The fibroblast growth factor receptor 2 (FGFR2) gene exons IIIb and IIIc are alternatively spliced in a mutually exclusive and cell type-specific manner. FGFR2 exon choice depends on both activation and silencing. Exon IIIb silencing requires cis-acting elements upstream and downstream of the exon. To examine the influence of transcription on exon IIIb silencing, the putative RNA polymerase II (RNAPII) pausing MAZ4 element was inserted at different positions within the FGFR2 minigene construct. MAZ4 insertions 5' to the upstream silencing elements or between exon IIIb and downstream silencing elements result in decreased silencing. An insertion 3' to the downstream silencing elements, however, has no effect on silencing. An RT-PCR elongation assay shows that the MAZ4 site in these constructs is likely to be a RNAPII pause site. Insertion of another RNAPII pause site into the minigene has a similar effect on exon IIIb silencing. Transfection of in vitro transcribed RNA demonstrates that the cell type specificity of FGFR2 alternative splicing requires co-transcriptional splicing. Additionally, changing the promoter alters both FGFR2 minigene splicing and the MAZ4 effect. We propose that RNAPII pauses at the MAZ4 elements resulting in a change in the transcription elongation complex that influences alternative splicing decisions downstream.

The processes of transcription and splicing are spatially and temporally linked within the cell (1, 2), and these links may be critical for both constitutive splicing and for the regulation of alternative splicing. Recruitment of several different transcriptional activators to a promoter controls the splicing efficiency of a constitutively spliced Drosophila doublesex intron; strong activators induce higher levels of splicing than weak activators (3). This result is not merely an indirect effect of the relative concentration of splicing precursors since the efficiency of splicing does not correlate with the level of transcripts (3). Similar promoter-dependent effects on splicing have been demonstrated for the alternatively spliced ED1 exon of the fibronectin gene (4–8). Transcription factors that alter the rate of transcript initiation have no effect on splicing, but transcription factors that alter elongation rate affect ED1 inclusion (6, 7). Conversely, splicing can also influence the efficiency of transcription. Promoter proximal splice sites can enhance gene transcription in vivo, an effect dependent upon the splicing factor U1snRNP (9). Additionally, transcript initiation and elongation can both be enhanced by splicing factors in vitro (10–12). Thus there is a two-way functional influence between transcription and splicing, each process is capable of influencing the other.

One possible mechanism for the effect of different transcriptional activators on splicing is that transcription factors may recruit splicing factors to the transcription complex thus impacting splicing decisions. Indeed, several splicing factors are associated with general transcription factors (e.g. hnRNPF with TBP), transcriptional activators (e.g. ASF/SF2 with p52) and transcriptional co-activators (e.g. SF1 with CA150) (13, 14). Alternatively, different transcriptional activators may alter transcript elongation rate, thus impacting alternative splicing decisions. Alterations in elongation rate have been shown to impact splicing in both yeast (15) and humans (16). In both cases mutations in RNA polymerase II (RNAPII) that reduce elongation rate alter alternative splicing decisions.

Alterations in transcription elongation rate may impact alternative splicing of the α-tropomyosin gene (17). Roberts et al. (17) demonstrated that insertion of a MAZ4 sequence element between the alternatively spliced α-tropomyosin exon 3 and its downstream regulatory element (DRE) resulted in increased exon 3 inclusion. The MAZ4 sequence element contains four copies of the MAZ protein binding sequence that is part of a RNAPII pause element downstream of the complement gene C2 (18). The MAZ4 sequence has been shown to induce RNAPII pausing in vitro, however there is no direct evidence that the MAZ4 element is capable of inducing RNAPII pausing in vivo (18–20). Using these data Roberts et al. (17) proposed a model in which a delay of synthesis of the DRE induced by the MAZ4 site results in more time for exon definition, thus increasing exon 3 inclusion.

The effect of transcription on both silencing and activation of alternatively spliced exons can be studied using the fibroblast growth factor receptor 2 (FGFR2) gene. Within this gene two exons are alternatively spliced in a mutually exclusive and cell type-specific manner. Exon 8 (IIIb) is included in epithelial cells and exon 9 (IIIc) is included in mesenchymal cells (Fig. 1A) (21, 22). Inclusion of exon IIIb or IIIc determines the ligand...
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binding specificity of the receptor (21, 23). The IIIb form binds preferentially to FGFR10 and FGFR7 whereas the IIIc form binds preferentially to FGFR2 (21). Loss of expression of either FGFR2 isoform leads to severe developmental defects in mice, and mutations in exon IIIc have been associated with several human syndromes (24–28). Additionally, alterations in exon inclusion have been correlated with progression from androgen-sensitive to androgen-insensitive prostate tumors in both rats and humans (24, 25). DT3 cells, derived from the Dunning rat prostate tumor model, are well differentiated, androgen-sensitive, and express FGFR2(IIIb). In contrast, AT3 cells are poorly differentiated, are androgen-insensitive, and express FGFR2(IIIc) (24).

