The Nonsense-mediated Decay Pathway and Mutually Exclusive Expression of Alternatively Spliced FGFR2IIIb and -IIIc mRNAs*

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Exons IIIb and IIIc of the FGFR2 gene are alternatively spliced in a mutually exclusive manner in different cell types. A switch from expression of FGFR2IIIb to FGFR2IIIc accompanies the transition of nonmalignant rat prostate tumor epithelial cells (DTE) to cells comprising malignant AT3 tumors. Here we used transfection of minigenes with and without alterations in reading frame and with and without introns to examine how translation affects observed FGFR2 splice products. We observed that nonsense mutations in other than the last exon led to a dramatic reduction in mRNA that is abrogated by removal of downstream introns in both DTE and AT3 cells. The mRNA, devoid of both IIIb and IIIc exons (C1-C2), is a major splice product from minigenes lacking an intron downstream of the second common exon C2. From these observations, we suggest that repression of exon IIIc and activation of exon IIIb inclusion in DTE cells lead to the generation of both C1-IIIb-C2 and C1-C2 products. However, the C1-C2 product from the native gene is degraded due to a frameshift and a premature termination codon caused by splicing C1 and C2 together. Derepression of exon IIIc and repression of exon IIIb lead to the generation of both C1-IIIc-C2 and C1-C2 products in AT3 cells, but the C1-C2 product is a premature termination codon in exon IIIc mRNA containing a premature termination codon in exon IIIc was present, but at apparently trace levels in both cell types. The nonsense-mediated mRNA decay pathway and cell type-dependent rates of inclusion of exons IIIb and IIIc result in the mutually exclusive expression of FGFR2IIb and IIIc.

The FGF1 signal transduction system is ubiquitous in multicellular organisms and mediates communication between cell compartments during development and in adult tissues (1–3). The system comprises activating FGF, heparan sulfate proteoglycan, and transmembrane receptor tyrosine kinase. Functional diversity and tissue specificity of the FGF signaling system results from combinations of at least 22 genetically distinct homologues of FGF polypeptides, heterogeneity of heparan-sulfate oligosaccharide chains in FGF proteoglycans, and alternative splicing of the FGFR kinase genes. The best characterized example of regulated alternative pre-mRNA splicing with high biological impact in the FGFR family is the cell type-specific, mutually exclusive expression of FGFR2 mRNAs containing either exon IIIb or IIIc from the FGFR2 gene (4–11). In parenchymal tissues, which exhibit epithelial and stromal compartments, only FGF receptor 2IIb (FGFR2IIib) is expressed in the epithelial cells and recognizes activating FGF-7 or FGF-10, which are expressed only in the stromal compartment (1, 3, 11–14). In contrast, only FGFR2IIic, which cannot recognize FGF-7 or FGF-10, is expressed in stromal cell types (4, 14–19). Paracrine signaling from FGF-7/FGF-10 in the stroma to FGFR2IIib in the epithelium has been implicated in the maintenance of homeostasis between compartments and has an overall effect of limiting cell proliferation and maintaining cell differentiation (11, 16, 17). The loss of FGFR2IIib activity severs the epithelial cells from the controlling signals of the stroma. A loss of expression of FGFR2IIib in epithelial cells concurrent with a switch to exclusive expression of FGFR2IIic has been observed during the progression of transplantable rat prostate tumors from a nonmalignant (DT) to a malignant (AT) phenotype (4, 14, 16, 17). The switch from exclusive expression of exon IIIb- to IIIc-containing mRNA in epithelial cells appears to be clonal, unidirectional, and irreversible.

