Characterization of the Intronic Splicing Silencers Flanking 
FGFR2 Exon IIIb*

The cell type-specific alternative splicing of FGFR2 pre-mRNA results in the mutually exclusive use of exons IIIb and IIIc, which leads to critically important differences in receptor function. The choice of exon IIIc in mesenchymal cells involves activation of this exon and repression of exon IIIb. This repression is mediated by the function of upstream and downstream intronic splicing silencers (UISS and DISS). Here we present a detailed characterization of the determinants of silencing function within UISS and DISS. We used a systematic mutational analysis, introducing deletions and substitutions to define discrete elements within these two silencers of exon IIIb. We show that UISS requires polypyrimidine tract-binding protein (PTB)-binding sites, which define the UISS1 sub-element, and an eight nucleotide sequence 5'-GCAGCACG-3' (UISS2) that is also required. Even though UISS2 does not bind PTB, the full UISS can be replaced with a synthetic silencer designed to provide optimal PTB binding. DISS is composed of a 5'-conserved sub-element (5'-CE) and two regions that contain multiple PTB sites and are functionally redundant (DISS1 and DISS2). DISS1 and DISS2 are separated by the activator sequence IAS2, and together these opposing elements form the intronic control element. Deletion of DISS in the FGFR2 exon IIIb context resulted in the near full inclusion of exon IIIb, and insertion of this silencer downstream of a heterologous exon with a weak 5' splice site was capable of repressing exon inclusion. Extensive deletion analysis demonstrated that the majority of silencing activity could be mapped to the conserved octamer CUCGGUGC within the 5' CE. Replacement of 5' CE and DISS1 with PTB-binding elements failed to restore repression of exon IIIb. We tested the importance of the relative position of the silencers and of the sub-elements within each silencer. Whereas UISS1, UISS2, DISS1, and DISS2 appear somewhat malleable, the 5'CE is rigid in terms of relative position and redundancy. Our data defined elements of function within the ISSs flanking exon IIIb and suggested that silencing of this exon is mediated by multiple trans-acting factors.

Fibroblast growth factor receptor 2 (FGFR2),1 one of four receptors that bind fibroblast growth factors (FGFs), contains an intracellular tyrosine kinase domain, a transmembrane domain, and an extracellular FGF-binding domain. This ligand binding region is composed of immunoglobulin-like domains Ig-II and Ig-III (1). Two variants of the Ig-III domain, with different carboxyl-terminal halves, are produced by alternative inclusion of exon IIIb or exon IIIc (2–5). These variants have remarkably different ligand specificity and cell type distribution; FGFR2(IIIb) binds FGF10 and FGF7 and is expressed in epithelial cells, whereas FGFR2(IIIc) binds FGF2 and is the predominant isoform in mesenchymal cells (6, 7). Proper cell type-specific expression of each isoform is required for the FGF/FGFR2 signaling that governs epithelial-mesenchymal interactions required for organogenesis in mouse embryos (8–10). Moreover, mutations that alter the ligand specificity of FGFR2(IIIc) or those that lead to inappropriate expression of exon IIIb in the mesenchyme have been linked to several developmental syndromes in humans (9–12). The physiological importance of FGFR2 isofom choice is underscored by the switch from FGFR2(IIIb) to FGFR2(IIIc) during progression of prostate carcinomas (7, 13), where the loss of FGFR2(IIIb) appears to be required for this progression (14).

The mutually exclusive incorporation of exon IIIb or exon IIIc into FGFR2 mRNA is regulated by the complex interplay of cis-acting elements in the FGFR2 pre-mRNA and trans-acting factors. To study the mechanism of regulation, we have employed two cell lines derived from Dunning rat prostate tumors. The DT3 cell line is a well differentiated carcinoma and expresses FGFR2(IIIb) mRNA exclusively, whereas the AT3 cell line is poorly differentiated and solely expresses FGFR2(IIIc) (7). We have also used human embryonic kidney 293T (HEK293T) cells, although of uncertain cell type provenance include exon IIIc exclusively (15, 16). Regulation of exon choice depends on both activation and silencing of the appropriate exon. Silencing of exon IIIb is facilitated by the presence of weak flanking splice sites and an exonic splicing silencer (ESS) in exon IIIb (17). Silencing absolutely requires two complex intronic splicing silencers (ISSs) that flank exon IIIb, the upstream ISS (UISS) and the downstream ISS (DISS) (Fig. 1A) (16, 18). UISS was shown previously to be composed of two regions, UISS1 and UISS2, both required for full UISS activity (18). DISS is embedded within the intronic control element...
(ICE), which also contains sequences responsible for cell type-specific activation of exon IIIb (Fig. 1A) (16). The characterization of the critical cis-acting elements within UISS and ICE is the focus of this paper.

