Sequence Requirements for Regulated RNA Splicing of the Human Fibroblast Growth Factor Receptor-1 α Exon*

(Received for publication, May 25, 1996, and in revised form, August 20, 1996)

Gilbert J. Cote‡§, Eileen S-C. Huang‡, Wei Jin‡, and Richard S. Morrison¶

From the §Section of Endocrinology, The University of Texas, M. D. Anderson Cancer Center, Houston, Texas 77030 and ¶Department of Neurological Surgery, University of Washington, Seattle, Washington 98195

Progression of astrocytes from a benign to a malignant phenotype is accompanied by a change in the RNA processing of the fibroblast growth factor receptor 1 (FGFR-1) gene. The level of a high affinity form of the FGFR-1 is dramatically elevated as a result of α-exon skipping during RNA splicing. In this paper we have been able to duplicate this tumor-specific RNA processing pathway by transfection of a chimeric minigene containing a 4-kilobase fragment of the human FGFR-1 gene (including the α-exon) into a variety of cell lines. In a transfected human astrocytoma cell line, α-exon skipping was consistently observed for RNA transcripts derived from both the chimeric minigene and endogenous gene expression. This exon skipping phenotype was dependent on the size of the flanking intron as deletions which reduced the introns to less than ~350 base pairs resulted in enhanced α-exon inclusion. Increased exon inclusion was not sequence-specific as exon skipping could be restored with insertion of nonspecific sequence. Cell-specific exon recognition was maintained with a 375-nucleotide sequence inclusive and flanking the α-exon, provided that intron size was maintained. These results identify the minimal cis-regulatory sequence requirements for exclusion of FGFR-1 α-exon in astrocytomas.

There are several mechanisms known to be involved in the malignant transformation of cells. One mechanism frequently overlooked is the disruption of pathways involving regulated RNA processing. Several different genes in multiple tumor types have demonstrated alterations in their RNA splicing pathways. Alterations in RNA processing fall into two broad categories, those involving cis effects where a specific gene is mutated and those believed to be trans-related because they lack a detectable mutation. The p53, BRCA1, hMLH1, and hMSH2 genes are examples of the former (1–7). Mutations in the splice site sequences of these genes have been associated with Li Fraumeni cancer syndrome, breast and ovarian cancer, and hereditary non-polyposis colorectal cancer (1–7). Genes without definable mutations but which have RNA processing changes associated with malignancy serve primarily as tumor markers or indicators of metastatic potential. In many cases these changes are peripheral and believed to result from the transformation process rather than be a contributing factor. For example, mutations in the RET protooncogene are the initiating event in transformation of the thyroid C-cell, but one outcome of transformation is a deregulation of calcitonin/calcitonin gene-related peptide alternative RNA processing (8–10). In other genes it is possible that alterations in RNA processing pathways may specifically contribute to the initiation or maintenance of the malignant phenotype. In breast cancer, several aberrant mRNA forms are observed for the estrogen receptor gene (11–13). Exon skipping results in receptor forms without DNA-binding or ligand-binding domains. The presence of dominant negative and positive receptor forms are believed to play a key role in estrogen-dependent cell proliferation as well as chemotherapeutic intervention with anti-estrogens (11–13). Finally, the CD44 gene product is believed to play a key role in tumor cell metastasis. Products of CD44 gene created by alternative RNA splicing are associated with increase tumor invasiveness and have been shown to enhance tumorigenicity (14, 15).

The focus of this paper involves changes in the RNA splicing patterns associated with astrocyte-derived neoplasms of the central nervous system. It is hypothesized that the genesis of these tumors occurs as a multistep progression from benign astrocytoma to anaplastic astrocytoma, and finally, to glioblastoma multiforme. In this transformation numerous and, as of now, poorly understood cytogenetic and biochemical changes take place (16). Recent studies have been directed toward defining the genes and gene products responsible for glial tumorigenesis and progression. Among the several growth factors and growth factor receptors that are activated or overexpressed in glial tumors, the fibroblast growth factors (FGF§) and their cognate receptors (FGFRs) are believed to play key roles in the maintenance and possibly progression of tumorigenicity (17–20). Four structurally related genes encoding high affinity receptors have been identified (21–23). Features common to members of the FGFR family include a signal peptide, two or three immunoglobulin (Ig)-like loops in the extracellular domain, a hydrophobic transmembrane domain, and a highly conserved tyrosine kinase domain split by a short kinase insert sequence. Given the structural similarities, it is not surprising that multiple FGFRs can bind multiple FGFs. However, there are several reports of cell- and tissue-specific expression and responsiveness to different FGF family members (21–25). One mechanism responsible for generating selective responsiveness

* The abbreviations used are: FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; hMT, human metallothionein; RSV, Rous sarcoma virus; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; bp, base pair(s); kb, kilobase pair(s).