Cell type-specific transfection of FGFR2 minigenes and trace analysis of mRNA products by the PCR has been employed by others to screen for cis-acting sequences and trans-acting factors that are involved in cell type-specific alternative splicing of FGFR2 pre-mRNA (5–8, 20). These studies have employed diverse minigene constructs in which partial FGFR2 or viral cDNAs flank the genomic sequence containing exons IIIb and IIIc. The studies noted that transient or permanent transfection and the reading frame of the constructs affected apparent exon inclusion, which complicated interpretation of results (6). In this study, we examined in detail how the translational reading frame affects the alternative splicing of the FGFR2
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PRE-MRNA and the stability of the spliced FGFR2 mRNA. Experimental minigenes were constructed from FGFR2 sequences and gave rise to translatable mRNAs containing either an artificial last exon harboring the termination codon or an upstream exon harboring a premature termination codon (PTC). Modes of transfection and analysis of mRNA products by the reverse transcription-polymerase chain reaction (RT-PCR) and ribonuclease protection assay (RPA) were compared. Our results confirm those of others that suggested that inclusion and exclusion of both exons IIIb and IIIc can occur during pre-mRNA splicing in addition to inclusion of only one or the other (5). Although results based solely on PCR analysis could be interpreted as PTC-dependent shifts in exon recognition during FGFR2 pre-mRNA splicing, the collective results after quantitative analysis suggested that the effect of a PTC was simply to depress the particular transcript containing the PTC. The results suggest that the mutually exclusive expression of only FGFR2 exon IIIb and IIIc is a result of cooperation between cell-specific splice-site regulation (5–8) and nonsense-mediated decay (NMD) of spliced mRNA products containing a PTC (21–24).

EXPERIMENTAL PROCEDURES

Construction of Functional FGFR2 Minigenes with Nonsense and Frameshift Mutations—Minigenes were constructed by generating fragments of rat genomic DNA in the PCR followed by ligation with the luciferase gene into the pcdNA3.1/Zeo+ vector (Invitrogen, Carlsbad, CA). FGFR2 cDNA fragments were then inserted 5’ and 3’ to the rat genomic DNA such that following pre-mRNA splicing, the constructs would encode a functional membrane-bound FGFR2 extracellular domain fused to an intracellular luciferase. These constructs are preceded by the designation, “M.” cDNA constructions identical to the minigenes, except for the absence of introns, are preceded by the designation, “C.” The pcDNA3.1/Zeo+ vector contains a cytomegalovirus promoter and an SV40 polyadenylation signal, but codes for no Kozak consensus.

RT-PCR and Restriction Enzyme Analysis of Minigene Expression Product—Total RNA was isolated from transfected rat prostate tumor cells using the Ultraspec RNA isolation reagent (Biotecx, Houston, TX) per the manufacturer’s instructions. Total RNA (5 μg) was used for generation of cDNA template by reverse transcription with a random 6-mer as primer in a total reaction volume of 25 μl. A 5-μl portion was used for subsequent PCR reactions. The PCR was performed at 94°C for 1 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with 20 μl of the PCR mixture, 2 μl of React 2 buffer (Life Technologies) was added along with the restriction enzymes Aval, EcoRV, or SacII for restriction analysis and were incubated for 1 h. This method reproducibly digested the products to completion. Each transient transfection, RT-PCR, and restriction enzyme analysis was carried out at least three times, and the figures displayed are a single representative of the three. RT-PCR and restriction analysis of hybrids with the corresponding contructs from stably transfected cell lines were repeated at least three times. The figures are all single representative of three reproductions.

Ribonuclease Protection Analysis of Minigene Products—Total RNA was isolated as described above and subjected to ribonuclease protection using the Hyspeed RPA kit (Ambion, Austin, TX) using the following probes transcribed with the Maxiscript kit (Ambion) according to the manufacturer’s protocol. The probes described previously were radiolabeled with [α-32P]UTP during transcription by either T3 or T7 RNA polymerase. The FGFR2IIIb probe was constructed by excising a 224-bp HaeIII-SacII fragment from the CIIIb construction and ligation into Bluescript SK that was digested with SmaI and SacII. Following digestion by HindIII, the probe was transcribed by T3 RNA polymerase. The FGFR2IIIc probe construction was made by excising a 218-bp SacII-AvaII fragment from the CIIIc construction and ligation into Bluescript SK digested with SmaI and AvaII. Following digestion by EcoRI, the construct was transcribed by T3 RNA polymerase. Rat β-actin probe was made by ligation of a 117-bp AluI fragment from a rat β-actin cDNA into pBluescript SK digested by EcoRV. Following linearization with EcoRI, the construct was transcribed by T7 RNA polymerase. The predicted products of the two probes and their location within FGFR2 minigenes are illustrated schematically. cDNA was isolated from stably transfected cell lines by reverse transcription with a random 6-mer and amplified with 20 cycles. The PCR was carried out in a total volume of 100 μl containing 50 ng of genomic DNA such that following pre-mRNA splicing, the constructs were quantitated by phosphorimaging analysis using the ImageQuaNT program. Unless otherwise noted, RPA experiments were represented at least three times.