Although the precise mechanism of exon IIIb silencing is not understood, some of the trans-acting factors that mediate silencing have been identified. The ESS functions to recruit an activator sequence IAS2, and together these operate redundantly (DISS1 and DISS2). DISS1 and DISS2 are separate regions that contain multiple PTB sites and are functionally equivalent. The UISS also function to repress exon IIIc inclusion in an epithelial specific manner (21, 26); hence these elements are known as intronic splicing activators and repressors (ISARs) (reviewed in Ref. 27).

Here we present a detailed characterization of the determinants of silencing function within UISS and DISS. We used a systematic mutational analysis, introducing deletions and substitutions to define discrete elements within these two silencers of exon IIIb. Previously, we had determined that UISS contained two important regions, UISS1 and UISS2. Here we show that an 8-nucleotide sequence 5′-GCAGCACCC-3′ at the 3′ end of the UISS2 is required for silencing. UISS1 binds PTB but UISS2 does not; however, UISS function can be replaced with a synthetic silencer designed to provide optimal PTB binding. DISS is composed of a 5′-conserved sub-element (5′CE) and two regions that contain multiple PTB sites and are functionally redundant (DISS1 and DISS2). DISS1 and DISS2 are separable by the activator sequence IAS2, and together these opposing elements form ICE. Deletion of DISS in the FGFR2 exon IIIb context resulted in the near full inclusion of exon IIIb, and insertion of this silencer downstream of a heterologous exon with a weak 5′ splice site was capable of repressing exon inclusion. Extensive deletion analysis demonstrated that although the PTB-binding sites within DISS were required for full repression, the majority of silencing activity could be mapped to the conserved octamer CUCGGUGC within the 5′CE. Substitution of any position within this octamer resulted in a dramatic reduction in IIIb repression with the antepenultimate U being acutely critical. Replacement of 5′CE and DISS1 with a synthetic silencer with consensus PTB-binding elements failed to restore repression of exon IIIb. Additionally, we tested the importance of the relative position of the silencers and of the sub-elements within each silencer. Whereas UISS1, UISS2, DISS1, and DISS2 appear somewhat malleable, the 5′CE is rigid in terms of relative position and redundancy. Our data defined elements of function within the ISSs flanking exon IIIb and suggested that silencing of this exon is mediated by multiple trans-acting factors.

**MATERIALS AND METHODS**

**Plasmid Construction**—All plasmids were constructed by using standard PCRs with Pfu polymerase (Stratagene) and were cloned by using standard cloning techniques. In all cases, plasmids were sequenced to confirm identity. Oligonucleotide sequences will be provided using standard PCRs with Pfu polymerase (Stratagene) and were cloned by using standard cloning techniques. In all cases, plasmids were sequenced to confirm identity. Oligonucleotide sequences will be provided (reviewed in Ref. 27).

**Intronic Silencers of FGFR2 Exon IIIb**

**Cell Culture and Transfection**—Rat DT3 and AT3 cells, as well as human embryonic kidney 293T cells, were cultured in low glucose Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone). Transfections and selections of stable cell lines were performed as described previously (21).

**RNA Isolation and RT-PCR Assay of Transfected Minigenes**—Cellular mRNA was isolated using a method described previously (16). Rat 293T cells were cultured in low glucose Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone). Transfections and selections of stable cell lines were performed as described previously (21).

**RNA Invasive Cleavage Assay**—The RNA invasive cleavage assay (Invader RNA assay) (29) was carried out as described previously (25, 28). In the analysis of double exon constructs, the Invader RNA assays were run in the biplex format using the probe set combinations IIIb-D/IIIb and IIIb/IIIc-I/IIIc as described previously (25, 28). By using

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*Intronic Silencers of FGFR2 Exon IIIb*
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FIG. 1. Intronic splicing silencers flank exon IIIb. A schematic of exonic and intronic silencers of FGFR2 exon IIIb. Silencers (red) shown as arrows: the upstream intronic silencer (UISS), composed of UISS1 and UISS2, where the exonic silencing element (ESS) and components of the intronic control element (ICE), the 5′ CE, DISS1 and DISS2. Splicing activating sequence 1 (green) is an intronic silencing enhancer (ISE). The stem-forming sequences IAS2 and ISAR (yellow) have both ISS and intronic silencing enhancer function.

previously defined standards (Ibid.), absolute levels of each splice variant were calculated.

Alignment of Nucleic Acid Sequences—Mammalian nucleic acid sequences were aligned with ClustalW (30) using the following alignment parameters: slow pairwise alignment with an open gap penalty of 50.0 and an extended gap penalty of 10.0. Multiple alignments were with an open gap penalty of 3.7, extended gap penalty of 1.0, delayed divergence of 30%, and weighted transitions.