† This work was supported in part by United States Public Health Service Grant CA67946 (to G. J. C.) and an American Cancer Society grant (to R. S. M.). DNA sequencing and oligonucleotide synthesis was provided by M. D. Anderson Core Facilities, and additional funds (to G. J. C.) were derived from United States Public Health Service Grant CA67946 (to G. J. C.) and an American Cancer Society Research Grant 2P30-CA16672. The costs of publication of this article were U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Section of Endocrinology, Box 15, Houston, TX 77030. Tel.: 713-792-2840; Fax: 713-794-4065; E-mail: gilbert_cote@isqm.mda.uth.tmc.edu.

§Towhomcorrespondenceshouldbeaddressed:M.D.AndersonCancerCenter,1515HolcombeBlvd.,SectionofEndocrinology,Box15,Houston,TX77030.Tel.:713-792-2840;Fax:713-794-4065;E-mail:gilbert_cote@isqm.mda.uth.tmc.edu.
to different FGF family members is alteration of the ligand binding domain by alternative RNA splicing (21–23). It is clear that for FGFR-1 and FGFR-2 alternative processing of the RNA precursor generates multiple mRNAs which produce receptors manifesting different ligand binding specificities and affinities (21–23). The role that these receptor forms play in development and cell growth is currently an active area of investigation.

Alternative splicing of the FGFR-1 RNA has recently been associated with astrocyte malignancy (26). For this gene, changes in the number and not the amino acid composition of the extracellular Ig-like loops correlates with astrocyte malignancy. An examination of graded astrocytic tumors revealed that there was a switch from a three Ig-like domain form (FGFR-1α) to a two Ig-like domain form (FGFR-1β) during the progression to malignancy (26). The production of the two Ig-like domain form of FGFR-1 (FGFR-1β) is the result of exon skipping (see Fig. 1). Normal human adult and fetal brain expressed a form containing three Ig-like disulfide loops (FGFR-1α). While the functional consequence of a shift in alternative RNA splicing from the α-form to the β-form of the FGFR is not entirely understood, FGFR-1β has been shown to exhibit a 10-fold greater affinity for acidic and basic FGF than FGFR-1α (27, 28). If this is true, then glioma cells expressing FGFR-1β would be more responsive to FGF than nontransformed cells or low-grade astrocytoma cells expressing primarily FGFR-1α. These types of structural differences may impart a growth or invasiveness advantage to glioma cells expressing FGFR-1β.

A better understanding of the mechanism(s) involved in recognition of the α-exon may lead to the identification of factor(s) directly involved in astrocyte transformation. We believe that the change in α-exon recognition results from a change in RNA processing factors and not gene mutation. Analysis of DNA sequence derived from the SNB 19 human astrocytoma cell line failed to find mutations within the α-exon and its flanking introns. Therefore, in an effort to begin clarification of the mechanisms involved in FGFR-1 RNA splicing, we have established a cell culture model system which mimics the RNA processing decisions observed in normal and malignant brain tissue. With this system we have determined that intron size plays a key role in the α-exon skipping phenotype and have defined a 375-bp minimal region that is required to maintain cell-specific RNA splicing.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—The following human cell lines T98G (glioblastoma), NTERA-2 cl.D1 (NT2/D1) (Pluripotent embryonal carcinoma), FPSK-1 (Primitive neuroectodermal tumor), and JEG-3 (Choriocarcinoma) were obtained from American Type Culture Collection (Bethesda, MD). These cultures were all maintained in monolayer cultures using standard methodology and conditions recommended by the ATCC. The human astrocytoma cell line SNB 19 was maintained as described previously (26).

