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Control of BEK and K-SAM Splice Sites in Alternative Splicing of the Fibroblast Growth Factor Receptor 2 Pre-mRNA

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The fibroblast growth factor receptor 2 gene pre-mRNA can be spliced by using either the K-SAM exon or the BEK exon. The exon chosen has a profound influence on the ligand-binding specificity of the receptor obtained. Cells make a choice between the two alternative exons by controlling use of both exons. Using fibroblast growth factor receptor 2 minigenes, we have shown that in cells normally using the K-SAM exon, the BEK exon is not used efficiently even in the absence of the K-SAM exon. This is because these cells apparently express a titratable repressor of BEK exon use. In cells normally using the BEK exon, the K-SAM exon is not used efficiently even in the absence of a functional BEK exon. Three purines in the K-SAM polypyrimidine tract are at least in part responsible for this, as their mutation to pyrimidines leads to efficient use of the K-SAM exon, while mutating the BEK polypyrimidine tract to include these purines stops BEK exon use.

Multiple alternative splicing events lead to synthesis from the fibroblast growth factor (FGF) receptor 2 (FGFR-2) gene of a family of receptors differing in defined parts of their extra- and intracellular domains (reviewed in references 19 and 21). In its first described version, FGFR-2 contains an extracellular domain made up of three immunoglobulin-like domains (Ig domains), with a stretch of consecutive acidic residues, the acid box, separating the first two Ig domains. A particularly interesting alternative splice concerns sequences of the mRNA coding for the carboxy-terminal half of the third Ig domain, as this region of the receptor appears to be part of the ligand-binding site. Two alternative exons (K-SAM and BEK) code for this part of FGFR-2 (4, 20, 29, 40). Use of the K-SAM exon results in synthesis of a high-affinity receptor for acidic FGF and keratinocyte growth factor (KGF), while use of the BEK exon yields a high-affinity receptor for acidic FGF and basic FGF (16, 29, 40). Correct control of the BEK–K-SAM splicing choice appears important, since this choice can influence a cell's response to growth factors that it produces itself as well as to those present in its environment. Consistent with this view, we and others have shown that a given cell line uses predominantly one of the two alternative exons, use of the other exon being sufficiently rare that it cannot be detected in a reverse transcriptase (RT)-polymerase chain reaction (PCR) analysis (4, 29). Thus, epithelial cells express the K-SAM form of FGFR-2 but do not produce KGF, an epithelial cell-specific growth factor, while fibroblasts, which secrete KGF, express the BEK form. The consequences of a "wrong" choice can be disastrous: forced expression of the K-SAM receptor form in fibroblasts producing KGF leads to transformation (30).

We are interested in determining the mechanisms involved in discrimination between the BEK and K-SAM exons. Pre-mRNA sequence elements representing potential targets for control of splicing include the 5′ and 3′ splice sites, the branch point sequence, and the associated polypyrimidine tract (reviewed in references 3, 13, and 28). These elements are known to interact early in splicing with different spliceosome components, the 5′ splice site with the U1 small nuclear ribonucleoprotein (snRNP), the branch point sequence with the U2 snRNP, and the polypyrimidine tract with a variety of proteins such as U2 auxiliary factor (U2AF), PBP/PTB, and IBP (reviewed in references 13 and 28). Inherent splice site strength reflects the importance of these interactions. Strong 5′ splice sites (11) and branch points (41) are capable of more extensive base pairing than are their weaker counterparts with defined regions of U1 and U2 snRNAs, respectively. Interruption with purines decreases the affinity of polypyrimidine tracts for the corresponding binding proteins (31).

In alternative splicing, one of a pair of alternative splice sites is often inherently stronger. Use of the weaker splice site requires either its activation or repression of the stronger splice site by *trans*-acting factors (reviewed in references 3, 13, and 28). Alternatively, modulation of the stability of secondary structures in the pre-mRNA, presumably by *trans*-acting factors, can change splice site accessibility (5, 14, 24). As a first step toward identifying possible *trans*-acting factors controlling alternative splicing of the FGFR-2 pre-mRNA and their targets, we report here that the weak polypyrimidine tract associated with the K-SAM exon stops its efficient use in HeLa cells, while use of the BEK exon is repressed in a keratinocyte cell line.

MATERIALS AND METHODS

Cell lines. SVK14, human keratinocytes transformed by simian virus 40 (SV40), were as described in reference 36. The HeLa cell line from an epidermal carcinoma of the cervix was as described in the American Type Culture Collection catalog. Cells were maintained by standard cell culture techniques.

Plasmids. pSG1, a pKCR3 (25) derivative in which the pBR322 backbone has been replaced by pBluescribe sequences, was a gift from S. Green. The BK1 minigene was obtained by placing 4.8 kb of FGFR-2 genomic gene sequences (4) between the SV40 promoter and the globin gene polyadenylation site of pSG1. These sequences include 150

