Polypyrimidine Tract-binding Protein Represses Splicing of a Fibroblast Growth Factor Receptor-2 Gene Alternative Exon through Exon Sequences*

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The fibroblast growth factor receptor (FGFR)-2 gene contains two mutually exclusive exons, K-SAM and BEK. We made a cell line designed to become drug-resistant on repression of BEK exon splicing. One drug-resistant derivative of this line carried an insertion within the BEK exon of a sequence containing at least two independent splicing silencers. One silencer was a pyrimidine-rich sequence, which markedly increased binding of polypyrimidine tract-binding protein to the BEK exon. The BEK exon binds to polypyrimidine tract-binding protein even in the silencer’s absence. Several exonic pyrimidine runs are required for this binding, and they are also required for overexpression of polypyrimidine tract-binding protein to repress BEK exon splicing. These results show that binding of polypyrimidine tract-binding protein to exon sequences can repress splicing. In epithelial cells, the K-SAM exon is spliced in preference to the BEK exon, whose splicing is repressed. Mutation of the BEK exon pyrimidine runs decreases this repression. If this mutation is combined with the deletion of a sequence in the intron upstream from the BEK exon, a complete switch from K-SAM to BEK exon splicing ensues. Binding of polypyrimidine tract-binding protein to the BEK exon thus participates in the K-SAM/BEK alternative splicing choice.

Many eucaryotic genes are composed of exons and introns, and RNA splicing converts their primary transcripts into mRNAs suitable for translation (1). Some pre-mRNAs contain only constitutively spliced exons and yield a single mRNA. Others contain subsets of exons that undergo alternative splicing and generate several different mRNAs, depending on which combination of the available exons are actually spliced. Most often, the different mRNAs code for similar yet distinct forms of a protein. This means that alternative splicing can be used to ensure that a cell synthesizes the particular version of a protein adapted to its needs, which may differ from those of a neighboring cell of another type (2–5). Controlling alternative splicing correctly is thus of fundamental importance.

One question posed by both constitutive and alternative splicing is how exons are recognized on pre-mRNAs. Specific sequences at or close to splice sites are important for this recognition and are known to interact with components necessary for spliceosome assembly (for reviews, see Refs. 1, 6, and 7). The 5′ splice site sequence, for example, is important for U1 snRNP binding, and the polypyrimidine tract associated with the 3′ splice site binds U2AF. In addition, bridging interactions between the proteins bound to the two splice sites are believed to exist (8). However, sequences distinct from those at intron/exon junctions can also influence splicing. Frequently, exons contain exon splicing enhancers required for their splicing. Many exon splicing enhancers bind members of the SR protein family (7). SR proteins are known to interact with both U1 snRNP and U2AF, so they could clearly be involved in splicing activation (2, 9, 10). Certain exons, including some exons with an exon splicing enhancer, contain exon splicing silencers (ESSs); some of these bind hnRNP A1 (7). Intron sequences that activate or repress splicing of an adjacent exon have also been described, and in certain cases proteins interacting with them have been identified (for reviews, see Refs. 7 and 11). One such protein is polypyrimidine tract-binding protein (PTB).1 Repression by PTB generally involves PTB binding to multiple intronic sites. Frequently, sites in both the introns flanking an exon are required, and it has been proposed that interactions between several bound PTB molecules may organize a zone of repression around a regulated exon (7, 11). As an individual exon can be linked to several different control sequences, the decision to splice or not to splice an exon can be quite complex. For example, splicing of a short src gene exon in neuronal cells requires binding of a multiprotein complex including hnRNP F, hnRNP H, and the protein KSRP to a downstream intron splicing enhancer (12–14). In addition, a neural polypyrimidine tract-binding protein isoform may act to reduce the repression of the exon’s splicing, which is normally exerted in non-neuronal cells by polypyrimidine tract-binding protein (14, 15).

Some genes contain alternative exons that are mutually exclusive. This is the case, for example, for the FGFR-2 gene K-SAM (or IIb) and BEK (or IIe) exons (16). Epithelial cells splice the K-SAM exon, whereas cells of mesenchymal origin prefer the BEK exon (17). Abrogation of either specific receptor form in mice leads to severe developmental defects (18–20), showing how important it is to get the splicing choice right. Furthermore, progression of prostate cancer has been shown to be accompanied by a change in FGFR-2 alternative exon choice in a rat model (21, 22). The marked tissue-specific control of the

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1 The abbreviations used are: PTB, polypyrimidine tract-binding protein; FGFR, fibroblast growth factor receptor; hnRNP, heterogeneous nuclear ribonucleoprotein; kh, kilobase pairs; hp, base pair(s); PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; ESS, exon splicing silencer; NEO, neomycin phosphotransferase; oligo, oligonucleotide; snRNP, small nuclear ribonucleoprotein.
K-SAM/BEK choice and its evident biological importance have encouraged work aiming to describe the underlying mechanism. Much of this work has been directed toward the K-SAM exon. It has been shown that multiple elements control splicing of this exon. Elements repressing splicing include an ESS that binds hnRNP A1 (23, 24) and intron splicing silencers in the upstream and downstream introns that bind PTB (11, 25). The downstream intron contains three sequences that activate K-SAM exon splicing (26–28). One of these binds the recently identified splicing activator TIA-1 (29, 30), which appears to assist U1 snRNP binding to the exon’s 5′ splice site. Proteins binding to the other two intron-activating sequences, which appear to act together (28), have not yet been identified. Compared with the K-SAM exon, little information is available about splicing control of the BEK exon. Splicing of this exon can be repressed (26, 31, 32), and among the sequences involved are some intron sequences also implicated in activation of K-SAM exon splicing. The proteins working to repress BEK exon splicing remain, however, to be identified. We set out to use a protocol based on gene transfer and drug selection (33) to clone cDNAs coding for BEK exon splicing repressors. This approach did not lead to the cloning of such cDNAs but did lead us indirectly to the observation that binding sites for poly(pyrimidino)tract-binding protein within the BEK exon itself participate in repressing splicing of this exon.

