAATTGCTACTCTGATGAAATTTA-3′; FP55, 5′-GGAGATCCAAGCTTTCCCCTTAG-3′; FP75, 5′-GGAGATCCAAGCTTTCCCCTTAG-3′; FP38, 5′-CTAATTGCTACTCTGATGAAATTTA-3′; FP81, 5′-ATGCTGCTACTCTGATGAAATTTA-3′; FP82, 5′-ATGCTGCTACTCTGATGAAATTTA-3′; FP83, 5′-ATGCTGCTACTCTGATGAAATTTA-3′; FP91, 5′-ATGCTGCTACTCTGATGAAATTTA-3′; FP99, 5′-CTCCTCCCCCCCTTCAGATCTGGCCACTCTCG-3′; FP101, 5′-CTCCTCCCCCCCTTCAGATCTGGCCACTCTCG-3′; FP105, 5′-GAATTCCTAGGGAGGAGGAGGAG-3′; FP113, 5′-ATGCTGCTACTCTGATGAAATTTA-3′; FP151, 5′-ATGCTGCTACTCTGATGAAATTTA-3′; or, 5′-ATGCTGCTACTCTGATGAAATTTA-3′.

**RESULTS**

The Exclusion of the a-Exon in SNB-19 Cells Requires an Upstream Intron Sequence—Previous studies have shown that the exclusion of the a-exon from the final FGFR-1 mRNA strongly correlates with glial-cell malignancy (8). To test this finding, we established a cell culture model system capable of cell-specific recognition of the a-exon during RNA processing of minigene-derived transcripts. The construct pFGFR-17 contained a 4-kilobase fragment of the FGFR-1 gene inserted into the splicing reporter RSVP1MT2 (Fig. 1A) (13). Transcripts derived from this construct showed a-exon-specific inclusion in JEG-3 cells and a-exon-specific exclusion in SNB-19 cells (glioblastoma cells); the a-exon was included in only 20% of the transcripts (Fig. 1B). Deletion of the intron sequence upstream of the a-exon in pFGFR-17 was previously shown to dramatically up-regulate a-exon recognition in SNB-19 cells (13). This result was thought to be largely nonspecific, because substitution of the deleted sequence with nonspecific sequence resulted in a rescue of the phenotype. We decided to reexamine the regulatory role of the flanking intron sequences when quantitative analysis revealed a consistent enhancement of a-exon inclusion in sequence replacement constructs with pFGFR-17 (not shown). Three constructs containing progressive deletions beginning at the 5′ end of the FGFR-1 insert were examined for a-exon inclusion in transfected SNB-19 and JEG-3 cells (Fig. 1B). In the SNB-19 cell line, we observed a biphasic increase in a-exon inclusion (Fig. 1B). For constructs pFGFR-22 and pFGFR-50, a 20% increase in a-exon inclusion was observed. This increase may have resulted from the decrease in intron size (from about 2500 nucleotides to about 500 nucleotides). Further deletion of the a-intron sequence resulted...
in a dramatic increase in α-exon inclusion in SNB-19 cells to a level comparable with that observed in JEG-3 cells (construct pFGFR-D1) (Fig. 1B). In the JEG-3 cells, the level of α-exon inclusion was modestly affected by the reduction of intron size, from 80% (pFGFR-17) to 92% (pFGFR-51) (Fig. 1B).