The alternative splicing of FGFR2 exons IIIb and IIIc is regulated by both exonic and intronic splicing control elements (Fig. 1A). Silencing of exon IIIb in AT3 cells is dependent upon weak splice sites, an exonic splicing silencer (ESS), upstream intronic splicing silencers (UISS1 and UISS2), and the downstream intronic control element (ICE). ICE includes downstream intronic silencing elements (DISS1 and DISS2) as well as the intronic activator sequence 2 (IAS2) (22, 29–31). Deletion of the upstream or downstream intronic silencers results in increased exon IIIb inclusion (30, 31). In DT3 cells, activation of exon IIIb is required to overcome the silencing of this exon. Exon IIIc is activated, and IIIc is repressed by ISAR and the intronic splicing activator and repressor (ISAR, also called IAS3) (32–34). Mutation of either IAS2 or ISAR results in the default state of exon IIIb silencing, even in cells that would normally include this exon (32, 33).

Here, we examine the link between transcription and splicing in the silencing of FGFR2 exon IIIb. The MAZ4 element and three random sequence inserts (from 100 to 300 bp in length) were introduced at different positions within the FGFR2 minigene construct. While the random sequence inserts had no effect on splicing, the MAZ4 inserts upstream of the exon IIIb silencing elements and within the downstream silencing region resulted in an increase in exon IIIb inclusion. MAZ4 inserts downstream of the silencing elements had no effect on exon IIIb repression. Furthermore, the MAZ4-dependent effect on exon IIIb inclusion was not cell type-specific. In fact, the same effect was observed in DT3 cells when the inserts were introduced into minigenes with ISAR deleted. When minigen RNAs were transcribed in vitro and subsequently transfected into cells, there was no MAZ4-dependent effect on splicing. Instead, normal control of splicing was compromised, suggesting that FGFR2 alternative splicing control requires co-transcriptional splicing. The MAZ4 effect was recapitulated by only one of the three other RNAPII pause sites, suggesting that the effect cannot be generalized to all pause sites. FGFR2 minigene splicing and the MAZ4 effect can be altered by changing the promoter driving transcription, supporting the idea that transcription is crucial to the regulation of FGFR2 alternative splicing. Our data suggest a new model for the MAZ4 effect on alternative splicing— one in which the MAZ4 element alters splicing decisions by inducing a change in the transcription elongation complex.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The rat FGFR2 minigene constructs, p12DE-WT-FS and p12DE-FS/ISAR, have been previously described as p11-1S and p11-1S dNeDE/ISAR, respectively (33). Insert sequences were introduced into the p12DE-WT-FS constructs by standard cloning techniques. Unique ClaI restriction sites were introduced at positions 138-bp upstream of exon IIIb (UpUISS), 63-bp downstream of exon IIIb (DISS/ISS1), and 456-bp downstream of exon IIIb (DISS/ISAR). Insert sequences were subsequently introduced into each ClaI site. The MAZ4 element, a-tropomycin heterologous sequences and a-globin pause site were derived from plasmids pTS3at MAZ4, pTS3at +391, and pTS3at +68, respectively (Plasmids kindly provided by Christopher Smith, Ref. 17). The immunoglobulin mu pause element was derived from plasmid pUCPatM (kindly provided by Martha Peterson, Ref. 35). The H3.3 pause site was generated by annealed oligo cloning (36). Plasmid pB685.8, used for generating RNase protection assay probes, was generated by cloning the U-IIIb-IIIc-D splice product cDNA (from an EcoRI site in the U exon to an Acc65 site in the D exon) into pDP19 (Ambion). CMV-TAR and ADM-L-TAR promoters were introduced into p12DE-WT-FS and insertion constructs by standard cloning techniques. CMV-TAR and ADM-L-TAR promoters were derived from plasmids pCA10 and pAd10, respectively. pCA10 and pAd10 were cloned by replacing the HIV promoter of pHA10 with PCR products containing the new promoters (37). Details of plasmid construction are available upon request.

Tissue Culture—AT3, DT3, and 293 cells were grown in Dulbecco’s modified Eagle medium, low glucose (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone) at 37 °C and 5% CO2. Transfections were performed with LipofectAMINE (Invitrogen) following the manufacturer’s recommendations. Briefly, cells were plated (~4 × 105 AT3, 2 × 106 DT3, and 4 × 105 293 cells per 10 cm2 well) in a 6-well culture dish the day before transfection. 24 h later, 1.5 μg of plasmid was mixed with 5 μl of LipofectAMINE in 200 μl of Opti-MEM (Invitrogen) and incubated 30 min at room temperature. 0.8 ml of Opti-MEM was added, and the mix was transferred to the plated cells. Cells were transfected for 4 h, and media was replaced with regular media. Two days after transfection, media containing 0.5 mg/ml genetin (Invitrogen) was added to select for stable cell populations, or RNA was isolated from transient transfections as described below.

Isolation of RNA—Total RNA was isolated from tissue culture cells using TRIzol reagent (Invitrogen) as recommended by the manufacturer. To isolate nuclei, cells washed in phosphate-buffered saline were collected in a microcentrifuge tube. Cells were lysed in 50 mM Tris, pH 8.0, 100 mM NaCl, 5 mM MgCl2, and 0.5% Nonidet P-40 for 5 min on ice. Nuclei were pelleted in the cold room for 2 min at 14,000 rpm (38). Nuclei were resuspended in TRIzol, and nuclear RNA isolated as above.