**EXPERIMENTAL PROCEDURES**

**Plasmids—** pN5 (33) contains two NEO-coding exons separated by a globin intron under control of the SV40 early gene promoter. pNβBEK was made by inserting into the Nβ intron a 2.1 kb fragment of the human FGR2 gene containing the BEK exon flanked by 1.0 kb of upstream and 0.95 kb of downstream intron flanking sequences. Vectors using the human cytomegalovirus immediate early gene promoter for expression of TIA-1 (pTIA-1), hnRNP C1 (phnRNP C1), and baxotrophage MS2 coat protein (pcoat) were based on pCI-neo (Promega) and have been described previously (29). An expression vector for PTB1 was a gift from Christopher Smith. RK3 is an FGR2-2 minigene containing the alternative K-SAM and BEK exons together with flanking intron sequences and has been described elsewhere (28).

**Insert Identification—** HeLa cells were cotransfected with the calcium phosphate precipitation technique as described previously to clone BEK transcripts, or FGR2-2 exon C2 sequences (for analysis of pNβBEK transcripts), or FGR2-2 exon C2 sequences (for analysis of RK3 transcripts). For quantification, radioactivities present in bands were determined using a Molecular Dynamics PhosphorImager and used to calculate splicing percentages. For ΔIAS3 and ΔIAS3 Mut R, the relative contributions of the b1 and s products to the intensity of the b1 + s band was determined using data from the Acoil digests, where the b1 band and the s + band (which is derived from the s band) are separated. All RT-PCR experiments were carried out at least in triplicate, and representative results are shown here.

**Cross-linking and Immunoprecipitation—** RNA probes were synthesized in vitro from pBluescript SK+ based plasmids containing different BEK derivatives with expression vectors for TIA-1, hnRNP C1, and baxo. Both probes (carrying mutations, or insertions of different oligonucleotides) downstream of the T7 promoter, for generation of the α-actin probe, a plasmid containing the NM and SM exons plus the intron between them, with a 30-bp spacer between the NM 5′ splice site and the SM branch point, was used (34). RNAs were uniformly labeled to high specific activity (2–3 × 10^6 cpm/μg for probes) or low specific activity (1–2 × 10^5 cpm/μg for competitors) using T7 RNA polymerase in vitro (Ambion) using manufacturer’s instructions. Unincorporated nucleotides were removed using a MicroSpin G-25 column (Amersham Pharmacia Biotech). For each cross-linking reaction, 3 μl of HeLa nuclear extract (a gift from J. Steväenin) was preincubated for 15 min at 31 °C in a final volume of 10 μl containing 10 μM Hepes, pH 7.4, 50 μM KCl, 0.1 mM EDTA, 0.25 mM dithiothreitol, 0.76 μM ATP, 25 μM creatine phosphate, 1 mM MgCl2, and 0.25 μM of RNA. When appropriate, competitor RNA was added during the preincubation. 10 μl of a buffer containing 10 μM Hepes, pH 7.4, 50 μM KCl, 0.1 mM EDTA, 0.76 μM ATP, 25 μM creatine phosphate, 1 mM MgCl2, 1.25 mM dithiothreitol, 44 units of RNasin (Ambion), 0.1% Nonidet P-40, 60 μg/ml bovine serum albumin, and 10% glycerol was then added, together with 1 μl of probe RNA (250,000 cpm). Samples were incubated for 15 min at 31 °C and then irradiated on ice for 15 min, prior to the addition of RNase T1 (200 units) and incubation for 40 min at 37 °C. Samples were either analyzed directly by electrophoresis on an SDS-8% polyacrylamide gel or immunoprecipitated first. In the latter case, 5 μl of protein A-Sepharose beads (Amersham Pharmacia Biotech) was added, and samples were mixed at 4 °C for 90 min. Beads were removed by centrifugation, and to 10 μl of the supernatant, 1.85 μl of a rabbit polyclonal antibody against poly-pyrimidine tract binding protein (gift from Christopher Smith). Samples were mixed gently at 4 °C for 90 min, before the addition of 7.5 μl of protein A-Sepharose beads and further gentle mixing at 4 °C for 90 min. Beads were washed three times in IPP buffer containing 0.25% Nonidet P-40, and bound...
C1  K-SAM  BEK  C2

BEK skipped:  NEO  NEO

SV40  NEO  BEK  NEO

BEK spliced:  NEO  BEK  NEO

SV40  NEO  Insert  BEK  NEO

NβBEK:

Fig. 1. Structure of the NβBEK gene. Part of the human FGFR-2 gene is shown, with the alternative K-SAM and BEK exons between the constitutively spliced flanking C1 and C2 exons. The BEK exon and the flanking intron sequences, represented by a heavy black line on the FGFR-2 gene fragment, were inserted into a hybrid intron interrupting the sequences coding for NEO, which are under control of an SV40 early promoter to obtain the NβBEK gene. The structures of proteins obtained from this gene if the BEK exon is skipped or spliced are represented schematically above and below the gene, respectively. The positions of primers used for the RT-PCR analyses of Figs. 3, 4, and 6 are indicated by arrows. Also shown is the structure of the NβBEK ins+gene from G418-resistant H7 cells.

proteins were then eluted in 20 μl of SDS loading buffer at 100 °C for 5 min and loaded on an SDS-8% polyacrylamide gel.