**Gene Mapping**—All gene mapping studies were performed on the human genomic P1 clone DMPC-HFF#1-4690E obtained from Genome Systems, Inc. (St. Louis, MO). The oligonucleotide primers that were provided to Genomic Systems map to the α-specific exon for FGFR-1 gene (αF and αR). DMPC-HFF#1-4690E was one of three clones received from their screening. This clone was subjected to BanHI and BglII digestion, and the resultant fragments were subcloned into pGEM 4 or pUC 19 by standard methodology. Three subclones were obtained by digestion, and the resultant fragments were subcloned into pGEM 4 or pUC 19. pGFR-3 contains an ~4-kb BamHI fragment containing the α-exon inserted into pGEM 4. All the above inserts were derived from DMPC-HFF#1-4690E. The parental minigene splicing construct pFGFR-17 was generated using the BamHI fragment of pGFR-3 containing the α- and γ-exons into the BglII site in intron 1 of a human metallothionein 2A minigene (pSVShM2A) (see Fig. 2A) (29). Deletions of the 3′ end of the insert were created using restriction sites within the BamHI insert. The clones pGFR-18 (XhoI to Smal) and pGFR-19 (SacI to Smal) were deletions of pGFR-17. The clone pGFR-20 is a BamHI to BglII fragment constructed by inserting into the BglII site of pSVShMT2A. In the “suffer” clone pGFR-21, the ApaI to Smal region containing the γ-exon has been replaced with an ApaI to Smal fragment from pGFR-3. The placement sequence is derived from intronic sequence located ~1.4 kb upstream of the α-exon. The fragment is inserted in the antisense orientation. Deletions of the 5′ end of the BamHI insert were created using the following restriction sites: BglII (pGFR-22), BstXI (pGFR-23), MseI (pGFR-24). These clones are a BamHI to Smal fragment from pGFR-3. The replacement sequence is derived from intronic sequence located ~1 kb upstream of the α-exon. The fragment is inserted in the antisense orientation. The construct pGFR-26 has a BglII fragment derived from pGFR-3 inserted into the BglII site of pSVShMT2A. Constructs pGFR-27, pGFR-28, pGFR-29, and pGFR-30 all contain the same stuffer sequence used to create pGFR-20. A second series of stuffer sequences made was used to derive the 5′-flanking region of the FGFR-1 gene (approximately -4200 to -700). The construct pGFR-31 was made by insertion of an ~3500-bp BamHI to SacI fragment of pGFR-1 into the BglII and Smal sites of pSVShMT2A, followed by deletion of a HindIII site contained within vector multilinker. The plasmids pGFR-32 and pGFR-33 were constructed by insertion of PCR-generated fragments containing α-exon (primers FP-2 and FP-4) and FP-11 and FP-12) sequences, respectively, into the HindIII and Smal sites of pGFR-31 (see Fig. 7). In some cases multiple cloning steps and/or partial restriction digestions were required to obtain the desired plasmid construct. Specific details describing the construction of each plasmid are available upon request.

**Transfections**—All transfections were performed using Lipofectamine® according to protocols suggested by the manufacturer (Life Technologies, Inc.). Briefly, cells were plated in duplicate on 100-mm dishes and allowed to grow to ~80% confluency prior to transfection. The DNA/Lipofectamine® mixture (10 µg/40 µl) was allowed to associate at room temperature for 40 min. This mixture was then added to 4 ml of serum-free medium, which then replaced the culture medium. Transfection was allowed to proceed for 6 h prior to switching to medium containing serum. Total RNA was isolated 72 h after transfection by RNAzol® extraction as suggested by the manufacturer (TEL-TEST, Friendswood, TX). All results are representative of at least three transfections for each plasmid.

**Reverse Transcription-Polymerase Chain Reactions (RT-PCR)**—All RT-PCR reactions were performed as described previously with minor modifications (30, 31). Both the reverse transcription and PCR steps were prepared in the same tube. Five µg of total RNA was used for each RT-PCR. The reverse transcription reaction was performed using the downstream PCR primer (Endo R, HMT3, or HMT2/3). PCR reactions were performed by diluting the RT reaction with a mixture containing 20-P-end-labeled upstream primer (Endo F or DS8). Each amplification cycle consisted of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min. PCR reactions were performed for 16 amplification cycles which were empirically determined to be in the linear range for the downstream primers and target sequence. PCR products were analyzed by polyacrylamide gel electrophoresis and visualized by autoradiography. The identity of all PCR products was confirmed by both restriction enzyme analysis and DNA sequencing.

**Primers**—αR, GGAGCCGGCGTTGGAGAGTGAG; αG, CTCCTCCTCCTCCTGTAGTCCGGC; R1, GAAACCAAGGATTTTCTC; PA, CGAGCTCACGTGGAGATTCATGAT; FP-1, CCAAAAGAATACGGCAAAAG; FP-2, TGGCGAGCTCGTTCAGTGTGG; FP-3, GCTGT; HMT2/3, GCAGCAGGAGCAGCAGCTTT.
FIG. 1. Genomic organization of the 5′ region of the human fibroblast growth factor-1 (FGFR-1) gene. A, schematic representation of the partial gene structure. The first 5 exons of the FGFR-1 are depicted as boxes, and intervening sequences are shown as thin lines. Exon and intron size is shown in base pairs. Thick lines show the relative positions of the plasmids constructs pGFR-1, pGFR-2, and pGFR-3. Arrows indicate the positions of the oligonucleotide primers used in screening. RNA splicing pathways observed in normal glia and glioblastoma are shown by dotted lines. B, sequences found at all intron/exon boundaries are shown. Intron sequence is given in lowercase letters.

