Alternative RNA processing of the human fibroblast growth factor receptor-1 transcript results in receptor forms that vary in their affinity for fibroblast growth factor. An alternative RNA processing event involving recognition of the α-exon is deregulated during neoplastic transformation of glial cells. We have previously established a splicing reporter/transfection cell culture model system to identify sequences involved in recognition of this exon. In this study, the system was used to identify two sequence elements that differentially function to regulate splicing of this exon. Exclusion of the α-exon in glioblastoma cells specifically required the downstream intron sequence comprising the 5′-splice site. Replacement or mutation of this sequence increasing complementarity to U1 RNA resulted in enhanced exon recognition in SNB-19 glioblastoma cells. Sequences within the exon were found to be required for α-exon inclusion. Deletion and gain-of-function experiments identified a 69-nucleotide exon sequence that was specifically required for α-exon inclusion. These studies indicate that multiple sequences are required for the regulated recognition of the α-exon.

Alternative processing of mRNA precursors provides an important method for creating genetic diversity. In many cases, the process is highly regulated such that unique mRNA forms are found to exist only in specific tissues or at a single developmental stage (1–3). The mechanisms involved in regulation of alternative RNA processing remain unclear for the most part. However, it has been speculated that different RNAs are likely to share common regulatory factors during alternative splicing. Therefore, deregulation of a single alternative splicing event would be predicted to have widespread cellular effects by disruption of other RNA splicing pathways employing the same regulatory factors.

We have continued to examine the mechanisms involved in alternative RNA splicing of transcripts derived from the fibroblast growth factor receptor-1 (FGFR-1) gene. This tyrosine kinase receptor is part of a four-gene family whose members are differentially activated by one or more of at least nine fibroblast growth factor (FGF) peptide ligands (4–6). The FGFRs have been implicated in many patterning events, including limb formation, keratinocyte organization, and brain development (7–9). The great diversity of function is created in part by alternative RNA processing of the FGFRs and ligands. For the FGFR-1 gene, alternative RNA processing of the primary transcript differentially alters ligand affinity, ligand specificity, membrane association, and tyrosine kinase activity (4–6, 10). Our interest has been in a regulatory event that affects ligand affinity. The regulated inclusion of a single exon termed “α” results in the production of a receptor with reduced affinity for FGF-1 and FGF-2 (11). This receptor form (FGFR-1α) contains three extracellular, immunoglobulin-like disulfide loops and is expressed predominantly in the normal adult brain. Recognition of the α-exon is disrupted by glial cell malignancy (12, 13). Instead, a receptor form lacking the α-exon (FGFR-1β) and containing only two extracellular, immunoglobulin-like disulfide loops is expressed. There appears to be a strict relationship between the degree of glial cell malignancy and RNA splicing to exclude the α-exon. The result is the predominant expression of a receptor form with higher ligand affinity in glioblastoma cells. This strong association of malignancy with disruption of an RNA processing pathway, as well as the potential involvement of the higher affinity receptor in tumor progression, prompted us to examine the mechanisms involved in greater detail.

We previously established a cell culture model to examine the regulatory sequences involved in regulated recognition of the FGFR-α-exon (14). A chimeric minigene containing 4 kilobases of the human FGFR-1 gene encompassing the α-exon was inserted into a splicing reporter construct. This chimeric gene displayed cell-specific recognition of the α-exon in JEG-3 cells and exclusion in SNB-19 glioblastoma cells. With this model, we narrowed the sequences required to maintain α-exon-regulated recognition to a 375-bp fragment inclusive of the exon. Here we have extended these studies to identify the specific regions of this sequence required for the inclusion and exclusion of the α-exon.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The human astrocytoma cell line SNB-19 was provided by Dr. Richard Morrison (University of Washington, Seattle, WA) and maintained as described previously (12). The human choriocarcinoma cell line JEG-3 was obtained from American Type Culture Collection (Rockville, MD) and maintained according to the suggested protocol.