RESULTS

A highly conserved intronic splicing silencer lies upstream of exon IIIb. We have shown previously significant phylogenetic conservation of sequences within the FGFR2 intron between exons IIIb and IIIc and also in the intron upstream of exon IIIb (18, 24). The conserved sequences upstream of exon IIIb corresponded to the silencer element ISS (18), which was renamed upstream ISS (UISS) in light of the identification of silencer elements downstream of exon IIIb (16). UISS is composed of two dissimilar regions as follows: a pyrimidine-rich region that could be readily cross-linked to PTB was named UISS1, and a second region, characterized by a G-T repeat, was named UISS2 (Fig. 1). Alignment of six mammalian FGFR2 sequences confirmed and extended our previous phylogenetic analysis (Fig. 2A). Our observations can be summarized as follows. Although the 10-nucleotide sequence previously shown to be required for PTB binding in rat FGFR2 (18) was not precisely conserved, all of the UISS sequences had poly-pyrimidine (Py)-rich tracts. Sequence conservation over UISS2 was significantly higher than that observed over the rest of the intronic regions. Conservation of UISS2 extended over a 30-nucleotide region that included a T-G-rich segment that spanned ∼20 nucleotides in rat FGFR2. This segment was also conserved in chicken (Gallus gallus) FGFR2 and partially conserved in the single FGFR gene in sea urchin (Strongylocentrotus purpuratus) (24). The UISS2 sequence from dog (Canis familiaris) FGFR2 showed an expansion of the T-G-rich segment. The predicted branch point sequence and Py tract for intron 7 lie immediately downstream of UISS2, and the Py tract in these mammalian introns is predicted to be weak (31).

In order to further dissect the elements of UISS function and to characterize the relative contributions of UISS1 and UISS2 to overall silencing function, we constructed a series of forward and reverse nested deletions of the UISS. These deletions were made in the context of the construct p12IIb, which was shown previously to report accurately on the silencing of exon IIIb (Fig. 2B) (18, 26). Transcripts derived from p12IIb and all variants shown on Fig. 2 do not contain the exon IIIb-activating sequence ISAR, and therefore inclusion of this exon was solely a reflection of the loss of silencing. Exon IIIb inclusion for the p12IIb-WT transcript, which contains the full UISS sequence, was measured in DT3 cells to be ∼10% (Fig. 2C). Forward deletions from FΔ1 to FΔ3.5 showed increasing levels of exon IIIb inclusion, which correlated well with the sequential deletion of PTB-binding sites (underlined in the WT sequence in Fig. 2A). Most surprisingly, deleting into the T-G-rich sequence led to a decrease in exon IIIb inclusion, perhaps revealing the existence of a weak intronic silencing enhancer (FΔ4 and FΔ4.5 in Fig. 2C). Deletion of the last 15 nucleotides in the UISS resulted in a dramatic increase in exon inclusion, suggesting that this is the location of UISS2 (ΔUISS). This is consistent with the results obtained with the first two reverse deletions, which resulted in a 3–4-fold increase in exon IIIb inclusion (RΔ2 and RΔ3 in Fig. 2C). As was observed for the forward deletions, insertions into the T-G repeat segment led to a slight decrease in exon inclusion. Finally, the reverse deletions through UISS1 showed increasing levels of inclusion as PTB sites are deleted (RΔ5, RΔ8, and RΔ10). These data suggest that UISS1 has multiple redundant elements, which, based on results obtained with these deletions and on previous data (16, 18), are likely PTB-binding sites. The UISS2 activity resided within the last 10 nucleotides at the 3′ end of UISS.

In order to map critical elements within UISS2, we created a series of 4-nucleotide substitution mutants spanning 16 nucleotides near the 3′ end of UISS. We introduced these changes in the context of the p12DE construct, which reports on activation and silencing of exon IIIb, as well as inclusion of exon IIIc (Fig. 3A) (21). The reporter constructs were transfected into AT3 cells, which were expected to include exon IIIc and silence exon IIIb. As we have observed previously by using p12DE, the WT transcripts showed predominantly (70%) exon IIIc inclusion and low (20%) levels of dual IIIb-IIIc inclusion (28). A disruption of exon IIIb silencing is expected to increase the frequency of IIIb-IIIc double inclusion. As expected from the results obtained above, mt-3 and mt-4, which altered the 3′ end of UISS2, led to a 2-fold increase in IIIb-IIIc double inclusion (Fig. 3B).

The data from the nested deletions together with these results confirm and refine our prior mapping of two elements within UISS. An upstream element, UISS1, was composed of redundant PTB-binding sites, and a downstream element, UISS2, minimally required the sequence 5′-GCAGGCACC-3′.