The deletion constructs localize a potential SNB-19 cell-specific inhibitor of α-exon inclusion to an upstream intron region between nucleotides −242 and −322. To definitively assign an inhibitory element to this region, we tested constructs containing smaller deletions targeted to this region (Fig. 2). The pFGFR-57 construct contains a 77-bp internal deletion mapped to the region identified in Fig. 1. In transfected SNB-19 cells, this construct showed the same dramatic increase in α-exon inclusion seen for deletion construct pFGFR-51 (from 20 to 67%) (Fig. 2). Similarly sized deletions in sequence flanking the 77-nucleotide region had no effect on the level of α-exon inclusion in transfected SNB-19 cells (pFGFR-56 and pFGFR-58) (Fig. 2). No significant difference in the level of α-exon inclusion was observed for any of these constructs in transfected JEG-3 cells (Fig. 2). In addition, similar levels of α-exon inclusion were also seen in cells stably transfected with pFGFR-17 and pFGFR-57 (data not shown). Two final deletion constructs narrowed the inhibitory element to a 40-nucleotide region located 251 nucleotides upstream of the start of the α-exon. This small deletion had a dramatic effect on exon inclusion in SNB-19 cells (an increase from 20 to 68%), with little change in exon inclusion in JEG-3 cells (80 versus 84%). Therefore, these constructs identify a role for a specific sequence, which we have termed ISS-1, in addition to intron size, as a cell-specific inhibitor of α-exon inclusion.

Fine Mapping of the Intronic Repressor of Splicing—To aid in the search for cis-regulatory elements, we concurrently obtained clones of the rat FGFR-1 gene in order to perform a sequence comparison. The conservation of sequences has been noted among many reported cis elements involved in the regulation of alternative splicing (17–20). This turned out to be the case for the FGFR-1 gene as well. The region deleted from pFGFR-D1 had 37 of 40 nucleotides conserved in both the human and rat genes, whereas the sequence flanking this region was considerably less conserved (data not shown). This suggested that the entire 40-nucleotide region might serve as a functional element. Comparison of this region with previously identified intronic regulatory elements revealed two functional possibilities: the repeated UGC motif and a possible PTB-binding motif (UCUU in a pyrimidine-rich environment) (15, 21–23). Another potential sequence of interest is the direct repeat GCUGCUacaGCUGCU (Fig. 3). To distinguish between the functional significance of these motifs, we generated additional deletion and mutation constructs. Deletion of the 5′ half of the region (pFGFR-D2) (Fig. 3) removed three of the four UGCs and the GCUGCUacaGCUGCU repeat while leaving the PTB-binding region intact. This deletion enhanced α-exon inclusion to a level similar to that observed for pFGFR-D1 (Fig. 3), suggesting that PTB is not directly responsible for the inhibition of α-exon inclusion recognition. However, a second deletion removing two of the four UGCs, half of the GCUGCUacaGCUGCU repeat, and the PTB-binding motif was also observed to elevate α-exon inclusion in transfected SNB-19 cells (pFGFR-D3) (Fig. 3). Unfortunately, the UGCs elements interspersed within the GCUGCUacaGCUGCU repeat are difficult to target specifically. Therefore, five additional constructs containing mutations scanning the 40-nucleotide region were created. Muta-
Alternative Splicing of the FGFR-1 RNA Precursor

**Fig. 2. Identification of a 40-nucleotide region that inhibits a-exon inclusion in SNB-19 cells.** Intronic deletions were performed in pFGFR-17 without changes to the flanking sequence (see Fig. 1). Construct deletions are depicted schematically, showing the size and location of the deletions relative to the beginning of the a-exon (size shown in nucleotides). The black box and gray shading localize a 40-nucleotide region that was deleted from constructs displaying elevated a-exon inclusion in SNB-19 cells. To the right of each diagram is the pFGFR clone number and the quantification of a-exon inclusion in JEG-3 (□) and SNB-19 cells (■) performed as described in Fig. 1. The values presented are for three independent transfections ± S.D. Representative autoradiographs of the transfection results are shown on the left.

**Fig. 3. Mutation of a repeat sequence within the intronic splicing inhibitor results in enhanced a-exon inclusion in SNB-19 cells.** Intronic deletions and mutations were performed in pFGFR-17 without changes to the flanking sequence (see Fig. 1). The schematic localizes and provides the specific sequence of the 40-nucleotide element required for a-exon exclusion. Three different sequence UGC motifs (underlined), a direct repeat (double line), and a PTB-binding site (solid line above sequence) are highlighted. Below the motifs are the specific deletions (−) and mutations (uppercase letters) found in the RNA of each construct. To the right of each diagram is the pFGFR clone number and the quantification of a-exon inclusion in JEG-3 (□) and SNB-19 cells (■) performed as described in Fig. 1. The values presented are for three independent transfections ± S.D.