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bp of upstream flanking exon C1 (nucleotides 801 to 951 [16]), 1,148 bp of intron, the 147-bp K-SAM exon, 1,220 bp of intron, the 144-bp BEK exon, 1,997 bp of intron, and 37 bp of the downstream flanking exon C2 (nucleotides 1097 to 1134 [16]). The minigene BK3 was obtained by placing nucleotides 1 to 800 of the FGFR-2 cDNA (16) immediately upstream of exon C1 in BK1. ΔK-SAM was obtained by deleting a 0.84-kb *Bgl*II-*Xba*I fragment from BK3, i.e., from the *Bgl*II site lying 0.48 kb upstream of the K-SAM exon to the *Xba*I site lying 0.21 kb downstream from it. Δ252-918 was obtained by deleting a 666-bp *Apa*I fragment from BK3. Δ404-582 was made by digesting BK3 with *Bsp*EI and *Pfl*MI and recircularizing in the presence of the oligonucleotide 5′-CCGGGCG-3′. BK3ΔNcoI was made by deleting a 42-bp *Nco*I fragment from BK3. ΔBEK was prepared by deleting a 73-bp *Xho*I-*Hpa*I fragment including the BEK exon polypyrimidine tract and 3′ splice site and the first 12 bp of the BEK exon from sSb(g)B. Other BK3 deletions were made by using standard PCR technology (1). BK3+1, Cys2→Ser, and Cys4→Val were made by using the Transformer mutagenesis kit from Clontech. The selection primer was 5′-GCAAAAAGCTCGATCCCCCGG-3′, designed to eliminate a unique *Bam*HI site lying in the polylinker between the SV40 promoter and the FGFR-2 sequences (the mutation is underlined). The mutation primers were 5′-GTAACCATG GTCGAGCTGGGGTCGT-3′ for BK3+1, 5′-CCTCTATGC TAGCACTGCCAGTAG-3′ for Cys2→Ser, and 5′-GGAAA TTATACCCGTGTGGTGGAG-3′ for Cys4→Val. Standard PCR technology (1) was used for the introduction of *Sal*I, *Xho*I, and *Xba*I sites into BK3 to make sSbB and for construction of s(c)SbB, s(t)SbB, s(a)SbB, s(g)SbB, and sSb(g)B. bSsB, sSsB, and bSbB were obtained by exchanging appropriate parts of BK3 and sSbB. Mutations were verified by sequencing.

PCR analysis. SVK14 or HeLa cells were transfected by the calcium phosphate coprecipitation technique (1). RNA was harvested 60 h posttransfection (or directly from untransfected cells) by using a rapid cytoplasmic RNA extraction technique (12). cDNA was synthesized from RNA (2 μg) with an oligo(dT) primer in a final volume of 50 μl; Moloney murine leukemia virus RT was used under the conditions specified by the manufacturer (GIBCO-BRL). PCR was carried out in a final volume of 25 μl, using a 1-μl aliquot of cDNA with reaction conditions specified in the GeneAmp kit and a DNA Thermal Cycler, both from Perkin-Elmer. PCR parameters (30 cycles) were as described in the RACE protocol (8), with an annealing temperature of 58°C in all cases. For analysis of the endogenous gene, primers used were P3 (5′-CGCCTTCGGTTCCTGAG-3′) and P4 (5′-GTC TGGGGAAGCTGTAAT-3′). For analysis of minigenes, primers used were P1 (5′-CCAGAAGTAGTGAGGAGG-3′; from the SV40 sequence) and P2 (5′-TTGTGAGCCAGGG CATTG-3′; from the globin gene sequence). Aliquots (4 μl) of PCR products were digested in a final volume of 10 μl with *Ava*I, *Hin*cII, or *Eco*RV, using conditions specified by the manufacturer (Boehringer Mannheim). K-SAM fragments should decrease in size by 0.25 kb following *Ava*I digestion; BEK fragments should decrease in size by 0.15 and 0.24 kb following digestion by *Eco*RV and *Hin*cII, respectively. Digested fragments were separated by electrophoresis on agarose gels and transferred to a Hybond-N nylon filter, using the alkaline transfer protocol given by the manufacturer (Amersham) with a VacuGene apparatus from Pharmacia LKB. Blots were hybridized to a fragment of the FGFR-2 gene (nucleotides 1 to 2291 [16]) ^{32}P labeled by using a Multiprime DNA labeling system from Amersham. In all cases, at least three independent transfections were carried out and analyzed. When mutated BK3 minigenes were analyzed, BK3 was always transfected in parallel as a positive control. In some cases, to facilitate cloning of amplified fragments, reamplifications of minigene samples were carried out with a primer just downstream from P3, 5′-GGAGGCTTTTTGGAGGCC-3′, and a primer just upstream from P4, 5′-CTGATAGGCAGCCTGCACC-3′. Reamplification did not affect the distribution of products. Reamplified material was cut by *Bam*HI (in the SV40 sequence) and *Eco*RI (in the globin sequence) and introduced between the corresponding sites of pBluescriptSK− (Stratagene) for sequencing by the dideoxy method with a Sequenase 2.0 kit from the United States Biochemical Corp.