RNase Protection Assay—pB685.8 (described above) was linearized with NruI, and annealed probes were transcribed with T7 RNA polymerase (Ambion). Approximately 5,000–10,000-cpm probe and 10–15 μg of each sample RNA were coprecipitated and resuspended in 20 μl of 40 mM PIPEs pH 6.4, 500 mM NaCl, 1 mM EDTA, and 80% formamide. Samples were heated to 95 °C for 5 min and incubated at 65 °C overnight. Annealed samples were digested in 150-μl reactions containing 300 μg/ml RNase A, 10 μg/ml salmon sperm DNA, and RNase Mixture (Ambion) for 1 h at 37 °C. RNases were inactivated, and RNA was precipitated with 800 μl of inactivation/precipitation solution (1.75 mM guanidine isothiocyanate, 0.22% n-lauroyl sarcosine, 11 mM sodium citrate, 44 mM β-mercaptoethanol and 50% isopropl alcohol) (39). Protected fragments were resolved on 6% denaturing gels. ImageQuant software (Molecular Dynamics) was used to quantify the bands with phosphorimager and ImageQuant software. The molar equivalents of a splice product were determined by dividing the value for the band representing that product by the number of uracils in that protected fragment. The percentage of each splice product was determined by dividing the molar equivalents of a splice product by the total molar equivalents of the U-IIIb-D, U-IIIc-D, and U-IIIb-IIIc-D splice products. Each data set are from experiments performed in triplicate with RNA samples from separate transfections.

In Vitro Transcribed RNA Transfections—In vitro transcribed minigen RNAs were made with T7 polymerase from FGFR2 minigene inserts (described above) linearized with HindIII. Note that the natural splicing donor is 5′ to the AAUAAA poly(A) site, thus the first reaction. Reactions contained 1× T7 reaction buffer (Ambion), 1 mM ATP, 1 mM UTP, 1 mM CTP, 0.1 mM GTP, 1 μg mG35′ppp(5′)G (New England Biolabs), 10 units of T7 polymerase, and 1 unit of RNase OUT (Invitrogen) (40). After 2 h at 37 °C, 10 units of DNase (Ambion) were added and incubated for 30 min. RNA was phenol/chloroform extracted, ethanol-precipitated, and resuspended in H2O. In control experiments a poly(A) tail was added using recombinant poly(A) polymerase (Ambion) as recommended by the manufacturer. 0.1–1.0 μg of RNA was transfected into 293 cells (plated at 4 × 105 cells per well of 6-well culture dish the day before) or DT3 cells (plated at 2 × 105 cells per well) with DMECR-C (Invitrogen) as recommended by the manufacturer. 48 h later, transfected cells were collected in a microcentrifuge tube. RNA was immediately added to cells and was left for 4 h before being replaced by normal growth media. RNA was isolated from transfected cells 24 h after transfection as described above.

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RT-PCR Pausing Assay—The quantitative RT-PCR assay to examine elongation of RNAPII has been previously described (43). Briefly, reverse transcription was performed using primers specific to sequences before and after each insert site. 1.5 pmol of primer was annealed to 2 

\[\text{dissociation constant (K_{diss})} = 1 \text{ mM dNTPs} \]

by heating to 95 °C for 5 min and incubating at 30 °C for 1 h. 12 

\[\text{mM dNTPs} \]

in reverse transcriptase mixture (1.6× first strand buffer, 16 

\[\text{mM dNTPs} \]

mM MgCl2, 0.2 mM dATP, 0.2 

\[\text{mM dNTPs} \]

mM MgCl2, 0.2 mM dCTP, 0.5 

\[\text{mM dNTPs} \]

dNTP, 50 

\[\text{mM dNTPs} \]

of Taq DNA polymerase. 25 cycles of PCR were performed with an annealing temperature of 50 °C and 30 s extension time. PCR products were resolved on 6% acrylamide gels and quantified using a phosphoimager and ImageQUANT software. Intensities of each PCR set were plotted (intensity versus dilution factor) to ensure linearity and to determine the slope of the line. Data were calculated as follows in Equation 1,

\[
\text{[Longmax]}/[\text{Shortmax}] = \frac{\text{[Long200]}/[\text{Short200}]}{\text{[Long316]}/[\text{Short316}]} \tag{Eq. 1}
\]

where [Long] or [Short] is the slope of the line from RT-PCR reactions using the RT primer after or before the insert site, respectively. Primers for reverse transcription of IIIb/DISS insert constructs were S1 (MO572): 5′-AATGTCCAGATCTGATCTAAAATAAGAGAAATGGG-3′, and L1 (MO393): 5′-GAGCTATGTACATTCCCTGAGAAATGTCTTACTCATATT-3′. Primers for reverse transcription of DISS/ISAR insert constructs were S2 (MO411): 5′-GAGCCATGTCAAGCTCACTATTGTGTGAGA-3′. PCR primers were P1 (MO791): 5′-CCCCAGCTGTGGTACCGATACCT-3′, and P2 (MO653): 5′-GGGATCGATAGAGCCGTTGAGTGTCAAGAATT-3′.

RESULTS

Construction of Co-transcriptional Splicing Reporter Constructs—Static models for the regulation of alternative splicing consider the binding rate and stability of both constitutive splicing complexes and regulatory factors. However, co-transcriptional splicing forced us to consider two additional issues. First, because the structure of the splicing substrate is dynamic, the rate of binding of splicing factors relative to the rate of transcript synthesis could be important. Second, the direct interaction of transcription factors and splicing factors may influence alternative splicing decisions.