RESULTS

An Insert in the BEK Exon Represses Its Splicing—A gene transfer and selection strategy designed to detect proteins capable of changing specific splicing patterns has been described previously (33). We adapted this strategy to study BEK exon splicing. In the NβBEK gene (Fig. 1), sequences coding for the enzyme neomycin phosphotransferase (NEO) are interrupted by a hybrid intron into which the BEK exon and flanking intron sequences have been inserted. When introduced into cells that splice the BEK exon, the NβBEK gene should induce synthesis of an inactive enzyme (NEO/BEK/NEO) containing an insert of BEK protein sequence. Such cells will be sensitive to the drug G418. Any event that leads to repression of BEK exon splicing in the cells (e.g. expression of a repressor following gene transfer) should lead to synthesis of an active enzyme (NEO/NEO), and cells will become G418-resistant.

The NβBEK gene was stably cotransfected into HeLa cells, which normally splice the BEK exon, together with a marker plasmid conferring resistance to puromycin. Several puromycin-resistant colonies were obtained and analyzed. One of these, H7, was G418-sensitive and contained and expressed the NβBEK gene. NβBEK transcripts in H7 cells contained the BEK exon as expected (data not shown). H7 cells were transfected with a cDNA library made from SVK14 cells, which do not splice the BEK exon and should thus express the putative BEK exon splicing repressor. Cells taking up a repressor-encoding cDNA should become G418-resistant. A number of G418-resistant colonies were indeed isolated from the transfected cell population. In most of these, little detectable repression of BEK exon splicing was observed, and we did not investigate the reasons for their resistance to G418. In one of the colonies, BEK exon splicing was completely repressed (data not shown). However, this did not appear to be the result of expression of a repressor, since the endogenous FGFR-2 gene transcripts still contained BEK sequences. Analysis of DNA from this colony showed that its NβBEK gene contained a 192-bp insert within the BEK exon (NβBEK ins, Fig. 1). The sequence of the BEK exon containing this insert is shown in Fig. 2. The insert itself (shown in capital letters) corresponds to a DNA sequence from a region of human chromosome 9 devoid of any known gene. Its insertion in the BEK exon has led to the duplication of three BEK exon base pairs (ttg) flanking the insertion site.

The BEK Insert Contains ESS Motifs—The insert is in some way responsible for blocking BEK exon splicing, so that NβBEK transcripts no longer contain BEK exon sequences and thus code for a functional enzyme. We reasoned that the insert must contain an ESS. A plasmid containing a NβBEK gene with the insert (pNβBEK ins) was prepared and transfected into 293-EBNA cells (which normally splice the BEK exon) in parallel with a plasmid containing the normal NβBEK gene. An RT-PCR analysis was carried out on RNA from transfected cells using primers shown in Fig. 1. As shown in Fig. 3A, NβBEK RNA contains the BEK exon as expected (lane 1), whereas NβBEK ins RNA does not (lane 2). This result strongly suggests that the insert contains an ESS, and we set out to try to localize it.

Deletion of a HpaI-BseRI fragment, a BseRI-Thh1111 fragment, or a BbsI-EcoRV fragment (Fig. 3B, deletions 1, 2, and 4, respectively) from pNβBEK ins did not lead to relief of BEK exon splicing repression (Fig. 3A, compare lanes 3, 4, and 6 with lane 2). However, deletion of a Thh1111-EcoRV fragment (deletion 3, Fig. 3B) restored BEK exon splicing (Fig. 3A, lane 5). (We use the term BEK exon splicing here and below to refer to splicing of the BEK exon containing any residual insert sequences.) These results suggest that the ESS lies between the Thh1111 and BbsI sites. Effectively, deletion of the Thh1111-BbsI fragment (deletion 5, Fig. 3B) led to generation of two RT-PCR products containing BEK exon sequences (Fig. 3A, lane 7). Sequencing of cloned products showed that the larger of these corresponds to splicing of the BEK exon with residual insert sequences, whereas the smaller (marked by a star) corresponds to splicing of the BEK exon using a 5′ splice site (aca ↓ gtaag) created upon ligation of the repaired Thh1111 and BbsI extremities. However, deletion of neither the left half (deletion 6, Figs. 2 and 3B) nor the right half (deletion 7, Figs. 2 and 3B) of the Thh1111-BbsI fragment led to relief of BEK exon splicing repression (Fig. 3B, lanes 8 and 9, respectively). This suggests that each half of the Thh1111-BbsI fragment contains an independent ESS.

One ESS Motif Is a Pyrimidine-rich Sequence—We searched for the putative ESS remaining in pNβBEK ins after deletion 7. This ESS should be contained within a sequence made up of the nucleotides between the Thh1111 site and the start of deletion 7 plus the nucleotides between the end of deletion 7 and the end of the insert. Oligo 2 (Fig. 4A) contains most of this sequence. Oligo 2 was inserted in the BEK exon of pNβBEK five base pairs downstream of the original insert. Oligo 1, a sequence of the same size (Fig. 4A), was inserted in parallel. Oligo 1 is derived from insert sequences and partially overlaps oligo 2, but the overlapping sequences contain several point mutations (Fig. 4). 293-EBNA cells were transfected with the resulting plasmids, and RT-PCR analysis was carried out on RNA harvested from them. Insertion of oligo 2 repressed BEK exon splicing as effectively as the entire insert (Fig. 4B, compare lanes 4 and 2). In contrast, insertion of oligo 1 had a very limited effect (lane 3). We wished to determine whether oligo 2’s action was position-dependent and decided to insert it into
The sequence of the BEK exon is shown in exon with an insert is shown. The insert the BEK exon.