RESULTS

The fibroblast growth factor receptor 1 (FGFR-1) RNA precursor is known to undergo several alternative RNA processing events affecting both the extracellular and intracellular domains of the protein. The production of a receptor containing three extracellular Ig-like binding domains results from the inclusion of a single exon termed α. Deregulation of this RNA splicing has been observed in astrocyte malignancy with a predominant α-exon skipping pathway observed (Fig. 1A) (26). Exon skipping is likely to result from changes in trans-factor composition as analysis of glioblastoma DNA failed to demonstrate mutations within the α-exon and flanking intron sequence (data not shown). The goal of these studies was to develop a model for examining the regulatory event(s) involved in α-exon recognition. While much of the structure of the FGFR-1 gene has been well characterized, the genomic organization of the 5′ end of the human gene as well as the sequences surrounding the α-exon remain to be defined. Because the genomic structure and intronic sequences frequently play a role in alternative RNA processing decisions, we sought to first clarify the genomic organization in this region.

Structure of the 5′ Region of the FGFR-1 Gene—A P1 clone was obtained from Genome Systems, Inc., using PCR primers specifically mapping to the α-exon (αF and αR). Southern analysis of the P1 clone using a primer mapping to the 5′-most end of a cDNA (R1) suggested the presence of the transcription start site in our clone (31). Three nonoverlapping subclones were obtained from the P1 clone by colony hybridization using cDNA-derived oligonucleotide probes (R1, P1B, and αF) (Fig. 1A). Comparison of genomic sequence with reported cDNA sequence confirmed the location of the α-exon boundaries and, like mouse, two exons are located upstream of the α-exon (32) (Fig. 1). DNA sequence obtained from the 5′-most end of exon 1 allowed the determination of the exon/intron boundary. Our genomic DNA sequence diverged from the cDNA sequence 641 bp from the predicted transcription start site. As expected, this new intron sequence began with a 5′ splice site consensus (33). This observation would place the translation initiation site in exon 2, as has been reported for the mouse gene (32). The size of the introns flanking exon 2 are estimates based on Southern and PCR analysis of the P1 clone (data not shown).

Constructs and RNA Splicing of a Chimeric FGFR-1 Minigene—Based on our genomic clones and previously published data (34), we estimate the FGFR-1 gene to contain 20 exons that map to a region in excess of 30 kb. Therefore, it is impractical to perform cis element mapping in the intact gene. To overcome this problem we employed a strategy similar to that used to map the cis-regulatory regions of the human calcitonin gene (30, 31). The ~4-kb insert of pGFR-3 was inserted into intron 1 of the human metallothionein 2A (MT 2A) splicing reporter gene construct to create pGFR-17 (Fig. 2A). Splicing of this minigene is driven by the Rous sarcoma virus LTR enhancer/promoter. This provides both a high level of expression and a means of distinguishing transcripts derived from the minigene from endogenous MT 2A expression. The construct maintains the alternatively spliced downstream γ-exon but replaces the upstream exon 2. pGFR-17 was transiently transfected into several cell lines (primarily of central nervous system origin) in order to determine whether RNA processing of the minigene transcripts mirrored the processing observed for endogenous FGFR-1 precursor RNA. Splicing pattern was determined by quantitative RT-PCR analysis for both endogenous FGFR-1 and pGFR-17 RNA transcripts. As seen in Fig. 2 the pattern of α-exon inclusion for transcripts derived from the minigene was similar to those observed for endogenous RNA precursor splicing. The glioblastoma-derived cell lines, T98G and SNB 19, showed almost exclusive α-exon skipping. The neuroectodermal-derived cells PFSK-1 also exhibited a predominant α-exon skipping pattern for both the endogenous and transfected FGFR-1 gene transcripts. In contrast, the embryonal carcinoma cell line (NTERA-2 cl.D1) and choriocarcinoma cell line (JEG-3) both displayed predominant α-exon inclusion pathways. The level of endogenous gene expression did not differ between the cell lines, except for the JEG-3 cells which had significantly lower levels. Differences between the level of transcript derived from the minigene likely result from differences in splicing efficiency.