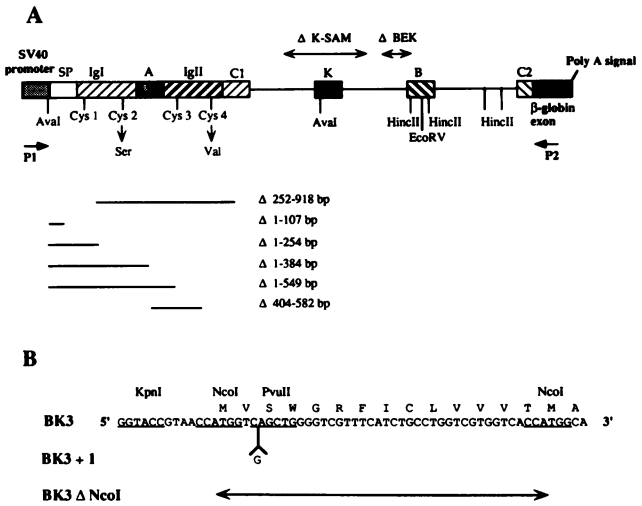

FIG. 1. Schematic representation of the BK3 minigene and mutations thereof. (A) The BK3 minigene. BK3 contains the K-SAM (K) and BEK (B) exons (their characteristic restriction enzyme sites are shown) as well as the upstream flanking exon C1 and part of the downstream flanking exon C2. In addition, sequences coding for the signal peptide (SP), the first two Ig domains (IgI and IgII), and the acid box (A) of FGFR-2 are present. Primers P1 and P2, used for specific amplification of BK3 cDNAs, are shown. Cys1 and -2 and Cys3 and -4 refer to codons for cysteine residues believed to hold together the first and second Ig domains, respectively. In one BK3 mutant, the Cys-2 codon has been changed to a serine codon, and in another, the Cys-4 codon has been changed into a valine codon, as shown. The extents of several deletion mutants of BK3 are indicated by horizontal lines. (B) Mutations of the BK3 coding sequence. In BK3+1, a G has been added immediately after the second codon. In BK3ΔNcoI, the first 14 codons have been deleted.

RESULTS

Correct splicing of a minigene pre-mRNA. The FGFR-2 minigene BK3 (Fig. 1) contains linked FGFR-2 gene and cDNA sequences placed between the SV40 early gene promoter and a rabbit β-globin gene polyadenylation site. The FGFR-2 gene fragment includes the K-SAM and BEK exons coding for alternative carboxy-terminal halves of the third Ig domain, as well as the upstream flanking exon C1 and part of the downstream flanking exon C2. Exon C1 codes for the amino-terminal half of the third Ig domain of FGFR-2. FGFR-2 cDNA sequences coding for the signal peptide, the first two Ig domains, and the acid box are joined directly to exon C1. We wished to determine whether BK3 pre-mRNA can be correctly spliced in HeLa and SVK14

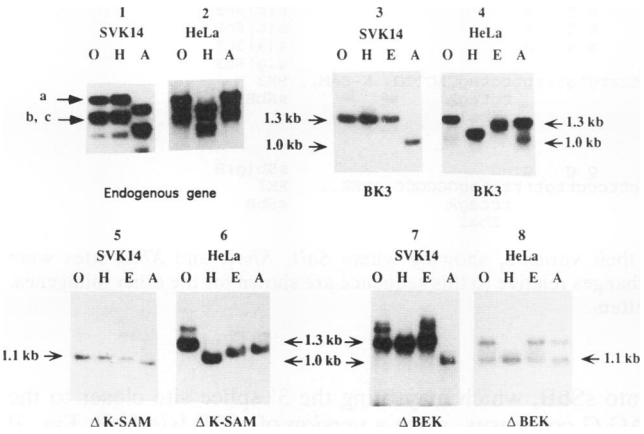

FIG. 2. RT-PCR analysis of splicing in HeLa and SVK14 cells of pre-mRNAs from either the endogenous FGFR-2 gene, the BK3 minigene, or versions thereof lacking either the K-SAM or the BEK exon. Where appropriate, cells were transfected with 20 μg of DNA, and RNA was harvested and reverse transcribed into cDNA. cDNA was amplified by using primers P1 and P2 of Fig. 1A (minigene experiments) or primers P3 and P4 (endogenous gene experiments); samples were digested by *Hin*cII (H), *Eco*RV (E), or *Ava*I (A) or were not digested (0) prior to Southern blotting analysis using an FGFR-2 probe. For the endogenous gene experiments, P3 corresponds to a part of the FGFR-2 mRNA's 5′ untranslated region, while P4 is complementary to sequences coding for the last six amino acids of the extracellular domain. Use of these primers thus amplifies fragments coding for the entire FGFR-2 extracellular domain. a, b, and c identify fragments of 1.26, 0.99, and 0.92 kb. The 1.26-kb fragments represent FGFR-2 gene transcripts coding for three Ig domains and the acid stretch. The 0.99- and 0.92-kb fragments represent FGFR-2 gene transcripts lacking sequences coding for the first Ig domain and for the first Ig domain and the acid stretch, respectively.

cells, which normally use predominantly the BEK exon and the K-SAM exon, respectively (4).

As a general technique to distinguish between BEK and K-SAM mRNAs, we prepare cDNA and amplify it by using primers derived from exon sequences flanking the BEK and K-SAM exons. It is possible to distinguish (4) between amplified cDNAs containing BEK and K-SAM sequences, as the K-SAM exon contains an *Ava*I site absent from the BEK exon, while the BEK exon has two *Hin*cII sites and an *Eco*RV site absent from the K-SAM exon (Fig. 1). Control experiments using in vitro-transcribed BEK or K-SAM RNA showed that neither RNA was favored with this protocol (our unpublished results). When this RT-PCR approach was used to analyze HeLa and SVK14 mRNAs derived from the endogenous FGFR-2 gene, results obtained confirmed our previous observations (4): for SVK14 cells, a large majority of amplified fragments contained an *Ava*I site, reflecting predominant use of the K-SAM exon in these cells, while for HeLa cells, a large majority of amplified fragments contained *Hin*cII sites, reflecting predominant use of the BEK exon (Fig. 2, panels 1 and 2; the figure legend identifies the various fragments).