The ISSs Are Functionally Redundant—We previously identified a 62-bp sequence (ISS-2) beginning 97 bp downstream of the a-exon, which, when deleted, also resulted in enhanced exon inclusion in SNB-19 cells (Fig. 4) (15). Like the ISS-1 sequence, mutation analysis found that exclusion of the a-exon in SNB-19 cells required the presence of a repeated UGC motif (Fig. 4). A comparison of the two elements found that they contained remarkable sequence homology in addition to the UGC motif (Fig. 4). This suggested that these two elements might be functionally redundant. To address this possibility, constructs were created in which the ISS-1 and ISS-2 elements were interchanged (Fig. 5). We first tested to see whether the ISS-2 would substitute for the ISS-1 element (pFGFR-101) (Fig. 5). In transfected SNB-19 cells, a-exon inclusion was nearly identical to that of the parent construct pFGFR-17 (22 versus 20%) (Fig. 5). This confirmed that the ISS-2 element could substitute for ISS-1. To determine whether the reverse was also true, an analogous replacement of the ISS-2 element with ISS-1 was performed (pFGFR-103) (Fig. 5). Again, the level of a-exon inclusion observed was nearly identical to the parent construct pFGFR-17 (26 versus 20%) (Fig. 5). In JEG-3 cells, the level of a-exon inclusion for both constructs was similar to pFGFR-17, and slightly lower compared with their deletion clones (Fig. 5). Finally, we simultaneously swapped the positions of the two elements (Fig. 5, pFGFR-105). Once again, the cell-specific pattern of a-exon inclusion continued to be maintained. Therefore, the ISS-1 and ISS-2 elements are clearly functionally redundant and function in a position independent manner. Furthermore, both elements appear to be required for the maximal exclusion of the a-exon in SNB-19 cells. Deletion of either the ISS-1 or ISS-2 element significantly enhances a-exon inclusion in SNB-19 cells, but removal of both elements results in a nearly 100% level of a-exon inclusion in both cell types (pFGFR-104) (Fig. 5). Therefore, it appears that in the context of the pFGFR-17 construct, each individual element does maintain some degree of its inhibitory function.

The Action of the ISS Elements Is Not Cell-specific—The
cells, the level of exon 4 inclusion dropped when both elements were simultaneously included (pPPT-109) (Fig. 6). In SNB-19 cells, a similar decrease in exon 4 inclusion was observed when the ISS-2 element was placed in the upstream intron did not decrease exon 4 inclusion in either cell line (pPPT-107) (Fig. 6). A similar decrease was seen in JEG-3 cells, indicating that the action of these elements is not cell-specific. The inhibition was specific to the a-exon. To the right of each diagram is the pFGFR clone number and the quantification of a-exon inclusion in JEG-3 (■) and SNB-19 cells (□) performed as described in Fig. 1. The values presented are for three independent transfections ± S.D. Representative autoradiographs of the transfection results are shown on the left.