Downstream Intron Size and Not the Presence of γ-Exon Plays a Role in α-Exon Selection—The observed RNA processing pattern for the pGFR-17 minigene in several cell lines confirmed that minigene transcripts are capable of mimicking the RNA processing pathway observed for the endogenous gene. However, the FGFR-1 gene insert size of this construct exceeds 4 kb. A series of 3′ end insertion deletions were introduced into the pGFR-17 construct to determine the role of downstream intron and γ-exon sequence on α-exon skipping (Fig. 3). Constructs pGFR-17, pGFR-18, pGFR-19, and pGFR-20 were transiently transfected into the SNB 19 glioblastoma cell line. RNA splicing pathways were determined by 16 cycle RT-PCR (see “Experimental Procedures”). As previously observed pGFR-17-derived transcripts predominantly excluded the α-exon (Fig. 3, lane 1). The stepwise removal of downstream sequence resulted in a gradual increase in the level of α-exon inclusion, with the highest level observed for pGFR-20. This effect appeared to be nonspecific with no significant increase coinciding with the removal of the γ-exon (Fig. 3, lane 3 versus lane 4). To confirm the nonspecific effect, the downstream intron size was expanded with antisense sequence derived from intron 2 (pGFR-21). Transfection of pGFR-21 into SNB 19 cells demonstrated that the intron expansion was capable of
restoring the RNA splicing phenotype (compare lanes 1 and 5). These results suggest that downstream intron size and not the presence of specific sequence or the γ-exon plays a role in α-exon selection. The results obtained above from the 3′ end deletion clones suggest that while the ~950 bp of deleted sequence is necessary for appropriate α-exon skipping, the specific sequence is not required as it can be substituted.

Upstream Intron Size Also Plays a Key Role in α-Exon Selection—The intronic sequence upstream of the α-exon in the pFGFR-17 clone accounts for the majority of the FGFR-1 intron. In order to define further the sequence requirements for regulated α-exon recognition, additional deletions were made in this upstream intron sequence. As above, series of 5′ stepwise deletions of ~1500 bp (pFGFR-22), ~2250 bp (pFGFR-23), and ~2300 bp (pFGFR-24) were introduced into pFGFR-17. The constructs were then transfected into SNB 19 cells and total RNA analyzed by RT-PCR for splicing products (see “Experimental Procedures”). For these constructs, removal of intronic sequence resulted in a dramatic increase in α-exon inclusion (Fig. 4, lanes 2–4). The average ratio of α-exon inclusion/exclusion was 5.5 for pFGFR-23 and 8.1 for pFGFR-24 compared with 0.2 observed for pFGFR-17 (as determined by measurement using a PhosphorImager, Molecular Dynamics). This increase in α-exon inclusion suggested that an inhibitory element preventing exon recognition may have been deleted in these clones. To test this possibility the deleted sequence was replaced with a nonspecific stuffer to create pFGFR-25. Analysis of splicing from products derived from this clone showed α-exon skipping was restored to the wild-type level (compare lanes 1 and 5). Therefore, as above, these results again suggest a role for intron size and not specific sequence, with a dramatic increase in α-exon recognition occurring when the intron was reduced below ~330 bp.

Defining the Intronic Sequence Requirements for α-Exon Selection—Deletion of intronic sequence flanking the α-exon suggested that individually neither sequence was specifically required for exon inclusion and that only intron size was important. In order to define further the sequence requirements and test for redundancy of elements, additional constructs were created. The construct pFGFR-26 (Fig. 5) combines the 5′ and 3′ deletions of pFGFR-20 and pFGFR-22. As observed for the individual deletions, there was an enhancement of α-exon inclusion during RNA processing relative to pFGFR-17 (compare lanes 1 and 2, Fig. 5). However, the level of α-exon inclusion was not significantly greater than that observed for the pFGFR-22 deletion construct. Therefore, any redundancy or synergism involving downstream elements would involve sequences 5′ of the pFGFR-22 deletion point. To
FIG. 4. Deletion of upstream intron sequence increases α-exon inclusion.  

A, schematic representation of deletions introduced into pFGFR-17. Specific deletions used to create constructs pFGFR-22 through pFGFR-25 are described under "Experimental Procedures." In construct pFGFR-25 deleted sequence has been replaced (thicker line) to maintain intron size. B, examination of RNA splicing for transcripts derived from the minigene constructs transfected into SNB 19 glioblastoma cells. RT-PCR analysis was performed as in Fig. 2. Bands representing inclusion and exclusion products are indicated. Individual constructs transfected are shown above respective lanes.

test this possibility, the same 5' deletions introduced into pFGFR-17 were created in pFGFR-21 (constructs pFGFR-27, pFGFR-28, and pFGFR-29). As previously observed, there was a dramatic increase in the level of α-exon inclusion with the deletions leaving 151 (pFGFR-28) and 95 (pFGFR-29) nucleotide of intron sequence preceding the α-exon, even when stuffer sequence was included downstream (compare lanes 5 and 6 with lane 4, Fig. 5). Finally, a construct containing replacement sequence both 5' and 3' of the α-exon (pFGFR-30, Fig. 5) was made to confirm the role of intron size and rule out the possibility of redundant elements flanking the α-exon. Like pFGFR-21 (lane 3, Fig. 5) and pFGFR-25 (Fig. 4), exclusion of the α-exon was maintained in the absence of specific flanking sequence. This would suggest that the α-exon with 95 nucleotides of 5'-flanking intron and 191 nucleotides of downstream intron provides sufficient sequence information for regulated exclusion when provided with large intronic flanks.