The 5′ End of the Intronic Control Element Is Highly Conserved—In addition to an upstream ISS, we had shown previously the existence of an equally important ISS downstream of exon IIIb, and we named it downstream ISS or DISS (16). In prior work, we showed that DISS is embedded within a larger conserved sequence, which spans 239 nts of the rat FGFR2 gene. Because this sequence contained activators of exon IIIb as well as the DISS, we had previously defined it as the intronic control element (ICE) (16, 24). Two regions within the ICE, DISS1 and DISS2, were shown to contain multiple PTB sites, bind PTB in vitro, and be required for silencing of exon IIIb (16). DISS1 and DISS2 are separated by IAS2, which is involved in the cell type-specific activation of exon IIIb (20) and repression of exon IIC (28). All the evidence indicated that IAS2 did not play a role in silencing of exon IIIb. Within the 239-nt ICE, we also recognized a highly conserved sequence ~80 nts downstream of exon IIIb (16, 24). This sequence, which had not been functionally defined, was designated the 5′-Conserved Element (5′ CE).

We extended our phylogenetic analysis of ICE and compared these sequences in the FGFR2 gene of six mammals (Fig. 4). Although we noted conservation of multiple PTB sites over DISS1 and DISS2, the most impressive conservation of sequence was observed for IAS2 and for the 5′ CE (Fig. 4).

The Intronic Control Element Is Necessary and Sufficient for Silencing—Previously, we have shown that deletion of DISS1 and DISS2 (p12ΔDISS1/2) led to ~40% inclusion of exon IIIb relative to ~10–15% inclusion p12IIb transcripts (16). In order
FIG. 2. Mapping of UISS1 and UISS2. A, alignment of sequences upstream of the FGFR2 exon IIIb from six mammalian species: Homo sapiens (human), Pan troglodytes (chimpanzee), Canis familiaris (dog), Oryctolagus cuniculus (rabbit), Rattus norvegicus (rat), and Mus musculus (mouse). The approximate locations of UISS1 and UISS2 derived from our previous work (18) are indicated. B, schematics of the deletions of UISS in the context of pI12IIIb-WT. The SpeI/Xba and NotI sites are indicated in italics; proven and proposed PTB-binding sites are underlined; and deletions are labeled as F for forward deletions and R for reverse deletions. C, the effect of UISS deletions on exon IIIb inclusion. Exon IIIb inclusion was measured by RT-PCR and the % IIIb inclusion was calculated using the formula: \(((U-IIIb-D)/(U-IIIb-D + U-D)) \times 100.\) WT, wild type.
to investigate whether other sequences within the ICE contributed to the remaining silencing, we created a series of deletions and substitutions of the entire ICE in the context of pII12IIIb (Fig. 5A). As demonstrated previously (18), deletion of UISS dramatically increased the level of IIIb inclusion in both DT3 and AT3 cells (∆UISS in Fig. 5B). Equally, deletion of the ICE (∆ICE) resulted in near exclusive inclusion of exon IIIb in both DT3 and AT3 cells, which, when compared with only partial abrogation of silencing by deletion of only DISS1/DISS2 (16), suggested an important contribution of the 5′ CE to silencing. Given the profound effect of deleting either UISS or ICE, it was not surprising to find little additive effect of a double deletion acting factors required for exon inclusion (F4–F7) until the transcripts contained only two apparent (F2 and F3). Further deletions of ICE did not increase silencing of exon IIIb. In order to create the forward deletions, a ClaI restriction site was introduced immediately upstream of the sequence 5′-TATAAAA-3′ at the 5′ end of the 5′ CE (see Fig. 4). Insertion of the ClaI site had no effect on exon IIIb inclusion (+ICE versus F1, Fig. 6A); however, when the 5′ end of ICE was deleted, a drastic increase in IIIb inclusion was apparent (F2 and F3). Further deletions of ICE did not increase exon inclusion (F4–F7) until the transcripts contained only two of the seven UCUU motifs (F8). The position of the UCUU motifs, which are presumed PTB-binding sites, and define DISS1 and DISS2, are indicated as asterisks in the schematics in Fig. 6. Complete deletion of ICE resulted in high exon IIIb inclusion (86%) (F10 in Fig. 6A), which is equivalent to the results observed with the almost identical construct pII12-∆ICE (Fig. 5A; see also Fig. 6B). It is of interest to note that the F5 deletion appears to silence exon IIIb better than F6. This could suggest that F6 disrupts a general enhancing function of IAS2; however, we have previously shown that in the absence of ISAR, IAS2 has no function (25, 28). Another explanation seems plausible, F6 is the only deletion that places a UCUU motif in approximately the same position, relative to exon IIIb, as in the WT (126 nts downstream of the 5′ splice site) (see Fig. 4). It is possible that, given a minimally required number of UCUU motifs, there is an optimal position for the most proximal one.