**Plasmid Constructs**—Most constructs used in this paper were derived from pFGFR-17, pFGFR-31, pFGFR-32, and pFGFR-33, which have been described previously (14). Plasmids pFGFR-34 through pFGFR-39 were created by sequence-specific substitutions between pFGFR-32 and pFGFR-33 using PCR-generated products and intermediates. The construct pFGFR-40 introduced a two-nucleotide change into pFGFR-32, which improves the U1 snRNA complementarity to the 5′-splice site. In all cases, PCR was carried out in a 30-cycle reaction with an extended 5-min denaturing step and a 7-min elongation step. Each repeating cycle contained three steps: 1 min at 94 °C, 1 min at 30 °C.
55 °C, and 2 min at 72 °C. All cloning strategies took advantage of a unique upstream HindIII site and downstream Smal site in pFGFR-31, which were used to insert engineered exons into intron 1 of the splicing reporter construct. Changes in each clone were limited to the regions between these two sites.

The construct pFGFR-34 substitutes an α-specific upstream intron sequence with the analogous sequence found upstream of exon 4 in the FGFR-1 gene. The replacement was performed in multiple steps. Cloning took advantage of a PstI site comprising the α-exon 3′-splice site and a Smal site at the exon 4 splice site. The α-exon was amplified using primers FP15 and FP4 to replace the PstI site with a Smal site, and the PCR product was subcloned into pBluescript II KS (Stratagene, La Jolla, CA). Next, the intron sequence upstream of exon 4 was amplified with primers FP11 and FP12, digested with HindIII and Smal, and placed upstream of the α-exon in the clone described above. The α-exon clone containing the substituted 3′-splice region was then transferred to pFGFR-31 as a HindIII/Smal fragment. The generation of pFGFR-35 also required multistep cloning using a similar strategy. In this case, a PCR-generated fragment (FP13 + FP12) that replaced the Smal site in front of exon 4 with a PstI site was cloned into pBluescript II KS. The α-exon upstream intron was then introduced as an XbaI/PstI fragment.

Finally, a PCR product of FP2 and FP14 was inserted into pFGFR-31 to create pFGFR-35. Substitution of the α-specific downstream intron in pFGFR-36 was achieved by incorporation of the intron 4 sequence into pFGFR-31 in a direct insertion of the PCR product derived from FP9 and FP10. Insertion of the PCR product derived from FP11 and FP14 into pFGFR-31. Replacement of both upstream and downstream introns in pFGFR-38 was accomplished by PCR amplification of the cloning intermediate described above with primers FP11 and FP16, digested with HindIII and Smal, and inserted into the PstI/Smal sites of pBluescript intermediate containing the α-exon 3′-splice site. From this clone, a HindIII/Smal fragment was then transferred to pFGFR-31 to create the final construct.

The constructs pAVWT, pAVWT.BSC, pAVWT.GAR4, and pAVWT.MGAR4 were a generous gift of Dr. Thomas Cooper (Baylor College of Medicine) and have been described previously (15). The constructs pAVWT.a-69, pAVWT.a-72, and pAVWT.a-24 were created by insertion of cDNA sequence between BglII and the BstEII restriction site (BglII site was engineered) of the constructs pAVWT.a-69, pAVWT.a-72, and pAVWT.a-24, respectively. The α-exon fragments used for insertion were generated by digestion with BglII and BstEII restriction sites in these plasmids were created by the introduction of an AVWT-69, AVWT-24, where the insert was synthesized (primers FP11 and FP12) that replaced the 3′-splice region was then transferred to pFGFR-31 as a HindIII/Smal fragment. The generation of pFGFR-36 was accomplished by PCR amplification of the cloning intermediate described above with primers FP11 and FP16, digested with HindIII and Smal, and inserted into the PstI/Smal sites of pBluescript intermediate containing the α-exon 3′-splice site.