The sequence of the BEK cells contains a 192-bp insert within the asterisk splice site). An splicing (taken to refer to splicing using the BEK exon indicating in Fig. 2 and 'A'). The N/H9252 and the N/H11032.

Fig. 3. Localization of exon splicing silencers within the insert. A, RT-PCR analysis of RNA from 293-EBNA cells transfected with the Nj/BEK gene (+ insert), the Nj/BEK gene containing the insert (+ insert), and the Nj/BEK gene containing deleted versions of the insert (Δ) as indicated in Fig. 2 and A. + BEK, an RT-PCR product corresponding to BEK exon skipping; + BEK, a product corresponding to BEK exon splicing (taken to refer to splicing using the BEK exon's site). An asterisk (lane 7) refers to a product originating from use of a 5' splice site created by deletion 5 and discussed here. B, representation of the BEK exon with its insert. The insert is identified by diagonal shading. Cleaveage sites for restriction enzymes used for creating some deletions are marked, and the extents of sequences deleted are indicated by continuous underlining.

Different oligonucleotides (Fig. 4A, oligos 3–8) whose sequences were derived from that of oligo 2 were also tested for their repression activity following insertion into the EcoRV site of the BEK exon mutated for 5' splice site 2. Oligo 3 (Fig. 4C, lane 2) did not repress BEK exon splicing significantly better than the control oligo 1 (Fig. 4B, lane 3). Oligo 4, however, was more effective (lane 1). Oligo 5 was significantly less active than oligo 4 (compare lanes 3 and 1). One of the differences between these two oligonucleotides is that oligo 5 does not contain a tagg motif present in oligo 4 (Fig. 4A). We have previously shown this motif to have ESS activity in the K-SAM exon (23). However, oligo 6, a version of oligo 4 in which the tagg motif has been changed to tagc, represses BEK exon splicing about as effectively as oligo 4 (Fig. 4C, compare lanes 4 and 1). Oligo 7, a shorter version of oligo 4 with the tagg motif intact, does not repress BEK exon splicing significantly (lane 5). These results suggest that the tagg motif is not responsible for repression activity here. Another difference between oligos 5 and 7, which do not repress BEK exon splicing efficiently, and oligo 4, which does, is that both of the former lack specific pyrimidine runs present in the latter (Fig. 4A). Oligo 5 lacks a tctt run, whereas oligo 7 lacks a tctt run. Both UCUU and UUCU in a pyrimidine-rich context have been identified previously as PTB binding sites (36, 37). Mutating two pyrimidine-rich stretches in oligo 4 to obtain oligo 8 (Fig. 4A) essentially abolishes its repressing activity (Fig. 4C, lane 6). These observations raise the possibility that PTB binding could be important for repression by oligo 4.

The Pyrimidine-rich ESS Binds PTB and Splicing Repression

The Pyrimidine-rich ESS Binds PTB—The experiments described above show that oligo 2 contains an ESS. We used UV cross-linking to investigate proteins that bind to the ESS present in oligo 2. In vitro generated transcripts of the BEK exon,
**A**

Repression of BEK exon splicing by oligonucleotides.

A, map of the BEK exon showing the sites of oligonucleotide insertion. The sequences of oligonucleotides used are marked below the map. The sequence of oligo 2 is marked in heavy type, as are parts of other oligonucleotides that can be found in the oligo 2 sequence. The sequence of oligo 5 represents a contiguous stretch of nucleotides of the insert (see the insert sequence, Fig. 2). This is also the case for oligo 3, except for its last two nucleotides (cgt). Residues mutated in individual oligonucleotides are shown as capital letters. For oligo 8, selected pyrimidines were mutated to purines. Other pyrimidines were interchanged (C to T or T to C) to avoid creating long purine-rich runs. B, RT-PCR analysis of RNA from 293-EBNA cells transfected with the NβBEK gene (– insert), the NβBEK gene containing the insert (+ insert), the NβBEK gene containing oligo 1 or 2 positioned 5 bp downstream from the original insert site (oligo 1 and oligo 2, respectively), or the NβBEK gene containing oligo 2 placed 46 bp downstream from the original insert site, in the BEK exon’s EcoRV site (oligo 2 EcoRV). For B–D, –BEK indicates an RT-PCR product corresponding to BEK exon skipping; +BEK indicates a product corresponding to BEK exon splicing (taken to refer to splicing using the BEK exon’s splice site). C, RT-PCR analysis of RNA from 293-EBNA cells transfected with versions of the NβBEK gene in which 5’ splice site 2 has been inactivated (Mut 5’ss-2 series). The different versions contain different oligonucleotides as marked placed in the BEK exon’s EcoRV site. For samples in B and C, the intensities of the +BEK and the −BEK bands were determined by PhosphorImager analysis. The percentage of BEK exon splicing for each sample was calculated by dividing the intensity of the +BEK band by the sum of the intensities of the +BEK and −BEK bands, before multiplying by 100%. Results are given as the averages of at least three independent experiments, and error bars represent the S.E. For lanes 2 and 4 in B, the +BEK product was below detectable levels. D, RT-PCR analysis of RNA from 293-EBNA cells transfected with the NβBEK gene (BEK), the NβBEK gene in which 5’ splice site 2 has been inactivated by mutation (Mut 5’ss-2), the NβBEK gene in which 5’ splice site 1 has been inactivated by mutation (Mut 5’ss-1), or the NβBEK gene containing oligo 4 in the unmutated BEK exon’s EcoRV site. +BEK products use either 5’ splice site 1 or 2 as marked.
the BEK exon containing oligo 1, and the BEK exon containing oligo 2 (Fig. 5A) were incubated in HeLa cell nuclear extract prior to UV cross-linking, ribonuclease digestion, and SDS-PAGE analysis. The BEK exon containing oligo 2 binds very strongly to a 60-kDa protein (Fig. 5B, lane 2). This protein also binds to the BEK exon and the BEK exon containing oligo 1, although much more weakly (lanes 1 and 3, respectively). The 60-kDa protein appeared as a doublet on shorter exposures (data not shown), suggesting that it might be PTB (38, 39). Using a polyclonal antibody specific for PTB, the doublet could be immunoprecipitated from the BEK, BEK + oligo 2, and BEK + oligo 1 samples (Fig. 5C, lanes 1–3, respectively). In the experiment shown in Fig. 5, B and C, an α-actinin gene transcript previously shown to bind PTB (34) was used as a positive control.
control. It cross-linked to a protein (Fig. 5B, lane 5) of similar size to that cross-linked to the BEK exon containing oligo 2 (lane 2), and both proteins were recovered with similar yields following immunoprecipitation with the anti-PTB antibodies (compare Fig. 5C, lanes 2 and 5). No cross-linked proteins were immunoprecipitated by monoclonal antibodies directed against U2AF65 (data not shown). Given the known splicing repression activity of PTB (7, 11), these results suggest that oligo 2 represses BEK exon splicing by recruiting PTB. This is consistent with the data discussed above showing that oligos 5 and 7 repress splicing less well than oligo 4 (Fig. 4C); both oligos 5 and 7 lack one of the PTB-binding motifs present in oligo 4.