SVK14 and HeLa cells were transfected with 20 μg of BK3. RNA was harvested and used for making cDNA. The cDNA obtained was amplified by using primers derived from the SV40 and globin sequences (P1 and P2; Fig. 1). Only cDNAs corresponding to minigene-derived transcripts should be amplified (the expected size of the amplification product is 1.3 kb). The amplified fragments were digested with *Ava*I, *Hin*cII, or *Eco*RV and subjected to a Southern blotting analysis using an FGFR-2 probe. Use of the K-SAM exon should result in fragments with no *Hin*cII or *Eco*RV sites but two *Ava*I sites, since apart from the *Ava*I site in the K-SAM exon, there is an *Ava*I site (the polylinker site) in BK3 in the junction region between the SV40 promoter and FGFR-2 sequences (Fig. 1). Use of the BEK exon should result in fragments with *Eco*RV and *Hin*cII sites and only the polylinker *Ava*I site. Results obtained (Fig. 2) were consistent with predominant use of the K-SAM exon in BK3 transcripts in SVK14 cells (panel 3), while the BEK exon was used predominantly in HeLa cell BK3 transcripts (panel 4). Our identification of the above-mentioned fragments was confirmed by the sequencing of subcloned fragments. We conclude that pre-mRNA from the BK3 minigene is spliced in a fashion similar to that for FGFR-2 gene pre-mRNA.

Control is exerted on both exons. To investigate control of each exon separately, we transfected cells with 20 μg of BK3 versions from which the K-SAM exon and flanking sequences had been deleted (ΔK-SAM; Fig. 1) or which lacked the BEK exon polypyrimidine tract and 3′ splice site (ΔBEK; Fig. 1). Results of the RT-PCR analysis for ΔK-SAM with use of P1 and P2 (Fig. 2) are consistent with predominant use of the BEK exon in HeLa cells (panel 6). In the SVK14 sample (panel 5), the PCR product is 1.1 kb in size, is not cut by *Eco*RV or *Hin*cII, and appears to contain only the polylinker *Ava*I site. We supposed that this product represents ΔK-SAM transcripts containing neither the K-SAM nor the BEK exon and in which the flanking exons C1 and C2 have been spliced together. This identification was confirmed by sequencing subcloned 1.1-kb fragments. Efficient use of the BEK exon is not made in SVK14 cells, even in the absence of the K-SAM exon.

The results of the RT-PCR analysis for ΔBEK are shown in Fig. 2, panels 7 and 8. In SVK14 cells, the K-SAM exon is used predominantly. In the HeLa sample, the major amplification products are 1.1 and 1.5 kb in size. Neither of these fragments contains BEK or K-SAM sequences (no digestion by *Ava*I or *Eco*RV). The 1.1-kb fragments represent transcripts in which the flanking exons C1 and C2 have been spliced together. We believe that the 1.5-kb fragments represent transcripts in which exon C1 has been spliced to 3′ splice sites upstream of that of exon C2, as these fragments hybridize to a probe corresponding to the intron upstream from the exon C2 3′ splice site (data not shown) and are cut by *Hin*cII (there are *Hin*cII sites in this intron; Fig. 1). This use of the splice sites upstream of the exon C2 3′ splice site may be a consequence of the particularly poor polypyrimidine tract associated with the exon C2 3′ splice site (4). We conclude from these experiments that efficient use of the K-SAM exon is not made in HeLa cells, even in the absence of a functional BEK exon, and that control is exerted on each exon.

A weak polypyrimidine tract hinders K-SAM exon use in HeLa cells. Why is the K-SAM exon not used efficiently in HeLa cells? A comparison of mouse, chicken, and human (4, 34, 40) BEK and K-SAM splice site sequences provides a clue. While neither of the two 5′ splice sites appears inherently stronger (both have two changes from the consensus AG GURAGU), the K-SAM polypyrimidine tract is interrupted by more purines in all of the available sequences (see Fig. 3 for the human sequences).