The cloning intermediate was then inserted into pBluescript II KS. The α-exon upstream intron was then introduced as an XbaI/PstI fragment. Finally, a PCR product of FP2 and FP14 was inserted into pFGFR-31 to create pFGFR-35. Substitution of the α-specific downstream intron in pFGFR-36 was achieved by incorporation of the intron 4 sequence into pFGFR-31 in a direct insertion of the PCR product derived from FP9 and FP10. Insertion of the PCR product derived from FP11 and FP14 into pFGFR-31. Replacement of both upstream and downstream introns in pFGFR-38 was accomplished by PCR amplification of the cloning intermediate described above with primers FP11 and FP16, digested with HindIII and Smal, and inserted into the PstI/Smal sites of pBluescript intermediate containing the α-exon 3′-splice site.

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From this clone, a HindIII/Smal fragment was then transferred to pFGFR-31 to create the final construct.
Splicing of the FGFR-1 RNA Precursor

RESULTS

Cell-specific Splicing of the FGFR-1 a-Exon Is Maintained by Minimal Sequence—Previous studies found that the exclusion of the a-exon from the final FGFR-1 mRNA shows a strong correlation with glial cell malignancy (12, 13). To examine the mechanism of this process, we established a cell culture model capable of cell-specific recognition of the a-exon during RNA processing of minigene-derived transcripts. The construct pFGFR-17 contains a 4-kilobase fragment of the FGFR-1 gene inserted into the splicing reporter RSV/nMT2 (14) (Fig. 1A). Transcripts derived from this construct show a-a-exon-specific inclusion in JEG-3 cells (75%) and exclusion in SNB-19 cells (the exon was included in only 20% of the transcripts) (Fig. 1B). Substitution of the majority of the FGFR-1 gene sequence flanking the a-exon was done to create the construct pFGFR-32 (Fig. 1). Examination of transcripts derived from this construct revealed no significant change in the inclusion phenotype observed in JEG-3 cells (75% versus 73%) and revealed an increase in the level of a-exon inclusion in SNB-19 cells (from 20 to 37%). The results support a role for flanking sequence a-exon exclusion and suggest that the key components of the cell-specific exon inclusion pathway are localized to the remaining sequence. Replacement of the a-exon and its flanking sequence with an analogous sequence from exon 4 of the FGFR-1 gene (pFGFR-33) resulted in a similar level of exon inclusion in transcripts derived from both cell types (66% versus 58%). Therefore, comparison of the splicing patterns for these clones suggests that the FGFR-1 sequence in pFGFR-32 contains elements required for cell-specific recognition of the a-exon, but additional sequences might function in the exclusion pathway. This observation is consistent with our previous findings for these three constructs (14).

FGFR-1 Gene Sequence Requirements for a-Exon Skipping in SNB-19 Glioblastoma Cells—The cell-specific RNA splicing pattern of transcripts derived from pFGFR-32 suggests that this process is regulated. It is unclear whether sequences within pFGFR-32 act to inhibit a-exon splicing in SNB-19 cells, enhance exon inclusion in JEG-3 cells, or both. To examine the possible role of FGFR-1 sequences in the regulation of a-exon recognition, we created a series of substitution constructs. The FGFR-1 sequence of pFGFR-33 served as a donor for these substitution constructs because exon 4 did not show the same cell-specific pattern of splicing, although the overall level of exon inclusion was reduced in the context of the splicing reporter (Fig. 1). In addition, the splicing of this exon was unaffected by malignant transformation (data not shown). Replacement of an upstream intron (pFGFR-34) or swapping of exon sequence (pFGFR-35) had no effect on the level of inclusion in SNB-19 cells (Fig. 2A). However, replacement of a downstream intron (pFGFR-36) resulted in ~20% enhancement of a-exon inclusion in SNB-19 cells (Fig. 2A).