RESULTS

Expression of Minigenes Containing Exons in-Frame with the FGFR2 Translational Initiation Site or with PTC—FGFR2 minigenes and cDNAs used in the study are summarized in Fig. 1A. Minigenes were designed using rat genomic DNA, employed the natural FGFR start codon, encoded the entire extracellular and transmembrane domains of FGFR2, and are predicted to yield FGF-binding transmembrane receptors following translation. Minigenes were also fused with the coding sequence for luciferase at the COOH terminus of full-length expression products. Initially, four minigenes were constructed. MWI contained no mutations. IIIb-stop contained mutations that caused a PTC in exon IIIb. IIb-frsh contained an insertionional mutation that caused a reading-frame shift within exon IIIb. The shift resulted in a termination codon in exon C2 of the C1-IIIb-C2 product but also resulted in stop codons in exon IIIa and 3′ end of the C2 transcript. The frameshift in exon C2 of the C1-IIIb-C2 product but also resulted in stop codons (PTCs) in exon IIIc of any C1-IIIb-IIIc-C2 transcripts. IIIb-stop contained mutations causing a PTC in exon IIIc. FGFR2 cDNAs (CIIIb and CIIIc) were constructed and employed as controls for the expression of minigenes in the absence of pre-mRNA splicing. Additionally, a construct, MWI-
A, minigenes were constructed with four exons and three introns as indicated. Constant exon C1 was comprised of coding sequence for the NH2-terminal signal sequence, immunoglobulin module II and the intronless minigenes were constructed with four exons and three introns as boxed TAA which mutations were made to generate stop codons (\textsuperscript{[125]}I)FGF-1 binding was used to assess binding to all FGF receptor products, whereas \textsuperscript{[125]}IFGF-7 binding was used to assess binding specifically to FGFR2IIIb (11, 14). COS cells transfected with all minigenes and cDNAs except those with a PTC in exon IIIc exhibited a 3- to 5-fold increase in \textsuperscript{[125]}IFGF-1 binding over untransfected cells (Fig. 2A). COS cells transfected with all minigenes except those with a stop codon or frameshift in exon IIIb exhibited a 5- to 15-fold increase in the binding of FGF-7 (Fig. 2B). Covalent affinity cross-linking confirmed that the binding reflected formation of specific radiolabeled FGFR-1-FGFR complexes of expected molecular mass of about 133 kDa (Fig. 2C). A separate analysis of luciferase activity from cells transfected by the minigenes yielded insufficient differences between constructs with and without upstream nonsense mutations to be of utility in monitoring cell type-specific alternative splicing. Luciferase activity was subsequently used to monitor transfection efficiency among minigenes and different cell types. Once minigenes were validated as capable of producing sufficient levels of translation product in COS cells, expression was then examined at the mRNA level in DTE and AT3 cells, which mutually exclusively express FGFR2IIIb and FGFR2IIIc, respectively (5, 14).