To begin examining the potential role of co-transcriptional splicing on the regulation of FGFR2 alternative splicing, we postulated that altering transcription elongation might affect the alternative splicing pattern of these transcripts. Thus, the putative RNAPII pause site MAZ4 was inserted at various positions within the FGFR2 minigene construct, p112DE-WT-FS (Fig. 1B) (33). The p112DE-WT-FS construct contains rat genomic FGFR2 sequences spanning exons IIIb and IIIc within the intron of an adenoviral derived vector driven by the CMV immediate early promoter. This minigene recapitulates the splicing pattern of the endogenous gene in both AT3 (IIIc) and DT3 (IIIb) cells (33). The MAZ4 element used in these experiments contained four copies of the MAZ sequence element within a total of 200 base pairs (17). To control for any spacing effects 108, 200, and 316 bp heterologous sequences derived from the α-tropomyosin intron 1 were inserted at the same locations as the MAZ4 element. The region of intron 1 used in the insertions has previously been demonstrated to have no effect on the splicing of α-tropomyosin (44). The first MAZ4 element was inserted upstream of U1SS (UpU1SS). An RNAPII pause at this location would occur before the exon and its control elements have been synthesized. In this case, there should be no effect on the relative rates of appearance of the exon and its control elements (as long as there is no decrease in the rate of elongation after the MAZ4 site), so the

UpU1SS insertion was not expected to alter splicing regulation. The second insertion, IIIb/DISS, was expected to delay the synthesis of the entire ICE, and was therefore similar to constructs tested by Roberts et al. (17). If this MAZ4 insertion delayed the synthesis of necessary downstream silencing elements, the insertion would be expected to decrease exon IIIb silencing. The third insertion, DISS/IAS, should delay the synthesis of the second half of ICE, which contains the activating sequences IAS2 and DISS2. This insertion was expected to have less of an effect than the IIIb/DISS insert because deletion of DISS2 has little effect on exon IIIb silencing, however, given the potential proximity of a paused RNA-PPI to critical DISS1 elements we could not clearly predict the results from this insert. A fourth MAZ4 insert downstream of DISS2 (DISS/ISAR) was not expected to have any effect on silencing of exon IIIb since the potential RNAPII pause site was placed far downstream of any known signal required for silencing.

Extending the Distance between Exon IIIb and the Downstream Silencers Did Not Reduce Silencing—Each insert containing minigenes was stably transfected into AT3 cells, RNA was isolated, and the spliced transcripts were analyzed by RNase protection assay (RPA). The RPA probe was derived from the cDNA of the U-IIIb-IIIC-D splice product (Fig. 2A). Protection of spliced RNA with this probe yielded unique products (Fig. 2, A and B). The U-D splice product could not be quantified using this probe since it did not yield unique protection products. A second RPA probe designed specifically to detect the U-D product showed very low levels of this product and these levels did not change with any of the inserts (data not shown).

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2 E. Wagner and M. A. Garcia-Blanco, unpublished data.
PI12DE-WT-FS, the wild-type FGFR2 minigene containing no insert sequences, gave the expected pattern of splicing (Fig. 2B, lanes 2 and 3 and Fig. 2C, WT). Greater than 70% of the splice products from this construct were U-IIIb-IIIc-D, 20–25% of the splice products were U-IIIb-D, and less than 5% of the splice products were U-IIIb-D. The insertion of a ClaI restriction site, which was used for cloning the inserts, had no significant effect on the splicing pattern at any of the insert sites (Fig. 2B, lanes 4 and 5 and Fig. 2C, ClaI). The insertion of the +108, +200, or +316 size controls into each of the four insert sites also had no significant effect on the pattern of splicing (Fig. 2B, lanes 8 and 9 and Fig. 2C, +108, +200, and +316). This result was expected for the UpUISS and DISS/ISAR inserts since they did not alter the spatial relationship of the exon relative to its repressive elements. Unexpectedly, when DISS1 and DISS2 were moved more than 300-bp downstream of their normal position by the IIIb/DISS +316 insert, these elements were still able to repress exon IIIb (Fig. 2C, IIIb/DISS). This result is different from that observed in the α-tropomyosin system where increasing the size of insertion between the exon and its downstream regulatory element had an increasing effect on exon inclusion (17).

**MAZ4 Sequences Affect Silencing of Exon IIIb**—In contrast to the insertion of unrelated sequences, insertion of the MAZ4 element at certain sites significantly disrupted silencing of exon IIIb. A decrease in silencing of exon IIIb resulted in an increase of the U-IIIb-IIIc-D splice product. As expected, the MAZ4 insertion downstream of DISS2 (DISS/ISAR) had no significant effect on the splicing pattern (Fig. 2C, DISS/ISAR). In contrast, MAZ4 inserts at IIIb/DISS and DISS/IAS resulted