**DISCUSSION**

In this study, we have identified a second intronic sequence that plays a key role in the regulated exclusion of the a-exon during processing of the FGFR-1 primary transcript. This element, which we have termed ISS-1, is located upstream of the a-exon, whereas the previously identified ISS-2 element is located in the downstream intron (see Fig. 4). The positioning of regulatory elements flanking the regulated a-exon seems appropriate. An emerging theme among exons that undergo alternative recognition is that their splice-site sequences are generally recognized less efficiently (1–4). Thus, regulation of exon recognition involves cell-specific enhancement or repression of a weak constitutive event. We have previously identified a unique exonic sequence that mediates cell-specific enhancement of a-exon inclusion (14). The data presented here indicate that recognition of the a-exon is also actively repressed using multiple elements to ensure exon skipping. Regulation of exon recognition by employing an active enhancer/repressor system has been noted for other alternatively recognized exons. Exonic silencing elements have been found in two human immunodeficiency virus genes and in bovine papillomavirus (26–28). In these examples, the repressor elements are juxtaposed with a splicing enhancer that is required for exon inclusion. It is thought that the binding of the repressor then acts to inhibit enhancer function. Multiple enhancer and repressor elements are also found for the FGFR-2 gene (29). One of the FGFR-2 elements also appears to have a dual function by acting to enhance inclusion of the upstream exon while simultaneously inhibiting downstream exon recognition (30). We have no evidence for either mechanism in a-exon splicing. Deletion analysis did not detect significant enhancer activity flanking the ISS-1 repressor (Fig. 2) or repressor sequences flanking the a-exon enhancer element (14). Additionally, both repressors function in a heterologous context suggesting that their inhibitory action is mediated through disruption of constitutive rather than enhancer-dependent exon recognition (Fig. 6).

A second class of repressors is mediated through the actions of PTB (31, 32). For a growing number of mammalian genes, a- and b-tropomyosin, GABA_A, receptor y 2, c-src, fibronectin, and a-actinin, the repression of exon recognition has been specifically linked to regulatory elements that bind PTB (22, 23,
ISS-1 and ISS-2 elements are functionally redundant. The prepro-tachykinin (PPT) gene expression clone is schematically depicted. The ISS elements are denoted by black boxes, and their insertion location is indicated relative to exon 4. In PPT-110 the ISS-1 contains the pPPT-M2 inactivating mutations, and the ISS-2 has the central TGCTGC repeat mutated (see Figs. 3 and 4). The numbers indicate the length of the exons or introns in base pairs. To the right of each diagram is the PPT clone number and the quantification of exon 4 inclusion in JEG-3 (□) and SNB-19 cells (■) performed as described in Fig. 1. The values presented are for three independent transfections ± S.D. Representative autoradiographs of the transfection results are shown on the left.

In this study, we have begun to address the underlying mechanisms involved in the regulated recognition of the α-exon during processing of the FGFR-1 RNA precursor. Whereas the FGFR-1 gene is widely expressed, recognition of the α-exon appears to be limited to the brain. It has been speculated that because inclusion of this exon results in a receptor form that has reduced affinity for ligands, this RNA splicing pattern may be important in end-stage differentiated cells (11, 12). This is further supported by the observation that glial cell malignancy is associated with a loss of RNA splicing regulation. Therefore, it is easy to infer that α-exon inclusion is highly regulated and that α-exon exclusion represents the default pathway. Our results support this conclusion; however, we have clearly established that recognition of the α-exon is actively repressed. The localization of repressor elements flanking the exon suggests a mechanism involving coordinated repression of both the 3′ and 5′ splice sites. This is also supported experimentally by the observations that deletion of a single ISS element is sufficient to enhance the level of α-exon inclusion and that both elements are required to inhibit PPT exon 4 inclusion. Because the ISS-1 and ISS-2 elements are functionally redundant, the trans-regulatory factors recognizing these elements would have to be capable of preventing productive association of both U2 snRNP and U1 snRNP at the their respective splice sites. Female-specific RNA splicing of the sex lethal protein in Drosophila utilizes just such a mechanism. Multiple repressor elements flanking the male-specific exon 3 are capable of binding the sex lethal protein. The RNA-bound sex lethal protein then interacts via sans-fille protein with U1 and U2 snRNP to prevent splicing (40). Inclusion of the male-specific exon 3 occurs in the absence of the "sex lethal" protein. For FGFR-1, an alternative mechanism must exist, as the ISS elements are capable of enhancing PPT exon 4 exclusion in JEG-3 cells, which predominantly include the α-exon. Because recognition of the α-exon requires the presence of an enhancer element, it would appear that this pathway is dominant over the inhibitory pathway. Precisely sorting out the regulatory mechanism by which the α-exon is differentially regulated will require determining the specific proteins that associate with these elements.

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