The enhanced inclusion observed for pFGFR-34 can be explained by the removal of a specific inhibitory element, a change in the strength of the 5′-splice site, or a combination of both. To provide additional support for the relevance of this sequence, a-exon sequences were substituted into pFGFR-33. Replacement of an upstream intron (pFGFR-37) or exon 4 (pFGFR-38) did not alter the level of exon inclusion in SNB-19 cells, suggesting that neither contained sequences inhibitory to splicing (Fig. 2B). However, replacement of the downstream intron sequence (pFGFR-39) resulted in a significant drop in the level of exon inclusion (Fig. 2B). This result is consistent with the findings for pFGFR-36 and supports a role for this sequence in a-exon exclusion. In an effort to distinguish between possible mechanisms, we introduced mutations into the a-exon 5′-splice site that increase complementarity to U1 snRNA (pFGFR-40) (Fig. 2C). Previous studies have demonstrated a direct correlation between the number of contiguous base pairs and the level of exon inclusion (16). We also transfected constructs into JEG-3 cells in order to differentiate cell-specific effects from generalized inhibitory effects. Transfection of pFGFR-40 into SNB-19 and JEG-3 cells showed that a-exon recognition was nonspecifically increased in both cell types (Fig. 2A), an observation consistent with enhanced U1 snRNA base pairing. Enhanced a-exon inclusion for pFGFR-36 was also nonspecific and observed in both cell types, as was the repression of exon 4 recognition for pFGFR-39 (Fig. 2, A and B). The predicted U1 snRNA base pairing for these 5′-splice sites is shown in Fig. 2. The 5′-splice site substitutions found in pFGFR-36 and pFGFR-40 increased U1 snRNA base pairing and decreased the U1 snRNA base pairing in pFGFR-39 (Fig. 2C). These results suggest that the “weaker” 5′-splice site is specifically required for exon skipping in SNB-19 cells. The finding that splice site changes had similar effects in both cell types indicates that this region is not likely to contain a cell-specific inhibitory element, although we cannot rule out a complex interaction requiring multiple sequences.

FGFR-1 Gene Sequence Requirements for a-Exon Inclusion in JEG-3 Cells—While the a-exon is weakly recognized in SNB-19 cells, the same 5′-splice site is efficiently utilized in JEG-3.
cells. In the absence of a cell-specific inhibitory element, 5'-splice site recognition in JEG-3 cells is likely to be facilitated by an enhancer element. To address this possibility, the substitution constructs were transfected into JEG-3 cells, and the level of exon inclusion was examined. Substitution of the 5'-splice site (see “Experimental Procedures”) B, shown is the substitution of sequences in pFGFR-33 with regions from pFGFR-32. Substituted regions are indicated by single lines. Experiments were performed as described in the legend to Fig. 1. Results from the quantification of α-exon inclusion in SNB-19 (black bars) and JEG-3 (shaded bars) cells are shown on the right. The values presented are the means ± S.D. of three independent transfections. C, the sequences comprising the 5'-splice sites and the predicted base pairing with U1 snRNA are shown for key constructs. The exon/intron boundary is indicated by colons. Standard base pairing is indicated by vertical lines, and G/U base pairing is indicated by dots.

significant decrease in exon inclusion was seen for pFGFR-34 (Fig. 2A); however, we cannot rule out a complex regulatory effect, i.e., redundant regulatory elements. Therefore, it is not possible to conclusively assign enhancer activity to either the upstream intron or the α-exon.