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**Fig. 2. MAZ4 insertions altered FGFR2 splicing in AT3 cells.** A, diagram of the probe and expected products in the RNase protection assay. Boxes show possible splice products. The line at the top indicates the sequences of the U-IIIb-IIIc-D splice product that were used for the probe. Dashed lines indicate sequences not complementary to FGFR2 sequences. Under each splice product, lines represent predicted RPA protection products. The sizes of each protection product are indicated on the right in base pairs with unique, quantifiable products indicated in bold. B, representative RPA. RPA of RNA derived from AT3 stable cell lines transfected with UpUISS insert constructs. Each sample is shown in duplicate. The DNA marker is shown in lane 1 with sizes indicated on the left in base pairs. On the right, arrows indicate positions of each possible protection product. C, quantification of RPA data for all four insert sites. Percent inclusion was calculated as indicated under “Experimental Procedures.” Black bars represent U-IIIb-IIIc-D, gray bars represent U-IIIc-D, and white bars represent U-IIIb-D. These data are from a representative experiment performed in triplicate.
in a 4- or 2-fold increase in the U-IIIb-IIIc-D product, respectively, compared with the size-matched +200 insert (Fig. 2C, IIIb/DISS and DISS/IAS). The increase in the U-IIIb-IIIc-D splice product was accompanied by a corresponding decrease in the U-IIIc-D splice product. There was no significant change in the U-IIIb-D RNA levels for these insert sites. Surprisingly, insertion of the MAZ4 element upstream of UISS also resulted in a 2-fold increase in the U-IIIb-IIIc-D splice product (Fig. 2, lanes 6 and 7 and C, UpUISS). This result suggests a MAZ4-dependent effect on RNAPII that influences downstream splicing decisions.

The MAZ4 Effect Is Not Cell Type-specific—Unlike AT3 cells, DT3 cells normally include exon IIIb through both activation of exon IIIb and silencing of exon IIIc. In DT3 cells, the MAZ4 inserts had no effect on activation of exon IIIb or silencing of exon IIIc (data not shown). This result raised the possibility that the effect observed in AT3 cells was due to a cell type-specific factor. To test this possibility, insertion constructs were cloned into pI12DE-FS/H9004 ISAR, a pI12DE-WT-FS derivative that contains a deletion of ISAR (33). In the absence of ISAR, exon IIIb is silenced in DT3 cells (33). The /H9004 ISAR insertion constructs were transfected into DT3 cells and the spliced transcripts were analyzed by RPA as described above (Fig. 3). As with pI12DE-WT-FS in AT3 cells, pI12DE-FS/H9004 ISAR primarily splices the U-IIIc-D product in DT3 cells. There was, however, a higher level of exon IIIb containing products for this construct. U-IIIb-IIIc-D represented about 50%, U-IIIc-D represented about 30%, and U-IIIb-D represented about 20% of all splice products (Fig. 3, WT). There was no significant change in the splice pattern with either the ClaI or the +200 insert at any

FIG. 3. MAZ4 insertion effect was not cell type-specific. Quantification of RPA of RNA derived from /H9004 ISAR insertion constructs stably transfected into DT3 cells. Percent inclusion was calculated as indicated under “Experimental Procedures.” Black bars represent U-IIIb-IIIc-D, gray bars represent U-IIIc-D, and white bars represent U-IIIb-D. These data are from a representative experiment performed in triplicate.
insert site (Fig. 3, ClaI and +200). Although the level of U-IIIb-IIIc-D splice products for the +200 construct was higher when ISAR was deleted than that observed in AT3 cells, there was still a 1.5-fold increase in this splice product when the MAZ4 element was inserted upstream of UISS, between IIIb and DISS1, and between DISS1 and IAS2 (Fig. 3, +200 and MAZ). As in AT3 cells, there was no significant MAZ4-dependent effect when this insert was downstream of DISS2 (DISS/ISAR). A similar MAZ4-dependent effect on exon IIIb silencing was also seen with the p12DE-WT-FS-derived constructs in 293 cells, a cell line that normally silences exon IIIb (data not shown). Thus, the observed MAZ4 effect on exon IIIb silencing was not cell type-specific.

There Are Fewer Transcripts after the MAZ4 Site—The observed MAZ4 effect could have resulted from RNAPII pausing, RNAPII termination, a reduction in elongation rate, or a change in the protein composition of the RNAPII transcription complex after it encounters a MAZ4 site. To examine if the MAZ4 sequences led to pausing or termination, a semiquantitative RT-PCR assay was used to determine the relative level of transcripts past this sequence (43). Nuclear RNA was isolated from stable AT3 cell lines containing constructs with either MAZ4 or +200 inserts at the IIIb/DISS and DISS/ISAR insert sites. Reverse transcription was performed with primers specific to the template immediately before (short product) and after (long product) each insert site (Fig. 4A). Reverse transcription products were then amplified by semiquantitative PCR with a second set of primers upstream of those used for the reverse transcription (Fig. 4A). Thus, the relative levels of transcripts before and after each insert site were determined. Data are represented by the ratio shown in Equation 1. The results from a representative experiment are shown in Fig. 4B. For both insert sites, there were fewer transcripts after the MAZ4 insert than after the +200 insert.

The inherent error in the experimental protocol was frequently greater than the observed MAZ4-dependent effect on transcription. Despite this level of error, in three separate experiments (each performed in triplicate) there were fewer transcripts after the MAZ4 insert than after the +200 insert at the DISS/ISAR insertion site. In one of three experiments, there were more transcripts after MAZ4 than after +200 at the IIIb/DISS insert site.