To investigate a possible influence of polypyrimidine tract sequences on the K-SAM/BEK choice, we constructed a version of BK3 designed to allow permutations of sequences upstream of the K-SAM and BEK 3′ splice sites (sSbB; Fig.

```
gtcgac                                              c c   c                    s(c)SbB
gtcgac                                              t t   t                    s(t)SbB
gtcgac                                              a a   a                    s(a)SbB
gtcgac                                                                         s(g)SbB
atagacaatgctaagaccttcctggttggccgttatattgttctcctgtgtctgtttctagCACTCG..K-SAM.. BK3
gtcgac                                                        tctagA           sSbB
 SalI                                                          XbaI

ctcgag                                              g g   g                    sSb(g)B
ccacagccccctccacaatcattcctgtgtcgtctagccttttcttttgcttcccttgttttctagGCCGCC..BEK.. BK3
ctcgag                                                        tctagA           sSbB
 XhoI                                                          XbaI
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FIG. 3. Sequences of K-SAM and BEK polypyrimidine tracts and their variants, showing where *Sal*I, *Xho*I, and *Xba*I sites were introduced by mutation. The BK3 minigene sequence is shown; only the changes relative to this sequence are shown for the other minigenes. Intron sequence is in lowercase letters; exon sequence is in uppercase letters.

3 and 4A). sSbB has *Xba*I sites at K-SAM and BEK 3′ splice sites and a *Sal*I or *Xho*I site around 60 bp upstream of these splice sites. Use of the RT-PCR protocol showed that transfection of 20 μg of sSbB into SVK14 cells yielded transcripts containing predominantly the K-SAM exon (Fig. 4B, panel 1). However, transfection into HeLa cells yielded transcripts using the BEK exon as well as transcripts using the K-SAM exon (Fig. 4B, panel 5; some fragments digested by *Ava*I and others digested by *Hin*cII). Activation of the K-SAM exon appears to be due to the *Xba*I site introduced into sSbB, which may bring the 3′ splice site closer to the AG G consensus, since a version of sSbB [s(g)SbB; Fig. 3] without the *Xba*I site uses predominantly the BEK exon in HeLa cells (data not shown).

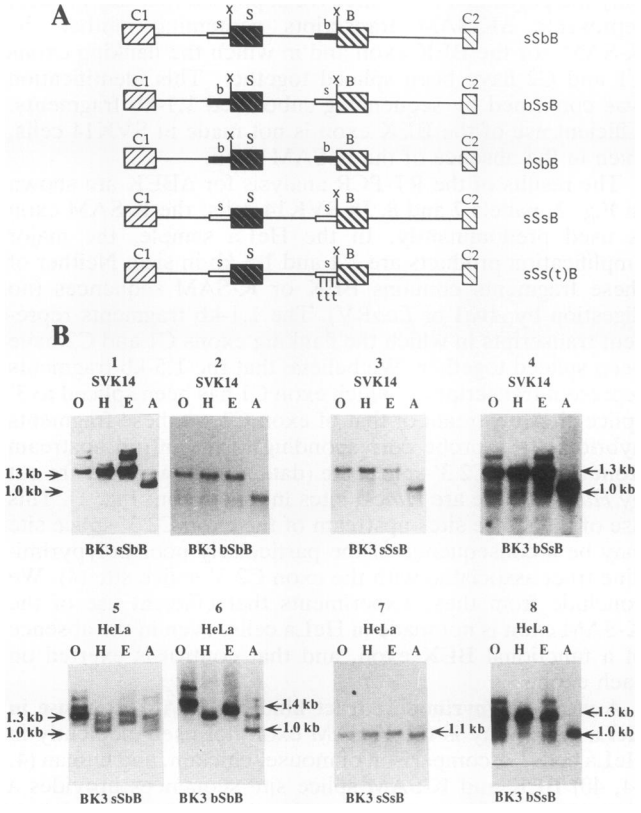

FIG. 4. Effect on splicing of manipulation of polypyrimidine tracts. (A) Schematic representation of parts of the BK3 derivatives sSbB, bSsB, sSsB, and bSbB. The K-SAM polypyrimidine tract (in fact a 60-bp fragment; see Fig. 3) is referred to as s; the BEK polypyrimidine tract (in fact a 66-bp fragment; see Fig. 3) is referred to as b. X, *Xba*I. (B) RT-PCR analysis of splicing of pre-mRNA from the above-described BK3 derivatives. Cells were transfected with 20 μg of the various BK3 mutants, and RNA was harvested and analyzed as described in the legend to Fig. 2.

Exploiting the *Xba*I, *Sal*I, and *Xho*I sites of sSbB, we made a series of minigenes in which the sequences immediately upstream of the K-SAM and BEK exons had been exchanged. We obtained the minigenes bSsB, sSsB, and bSbB (Fig. 4A; s and b refer to the 60- and 66-bp sequences immediately upstream of the K-SAM and BEK 3′ splice sites, respectively, while S and B refer to the K-SAM and BEK exons, respectively). Results of the RT-PCR analysis following transfection of 20 μg of these minigenes into SVK14 cells are shown in Fig. 4B. In all cases, the K-SAM exon is used predominantly, consistent with the notion that use of the BEK exon is repressed in these cells. Results from HeLa cell transfections can be summarized by saying that exons associated with the b sequence are used efficiently, while exons associated with the s sequence are not (except for sSbB, in which, as discussed above, the 3′ splice site has also been improved). In particular, with bSbB, both K-SAM and BEK exons are used and are indeed spliced together. The PCR product (panel 6) is of the form C1–K-SAM–BEK–C2 (1.4 kb, with sites for *Ava*I, *Hin*cII, and *Eco*RV). This identification was confirmed by sequencing subcloned 1.4-kb fragments. For bSsB (panel 8), the normal splicing pattern is completely inverted: only the K-SAM exon is used. For sSsB (panel 7), however, the PCR product is of the form C1-C2 (1.1 kb, with only the polylinker *Ava*I site), this identification again being checked by sequencing. This result confirms our previous observation that HeLa cells do not use the K-SAM exon efficiently even when the BEK exon is not used.