**Binding of PTB to the BEK Exon**—Whereas oligo 2 enhances PTB binding to the BEK exon, PTB binds somewhat to the BEK exon even in the absence of oligo 2 (Fig. 5, B and C, lanes 1). The BEK exon contains five runs of pyrimidines, which are **underlined** in Fig. 2. Four of these are represented schematically in Fig. 5A (marked as pairs L and R). In vitro generated transcripts of a BEK exon (Fig. 5A, Mut L + R) in which these runs have been interrupted by the inclusion of purine residues no longer cross-link detectably to PTB (Fig. 5, B and C, lanes 4), showing that at least some of these pyrimidine runs are important for PTB binding. To determine which runs are important, we constructed BEK exons in which either the first two runs (Fig. 5A, Mut L) or the last two runs (Fig. 5A, Mut R) were interrupted. In vitro generated transcripts from the BEK exon or the BEK exon with the L, R, or L + R mutations were incubated in HeLa cell nuclear extract prior to UV cross-linking, ribonuclease digestion, and immunoprecipitation with the polyclonal PTB-specific antibody. The results show that both the L (Fig. 5D, compare lane 2 with lane 1) and the R mutation (Fig. 5D, compare lane 3 with lane 1) diminish PTB cross-linking to the BEK exon, suggesting that at least one of the first two and at least one of the last two pyrimidine runs in the BEK exon are implicated in PTB binding.

The results described above suggest that the Mut L + R BEK exon should not be able to compete with the BEK exon or the BEK exon containing oligo 2 for binding to PTB. This was confirmed by the experiments shown in Fig. 5E. A 150-fold excess of a very low specific activity transcript of the BEK exon containing oligo 2 competes effectively with the corresponding high specific activity transcript for cross-linking to PTB (compare lanes 3 and 1). In contrast, a 150-fold excess of very low specific activity transcript of the BEK exon with the L + R mutation is not a competitor (compare lanes 5 and 1). A 150-fold excess of very low specific activity transcript of the BEK exon competes effectively with the corresponding high specific activity transcript for PTB cross-linking (compare lanes 11 and 9). However, a 150-fold excess of very low specific activity transcript of the BEK exon with the L + R mutation has little effect on PTB cross-linking to the high specific activity BEK exon transcript, while significantly reducing cross-linking to other proteins (compare lanes 8 and 6).

**Repression by PTB Overexpression Requires BEK Exon Pyrimidine-rich Sequences**—As shown above, insertion of the pyrimidine-rich oligo 2 into the BEK exon leads to increased PTB binding to the exon and repression of its splicing. However, the BEK exon already contains several pyrimidine-rich stretches (Fig. 2), some of which we have shown to interact with PTB. We wondered if increasing PTB levels in 293-EBNA cells, which normally splice the BEK exon, would suffice to repress BEK exon splicing. To address this question, 293-EBNA cells were cotransfected with pN6BEK together with expression vectors for bacteriophage MS2 coat protein (a negative control), PTB1, TIA-1, or hnRNP C1. Like PTB, the latter two proteins are known to bind to pyrimidine-rich sequences involved in splicing control (29, 30, 40). RT-PCR was carried out on RNA from transfected cells. As shown in Fig. 6A, PTB1 overexpression, but not overexpression of TIA-1 or hnRNP C1, led to efficient BEK exon repression (compare lane 2 with lanes 1, 3, and 4). Repression of BEK exon splicing by PTB overexpression requires the pyrimidine-rich stretches present in the BEK exon, which bind PTB, since splicing of a BEK exon with the L + R mutation (Fig. 5A) is not efficiently repressed by PTB1 overexpression (Fig. 6B, compare lane 4 with lane 2). Note that 18 μg of PTB1 expression vector has little detectable effect on splicing of a BEK exon with the L + R mutation (lane 4), whereas 2 μg of PTB1 expression vector suffices to repress completely splicing of a normal BEK exon (lane 2).