We also analyzed a reverse deletion series (Fig. 6B). Deletion of the sequences that include the UCUU motifs resulted in ∼2-fold decrease in exon IIIb repression (R1–R7 in Fig. 6B). Most of the effect was noted when only two UCUU motifs remained in the transcripts (Fig. 6B, R5), which was consistent with the results of the forward deletions. A further 4-fold increase in exon inclusion was observed when the 5′ CE was deleted (Fig. 6B, R9), and this was indistinguishable from the results obtained with pII12IIIb-∆ICE. The results shown here were obtained in DT3 cells, and as expected, nearly identical results were obtained when the minigenes were transfected as follows: exon 8 of the rat fibroblast growth factor receptor 3 (FGFR3) gene and downstream of the chicken cardiac troponin T exon 5 (Fig. 5C). These exons, their adjacent intronic sequences, and the sequences to be tested for silencing action were cloned into the intron of pII12 as shown in Fig. 5C (see “Materials and Methods”) (21). In DT3 cells, the level of FGFR3 exon 8 inclusion was 47% (R3-8 in Fig. 5D), and this was suppressed by the presence of ICE to 3.1% (R3-8+ICE). Although intron size was not an issue with the FGFR2 minigenes, the same Bluescript substitution used previously resulted in about a 2-fold reduction in exon 8 inclusion suggesting that intron size did have a small effect on FGFR3 exon 8 inclusion (Fig. 5D, R3-8+blue); however, this was modest when compared with the ~15-fold effect of ICE. The presence of the UISS element upstream of exon 8 did not enhance silencing by ICE, but the analysis was complicated by the activation of a cryptic splice site within UISS (R3-8+UISS/ICE in Fig. 5D and data not shown). Placing ICE downstream of the cardiac troponin T exon 5 with or without UISS, did not change its inclusion pattern. Collectively, these data suggest that the ICE is both necessary to repress FGFR2 exon IIIb and sufficient to repress heterologous exons with weak 5′ splice sites.

Two Types of ISS Elements within Intronic Control Element; Redundant UCUU Motifs and a Potent Silencer within the 5′CE—The large size of ICE as well as phylogenetic data suggesting that ICE was composed of multiple elements compelled us to further dissect the silencers within this element. To this end, we constructed a series of forward and reverse deletions across the full ICE (Fig. 6, A and B, left panels) in the context of pII12IIIIb, where deletions of ICE would report exclusively on silencing of exon IIIb. In order to create the forward deletions, a ClaI restriction site was introduced immediately upstream of the sequence 5′-TATAAAA-3′ at the 5′ end of the 5′ CE (see Fig. 4). Insertion of the ClaI site had no effect on exon IIIb inclusion (+ICE versus F1, Fig. 6A); however, when the 5′ end of ICE was deleted, a drastic increase in IIIb inclusion was apparent (F2 and F3). Further deletions of ICE did not increase exon inclusion (F4–F7) until the transcripts contained only two of the seven UCUU motifs (F8). The position of the UCUU motifs, which are presumed PTB-binding sites, and define DISS1 and DISS2, are indicated as asterisks in the schematics in Fig. 6. Complete deletion of ICE resulted in high exon IIIb inclusion (86%) (F10 in Fig. 6A), which is equivalent to the results observed with the almost identical construct pII12-∆ICE (Fig. 5A; see also Fig. 6B). It is of interest to note that the F5 deletion appears to silence exon IIIb better than F6. This could suggest that F6 disrupts a general enhancing function of IAS2; however, we have previously shown that in the absence of ISAR, IAS2 has no function (25, 28). Another explanation seems plausible, F6 is the only deletion that places a UCUU motif in approximately the same position, relative to exon IIIb, as in the WT (126 nts downstream of the 5′ splice site) (see Fig. 4). It is possible that, given a minimally required number of UCUU motifs, there is an optimal position for the most proximal one.

We also analyzed a reverse deletion series (Fig. 6B). Deletion of the sequences that include the UCUU motifs resulted in ∼2-fold decrease in exon IIIb repression (R1–R7 in Fig. 6B). Most of the effect was noted when only two UCUU motifs remained in the transcripts (Fig. 6B, R5), which was consistent with the results of the forward deletions. A further 4-fold increase in exon inclusion was observed when the 5′ CE was deleted (Fig. 6B, R9), and this was indistinguishable from the results obtained with pII12IIIb-∆ICE. The results shown here were obtained in DT3 cells, and as expected, nearly identical results were obtained when the minigenes were transfected

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**Fig. 3. The determinants of specificity of UISS2.** A, schematic of the substitutions that were made in UISS2 in the context of the pl12DE-WT, which contains both exon IIIb and exon IIIc and the sequence elements required for cell type-specific exon choice. B, the effect of UISS2 substitutions was measured using RT-PCR, and the % of a specific splicing product was calculated using a formula exemplified here by the calculation for the double inclusion product U-IIb-IIIc-D: ((U-IIb-IIIc-D)/U-IIb-IIIc-D + U-IIb-D + U-IIIc-D + U-D)) × 100 as described previously (28).
into AT3 cells (not shown). Once again, this underscores our view that the silencing capability of the ICE is mediated by homologous factors in DT3 and AT3 cells. The overall conclusion of these deletion series is that the silencing activity of the ICE is mediated by two sub-elements: the 5\'/H11032 CE and an extended sequence with multiple and partially redundant UCUU motifs. The later bind PTB, and our previous data indicate that their action is mediated by this protein (16, 18).