FGFR2 Alternative Splicing Regulation Is Dependent on Co-transcriptional Splicing—It was possible that the effect of the MAZ4 element insertion was not due to changes in transcription, but rather a cis-acting effect of this sequence on the RNA. The G-rich MAZ4 sequence included sequences consistent with a consensus hnrNP H binding site (45). HnRNPH H has been shown to play a role in the control of alternative splicing of other transcripts, presumably by binding to the RNA (46–51). Previous reports have shown that in vitro transcribed RNA can be accurately processed when introduced into cells. RNA microinjected into cell nuclei can be spliced and polyadenylated (52, 53). To test whether the observed MAZ4 effect resulted from a cis-acting effect on the RNA instead of a transcription-dependent effect, minigene RNAs were transcribed in vitro with T7 polymerase and transfected into 293 cells. The transfected RNAs contained a 5′-cap, but were not polyadenylated and did not contain the polyadenylation signal. The splicing pattern of these transfected RNAs was analyzed by RPA. There was no difference in the splicing pattern of +200 and MAZ4 inserts at the UpUISS, IIIb/DISS, and DISS/ISAR insert sites (Fig. 5, A and B). Titration of the RNA had no effect indicating that this result was not due to overwhelming of the system with excess RNA (Fig. 5B, 0.1, 0.5, and 1 μg). Addition of the polyadenylation signal and/or addition of a poly(A) tail to the transfected RNA had no effect on the observed splicing pattern (data not shown). These results suggested that the MAZ4 effect was dependent upon co-transcriptional splicing and was not a cis-acting effect on the RNA.

293 cells normally display an FGFR2 splicing pattern similar to that of AT3 cells when both transcription and splicing occur in vivo (31). However, the pattern of splicing was dramatically altered when in vitro transcribed RNAs were transfected (Fig. 5, A and B). In this case there was almost equal amounts of U-IIIb-D and U-IIIc-D splice products suggesting that the normal control of alternative splicing was lost by uncoupling transcription and splicing. The same loss of splicing regulation was observed in DT3 cells (data not shown), indicating this result was not specific to silencing of exon IIIb. These results strongly suggest the importance of co-transcriptional splicing in the control of alternative splicing of the FGFR2 minigene.

At Least One Other RNA Polymerase Pause Site Affects Exon IIIb Silencing—To further test whether the MAZ4 effect was a general effect of RNAPII pausing and not a specific effect of the MAZ4 sequence, three other RNAPII pause sites were introduced into the FGFR2 minigene. Two of the pause sites were derived from sequences downstream of a poly(A) site: the 181-bp α-globin pause site was derived from the region downstream of the human α2-globin gene and the 176-bp μ pause site was derived from the region downstream of the mouse immunoglobulin μ gene (35, 54). Both of these sequences enhance the use of an upstream poly(A) site in a transcription-dependent manner and have been shown to induce RNA polymerase pausing by nuclear run-on (18, 35, 54). The third pause site was 105 bp from the first intron of the human histone H3.3 gene (55). This pause site induces RNAPII termination in in vitro transcription assays and pausing in vivo by nuclear run-on (36, 55). None of these RNAPII pause sites had any significant sequence similarity to the MAZ4 element. As for MAZ4, these pause sites were cloned into the FGFR2 minigene, stable AT3 cell lines made and splicing analyzed by RPA. There was no significant difference in FGFR2 splicing for the α-globin and H3.3 pause sites relative to their size matched controls at any insertion site (Fig. 6, A and C). In contrast, the μ pause site induced a 2-fold increase in the U-IIIb-IIIc-D splice product when inserted at the IIIb/DISS insertion site (Fig. 6B). There was however, no significant change in the splicing pattern at the UpUISS, and DISS/ISAR insertion sites. Thus, one of the three other RNAPII pause sites was able to change the FGFR2 splicing pattern in a way similar but not identical to MAZ4.

The MAZ4 Effect Is Promoter-dependent—Alternative splicing decisions have previously been shown to be dependent upon transcription by altering promoter structure (3–8). To confirm that the MAZ4 effect on splicing was dependent on transcription, the FGFR2 wild-type minigene and insertion constructs were cloned downstream of the adenovirus major late (ADML) promoter. Since the new constructs also included the first 41 bp of HIV transcripts that encode the TAR element, new CMV driven constructs were also constructed that included this sequence. Each of the CMV-TAR and ADML-TAR driven constructs was transfected into AT3 cells, stable cell lines selected and splicing analyzed by RPA. There was no significant change in the splicing pattern observed between the CMV driven constructs with and without the HIV sequence (Figs. 2C and 7A). The CMV-TAR constructs showed the same magnitude of MAZ4-dependent increase in U-IIIb-IIIc-D splice products relative to the +200 insertions as the CMV constructs without TAR when MAZ4 was introduced at the UpUISS, IIIb/DISS, and DISS/ISAR insertion sites. In contrast, the splicing pattern did change when the transcripts were driven by the ADML promoter.
FGFR2 minigene splicing was better regulated when driven by the ADML promoter than when driven by CMV. The ADML-TAR wild-type construct had nearly 80% U-IIIc-D splice product relative to the 65% observed in the CMV-TAR construct (Fig. 7, A and B). The increase in this splice product was likely due to tighter regulation of exon IIIb silencing, since there was a corresponding decrease in the U-IIIb-IIIc-D splice product. Most importantly, the effect of MAZ4 insertions was also changed in the ADML-TAR constructs. As with the CMV and CMV-TAR constructs, there was an increase in the U-IIIb-IIIc-D splice product with MAZ4 insertion at UpUISS and IIIb/DISS, however, the magnitude of this increase was reduced (Fig. 7, A and B). In the CMV-TAR constructs, the MAZ4 insertion at UpUISS resulted in a 2-fold increase in this splice product relative to the size matched /H11001200 insertion whereas this increase was less than 1.5-fold in the ADML-TAR constructs. Similarly, the MAZ4-dependent increase in the U-IIIb-IIIc-D splice product at the IIIb/DISS insertion site dropped from greater than 3-fold in the CMV-TAR constructs to about 2-fold in the ADML-TAR constructs. In contrast to the CMV-TAR constructs, there was no effect of the MAZ4 insertion at the DISS/IAS site. The 2-fold decrease in the U-IIIb-IIIc-D splice product when MAZ4 was inserted at DISS/IASR relative to the +200 insertion was puzzling. These results support the model that FGFR2 alternative splicing regulation is transcription-dependent.