To overcome problems associated with the pFGFR-35 construct, we chose to create constructs containing internal deletions of the α-exon. Deletion of the majority of the α-exon (pFGFR-41) resulted in a dramatic reduction in exon inclusion (Fig. 3). Sequence analysis confirmed that no mutations had been introduced into either splice site during cloning. To localize the region containing the enhancer activity, a series of constructs that added back portions of the α-exon were examined. The inclusion of 69 additional nucleotides 5' of α-exon (pFGFR-42) was found to restore exon inclusion to a near wild-type level (Fig. 3). However, when more sequence was included (pFGFR-43), a slight, but not significant, reduction in the level of inclusion was found (Fig. 3). A similar pattern was observed when α-exon sequence was added from the 3'-end of the deletion. The addition of 24 nucleotides (pFGFR-44) nearly restored α-exon inclusion, and the same central sequence (pFGFR-45) caused a slight reduction in inclusion (Fig. 3). Finally, independent deletion of the central region (pFGFR-46) confirmed that this sequence is not required for α-exon inclusion in JEG-3 cells (Fig. 3). Together, these results suggest a complex exon organization involving at least two enhancer elements and a possible inhibitory sequence. The presence of a single enhancer appears sufficient to restore α-exon inclusion as demonstrated by both addition and deletion of either the 69- or 24-nucleotide sequence. However, we cannot rule out the possibility that the differences in splicing might be mediated by changes in exon size.

**FGFR-1 Gene α-exon Sequence Is Capable of Functioning as a Splicing Enhancer in a Heterologous Gene**—The observation that deletions of the α-exon reduce inclusion of this exon supports a role for the presence of an enhancer element. To specifically characterize which α-exon sequence(s) were capable of enhancer function, we chose to look for “gain of function” in a previously described splicing reporter construct (15). The pa-
Fig. 4. α-exon sequence provides enhancer activity in a heterologous splicing reporter. A, schematic depiction of constructs used in the transfection experiments described below. The shaded boxes and thin lines represent sequence derived from the avian sarcoma virus env gene fused to the RSV enhancer/promoter and containing a downstream actin polyadenylation signal. Constructs are identical except for the sequences identified by the open boxes inserted 30 nucleotides downstream of the 3′-splice site. The purine-rich enhancer, GAR4, and mutated enhancer sequence, MGAR4, have been previously described (15). The inserts α-69, α-72, and α-24 were derived from the α-exon regions deleted in Fig. 3 (see "Experimental Procedures"). A 71-nucleotide fragment of multilinker DNA was used as a size control. B, examination of splicing enhancer activity in RNA isolated from cells transfected with these constructs. RT-PCRs were performed 72 h after transfection using a 32P-end-labeled primer (see "Experimental Procedures"). Reaction products were separated by polyacrylamide gel electrophoresis. A representative autoradiograph is shown. The RT-PCR bands representing the spliced and unspliced RNA products are indicated. Similar results were obtained for three independent transfection experiments.

Alternative processing of the FGFR-1 RNA transcript to include the α-exon is a pathway nearly exclusive to the brain. Therefore, it is curious that deregulation of this splicing event coincides with malignant transformation of glial cells. We had previously established a model system that demonstrated cell-specific recognition of the α-exon (14). That study identified a minimal sequence unit comprising the α-exon, with 97 bp of upstream sequence and 11 bp of downstream sequence (pFGFR-32), as containing all necessary elements for regulated exon recognition. In this paper, we have attempted to identify the sequences contained within this 375-bp fragment that are responsible for exon skipping in SNB-19 cells and exon inclusion in JEG-3 cells. One consideration in performing this analysis is that our fragment contains the 3′- and 5′-splice sites, which are constitutively required for RNA splicing. Therefore, it is not possible to perform a simple deletion of intron sequence flanking the α-exon, as it would remove these required elements. To avoid this problem, we performed substitutions. Exon 4 of the FGFR-1 gene is included in all cell types shown to express the gene (5). When this exon and analogous flanking sequence were placed in an identical context (pFGFR-33), the exon did not display cell-specific regulation. The exon was skipped at a constant level, suggesting that recognition was weakened by the context of our splicing reporter. As weakly recognized splice sites are a prerequisite for alternative splicing, these sequences provided an ideal candidate for our substitution experiments.