We first screened for the presence of the four possible mRNA products of the minigenes, C1-IIIb-IIIc-C2, C1-IIIb-C2, C1-IIIc-C2, and C1-C2, by RT-PCR using paired primers to C1 and C2. Expression was examined with a \(5'\)-specific primer for the upstream exon common to all FGFR2 cDNAs and a \(3'\)-specific primer for the luciferase sequence, a downstream sequence common to minigene-derived cDNAs, to amplify only minigene products. Restriction enzymes AvaI and EcoRV, which recognize sequences in FGFR2 exon IIIb and IIIc, respectively, were used to distinguish between exon IIIb-containing and exon IIIc-containing cDNAs in the PCR. Products generated in the PCR following treatment with the two restriction enzymes are summarized in Fig. 3.

Analysis by ribonuclease protection was subsequently employed to verify that mRNA products indicated by the PCR were present in greater than trace amounts and to quantify amounts of exon IIIb-C2 or IIIc-C2 products relative to other FGFR2 mRNAs. Amounts of mRNA were standardized by an internal \(\beta\)-actin control. Transfection efficiency, measured as intensity of minigene bands relative to \(\beta\)-actin bands, varied slightly among transfections but exon inclusion ratios did not (data not shown). All RPA were reproduced at least three times, unless otherwise noted, and inclusion ratio data varied less than 5% from transfection to transfection. Additionally, because the exon inclusion analysis involves quantitative comparison of bands within the lanes and not from different lanes, slight variability in transfection efficiency will not affect the analysis. The RPA probes and products that resulted from the four potential variants of the FGFR2 minigenes are summarized in Fig. 3. RPA probes were validated and conditions standardized with mRNA from cells transfected with intronless cDNAs, CIIIb and CIIIc, which were comprised of the identical coding sequences corresponding to the C1-IIIb-C2 and C1-IIIc-C2 products of the minigenes. The IIIb and IIIc cDNAs yielded the expected probe-specific bands at 224 and 218 nucleotides (nt), respectively, and bands that were 13 and 5 nt.

![Image](http://www.jbc.org/Downloaded from http://www.jbc.org)
shorter. This was presumably due to “breathing” of the RNA duplex at the 3'-end of the constant exon C2 in both probes and sensitivity to RNase at that site. The three bands were included in the quantitative analyses.

Expression of Exon IIIc and C1-C2 mRNAs in AT3 Cells and Reduction in Expression of Exon IIIc-containing mRNA by a PTC in Exon IIIc—About 67% of cell lines derived from malignant AT3 tumors express exclusively the FGFR2IIIc mRNA, whereas the other 33% express no FGFR2 products at all (14, 16). An initial analysis by RT-PCR of mRNA from AT3 cells following transient transfection with minigenes MWt, MIIIb-stop, and MIIIb-frsh revealed only the FGFR2IIIc cDNA band expected of the cell type (Fig. 4). The nonsense mutation in exon IIIc in the MIIIc-stop caused a reduction in the C1-IIIc-C2 product concurrent with an apparent increase in the C1-C2 product devoid of both exons. Employment of different primers complementary to C2 confirmed the predominant expression of the C1-IIIc-C2 mRNA from MWt, MIIIb-stop, and MIIIb-frsh, and the apparent increase in the C1-C2 product from the MIIIc-stop (results not shown). Minor bands that were resistant to both enzymes and correlated in size with the C1-C2 transcript
were sometimes detected. Dependent on primers and conditions, faint bands appeared from MWt that correlated with the expected size of the C1-IImb-IIce-C2 transcript. However, we failed to detect the presence of the C1-IImb-C2 cDNA by AvaI treatment. At first glance, these results suggested the predominant recognition of exon IIce by the splicing machinery in AT3 cells and that the nonsense mutation in exon IIce caused an increase in the C1-C2 splice product.