The results presented above suggest that the 60 bp immediately upstream from the K-SAM exon are sufficient to hinder use of the immediately downstream exon, be it the K-SAM or the BEK exon. What part of these 60 bp is responsible for this effect? Is it indeed the weak K-SAM polypyrimidine tract? To answer this question, we prepared two minigenes with K-SAM polypyrimidine tracts reinforced by point mutations: three g residues in the normal tract were replaced by t or c [s(t)SbB and s(c)SbB; Fig. 3]. When 20 μg of these minigenes was transfected into HeLa cells and the RT-PCR analysis was carried out, 1.4-kb fragments were obtained (Fig. 5, panels 2 and 3). They were cut by *Ava*I, *Hin*cII, and *Eco*RV and are thus of the form C1–K-SAM–BEK–C2, since the K-SAM and BEK exons had been spliced together. Full activation of the K-SAM exon was obtained only when all three g residues were mutated. When only one of any of the g residues was replaced by a t, PCR products were a mixture of the 1.4-kb C1–K-SAM–BEK–C2

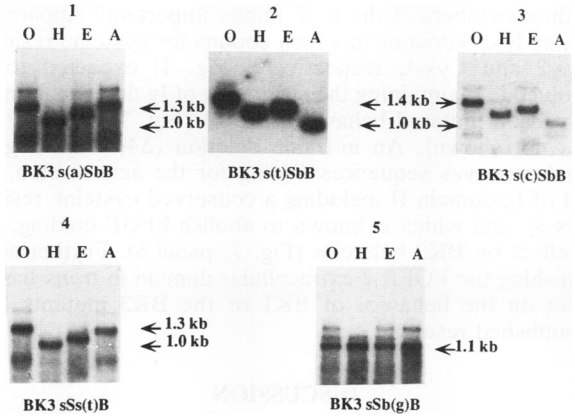

FIG. 5. Effect on splicing of manipulation of particular purine residues in polypyrimidine tracts. HeLa cells were transfected with various BK3 mutants (20 μg) as described in the legend to Fig. 3, and RNA was harvested and analyzed as described in the legend to Fig. 2.

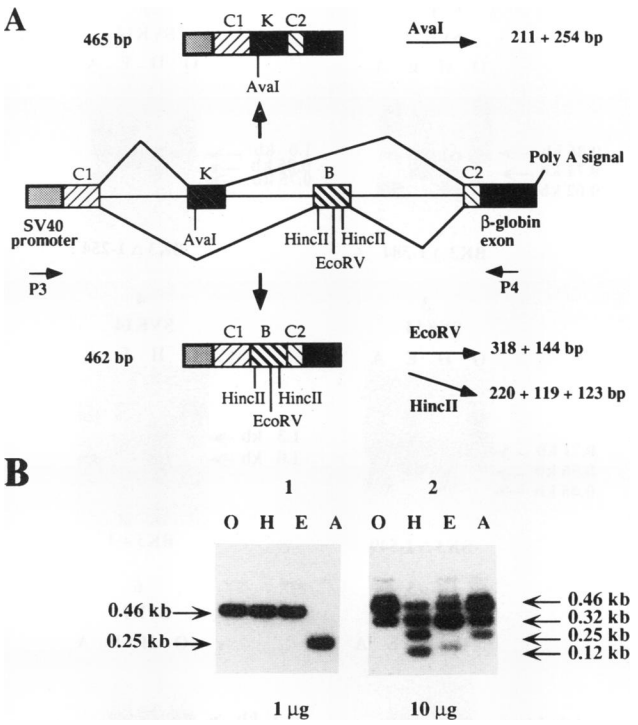

FIG. 6. The BK1 minigene and splicing of its pre-mRNA in SVK14 cells. (A) Schematic representation of the BK1 minigene showing the K-SAM (K) and BEK (B) exons with their characteristic restriction enzyme sites, as well as the upstream (C1) and downstream (C2) flanking exons. Also shown are primers P1 and P2, used for amplification of BK1 cDNAs, and schematic representations of possible amplification products and their behavior following digestion by *Eco*RV, *Ava*I, or *Hin*cII. (B) RT-PCR analysis of splicing of pre-mRNA from the above-described BK3 derivatives in SVK14 cells. Cells were transfected with 1 or 10 μg of BK1, and RNA was harvested and analyzed as described in the legend to Fig. 2.

fragment and the 1.3-kb C1-BEK-C2 fragment (data not shown). As a control, we also prepared a similar minigene retaining the three g residues and another in which the three g residues were changed to a [s(g)SbB and s(a)SbB; Fig. 3]. When these minigenes were transfected into HeLa cells and the RT-PCR analysis was carried out, the resulting 1.3-kb fragments reflected predominant use of the BEK exon (Fig. 5, panel 1, and data not shown). The three purine residues of the K-SAM polypyrimidine tract are thus indeed critical for silencing the K-SAM exon in HeLa cells.

The results obtained with sSsB and bSsB show that use of a BEK exon associated with an s sequence is blocked in HeLa cells. Evidence for the importance of the g residues of the K-SAM polypyrimidine tract in this phenomenon was obtained from a minigene in which the g residues in the s sequence upstream of the BEK exon had been converted into t residues [sSs(t)B; Fig. 3 and 4A]. When sSs(t)B was transfected into HeLa cells and the RT-PCR analysis was carried out, the resulting fragments reflected predominant use of the BEK exon (Fig. 5, panel 4). Further evidence for the importance of these g residues in blocking exon use in HeLa cells was obtained from the minigene sSb(g)B, in which the b sequence had been mutated to introduce g residues into the BEK polypyrimidine sequence at appropriate positions (Fig. 3). Neither the BEK nor the K-SAM exon was used, C1 being spliced directly to C2 (Fig. 5, panel 5).

Possible repression of BEK exon use in SVK14 cells. If the K-SAM exon's weak polypyrimidine tract stops its efficient use in HeLa cells, why do SVK14 cells not use the BEK exon efficiently? Results of experiments using a second minigene, BK1 (Fig. 6A), suggest that use of the BEK exon is repressed in SVK14 cells. In BK1, the FGFR-2 cDNA sequences coding for the signal peptide and the first two Ig domains, as well as part of exon C1, are missing. This minigene was transfected in parallel into HeLa and SVK14 cells. The results calculated for amplified fragments derived from BEK and K-SAM transcripts are shown in Fig. 6. For HeLa transfections using 10 μg of BK1, results obtained were consistent with predominant use of the BEK exon (data not shown). The results obtained for SVK14 transfections are shown in Fig. 6B. When 1 μg of BK1 was transfected, only K-SAM fragments, cut by *Ava*I but not by *Hin*cII or *Eco*RV, were detected. However, when 3, 5, or 10 μg of BK1 was transfected, a mixture of K-SAM and BEK fragments was detected (some fragments were cut by *Ava*I; others were cut by *Eco*RV and *Hin*cII). This result suggests that SVK14 cells contain a repressor of BEK exon use, which can be titrated by transfecting large amounts of BK1.