We first turned our attention to the question of why the α-exon is skipped in SNB-19 cells. The possibilities to consider are the presence of an inhibitory element, the absence of an enhancer, or both. The first mechanism would function through a unique silencer element, whereas an enhancer-based mechanism requires that the exon be weakly recognized through its splice sites. Sequence substitutions between the α-exon and exon 4 (Fig. 2) implicated the downstream intron sequence. When this region was replaced or mutated, a significant enhancement of the α-exon was observed. Consistent with this
Fig. 5. FGFR-1 flanking intron sequence modulates α-exon enhancer activity. A series of constructs containing deletions of the α-exon within pFGFR-17 (see Fig. 1) are depicted schematically on the left. Deletions are indicated by open regions, and the sizes of the deletions in the base pairs are shown. The remainder of the construct is unchanged. Experiments were performed as described in the legend to Fig. 1. The graph provides quantification of α-exon inclusion in transfected SNB-19 (black bars) and JEG-3 (shaded bars) cells. The values presented are the means ± S.D. of three independent transfections. A representative autoradiograph is shown on the right; arrows identify the RT-PCR products, which include (I) or skip (S) the α-exon.

observation, when the α-exon 3′-intron was placed downstream of exon 4, it was skipped (Fig. 2B). Furthermore, there is a direct correlation between the level of α-exon inclusion and predicted U1 snRNA base pairing. The constructs pFGFR-36 and pFGFR-40, which have the same number of U1 snRNA base pairs, show similar levels of α-exon inclusion in SNB-19 cells. The construct pFGFR-39, which has the lowest level of exon inclusion, also contains the least number of predicted U1 snRNA base pairs (Fig. 2A). Combined, these data support a role for a weak 5′-splice site in the α-exon skipping in SNB-19 cells. However, while there is good correlation with exon inclusion and base pairing, we cannot rule out that this effect is coincidental and that these changes are interfering with a negative regulatory domain.

While the 5′-splice site clearly plays an important role in α-exon skipping, it is not the sole element involved. Several observations suggest the presence of additional factors. First, while substitution or mutation of the 5′-splice site enhanced α-exon inclusion in SNB-19 cells, the same construct expressed in JEG-3 cells consistently showed higher inclusion. This may simply represent overall enhanced splicing ability of the JEG-3 cells or the presence of additional inhibitory factors in the SNB-19 cells. The gain-of-function experiments using a constitutive enhancer (GAR4) (Fig. 4) provide support for the former hypothesis. However, the presence of additional FGFR-1 flanking intron sequence clearly affects the level of α-exon inclusion. In SNB-19 cells, there is a significant and reproducible increase in the level of α-exon inclusion for pFGFR-32 compared with pFGFR-17 (37% versus 20%) (Fig. 1). An even greater difference in the level of α-exon inclusion can be attributed to flanking intron sequence when comparing pFGFR-45 (65% inclusion) (Fig. 3) with pFGFR-47 (25% inclusion) (Fig. 5). In support of these findings, we have recently identified a sequence within the upstream intron that appears to be required for maximum repression of α-exon inclusion in SNB-19 cells.2 These observations indicate that the additional repressor sequences may be required to enforce α-exon exclusion in the presence of reduced enhancer activity.

The weak 5′-splice site of the α-exon identifies the requirement for an enhancer element to promote inclusion in JEG-3 cells. Two regions were implicated by the substitution constructs. Replacement of the α-exon (pFGFR-35) reduced exon inclusion, whereas the α-exon 3′-intron was capable of enhancing recognition of a heterologous exon 4 (pFGFR-37) (Fig. 2). The inverse swaps did not support these findings. The α-exon was not included at a higher level than exon 4 (compare pFGFR-33 with pFGFR-38), and replacement of the α-exon 3′-intron did not cause a significant reduction in exon inclusion (compare pFGFR-34 with pFGFR-32) (Fig. 2). These contradictory results only serve to emphasize the complex relationship of splicing elements and splice sites. Exon enhancers typically function to facilitate 3′-splice site recognition when one or both splice sites are weak (15, 17, 18). Also, the need for these enhancers can be overcome by increasing the U1 snRNA complementarity to the downstream 5′-splice site, consistent with serine-arginine-rich our findings in both cell lines.