To confirm results from the PCR, we employed ribonuclease protection analysis (RPA) with IIib-C2 (Fig. 5A) and IIie-C2 (Fig. 5B)-specific probes. The results revealed that the C1-IIic-C2 mRNA comprised 63–80% of FGFR2 mRNAs from AT3 cells. The RPA analysis confirmed that expression of C1-IIic-C2 from the MIIic-stop was dramatically reduced, but yielded no evidence of a significant increase in either the C1-IIib-C2 or C1-C2 mRNA products. In contrast to RT-PCR, the RPA analysis suggested that the C1-C2 product comprised a constant 20–40% of the FGFR2 products in AT3 cells. The comparative analysis between RPA and RT-PCR approaches illustrate limitations in the RT-PCR analysis in estimate of minority products when common primers across alternative splice sites are employed. Pitfalls in interpretation of the increase in otherwise minority products due to a PTC in the majority product in RT-PCR analysis has been described and reviewed by others (27, 28).

**Mode of Transfection Alters the Pattern of Alternative Splicing in DTE Cells**—DTE cells, which express exclusively the IIib isoform of the FGFR2 receptor, were transfected transiently with minigenes MWt, MIIib-stop, MIIic-stop, and MIIib-frsh. Surprisingly, when the products were assessed by PCR, the transfection of DTE cells with all four minigenes yielded no apparent C1-IIib-C2 cDNA band expected of the cell type (Fig. 6). Instead, bands corresponding to C1-IIic-C2 cDNA and C1-C2 cDNA, in which both exons IIib and IIie were excluded, were the majority products from all minigenes except MIIic-stop. MIIic-stop gave rise to predominately the 589-bp band indicative of the C1-C2 cDNA devoid of both exons.

To determine whether this unexpected pattern was a consequence of transient transfection rather than the assay method, DTE cells were stably transfected (see “Experimental Procedures”), and expression products were again analyzed by RT-PCR. In contrast to the results from transiently transfected cells, DTE cells stably transfected with the MWt and MIIic-stop gave rise to exclusively the expected C1-IIib-C2 cDNA (Fig. 7). However, minigene MIIib-stop still gave rise to bands indicative of the C1-IIic-C2 and C1-C2 cDNAs, although the...
apparent levels of expression were reduced. As in AT3 cells, these PCR-based results again indicated that a PTC in the respective exon that is characteristic of the cell type potentially caused a shift to other alternatively spliced products.

The more quantitative RPA analysis was then applied to both transiently and stably transfected DTE cells. Consistent with the RT-PCR data, the analysis of mRNA with the R2IIIc-protection probe following transient transfection of minigenes MWt, MIIIb-stop, and MIIIb-frsh confirmed the unexpected expression of the C1-IIIc-C2 mRNA at levels from 40 to 42% (Fig. 8). However, in contrast to the analysis by PCR, which failed to indicate the presence of the C1-IIIb-C2 mRNA characteristic of DTE cells, analysis with the R2IIIb-protection probe revealed that the C1-IIIb-C2 product was significant. Expression of the expected C1-IIIb-C2 mRNA from minigenes MWt, MIIIc-stop, and MIIIb-frsh, all of which had no nonsense mutation in exon IIIb, was 11, 19, and 21% of total products, respectively (Fig. 9). These results show that expression from the minigenes in transiently transfected DTE cells is distributed between the C1-IIIb-C2, C1-IIIc-C2, and C1-C2 products at about 10–20, 40, and 40–50%, respectively, of total FGFR2 products if one assumes that these are collectively the significant mRNAs from the minigenes. The apparent 11–19% decrease in the C1-IIIb-C2 product relative to other products due to the PTC in exon IIIb could indicate a decrease in selection of the IIIb exon during pre-mRNA splicing. Otherwise, the PTCs in either exon IIIb or IIIc function only to decrease the respective product to 2 and 5% of the total without effect on relative amounts of other alternative products generated by the splicing machinery.