The splicing pathway observed for pFGFR-30 transcripts in transfected SNB 19 cells could result either from regulated skipping of α-exon or deletion of constitutive processing signals. To determine if the pFGFR-30 construct maintained sequences required for cell-specific α-exon inclusion, this construct was transfected into five cell lines described in Fig. 2. Total RNA was isolated 48 h post-transfection, and the processing pathways were determined by RT-PCR analysis (see "Experimental Procedures") (Fig. 6). The level of α-exon inclusion in each cell line was similar to that observed for pFGFR-17 (compare with Fig. 2B). The α-exon was predominantly excluded in T98G and SNB 19 glioblastoma cell lines, while predominantly included in NT-2 and JEG-3 cell lines. This observation strongly suggests that the key elements required for cell-specific α-exon recognition are contained within the 553-nucleotide sequence inclusive and flanking the α-exon. However, it does not rule out the possibility that the stuffer sequence may contain a cell-specific regulatory element. This might result fortuitously or because the stuffer sequence used to create the pFGFR-30 construct is derived from intron upstream of the α-exon. A palindromic element might still function in the antisense orientation. To address these concerns and narrow the regulatory region, additional constructs were created. The construct pFGFR-32 contains a 375-bp insert, deleting an additional 180 bp of the downstream intron while leaving the 5' splice site intact. The new stuffer sequences are derived from the 5'-flanking region of the FGFR-1 gene (Fig. 7A). To control for the possibility of an unforeseen regulatory element contained within this stuffer sequence, the α-exon and its flanking sequence in pFGFR-32 was replaced with analogous FGFR-1 exon 4 sequence to create pFGFR-33 (Fig. 7A). FGFR-1 exon 4 was chosen because like the α-exon it encodes Ig loop sequence but, unlike the α-exon, is not involved in cell-specific splicing (21–23, and data not shown).

The constructs pFGFR-17, pFGFR-32, and pFGFR-33 were transfected into SNB 19 and JEG-3 cells to compare the RNA processing of transcripts derived from these constructs. As was previously observed, transcripts derived from constructs pFGFR-17 showed α-exon exclusion in SNB 19 cells (~80%) and inclusion in JEG-3 cells (~70%) (Fig. 7B). The RNA processing
altering ligand affinity and specificity, subcellular localization, changes greatly impact upon the functionality of the protein, RNA forms. It is important to note that these RNA processing types, little is known about the distribution of the alternative the FGFR-1 gene is widespread, occurring in almost all cell six different exons (21–23, 29, 39, 40). While the expression of is quite complex and known to alternatively recognize at least processing event provides a mechanism for monitoring a change which would have a global effect and possibly lead to recognized in the two cell types, it was included at a similar ratio -exon is sufficient to mediate cell-specific recognition of this exon. However, they cannot rule out the possibility that additional elements located outside the defined region might play some role in modulating this event.

DISCUSSION

The alternative recognition of exons during RNA processing not only allows creation of genetic diversity but regulated processing decisions provide key switches to several developmental and tissue-specific processes. Changes in cell-specific exon recognition have been correlated to numerous cellular events in-including cell differentiation and the cellular transformation asso-
ciated with tumorigenesis (10–14, 35–38). Correlations involving a change in RNA processing are most often recog-
nized by examination of a single gene. However, it is easy to imagine that the identification of a single aberrant RNA processing event provides a mechanism for monitoring a change which would have a global effect and possibly lead to cellular transformation.

In this report we have focused on alternative RNA processing of FGFR-1 transcripts. Processing of this gene’s RNA precursor is quite complex and known to alternatively recognize at least six different exons (21–23, 29, 39, 40). While the expression of the FGFR-1 gene is widespread, occurring in almost all cell types, little is known about the distribution of the alternative RNA forms. It is important to note that these RNA processing changes greatly impact upon the functionality of the protein, altering ligand affinity and specificity, subcellular localization, and tyrosine kinase activity. For the α-exon, inclusion in the final transcript encodes for the production of a receptor with three Ig-like domains in the extracellular domain, while exclusion encodes a receptor with only two domains. This change has no effect on ligand specificity but has been demonstrated to reduce FGF-1 binding affinity (27, 28). The impact of the change in this splicing decision on glial cell growth is unclear and currently under investigation.