Exon enhancer elements are believed to function through the binding of serine-arginine-rich proteins, which then act to stabilize interaction between the factors associated with splice sites flanking the exon (3, 19, 21). Two major classes of non-specific exon enhancer elements have been identified, purine-rich and A/C-rich sequences (19, 21, 22). Examination of the α-exon sequence revealed several potential enhancer sequences of both types. Deletion of α-exon sequence confirmed the presence of enhancer elements within this exon. A 165-base pair internal deletion of the α-exon showed a dramatic reduction in exon inclusion in JEG-3 cells. Further deletion analysis failed to localize this effect to a single region, suggesting a redundancy of enhancer elements, although we cannot rule out an effect of exon size. Inclusion of the α-exon could be restored by the addition of as few as 24 bp, but the reciprocal deletion experiments did not result in exon exclusion (compare pFGFR-43 and pFGFR-44) (Fig. 3). A similar result was observed when the 69-bp sequence was deleted in pFGFR-45 (Fig. 3). To directly address the possibility of multiple enhancer elements, we examined the ability of the individual sequences to provide enhancer function in a previously characterized heterologous splicing reporter (μAVWT.BSC) (15). These gain-of-function experiments identified two regions as capable of enhancer function, a 69-nucleotide sequence and a 72-nucleotide sequence (Fig. 4). While deletion experiments suggest that the 72-nucleotide sequence is not required for α-exon recognition, this sequence clearly functions as a potent enhancer. This enhancer activity is not surprising as we identified five purine-rich elements within the sequence. It is intriguing, however, that in the absence of the 69-nucleotide element and in the presence of flanking intron sequence (pFGFR-47) (Fig. 5), this sequence does not function as an enhancer. The converse deletion of the 72-nucleotide sequence also had no effect on α-exon splicing.

2 W. Jin, E. S.-C. Huang, W. Bi, and G. J. Cote, unpublished observation.

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Therefore, it would appear that the 72-nucleotide enhancer is dispensable and that the 69-nucleotide enhancer is critical for α-exon inclusion.

The 69-nucleotide sequence does not contain purine- or A/C-rich sequences. Data base searches failed to identify any extensive sequence homologies for the 69-nucleotide region outside of the FGFRs. This is not unexpected given the relatively small size of most reported regulatory sequences. Whether the 69-nucleotide region contains sequences representing a new class of constitutive enhancer elements or is indeed a cell- or gene-specific splicing enhancer remains to be addressed. The gain-of-function experiments clearly suggest that the JEG-3 cell line has greater enhancer activity compared with the SNB-19 cell line. However, the deletion experiments performed in the pFGFR-17 construct indicate that intron sequences may modulate some of the enhancer effects. It will be interesting to determine if the FGFR-1 intron has similar effects in the context of the heterologous splicing reporter (μAVWT). It is also unclear if the 69-nucleotide sequence is the only element required for α-exon inclusion. There clearly are multiple sequences within the α-exon sequence that function as enhancers. This is not an uncommon theme among enhancer-regulated genes. The best characterized example is the female-specific inclusion of exon 4 of the Drosophila doublesex gene (23, 24). This exon contains six repeats that are recognized by the splicing factor TRA-2 and a single purine-rich enhancer that is thought to stabilize interactions through the recruitment of serine-arginine-rich proteins (23, 24). While maximal inclusion of exon 4 requires the presence of all enhancer sequences, this effect is additive, and only a single enhancer is required to maintain exon recognition (24). A similar complex relationship may exist for tissue-specific recognition of the α-exon.