**DISCUSSION**

In a general model for the silencing of an alternatively spliced exon, as transcription progresses different cis-acting elements required for silencing or splicing of an exon emerge from the elongating RNAPII complex and are available for protein binding. At any point several factors can influence whether silencing complexes or splicing complexes may form. The order of synthesis of each cis-element favors protein binding to those elements synthesized first. The relative rates of transcription, repressor binding, and splice factor binding, in addition to the relative stability of repressor and splice factor binding can also influence this decision. Lastly, proteins associated with the transcription complex may influence which factors can bind. The factors themselves may be associated with the transcription complex and be transferred to the RNA when brought into proximity of their binding site, or proteins associated with the transcription complex may activate or inhibit factor binding (14, 56).

To examine the link between transcription and control of alternative splicing, Roberts et al. (17) inserted the MAZ4...
element between the alternatively spliced α-tropomyosin exon 3 and a downstream silencer (DRE). Roberts et al. (17) observed an increase in exon 3 inclusion in the presence of the MAZ4 element, allowing more time for exon definition before silencing can be established. The data we have presented here, however, suggest that this model needs to be re-evaluated, at least in the case of FGFR2 transcripts. We propose that in addition to inducing RNAPII pausing, the MAZ4 element causes a long-term effect on the transcription complex that alters downstream splicing decisions.

We have shown that co-transcriptional splicing is essential for the correct alternative splicing control of the FGFR2 gene. Transfection of in vitro transcribed RNA resulted in equal quantities of the U-IIIb-D and U-IIIc-D splice products in two different cell types (Fig. 5 and data not shown). Surprisingly, there was very little detectable U-IIIb-IIIc-D splice product, as would be expected if there were a complete loss of splicing control. The preference for mutually exclusive splicing of either exon IIIb or IIIc is not dependent on co-transcriptional splicing; however, the cell type-specific inclusion of an exon requires this level of control.

The transcription-dependent silencing of FGFR2 exon IIIb in AT3 cells was examined by placing the putative RNAPII pause element MAZ4 at various positions within the FGFR2 minigene. At three of the four insert sites (UpUISS, IIIb/DISS, and DISS/IAS) the MAZ4 element resulted in an increase in the U-IIIb-IIIc-D splice product indicating a loss of exon IIIb silencing (Fig. 2). While our IIIb/DISS insert data are consistent with the kinetic model for the control of alternative splicing proposed by Roberts et al. (17), the UpUISS insert data suggest that this model will not work here. A purely kinetic model would predict that RNAPII pausing at the UpUISS insert would not alter the competition between splicing and silencing since the pause would occur before the exon and its repressors are transcribed. Instead the effect caused by this insertion suggests a long-term effect of the MAZ4 site on the RNAPII elongation complex. This effect could be a change in elongation rate of the polymerase, or a change in the protein constituents of the transcription complex.

In vitro transcription reactions show a build up of transcription products at the MAZ4 site (20). Additionally, our RT-PCR assay to examine RNAPII elongation in vivo suggests that there is a reduction in the number of polymerases after the MAZ4 site (Fig. 4). This result could be interpreted in three ways: the MAZ4 site could be inducing RNAPII pausing, inducing RNAPII termination, or reducing the RNAPII elongation rate. We do not believe that termination by MAZ4 sequences leads to a decrease in exon silencing. First, terminated products would not be detectable in our assay; and second, the reduction in transcript levels (i.e. titration effect) would be less than 2-fold. If the insert resulted in a reduction in the elongation rate of the polymerase, a significant decline in the number of transcripts at increasing distances after the pause site should be detectable. This was tested by comparing the RT-PCR ratio of long to short products observed when the long RT primer was immediately downstream of the insert and when the long RT primer was located in the intron between exon IIIc and exon D. There was no difference in the number of transcripts detected immediately after the pause site and far downstream of the MAZ4 site (data not shown). Thus the RNAPII elongation rate is not likely to have been altered by the MAZ4 site. Therefore, we believe that MAZ4 sequences inhibit exon

![Figure 6](http://www.jbc.org/)  
**FIG. 6. At least one other pause site affects exon IIIb silencing.** Quantification of RPA of RNA from AT3 cells transfected with FGFR2 minigenes containing three different RNAPII pause sites: A, α-globin; B, immunoglobulin μ; C, histone H3.5. Percent inclusion was calculated as indicated under “Experimental Procedures.” Black bars represent U-IIIb-IIIc-D, gray bars represent U-IIIc-D, and white bars represent U-IIIb-D. Data are from representative experiments performed in triplicate.