Determinants of Function at the Proximal End of the 5\'/H11032-Conserved Element—Although it appeared that the functionally important elements in the DISS1 and DISS2 region of ICE are the UCUU motifs, the elements of function within the 5'CE were not obvious. Given the functional importance of this element, we embarked on a detailed mutational analysis. We carried out scanning mutagenesis of the 5\'/H11032 CE by introducing seven 5-nucleotide substitutions across the regions of this element that were the most conserved (see Fig. 4) (24). Substitutions were introduced in the context of the R7 deletion, which eliminated all of DISS1 and DISS2 (Fig. 7A). Mutation of either the first or the second set of five nucleotides abrogated exon IIIb silencing as well as deletion of the entire 5'CE (compare A and B versus R9 in Fig. 7). Substitution of the first 10 nucleotides, which was a combination of mutants A and B, did not result in any further loss of exon IIIb inclusion. None of the other mutations tested resulted in as profound an inhibition of silencer function; however, several other substitutions (Fig. 7, C–E) led to some abrogation of silencing beyond that observed in R7, whereas substitution of the last 10 nucleotides of the 5'CE (Fig. 7, F and G) did not. Nucleotides upstream of the 5'CE, which in rat FGFR2 are 5'-AAAAAGA-3', were less well conserved and when mutated did not substantially affect exon IIIb silencing (data not shown). These data indicate that the first 10 nucleotides of the 5'CE are critical for effective exon IIIb silencing and that the next 15 nucleotides also play some role in this activity.

The first 10 nucleotides of the 5' CE were mutated individually, and the effect of these mutations was evaluated in the context of pI12IIIb transcripts in rat DT3 cells and human 293T cells (Fig. 8, A and B). Substitutions of any of the first eight nucleotides (A1–B3 in Fig. 8A) led to abrogation of silencing, whereas results with substitution of the 9th or 10th nucleotides were not significantly different from the WT (Fig. 8A, B4 and B5). This defined the sequence 5'-CUCGGUGC-3' as a critical element within the 5'CE. In order to confirm the importance of this sequence in a more authentic context, we introduced these changes in the context of the pI12DE construct (Fig. 8C). As mentioned above, the predominant products of this transcript in AT3 and 293T cells include exon IIC and the extent of IIIb silencing is determined by measuring IIIb-IIC double inclusion. Mutants A3 and B1, but not mutant B5, led to a sizeable increase in the levels of IIIb-IIC double inclusion, indicating that these residues were critical for the

![Fig. 4. The conservation of the intronic control element.](image-url)
FIG. 5. The intronic control element is both required and conditionally sufficient to mediate exon silencing. A, schematics of minigenes used to test ICE function in the context of the pI12IIIb-WT minigene. B, results of RT-PCR analysis of RNA from cells stably transfected with the minigenes described in A. The results are quantified as an average of a triplicate set of stably transfected cell lines. %IIIb inclusion is calculated as described in Fig. 2C. C, schematics of heterologous exons used to test the sufficiency of the ICE or UISS. Exon R3-8 is denoting rat FGFGR3 exon 8, whereas exon 75 represents chicken cardiac troponin T exon 5. D, results of RT-PCR analysis of RNA from DT3 cells transfected with the minigenes described in C. Results are quantified using the formula described in Fig. 2C with the exception of exon IIIb being replaced by the relevant heterologous exon. Results are the average of a triplicate set of stably transfected cells.

FIG. 6. Deletion analysis of the intronic control element. A, schematic of the forward deletion mutants within the context of the pI12IIIb-WT minigene. The asterisks represent consensus PTB-binding sites, whereas the two boxes with ? above represent the two blocks of conservation within the 5′CE (see Fig. 4). B, results of RT-PCR analysis from RNA isolated from DT3 cells transfected with the minigenes from A. Results are represented as an average from a triplicate set of stably transfected cells using the equation from Fig. 2C. C, schematic of the reverse deletion mutants within the context of the pI12IIIb-WT minigene. D, results of RT-PCR analysis from RNA isolated from DT3 cells transfected with the minigenes from C. Results are represented as an average from a triplicate set of stably transfected cells using the equation from Fig. 2C.
silencing of exon IIIb in the context of pl12DE transcripts. As expected, none of the mutations had any effect on overall exon IIIb inclusion.