The goal of this study was to develop a model system with which the mechanisms involved in alternative α-exon recognition might be elucidated. Similar model systems have been developed to examine RNA processing for several alternatively regulated genes, including FGFR-2 (41, 42). In the process of defining the human FGFR-1 gene structure, we found that like the mouse gene the first two introns are disproportionately large relative to other introns in the gene (the next largest, intron 10, is ~2100 bp) (Fig. 1) (34). Similar sized introns are observed for other receptor genes in this family. For example, the platelet-derived growth factor receptor has been found to have a 23-kb first intron (43). Therefore, it is possible that the size of these introns play a role in regulation of α-exon recognition. This concept is supported by our observation that a reduction in the size of the intron preceding the α-exon in the chimeric minigene had such a dramatic effect on RNA splicing (Fig. 4). However, cell-specific splicing can be restored by insertion of nonspecific sequence. Therefore, this regulatory mechanism is likely to be nonspecific.

Cell-specific RNA splicing of the α-exon was maintained with only 375 bp of FGFR-1 gene sequence. The sequence is comprised of the α-exon with 95 nucleotides of upstream and 11
nucleotides of downstream flanking sequence. This places the specific regulatory sequences either within or immediately adjacent to the α-exon. This finding is not unexpected. For several alternatively recognized exons where regulatory elements have been identified, these sequences are often found near the splice site regions. Splicing of the FGFR-2 gene RNA transcript provides a specific example. Mutually exclusive splicing of two exons encoding part of the third immunoglobulin-like loop determines ligand specificity. Inclusion of a K-SAM encoding exon in epithelial cells produces a receptor with high affinity for keratinocyte growth factor. Regulated splicing of this exon has been shown to require three different elements all located within or flanking the exon (41, 42). The three elements are suboptimal splice sites, a splicing enhancer (recognized in epithelial cells), and an inhibitory sequence (recognized in other cell types). For this RNA a purine-rich exon element functions to inhibit splicing of the K-SAM exon, whereas a pyrimidine-rich intrinsic element stimulates exon inclusion. Similar purine-rich sequences are present in the 375-bp FGFR-1 fragment on the pFGFR-32 minigene construct. Whether these sequences function in an analogous fashion remains to be addressed.

The observation that cell-specific RNA splicing of the FGFR-1 α-exon can be maintained in a chimeric minigene provides a useful tool for the further study of this splicing event. While a relatively small region (375 bp) is required to maintain regulated splicing of FGFR-1 α-exon, the mechanism(s) of exon recognition may be quite complex, involving several elements and possibly sequences outside the defined region which might modulate the response. The failure to include the α-exon as a result of astrocite transformation would suggest some alteration of factors that function through splicing enhancers. This could involve a pyrimidine-rich element, such as that used for FGFR-2 K-SAM or some other regulatory sequence. For example, the purine-rich exonic splicing enhancers have been identified in several exons and regulate the splicing of several transcripts (44). Sequences resembling the exonic splicing enhancer consensus can also be found in the α-exon. These elements function through interactions with a class of RNA binding proteins (SR proteins) to regulate exon inclusion (45). It is not known if neoplastic transformation is associated with a change in the expression of individual SR proteins, but they do show tissue-specific distribution. These proteins, therefore, become an attractive candidate for regulators of this splicing event. Additional experimentation to first define the specific cis-regulatory sequences, however, is required before a role for these proteins in α-exon splicing can be determined.