![Figure 7](http://www.jbc.org/)  
**FIG. 7. An alternative promoter changes FGFR2 splicing.** Quantification of RPA of RNA from AT3 cells transfected with FGFR2 wild type and insertion-containing minigenes with two different promoters: A, CMV-TAR; B, ADML-TAR. Percent inclusion was calculated as indicated under “Experimental Procedures.” Black bars represent U-IIIb-IIIc-D, gray bars represent U-IIIc-D, and white bars represent U-IIIb-D. Data are from a representative experiment performed in triplicate.
IIIb silencing by pausing the RNAPII elongation complex and in doing so altering its functional properties.

The splicing effect observed when the MAZ4 insertion was placed between DISS1 and IAS2 (DISS/IAS) is most easily explained by the presence of one or more stalled polymerases at the MAZ4 site. Since DISS2 can be deleted with no effect on exon IIIb silencing, any delay in synthesis or alteration of the polymerase that occurs after DISS1 and before DISS2 would not be expected to have an effect on splicing. Instead, a stalled polymerase at this MAZ4 site may interfere with the binding of proteins required for establishment of silencing. Trans-acting factors including hnRNP A1, which binds the ESS, and the polypyrimidine tract-binding protein (PTB), which binds U1SS1, DISS1, and DISS2, have been identified (31, 57). Both mutations within their respective binding sites and RNA-mediated knockdown of PTB results in increased IIIb inclusion (31). A pause of the RNAPII at the DISS/IAS MAZ4 site would leave the upstream DISS1 silencing elements on the emerging mRNA in close proximity to the transcription complex. The structure of the RNA or steric hindrance from transcription complex associated proteins may prevent PTB or other proteins required for silencing from binding. In contrast, the DISS/ISSAR insertion 270 bp farther downstream had no effect on splicing. This site should be far enough downstream of the DISS1 sequence that any blocks to PTB binding are resolved.

An alternative explanation for the effects of MAZ4 insertion observed here is that the MAZ4 sequence introduced a new splicing control factor binding site in the RNA. In fact, the MAZ4 sequence includes an hnRNP H consensus binding site, GGGAG (45). HnRNP H has been implicated in control of alternative splicing of several genes. HnRNP H is a negative regulator of splicing to the A3 3′-splice site in HIV, binds the negative regulator of splicing element in Rous Sarcoma Virus, and silences splicing of the rat B-tropomyosin exon 7 (46, 48, 50). HnRNP H is also an enhancer of splicing of the c-Src N1 exon and of the cryptic 6D exon in HIV (47, 51). We do not believe the MAZ4 effect observed here was due to binding of a cis-acting factor to the RNA, since splicing of in vitro transcribed RNA was not affected by the presence of the MAZ4 sequence (Fig. 5). This, however, does not exclude the possibility that such a regulatory factor (e.g. hnRNP H) must be loaded co-transcriptionally onto the RNA.

The importance of transcriptional pausing is supported by the fact that another pause site, mu, is capable of inducing a similar effect on splicing. As with MAZ4, insertion of the mu pause site at IIIb/DISS and DISS/IAS resulted in increased U-IIIb-IIIc-D splice product (Fig. 6). Interestingly, the mu pause site had no effect when inserted at UpUISS. The reason for this discrepancy with the MAZ4 result is not clear; however, it may be explained by differential effects of the pause sites on the RNAPII elongation complex. This explanation is supported by the fact that two other pause sites, α-globin, and histone H3.3, had no effect on FGFR2 splicing when inserted into the minigene.

Further support for our proposed model for the MAZ4 effect comes from the random sequence size controls. Roberts et al. (17) found that increasing insert size between α-tropomyosin exon 3 and its downstream regulatory element had an increasing effect on splicing. This result is consistent with their kinetic model for alternative splicing control. However, none of the random sequence size control insertions had any effect on alternative splicing of the FGFR2 minigene (Fig. 2). This is more consistent with a MAZ4-dependent change to the transcription complex, rather than a kinetic competition between splicing and silencing. It is interesting that the ICE can be moved more than 300-bp downstream with no effect on silencing of exon IIIb. PTB binding to sequences both upstream and downstream of exon IIIb has been proposed to establish a “zone of silencing” (58). Our data suggest that this zone of silencing can be established even when the PTB sites lie far downstream of the silenced exon.

An emerging theme in the link between transcription and alternative splicing control is that events at the promoter may influence splicing decisions far downstream. Alterations in promoter elements that influence the elongation rate of the polymerase impact alternative splicing decisions (3, 4, 6–8). Additionally, different splicing factors can be co-purified with both general transcription factors and with transcriptional activators, suggesting that splicing control could be determined by the proteins that are associated with the transcription complex at the time of transcript initiation (14). Another example occurs in the protocadherin genes, where a series of variable exons are alternatively spliced to downstream constant exons. The choice of variable exon is dictated by alternative promoter choice; each variable exon has its own promoter and only the promoter proximal variable exon is spliced to the constant exons (59). Similar promoter-dependent events may influence FGFR2 alternative splicing. Indeed, changing the promoter from the CMV immediate early promoter to the adenovirus major late promoter changes the splicing pattern of both the wild-type minigene and the MAZ4 insertion constructs (Fig. 7). Our data are most consistent with a model in which the MAZ4 element disrupts the transcription elongation complex in such a way as to eliminate exon IIIb silencing. This suggests that a factor associated with the elongation complex is required for correct FGFR2 alternative splicing.

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