**Position and Function Constraints of the 5’-Conserved Element**—The work above suggested an interesting topology of ISS elements surrounding exon IIIb. In order to address the importance of the location of the ISS elements relative to the exon and to each other, we made a series of constructs based on pl12DE, but we swapped the position of the ISS elements (Fig. 9A). We compared these swapped RNAs to pl12DE transcripts, which led to 20% IIIb-IIIC double inclusion, and to p112DE-DISS1/CE transcripts, which resulted in 75% IIIb-IIIC double inclusion (Fig. 9B). Switching the relative positions of UISS1 and UISS2 leads only to a moderate increase in double inclusion relative to pl12DE transcripts (Fig. 9B), suggesting that the relative position of these two elements is flexible. Swapping the relative positions of 5’CE and DISS1 had a much more detrimental effect (almost 60% exon IIIb inclusion) (DISS1/CE). Given the positional constraints encountered above and the distinct sequences of ISS elements flanking exon IIIb, it was reasonable to ask whether the different ISS elements could substitute for one another. Here we present the results of substitutions that asked two questions about the ISS elements. The first question was whether the UISS could be substituted with an artificial sequence predicted to strongly bind PTB (Fig. 9B). To that end we constructed the UISS→PTB minigene (Fig. 9A). When compared with pl12DE and ΔUISS, the level of exon IIIb inclusion among UISS→PTB transcripts was very close to that of the former (20% for pl12DE and 25% for UISS→PTB in Fig. 9D). Therefore, a strong PTB-binding sequence element could functionally substitute for the UISS. These data were obtained in AT3 cells where exon IIIb is normally silenced, so we asked whether the p112DE-UISS→PTB transcripts, which contain the ISAR element, could be activated to include exon IIIb in DT3 cells. Indeed, p112DE-UISS→PTB transcripts include predominantly exon IIIb in these cells, suggesting that replacement of the UISS with a PTB consensus sequence element can also be properly down-regulated by the cell type-specific factors that normally activate exon IIIb inclusion (not shown).

The second question asked was whether strong PTB sites could substitute for 5’CE and DISS1, and for that purpose we constructed a minigene that produced CE DISS1→PTB transcripts (Fig. 9A). When compared with pl12DE and ΔUISS, the level of exon IIIb inclusion among CE DISS1→PTB transcripts was even higher than the ΔUISS (70% for pl12DE and 80% for CE DISS1→PTB in Fig. 9D). These data indicate that the ISS elements within ICE cannot be replaced with strong PTB-binding sites and suggest a unique role for the 5’CE.

**DISCUSSION**

The Determinants of Function in the Intronic Silencers Flanking FGFR2 Exon IIIb—Phylogenetic and mutational analysis reveal the determinants of function of the ISS that flank FGFR2 exon IIIb. The upstream ISS contains two important elements as follows: UISS1, a Py-rich sequence punctuated by PTB-binding sites; and UISS2, which appears to reside within the sequence 5’-GCAGCACC-3’. The downstream ISS, which is embedded within the bifunctional ICE, can be divided into three elements. Two silenter elements within the partially redundant DISS1 and DISS2 are Py-rich and contain several PTB-binding sites, and a third element, which is a potent silencer, is within the 5’CE and is centered around the se-
The double inclusion product $U$-IIIb-IIIc-D is measured using the RNA invader assay, and the % specific product was calculated by using a formula exemplified here by the calculation for which contains both exon IIIb and exon IIIc and the sequence elements required for cell type-specific exon choice.

The level of double inclusion is an indicator of the loss of exon IIIb silencing.

**FIG. 8. Mapping the determinants of specificity of the 5′-conserved element.** A, schematics of single base substitutions in the 5′ end of the 5′CE in the context of p12IIb-WT. Mutants A and B are described in Fig. 7 and are five base substitutions of shaded residues 1–5 and 6–10, respectively. B, the effect of these single base substitutions on exon IIIb inclusion was measured by RT-PCR, and the % IIIb inclusion was calculated using the formula: $\frac{(U-IIIb-D)/(U-IIIb-D + U-D)}{} \times 100$. C, a schematic of substitutions A3, B1, and B5 in the context of the p12DE, which contains both exon IIIb and exon IIIc and the sequence elements required for cell type-specific exon choice. D, the effect of these substitutions was measured using the RNA invader assay, and the % specific product was calculated by using a formula exemplified here by the calculation for the double inclusion product $U$-IIIb-IIIc-D: $\frac{(U-IIIb-Ile-D)/(U-IIIb-Ile-D + U-IIIb-D + U-IIIc-D + U-D)}{} \times 100$, as described previously (28). The level of double inclusion is an indicator of the loss of exon IIIb silencing.