**BEK Exon Pyrimidine-rich Sequences Repress BEK Exon Splicing in Epithelial Cells**—The above results show that the exonic polypyrimidine runs can be involved in repressing splicing of the BEK exon. Having established this, we wished to determine whether they are implicated in the mutually exclusive splicing choice between the K-SAM and BEK exons. The BEK exon is not normally spliced in epithelial cells, the alternative K-SAM exon being spliced instead. The RK3 minigene contains the alternative K-SAM and BEK exons together with flanking constitutive exons C1 and C2 (Fig. 7A). The RK3 minigene and a version thereof containing a BEK exon with the L + R mutation (Fig. 7B) was carried out on RNA harvested from transfected cells using a minigene-specific primer pair. To distinguish between BEK and K-SAM exon splicing, RT-PCR products were analyzed by digestion with HpaI (there is one HpaI site in the BEK exon; Fig. 7A) or AvaI (there is one AvaI site in the K-SAM exon; Fig. 7A). As expected, RK3 pre-mRNA was spliced using the K-SAM exon (Fig. 7B, lanes 1–3; RT-PCR products are cleaved by AvaI and not by HpaI). RK3 Mut L + R pre-mRNA was also mainly spliced using the K-SAM exon (Fig. 7B, lanes 4–6), but there was a very modest increase in BEK exon splicing, as evidenced by the presence of some RT-PCR products resistant to AvaI digestion (lane 6) and some products cleavable by HpaI (lane 5).

This increase, albeit modest, encouraged us to investigate...
Effect of mutating the BEK exon’s pyrimidine runs on the splicing choice between the K-SAM exon and the BEK exon in epithelial cells. A, map of the RK3 minigene. RSV, the Rous sarcoma virus long terminal repeat promoter; BGH, the bovine growth hormone gene fragment providing the polyadenylation site. C1 and C2 are the FGFR-2 constitutive upstream and downstream exons, respectively. The normal BEK exon 5’ splice site is marked 5’ss-1; alternative sites described are marked 5’ss-2 and 5’ss-3. Positions of primers used for the RT-PCR analysis shown in B and C are marked by arrows. Below the map are marked the structures of various RT-PCR products obtained, corresponding to skipping of both exons (c), splicing of the K-SAM exon (s), and splicing of the BEK exon using the normal 5’ss-1 splice site (b1), or one of the alternative 5’ splice sites (5’ss-2 (b2) or 5’ss-3 (b3)). Digestion products of these fragments detectable with the probe used in B and C are marked by stars. A, AvaI; H, HpaI. B and C, RT-PCR analysis using primers shown in A of RNA from SVK14 cells stably transfected with the RK3 minigene, an RK3 minigene carrying the L+R mutation, an RK3 minigene from which IAS3 had been deleted (ΔIAS3), or versions of ΔIAS3 carrying the L+R, L, or R mutation. Products were left undigested (0), or were digested by HpaI or AvaI as marked before migration on a 2.5% agarose gel, transfer to a nylon filter, and hybridization with a probe corresponding to C2 sequences. RT-PCR products and their digestion products are identified by letters that correspond to structures shown in A. D, analysis of the RT-PCR data shown in C. For the four samples shown in C, the percentage of each RT-PCR product was determined by PhosphorImager analysis as described under “Experimental Procedures.” Results are given as the averages of three independent experiments, and error bars represent the S.E. For lanes 4-7 in C, the s and c products were below detectable levels. For lanes 1 and 10 in C, the b3 product was below detectable levels.
whether the exonic pyrimidine-rich sequences participate in BEK exon splicing repression, since even significant lifting of BEK exon repression might not suffice to render BEK exon splicing competitive with splicing of the preferred K-SAM exon in epithelial cells. Splicing of the K-SAM exon in SVK14 cells is under complex control. One control sequence is IAS3 (Fig. 7A), which activates K-SAM exon splicing (28). The equivalent of IAS3 in a rat FGFR-2 minigene also represses BEK exon splicing in a rat cell line in which the K-SAM exon is spliced normally (26). We set out to investigate the behavior of minigenes from which IAS3 has been deleted; K-SAM exon splicing should be less favored relative to BEK exon splicing on pre-mRNA from such minigenes. This did indeed prove to be the case when RNA from SVK14 cells stably transfected with genes from which IAS3 has been deleted; K-SAM exon splicing (s) and BEK exon splicing using 5’ splice site 1 (b1); BEK exon splicing using 5’ splice site 2 (b2); and skipping of both exons (c). Skipping of both exons is responsible for generation of the major RT-PCR product (Fig. 7D).