Consistent with the RT-PCR analysis, RPA analysis of RNA from stably transfected DTE cells revealed that C1-IIIb-C2 mRNA was negligible from cells transfected by all minigenes, independent of reading frame (Fig. 10). This property is a hallmark of the DTE cell type with respect to expression of the native FGFR2 gene and is consistent with the idea that recognition of exon IIIc is normally strongly repressed by the splicing machinery of DTE cells. Analysis with the R2IIIb-protection probe indicated that the C1-IIIb-C2 mRNA ranged from 55 to 61% in cells stably transfected with the MWt, MIIIb-stop, and MIIIb-frsh (Fig. 11). This suggested that, contrary to results from RT-PCR, DTE cells following stable transfection of minigenes without a nonsense codon in exon IIIb generated C1-IIIb-C2 and the C1-C2 mRNA variants in a 60/40 ratio (Fig. 11). In sum, the C1-C2 mRNA appears constant at about 40% of total splice products independent of the mode of transfection of DTE cells, whereas the mode of transfection affects the relative amounts of the alternative C1-IIIb-C2 and C1-IIIc-C2 products generated by the splicing machinery. The results are consistent with the saturation and neutralization of a repressor of exon IIIc inclusion in DTE cells by high levels of minigene expression per cell in transient transfections relative to the homogenous presumably lower expression per cell in stably transfected cell lines (5).

Finally, in contrast to results by RT-PCR, which suggested that the C1-IIIb-C2 mRNA was absent from DTE cells stably transfected by MIIIb-stop, RPA revealed that the exon IIIb-containing mRNA comprised 26% of the total FGFR2 mRNA. The fact that no change in the level of C1-IIIc-C2 and C1-C2 products was discernible again suggested that the consequence of the PTC in IIIb exon was destabilization of the C1-IIIb-C2 mRNA rather than alteration of alternative splicing. This was confirmed by restoration of the C1-IIIb-C2 mRNA to wild-type levels by mutation of a single base pair that converted the PTC into a sense codon (MIIIb-resc) (Fig. 11).
Pattern of PTC-containing Minigenes to That of MWt, Which Contains Normal Exons—A 4-bp mutation was initially employed to introduce nonsense mutations into the normal FGFR2 reading frame (Fig. 1). A single-base pair substitution at the site of the 4-bp mutation in MIIIb-stop and MIIIc-stop was used to restore the reading frame in the MIIIb-resc and MIIIc-resc constructs. Analysis by RT-PCR indicated that both the MIIIb-resc and MIIIc-resc minigenes (Fig. 12) exhibited the cell-specific expression pattern characteristic of the MWt minigene (Figs. 4 and 7). RPA analyses confirmed that the 1-bp substitutional mutation in most cases restored levels of mRNA to that of the MWt minigene with unmutated exons (Figs. 8, 9, and 11). These results strongly suggested that the 4-bp mutation in the respective minigene acted by generating PTCs, which elicit degradation of the mRNA product rather than alteration of exon recognition and disruption of a splicing enhancer.

To eliminate the possibility that the results of the 4-bp mutation were due to creation of a splicing silencer (the TTAAT-TAA sequence), two additional minigenes were constructed.
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Fig. 12. RT-PCR analysis of cells expressing minigenes with restored translational reading frames. A, DTE cells were transfected with minigene MIIIb-resc in which the reading frame was restored by a substitutional mutation into the coding sequence for exon IIIb within the MIIIb-stop, and products were analyzed as described in Fig. 4. B, AT3 cells were transfected with minigene MIIIc-resc in which the reading frame was restored by a substitutional point mutation into the coding sequence for exon IIIc within MIIIc-stop and products analyzed as described in Fig. 4.

Fig. 13. Ribonuclease protection analysis with the IIIc probe of expression of FGFR2 minigenes containing PTCs in transiently transfected DTE cells. The control MWt contained native exons IIIb and IIIc. MIIIc-stop contained a 4-bp mutation causing a PTC in exon IIIc; MIIIc1bp-stop contained a 1-bp mutation in exon IIIc causing a PTC, and MIIIc1bp-prev contained a 1-bp “revertant” mutation, which restored the reading frame in the MIIIc1bp-stop. The data presented are one of two independent experiments, and the quantitative data were the mean.