An alternative hypothesis is that UISS2 plays a general role in silencing that can be substituted by an unrelated ISS. The same strong PTB-binding sites were not sufficient to substitute for the 5′CE and DISS1, suggesting that elements within this ISS, likely the 5′CE, were uniquely required for silencing of exon IIIb. Although we have not definitively identified the factors that mediate the function of 5′CE, we do believe that members of the muscleblind (MBNL) protein family interact with this sequence (37). MBNL proteins have been shown to activate a regulated insulin receptor exon and to repress a cardiac troponin T exon 5 when overexpressed in chicken cells (38). The latter is consistent with an activity mediated by an ISS. The inability of one potent ISS to substitute for another, which has also been observed in other cases (39), suggests that specific mechanisms are required for silencing exons.

Spatial Constraints for the Silencers Flanking Exon IIIb—Mutational analysis of the ISS also demonstrated a requirement for specific spatial relationships between the silencer elements. Whereas UISS appears malleable, the silencer elements downstream of exon IIIb were positionally constrained. Our results showed that the relative position of UISS1 and UISS2 could be swapped but not so for the 5′CE and DISS1. This result is not likely a result of increasing the distance of 5′CE from exon IIIb because prior work has shown that a 300-nt insertion between exon IIIb and ICE did not affect IIIb silencing (32). Although we cannot formally rule out that approximation of DISS1 to the exon leads to a weakening of silencer function, the more likely explanation for the data is that there is a fixed topology for the 5′CE and DISS1. We also show here that 5′CE-DISS1 cannot be swapped with UISS suggesting further constraints. These could be interpreted in at least two ways, either the 5′CE must always occupy an exon...
proximal position relative to DISS1 or the 5' CE must be found downstream of the exon. The unique sequence and position constraints of the 5' CE and DISS1 are supported by phylogenetic analysis. The conservation of these elements and their relative position to each other, to the exon, and to IAS2 is remarkable and can be found in the FGFR gene in the Pacific sea urchin *S. purpuratus*, which shared an ancestor with mammals ~600 million years ago (24). In the urchin FGFR gene, the 5' CE does not have the 5'-CUCCGUGC-3' core conserved, but the PTB-binding sites in DISS1 (Fig. 4) are conserved (5'-UUUCGUUUCUUCUC-3') between the regulated exon and the *S. purpuratus* IAS2 (24). The architecture of ISSs flanking a regulated exon has been noted in many cases and suggests that these elements set up a zone of silencing around the exon (reviewed in Refs. 16, 40, and 41). These zones are also likely to be found in many introns to prevent the inappropriate inclusion of adjacent constitutive or positively regulated exons.

The Mechanism of Silencing—Mechanisms proposed for exon silencing must take into account the fact that within the regulated transcripts one exon is silenced, whereas an adjacent exon, even if weak, may be included efficiently. Although there are several ways to model silencing, we believe that silencing silencer elements form zones around the affected elements, which can be conventional exons (this work), a zero-length exon (43), a pseudoexon (44, 45), or isolated splicing elements such as a branch point sequence (46, 47). Silencing can be mediated by intrinsic (ISS) or exonic (ESS) cis-acting elements. The well studied protein hnRNPA1 can mediate silencing from both ISSs and ESSs (reviewed in Ref. 34). hnRNPA1 appears to mediate its action in vitro by interfering with the positive action of SR proteins, and at least in the case of the HIV-1 Tat exon 3 hnRNPA1 interacts differently with two SR proteins (48). This specificity and examples of regulation of exons by distant hnRNPA1 sites (some intrinsic) argues against a simple model of steric hindrance (49).

Two well studied ISSs are those that mediate their action via the PTB (see above) in mammals and SXL in *Drosophila* (50, 51). SXL inhibits the use of the male-specific 3' splice site in the transformer pre-mRNA, and this has been postulated to be due to a direct competition with U2AF at the Py tract (52). Steric encumbrance at both the 5' and 3' splice sites has also been implicated in the regulation of the male-specific 2 (msl2) RNA splicing by SXL (reviewed in Ref. 34). This simple steric hindrance does not explain the silencing action of SXL on the male-specific exon 3' within its own pre-mRNA (34, 53). This silencing requires distant ISS elements upstream and downstream of the regulated exon and is mediated by SXL interfering with exon definition, principally by down-regulating the male-specific 5' splice site (54). The requirement for distant ISSs and for other gene products (*e.g.* Snf) suggests a more complex mechanism for auto-regulation of the SXL transcript, one in which SXL leads to nonproductive interactions with U1 and U2 small nuclear RNAs. These nonproductive interactions block exon definition. Indeed, expression of an SXL fragment lacking the RNA binding domains and other data in flies sug-
gist that the simple block model for SXL action may not be correct even for tra and msl2 RNAs (55, 56). Parallels to SXL have been drawn for PTB, which also auto-regulates its levels by altering its own splicing (57). PTB can, in principle, silence exons by stericly hindering the binding of general splicing factors, namely U2A/F. Although this could explain the silencing of the 24-nt (N) exon of the γ-aminobutyric acid, type A, y2 mRNA (46, 47), it does not seem likely for the regulation of stable inhibitory RNP as proposed for S

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