When the analysis was carried out on cells transfected with a version of ΔIAS3 carrying a BEK exon with the L + R mutation, a different pattern of bands was observed (Fig. 7C, lanes 4–6). The L + R mutation derepresses BEK exon splicing, since now essentially no K-SAM exon splicing (s) or skipping of both exons (c) was observed (Fig. 7D). The major RT-PCR product corresponded to splicing of the BEK exon using 5’ splice site 1 (b1), although some products corresponded to use of 5’ splice site 2 (b2), or 5’ splice site 3 (b3), an additional BEK exon 5’ splice site hitherto undescribed. The identity of b3 was established by sequencing cloned b3 products. 5’ splice site 3 lies between the two polypyrimidine-rich sequences making up the L runs (Fig. 2), so it seemed likely that its use was a consequence of mutating these runs. Indeed, b3 (reflecting 5’ splice site 3 use) was the major RT-PCR product observed when the analysis was carried out on cells transfected with a version of ΔIAS3 carrying a BEK exon with just the Mut L mutation (Fig. 7, C (lanes 7–9) and D). Note that this mutation changes the cryptic 5’ splice site sequence from tgc gtaac to tgc gtaaca, actually rendering it a worse match to the 5’ splice site consensus (ag gtaagc). Mutation of the L runs stops PTB binding to them, and it is likely that this renders the cryptic site accessible. The Mut L mutation also derepresses 5’ splice site 1 and 2, since no K-SAM exon splicing or exon skipping was observed, whereas the amounts of products b1 and b2 (reflecting BEK exon 5’ splice site 1 and 2 use, respectively) increased relative to the ΔIAS3 sample (Fig. 7D).

Consistent with our previous observation that a pyrimidine-rich sequence is most effective at repressing a downstream 5’ splice site, the Mut R mutation appears to derepress mainly 5’ splice site 1. This effect on 5’ splice site 1 use can be seen by comparing 5’ splice site 1 use in the ΔIAS3 sample (20% of products; see Fig. 7, C (lane 1) and D) with 5’ splice site 1 use in the ΔIAS3 Mut R sample (43% of products; Fig. 7, C (lane 10) and D). It can also be observed by comparing 5’ splice site 1 use in the ΔIAS3 Mut L sample (25% of products; see Fig. 7, C (lane 7) and D) to 5’ splice site 1 use in the ΔIAS3 Mut L + R sample (48% of products; Fig. 7, C (lane 4) and D).

Exon Binding Sites for PTB and Splicing Repression

We describe the results of work with a cell line that normally splices the FGFR-2 gene alternative exon BEK but was designed to become drug-resistant if splicing of this exon was repressed. We hoped to use this cell line in an expression screening to isolate cDNA clones coding for a specific splicing repressor. This approach did not work. Perhaps an appropriate full-length cDNA was not present in the library screened, or it was not possible to maintain durably a sufficiently high level of repressor expression. However, during the course of the work, we identified a drug-resistant cell line that no longer spliced the BEK exon as a result of the insertion, within this exon, of a 192-bp fragment from human chromosome 9. This fragment contains at least two independent exon splicing silencers. We localized one of these to a pyrimidine-rich fragment, which represses BEK exon splicing by increasing PTB binding to the exon. The BEK exon itself binds to PTB even in the absence of the silencer, and this binding depends on some exonic pyrimidine runs. Overexpression of PTB1 blocks BEK exon splicing, and this again depends on the exon’s pyrimidine runs, suggesting that PTB overexpression represses splicing by increasing PTB binding to them. Our results show that PTB molecules bound to exonic pyrimidine runs can act as splicing silencers. This is of interest; splicing repression by PTB binding to intron sequences has been well documented (7, 11), but this is not the case for repression by PTB binding to exon sequences.

PTB binding to exon sequences has been described before but not shown to correlate with splicing repression. The bovine papilloma virus type 1 ESS (41) contains a U-rich part, which binds U2AF and PTB, and a C-rich part, which binds SR proteins. However, deletion or mutation of just the U-rich region did not significantly affect ESS activity. This was in marked contrast to the dramatic loss of ESS activity when the C-rich core was mutated, and it was concluded that the role, if any, of PTB binding in splicing repression was unclear in this case (41). Several PTB binding sites have been characterized in the intron upstream from a neuron-specific 24 nucleotide exon of the GABA\(_\alpha\) receptor \(\gamma2\) gene (36). Most of these sites are grouped around the exon’s branch point. Adding an RNA competitor containing the 3’ splice site region upstream of the exon to in vitro splicing reactions derepressed exon splicing (36), demonstrating the role exerted by PTB in repression. There is also a PTB binding site in the exon itself. However, it was not shown that the exon site is implicated in splicing repression.

Some other work has linked pyrimidine-rich exon sequences to splicing repression but led to the suggestion that U2AF binding rather than PTB binding might be involved. Thus, several pyrimidine-rich sequences were identified in a search for human genomic sequences capable of inhibiting splicing of a constitutively spliced exon following insertion therein (42). Whereas the proteins interacting with these sequences were not identified, it was shown that inhibition could be achieved by insertion of a U2AF65, but not a PTB, binding site consensus sequence.

How could PTB binding to the BEK exon repress its splicing? Most of the available data concerning PTB’s action on splicing describes the effects of its binding to intron sequences (7, 11, 43). In some cases, PTB may repress splicing via intron binding sites by a simple competition with U2AF (44, 45).

In many cases, however, there is no overlap between PTB binding sites and the U2AF binding site, and PTB binding sites often exist in both of the introns flanking an exon. In one model, PTB oligomerizes between the upstream and downstream binding sites and thus across the exon (for a review, see Ref. 11). Clearly, molecules of PTB bound to exon sequences would be particularly well placed for participation in this latter type of
mechanism. Binding sites for PTB have been characterized in the intron upstream from the BEK exon in the rat FGFR-2 pre-mRNA (25). It is not known if PTB binding sites exist in the downstream intron, although this intron does contain several candidate binding sites. It is thus possible, although we have not tested this here, that PTB molecules bound to the BEK exon interact with other PTB molecules bound to flanking intron sequences to repress BEK exon splicing. Other mechanisms for PTB repression via exon sequences must of course be taken into consideration. It is interesting to note that an ESS in the IgM exon M2, which forms an ATP-dependent complex containing U2 snRNP, contains several pyrimidine runs (46). PTB could thus conceivably be involved in its action and facilitate formation of this complex (46). Perhaps PTB binding to the BEK exon leads to formation of a similar complex on this exon.