DISCUSSION

Cell Type-dependent Expression of Exons IIIb and IIIc Assessed by Translatable Products from FGFR2 Minigenes—The aims of this study were to assess cell type-dependent expression of translatable mRNAs from minigenes comprised of FGFR2 cDNA flanking genomic sequences containing alternative exons IIIb and IIIc, and to examine the potential impact of reading frame on pre-mRNA splicing or mRNA stability. Consequently, we first confirmed that our minigenes could not only be transcribed and that the pre-mRNA was spliced into pre-mRNA sequences without regard to reading frame or use of intact FGFR2 reading frames on pre-mRNA splicing or mRNA stability. Consequently, we first confirmed that our minigenes could not only be transcribed and that the pre-mRNA was spliced into pre-mRNA sequences without regard to reading frame or use of intact FGFR2

The mode of transfection and the RT-PCR analysis of minigenes initially complicated the interpretation of results in DTE cells in which the exon IIIb-containing mRNA is exclusively expressed from the native gene. Using PCR analysis and a different transfection host, Breathnach and colleagues (6, 9) reported that FGFR2 minigenes lacking an open reading frame had to be stably transfected to detect exon IIIb-containing

Nonsense Mutations Do Not Affect Levels of Nonspliced FGFR2 mRNA Expressed from an Intronless cDNA—To determine whether the reduction in levels of exon IIIb or IIIc mRNA containing a PTC was dependent on splicing of pre-mRNA, we examined the levels of the C1-IIIb-C2 and the C1-C2 mRNAs were examined (Fig. 13). Similar to MIIIc-stop, MIIIc1bp-stop caused a significant reduction in relative abundance of the C1-IIIb-C2 product to that of MIIIc-stop. The fact that the single-base pair substitution generating a PTC caused a reduction in the level of C1-IIIb-C2 mRNA, whereas another single-base pair mutation had no effect, strongly suggests that nonsense-mediated destabilization of the mRNA occurred rather than creation of a splicing silencer by the TTAATTAA sequence.

Inclusion of Both IIIb and IIIc Exons Can Be Detected by PCR Using Paired Primers Specific for Exons IIIb and IIIc—To date, strategies using PCR primers common to alternative splice products of the native FGFR2 gene have failed to report either the C1-IIIb-IIIc-C2 or the C1-C2 products. Using the same strategy, we clearly demonstrated conditions under which the C1-C2 mRNA can be detected in both DTE and AT3 cells at up to 40% of products by use of our artificial minigenes where the termination codon in FGFR2 exon 10 (C2) is in the last exon. In contrast, neither the PCR nor the RPA analyses that were employed suggested the presence of the C1-IIIb-IIIc-C2 from the minigenes under the conditions and cell types tested. Yet the product has been detected by others using minigenes designed without regard to reading frame or use of intact FGFR2 reading frames on pre-mRNA splicing or mRNA stability. Consequently, we first confirmed that our minigenes could not only be transcribed and that the pre-mRNA was spliced into pre-mRNA sequences without regard to reading frame or use of intact FGFR2