Open reading frame requirement for BEK exon repression. Transfection of SVK14 cells with 20 μg of BK3 leads to predominant use of the K-SAM exon, while transfection with not much more than 1 μg of BK1 leads to use of both BEK and K-SAM exons. The explanation for this difference must lie in the extra sequences present in BK3. In an attempt to identify a particular part of BK3 responsible for this phenomenon, we constructed various deletion mutants of BK3 (Fig. 1) and transfected 20 μg of them into SVK14 cells. Removing the first 107, 254, 384, or 549 bp of FGFR-2 gene sequence in BK3 yielded variants which lacked the polylinker *Ava*I site and which behaved like BK1 (Fig. 7, panels 1 to 3, and data not shown). Typically, the RT-PCR protocol yielded fragments of the calculated sizes (0.86 kb for Δ1-384, 1.0 kb for Δ1-254, and 0.71 kb for Δ1-549), in which the K-SAM and BEK exons were about equally represented, as judged by the results of *Ava*I, *Hin*cII, and *Eco*RV digestion. Some larger fragments were also obtained. These larger fragments hybridize to a probe corresponding to the intron preceding exon C2 (data not shown). They appear to repre-

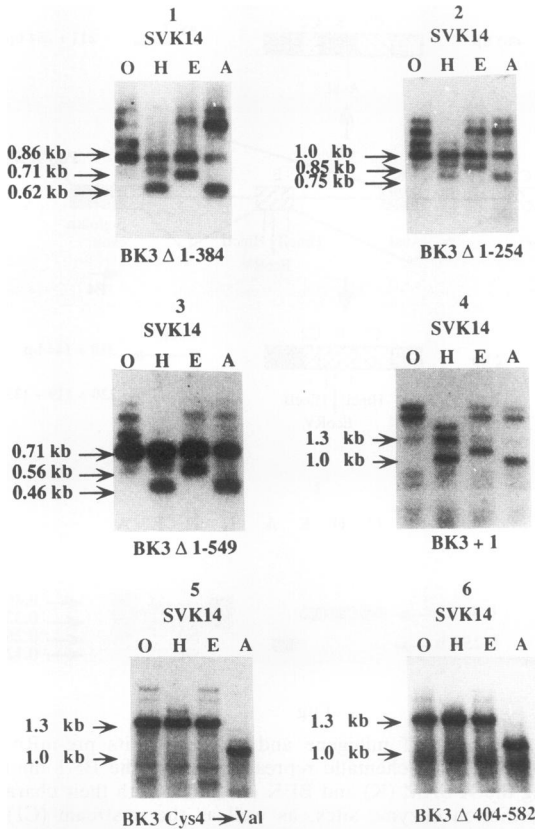

FIG. 7. RT-PCR analysis of splicing of pre-mRNA from truncated BK3 mutants in SVK14 cells. Cells were transfected with various BK3 mutants (20 μg) as described in the legend to Fig. 1, and RNA was harvested and analyzed as described in the legend to Fig. 2.

sent transcripts in which exon C1 has been spliced to the 3′ splice site of either the K-SAM or the BEK exon. However, the 5′ splice sites of these latter exons have been spliced to 3′ splice sites upstream of that of exon C2.

Having failed to identify a truncated version of BK3 which behaves like BK3, we wondered whether the open reading frame present in BK3 might be necessary for its characteristic behavior. We added a single base pair to BK3 just after the second codon of the signal peptide (BK3+1; Fig. 1). Results of the RT-PCR protocol used after transfection of 20 μg of BK3+1 into SVK14 cells are shown in Fig. 7, panel 4. A small amount of the 1.3-kb fragment is observed, together with more of the larger fragments discussed above. In these fragments, we detect approximately equal use of BEK and K-SAM exons, as judged by the results of *Ava*I, *Hin*cII, and *Eco*RV digestion. This frameshift mutation could exert its effect either by disrupting an essential *cis*-acting BK3 RNA sequence motif or by disrupting the open reading frame. To distinguish between these possibilities, we constructed a minigene lacking a short *Nco*I fragment covering the relevant region (BK3ΔNcoI; Fig. 1) and in which the open reading frame is maintained. This minigene behaves like BK3 (data not shown), suggesting that the open reading frame rather than a particular RNA sequence motif is what is important.

The BK3 open reading frame codes for the FGFR-2 extracellular domain. Is production of a protein capable of binding members of the FGF family important? Apparently not, as BK3 versions in which codons for cysteine residues (Cys-2 and Cys-4, respectively; Fig. 1) expected to be involved in maintaining the structures of Ig domains I and II have been mutated behave like BK3 (Fig. 7, panel 5, and data not shown). An in-frame deletion (Δ404-582; Fig. 1) which removes sequences coding for the acid stretch and part of Ig domain II including a conserved cysteine residue (Cys-3), and which is known to abolish bFGF binding, has no effect on BK3 behavior (Fig. 7, panel 6). Furthermore, furnishing the FGFR-2 extracellular domain in *trans* has no effect on the behavior of BK1 or the BK3 mutants (our unpublished results).

DISCUSSION

We present evidence that during splicing of the FGFR-2 pre-mRNA, use of both the alternative BEK and K-SAM exons is subject to control. Thus, inactivation of the BEK exon does not lead to efficient use of the K-SAM exon in HeLa cells, and deletion of the K-SAM exon does not lead to efficient use of the BEK exon in SVK14 cells, but in both cases, the modifications lead to splicing of the flanking exon splice sites together. This double control may explain why, when splicing the endogenous gene's pre-mRNA, some cells can skip both the BEK and the K-SAM exon (22). In principle, activation or repression of splice sites could be involved in the BEK/K-SAM choice. Can we distinguish between these possibilities?

Mutating three g residues in the K-SAM polypyrimidine tract to pyrimidines markedly increases use of the K-SAM exon in HeLa cells. These mutations could eliminate a binding site for a repressor of K-SAM exon use. However, introducing three g residues into the BEK polypyrimidine tract in analogous positions markedly decreases use of the BEK exon. It seems unlikely that we have constituted a repressor binding site in the BEK polypyrimidine tract in this way. On the other hand, the sequence composition of the polypyrimidine tract is known to be important in the selection of 3′ splice sites (7, 9, 31, 33). Both of these above results can be explained if the g residues render the polypyrimidine tracts so weak that efficient use of the associated exons is impossible in the absence of *trans*-acting factors. Such factors would normally activate the K-SAM exon, but only in SVK14 cells.