The BEK exon and the K-SAM exon are a pair of mutually exclusive FGFR-2 gene alternative exons. Cells of mesenchymal origin splice the BEK exon, whereas epithelial cells splice the K-SAM exon. We have shown that PTB binding to the BEK exon represses its splicing in epithelial cells. Splicing of the BEK exon is thus repressed in epithelial cells in at least two different ways. A sequence in the upstream intron termed IAS3 in the human gene (or ISAR in the rat gene) represses BEK exon splicing but also activates K-SAM exon splicing (26, 27). Deletion of the IAS3 sequence leads to a decrease in K-SAM exon splicing in epithelial cells and an increase in BEK exon splicing. However, skipping of both the BEK and the K-SAM exon is the major splicing choice. If in addition to the IAS3 deletion the BEK exon’s pyrimidine runs are mutated to stop PTB binding to the BEK exon, a complete switch to BEK exon splicing results. The pyrimidine runs appear to repress downstream 5’ splice site use, and runs toward the beginning of an exon may also be implicated in repressing cryptic splice site use. This repression may not be required in the epithelial cell line used here (mutating the pyrimidine runs leads to only a minimal increase in BEK exon splicing in epithelial cells when IAS3 is intact), but it could be of more importance in certain of the wide variety of other cells in which the FGFR-2 gene is expressed and in which BEK exon repression by IAS3 might be less effective. It is interesting to note that splicing of the rat equivalent of the K-SAM exon (exon IIb) is repressed by a sequence in the upstream intron (ISS1) that binds PTB (25). Deletion of ISS1 or a mutation of ISS1 that abolishes PTB binding increases IIb exon splicing from 14% to around 30% when pre-mRNA from a minigene carrying the IIb exon but lacking the IIc exon (the equivalent of our BEK exon) is transfected into cells that normally splice the IIc exon (25). It is not known, however, if the ISS1 mutations would suffice to induce significant IIb exon splicing if the IIc exon were available as a competitor. PTB may thus play comparable roles in repression of the K-SAM exon in cells that normally splice the BEK exon and in repression of the BEK exon in cells that normally splice the K-SAM exon.

What role does PTB play in the cell-specific choice between BEK and K-SAM exon splicing? Studies on the human and the rat FGFR-2 genes have unveiled multiple sequences controlling these exons (24–29). Some of these sequences are probably common to the human and rat systems, but this has not been investigated in detail. A map of the human FGFR-2 pre-mRNA region containing the K-SAM and BEK exons and identified human control elements is shown in Fig. 8. Splicing of the K-SAM exon is repressed by an ESS that binds hnRNP A1 (24). K-SAM exon splicing is activated by three intron sequences, IAS1, -2, and -3. IAS1 binds TIA-1 (29), which may help U1 snRNP-binding to the exon’s 5’ splice site (29). IAS2 and part of IAS3 form a secondary structure required for efficient K-SAM exon splicing (28). Splicing of the BEK exon is repressed by IAS3 and by pyrimidine runs within the BEK exon that bind PTB. It is not clear how these various sequence elements are used to control the cell-specific splicing of the K-SAM and BEK exons. There is no evidence that hnRNP A1 or TIA-1 can influence splicing of the K-SAM exon in a cell-specific manner. We have not detected any difference in PTB expression or PTB cross-linking to the BEK exon between the cells used here that splice the K-SAM exon and those that splice the BEK exon. It is thus possible that these proteins are used to set the stage for additional, yet to be discovered, proteins needed to impose the cell-specific splicing patterns. These proteins could conceivably act through IAS2/IAS3 in epithelial cells to activate K-SAM exon splicing and to repress BEK exon splicing. Our results demonstrate, however, that stopping such proteins from acting is not sufficient to obtain efficient BEK exon splicing; the BEK exon is also repressed by PTB, and this repression needs to be overcome too. There is some indirect evidence that the BEK exon’s normal 5’ splice site is under repression exerted by yet another system in epithelial cells. Thus, when both IAS3 and the BEK exon pyrimidine runs are mutated, a complete switch from K-SAM exon splicing to BEK exon splicing occurs, but use of two alternative 5’ splice sites for BEK exon splicing is more marked in epithelial cells than it is in cells that normally splice the BEK exon, where use of these sites is minimal.

Finally, it is interesting to note that repression of BEK exon splicing by the de novo insertion of additional sequences within the exon has been described before. The basis of one case of Apert syndrome is repression of BEK exon splicing by insertion of an Alu element within the exon (47). This element is associated with a long stretch of pyrimidines, which our results suggest may be implicated in the observed splicing repression. These pyrimidines presumably reflect the element’s origin as a reverse transcript. There is no evidence in favor of an RNA intermediate in the process that led to the insertion of the 192-bp fragment from human chromosome 9 into the BEK exon, and the underlying mechanism for this insertion is not clear. However, our results show that such insertions can take

\[^2^\] C. Le Guiner and R. Breathnach, unpublished results.
place. Inhibitory sequences may be frequent in the human genome (42), and the type of event we have described here, which results in silencing of an exon, may well have played a role in the evolution of our genes.

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Polypyrimidine Tract-binding Protein Represses Splicing of a Fibroblast Growth Factor Receptor-2 Gene Alternative Exon through Exon Sequences
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