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**DISCUSSION**

Cell Type-dependent Expression of Exons IIIb and IIIc Assessed by Translatable Products from FGFR2 Minigenes—The aims of this study were to assess cell type-dependent expression of translatable mRNAs from minigenes comprised of FGFR2 cDNA flanking genomic sequences containing alternative exons IIIb and IIIc, and to examine the potential impact of reading frame on pre-mRNA splicing or mRNA stability. Consequently, we first confirmed that our minigenes could not only be transcribed and that the pre-mRNA was spliced into predicted products, but also that the mRNA was translated. This distinguished our minigene expression system from those described previously.
mRNA. The dependence of IIb-containing mRNA expression on transfection mode was also observed by Carstens et al. (5) using the DTE cell host and a minigene with artificial exons flanking the FGFR2 genomic sequence. It has been suggested that this phenomenon is due to the high rate of minigene transcription in some cells during transient transfection, which titrates the activity of an exon IIIc repressor (5). Consequently, the inclusion of exon IIIb decreases as the inclusion of exon IIIc increases during pre-mRNA splicing. A potential difference in accessibility of the pre-mRNA to cis-acting splice factors in transiently transfected cells cannot be ruled out. In contrast, the more uniform lower expression across all cells in permanently transfected clones when the gene is stably integrated in the genome gives rise to the expected inclusion and exclusion of exons IIIb and IIIc, respectively, in mRNA from the DTE cell type. However, the C1-C2 product devoid of both exons appeared to be the majority product in DTE cells as well as a significant product in AT3 cells even after correction for the shortcomings of the PCR analysis and variations in mode of transfection. In toto, our results are consistent with the presence of a strong exon IIIb activator (or derepressor) and exon IIIc repressor in normal and premalignant epithelial cells (5). The DTE cells are a prototype of nonmalignant epithelial cells from prostate tumors in which the putative trans-acting factor is lost during and may contribute to progression to the malignant AT3 tumor phenotype. Our results raise the possibility that the C1-C2 transcript in which both exons are excluded may be a constitutive product that comprises 35–50% of products created following splicing of the FGFR2 pre-mRNA. The counterpart of the C1-C2 product is not significantly expressed in untransfected cells from the native FGFR2 gene. Our minigene differs from the native gene in that the termination codon generated by exclusion of both exons IIIb and IIIc is in the last exon of the resultant mRNA. This is in contrast to the premature termination codon (PTC) in exon 10 (out of 19) that arises from the native gene. Therefore, a mechanism must exist to reduce the translatable native C1-C2 as well as other aberrantly spliced FGFR transcripts that would result in an unproductive or potential dominant-negative fragment of an FGFR. Impact of Nonsense Mutations Causing a PTC on Observed FGFR2 mRNA Products—According to RT-PCR analysis, under all conditions where they were present, both exon IIb-containing and exon IIIc-containing mRNA products were quenched when nonsense mutations were introduced into a coding sequence for the respective exons in the minigenes. It was not apparent whether alterations in pre-mRNA splicing or stability of spliced mRNA was affected, because alternative transcripts that were otherwise very low or undetectable ap-
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level of transcription and splicing. However, its decay via the NMD pathway, possibly combined with a low level of product generated by the splicing machinery relative to the other three possibilities, results in a functionally insignificant level of the C1-IIIb-IIIc-C2 mRNA.

In summary, our results show that the partnership between NMD and cell type-specific regulation of inclusion of exons IIIb and IIIc at splicing underlies the cell-specific mutually exclusive expression of FGFR2IIIb and FGFR2IIIc mRNAs. To date, cis-acting sequence elements have been proposed, which appear to promote, derepress, or repress inclusion of exon IIIb, dependent upon cell type (6–8). In addition, a sequence element has been characterized that concurrently mediates both repression of exon IIIc and activation or derepression of exon IIIb in FGFR2IIIb-expressing cells (5). The C1-C2 mRNA devoid of both exons that is subject to NMD appears to be a constitutive default product of FGFR2 transcription. This is consistent with the presence of constitutive repressors of inclusion of both exons IIIb and IIIc that are overcome by cell-specific derepression (activation). Results of preliminary experiments to rescue the C1-IIIb-IIIc-C2 transcript by mutations, which eliminated the PTC in exon IIIc by shifting the termination codon into C2, failed to significantly increase the product. This suggests that generation of the C1-IIIb-IIIc-C2 mRNA may be severely limited at splicing prior to further degradation by NMD. The absence of repressors of both exons in a single cell type would be a requirement for generation of significant levels of the C1-IIIb-IIIc-C2 variant at splicing. To date, there is no evidence for this condition that would give rise to concurrent expression of both the C1-IIIb-C2 and C1-IIIc-C2 mRNAs in the same cell type. However, we have observed that up to 30% of malignant prostate tumor AT3 cell clones from cultures expressing exclusively the FGFR2IIIc mRNA express no detectable FGFR2 transcripts (16, 17). Whether this is due to loss of transcription of the FGFR2 gene or due to the dominance of repressors, e.g. the loss of derepressors, for both exons IIIb and IIIc in the tumor cells is under investigation.

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