REFERENCES

1. Warneford, S. G., Witton, L. J., Townsend, M. L., Rowe, P. B., Reddel, R. R., Dalla-Pozza, L., and Symonds, G. (1992) Cell Growth & Differ. 3, 839–846
2. Fribourg, T., Barbier, N., Yan, Y. X., Garber, J. E., Dreyfus, M., Fraumeni, J., Li, F. P., and Friend, S. H. (1994) J. Neurooncol. 20, 1022–1030
3. Friedman, L. S., Ostermeyer, E. A., Szabo, C. I., Dowd, P., Lynch, E. D., Donehower, L. A., and Berger, M. (1994) J. Neurooncol. 20, 207–216
4. Frebourg, T., Barbier, N., Yan, Y. X., Garber, J. E., Dreyfus, M., Fraumeni, J., Li, F. P., and Friend, S. H. (1994) Cancer Res. 54, 5760–5765
5. Wright, J. A., Turley, E. A., and Greenberg, A. H. (1993) Cell Growth & Differ. 4, 473–492
6. Fribourg, T., Barbier, N., Yan, Y. X., Garber, J. E., Dreyfus, M., Fraumeni, J., Li, F. P., and Friend, S. H. (1994) Mol. Reprod. Dev. 9, 39–61
7. Johnson, D. E., and Williams, L. T. (1990) Adv. Cancer Res. 50, 1–41
8. Hughes, S. E., and Hall, P. A. (1993) J. Biol. Chem. 268, 763–771
9. Reddel, R. R., and Friend, S. H. (1995) Cancer Res. 55, 288–293
10. Wright, J. A., Turley, E. A., and Greenberg, A. H. (1993) Cell Growth & Differ. 4, 473–492
11. Fribourg, T., Barbier, N., Yan, Y. X., Garber, J. E., Dreyfus, M., Fraumeni, J., Li, F. P., and Friend, S. H. (1994) Cancer Res. 54, 5760–5765
12. Wright, J. A., Turley, E. A., and Greenberg, A. H. (1993) Cell Growth & Differ. 4, 473–492
13. Cooper, D. L., and Dougherty, G. J. (1995) Mol. Cell. Biol. 15, 7135–7142
14. Cooper, D. L., and Dougherty, G. J. (1995) Mol. Cell. Biol. 15, 7135–7142
15. Harada, T., and Saito, H. (1995) Biochem. Biophys. Res. Commun. 205, 1057–1063
16. Mount, S. M. (1982) Nucleic Acids Res. 10, 459–472
17. Johnson, D. E., Lau, J., Chen, H., Werner, S., and Williams, L. T. (1991) Mol. Cell. Biol. 11, 4627–4634
18. Johnson, D. E., and Williams, L. T. (1990) Mol. Cell. Biol. 10, 4728–4736
19. Lou, H., Cote, G. J., and McKeehan, W. L. (1995) J. Biol. Chem. 270, 118, 705–706
20. Lou, H., Cote, G. J., and McKeehan, W. L. (1995) J. Biol. Chem. 270, 118, 705–706
21. Lou, H., Cote, G. J., and McKeehan, W. L. (1995) J. Biol. Chem. 270, 118, 705–706
22. Lou, H., Cote, G. J., and McKeehan, W. L. (1995) J. Biol. Chem. 270, 118, 705–706
23. Lou, H., Cote, G. J., and McKeehan, W. L. (1995) J. Biol. Chem. 270, 118, 705–706
24. Lou, H., Cote, G. J., and McKeehan, W. L. (1995) J. Biol. Chem. 270, 118, 705–706
25. Lou, H., Cote, G. J., and McKeehan, W. L. (1995) J. Biol. Chem. 270, 118, 705–706
26. Lou, H., Cote, G. J., and McKeehan, W. L. (1995) J. Biol. Chem. 270, 118, 705–706
27. Lou, H., Cote, G. J., and McKeehan, W. L. (1995) J. Biol. Chem. 270, 118, 705–706
28. Lou, H., Cote, G. J., and McKeehan, W. L. (1995) J. Biol. Chem. 270, 118, 705–706
29. Lou, H., Cote, G. J., and McKeehan, W. L. (1995) J. Biol. Chem. 270, 118, 705–706
30. Lou, H., Cote, G. J., and McKeehan, W. L. (1995) J. Biol. Chem. 270, 118, 705–706
31. Lou, H., Cote, G. J., and McKeehan, W. L. (1995) J. Biol. Chem. 270, 118, 705–706
32. Lou, H., Cote, G. J., and McKeehan, W. L. (1995) J. Biol. Chem. 270, 118, 705–706
33. Lou, H., Cote, G. J., and McKeehan, W. L. (1995) J. Biol. Chem. 270, 118, 705–706
34. Lou, H., Cote, G. J., and McKeehan, W. L. (1995) J. Biol. Chem. 270, 118, 705–706
35. Lou, H., Cote, G. J., and McKeehan, W. L. (1995) J. Biol. Chem. 270, 118, 705–706
36. Lou, H., Cote, G. J., and McKeehan, W. L. (1995) J. Biol. Chem. 270, 118, 705–706
37. Lou, H., Cote, G. J., and McKeehan, W. L. (1995) J. Biol. Chem. 270, 118, 705–706
38. Lou, H., Cote, G. J., and McKeehan, W. L. (1995) J. Biol. Chem. 270, 118, 705–706
39. Lou, H., Cote, G. J., and McKeehan, W. L. (1995) J. Biol. Chem. 270, 118, 705–706
40. Lou, H., Cote, G. J., and McKeehan, W. L. (1995) J. Biol. Chem. 270, 118, 705–706