The required splicing factor U2AF is likely to bind poorly to the weak K-SAM polypyrimidine tract. As this binding is a prerequisite for the U2 snRNP-branch point interaction, use of the downstream 3′ splice site will be hindered. A K-SAM exon activator could function by facilitating the binding of U2 snRNP or U2AF directly or indirectly. Precedents for activation of splice sites have been described in the literature (2, 13, 28). For example, the alternative rat preprotachykinin exon E4 has an inherently inactive 3′ splice site, whose use is enhanced by U1 snRNP binding to the downstream 5′ splice site. This binding targets U2AF to the 3′ splice site by a network of interactions spanning the exon (reference 15 and references therein). U1 snRNP can also enhance 3′ splice site use by binding to internal exon sequences (39). In drosophila sex determination, efficient use of a female-specific 3′ splice site on the doublesex pre-mRNA which is associated with a weak polypyrimidine tract requires activation by the products of the transformer and transformer-2 genes (17, 37). These proteins bind to exon sequences and are presumed to target essential splicing factors to the upstream 3′ splice site.

For the BEK exon, our data are most easily interpreted by invoking the existence of a repressor. Transfection of SVK14 cells with 20 μg of the minigene BK3 leads to predominant use of the K-SAM exon. The same result is obtained for a second minigene BK1 only if small amounts of DNA are transfected. Transfection with not much more than 1 μg leads to use of both BEK and K-SAM exons. The BK1 results may reflect changes in the relative amounts of general splicing factors such as SF2/ASF and heterogeneous nuclear RNP A1. Such changes are known to alter the relative use of alternative splice sites (10, 23, 27). Alternatively, SVK14 cells may contain limited amounts of a specific BEK exon repressor, which can be titrated by transfection of large amounts of BK1. If so, we may note that unlike another well-characterized splicing repressor, the sxl protein (18, 35), the BEK exon repressor does not act uniquely through sequences immediately upstream of the BEK exon, as 66 bp of these sequences are neither sufficient nor indispensable for repression.

Why do we not observe the same titration effect with BK3? Perhaps less BK3 pre-mRNA is made or accumulates. Alternatively, BK3 and BK1 pre-mRNAs could accumulate in similar amounts, but the BK3 pre-mRNA may be spliced under conditions in which more repressor is available. We have not distinguished between these two possibilities. It is, however, intriguing to note that the difference in behavior between BK1 and BK3 is linked to an open reading frame on BK3, even though production of a functional product of this open reading frame (the FGFR-2 extracellular domain) is not necessary. Other examples of the effect of blocking open reading frames on splicing have been described (references 6, 32, and 38 and references therein). Although the basis of these effects remains to be elucidated, one proposed explanation (38) is that coupling is necessary between (i) translation of part of the mRNA already spliced and exported to the cytoplasm and (ii) splicing of the rest of the pre-mRNA still in the nucleus. Another possibility evoked (38) is that scanning (by ribosome-like molecules?) takes place in the nucleus to ensure that splicing conserves open reading frames. Either of these mechanisms can distinguish between different pre-mRNAs and could be exploited to control alternative splicing. Certain pre-mRNAs could be directed to regions more or less rich in particular splicing factors or factor-rich regions created around them, for example.

In conclusion, we favor a model in which predominant use of the K-SAM exon requires both its activation and repression of the BEK exon by *trans*-acting factors. In the absence of these factors, predominant use is made of the BEK exon. If specific *trans*-acting factors are involved in the control of alternative splicing of the FGFR-2 pre-mRNA, it is tempting to speculate that the same factors control the splicing of other pre-mRNAs (several of the well-defined splicing regulators are known to control the expression of more than one gene [26]). As epithelial cells make the K-SAM choice, while fibroblasts make the BEK choice, candidate pre-mRNAs would code, for example, for proteins with epithelial cell-specific or fibroblast-specific versions. The splicing regulators might even play a role in differentiation, by coordinate control of a group of tissue-specific genes.

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