In this study, we used a reporter/transfection model system to identify cis-elements involved in the “cassette-type” recognition of the FGFR-1 α-exon. The exon skipping phenotype in SNB-19 glioblastoma cells requires a “weak” 5′-splice site; enhancers within the α-exon facilitate inclusion in JEG-3 cells. Both elements are required, but are not sufficient for cell-specific recognition of the α-exon. Clearly, no single sequence directly mediates the regulation of α-exon splicing. However, we feel justified to suggest a mechanism in which a reduction of enhancer function in glioblastoma cells results in α-exon skipping. Both the constitutive (purine-rich) and the α-exon (α-69) enhancer activities were found to be significantly reduced in SNB-19 cells (Fig. 4). The exclusion of α-exon in SNB-19 cells is likely to be enforced by repressor regulatory sequences acting in addition to the suboptimal 5′-splice site. A complex mechanism involving multiple intron enhancer and repressor elements has been proposed to explain the regulated inclusion of the exons that alternatively code for the third Ig domain of the FGFR-2 gene (20, 25). Whether a similar complexity is required for inclusion of the α-exon remains to be elucidated. Identification of the exon enhancer sequences, however, provides us with key information to begin to address this possibility.

REFERENCES

1. Adams, M., Rudner, D., and Rio, D. (1996) *Curr. Opin. Cell Biol.* 8, 331–339
2. Chabot, B. (1996) *Trends Genet.* 12, 472–478
3. Manley, J. L., and Tacke, R. (1996) *Genes Dev.* 10, 1569–1579
4. Green, P., Walsh, F., and Dobert, P. (1996) *Bioessays* 18, 639–646
5. McKeehan, W. L., and Kan, M. (1994) *Mol. Reprod. Dev.* 39, 69–81
6. Johnson, D. E., and Williams, L. T. (1993) *Adv. Cancer Res.* 60, 1–41
7. Rubin, J., Bottaro, D., Chedid, M., Miki, T., Ron, D., Cheon, G., Taylor, W., Fortney, E., Sakata, H., and Finch, P. W. (1995) *Cell Biol. Int.* 19, 399–411
8. Mason, I. (1996) *Curr. Biol.* 6, 672–675
9. Webster, M. K., and Dimmohwee, D. J. (1997) *Trends Genet.* 13, 178–182
10. Fernig, D. G., and Gallagher, J. T. (1994) *Prog. Growth Factor Res.* 5, 353–377
11. Wang, F., Kan, M., Yan, G., Xu, J., and McKeehan, W. (1995) *J. Biol. Chem.* 270, 10251–10255
12. Yamaguchi, F., Saya, H., Bruner, J., and Morrison, R. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 484–488
13. Morrison, R., Yamaguchi, F., Saya, H., Bruner, J., Yahanda, A., Donehower, L., and Berger, M. (1994) *J. Neuro-oncol.* 18, 207–216
14. Cote, G. J., Huang, E. S.-C., Jin, W., and Morrison, R. S. (1997) *J. Biol. Chem.* 272, 1054–1060
15. Xu, K., Teng, J., and Cooper, T. (1993) *Mol. Cell. Biol.* 13, 3660–3674
16. Kuo, H., Nasim, F., and Grabowski, P. (1991) *Science* 251, 1045–1050
17. Inoue, K., Ohno, M., and Shimura, Y. (1995) *Gene Expr.* 4, 177–182
18. Watakebe, A., Tanaka, K., and Shimura, Y. (1995) *Genes Dev.* 7, 407–418
19. Fu, X. (1995) *RNA* 1, 663–680
20. Carstens, R. P., McKeehan, W. L., and Garcia-Blanco, M. A. (1995) *Mol. Cell. Biol.* 15, 2205–2217
21. Hertel, K., Lynch, K., and Maniatis, T. (1997) *Curr. Opin. Cell Biol.* 9, 350–357
22. Coulter, L., Landree, M., and Cooper, T. (1997) *Mol. Cell. Biol.* 17, 2143–2150
23. Lynch, K. W., and Maniatis, T. (1996) *Genes Dev.* 10, 2089–2101
24. Hertel, K. J., and Maniatis, T. (1998) *Mol. Cell.* 1, 449–455
25. Del Gatto, F., Plet, A., Gesnel, M.-C., Fort, C., and Breathnach, R. (1997) *Mol. Cell. Biol.* 17, 5106–5116