MOLECULAR AND CELLULAR BIOLOGY, June 1988, p. 2610–2619
0270-7306/88/062610-10$02.00/0

Vol. 8, No. 6

Comparison of In Vitro and In Vivo Splice Site Selection in κ-Immunoglobulin Precursor mRNA

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Received 17 December 1987/Accepted 25 February 1988

The processing of a number of κ-immunoglobulin primary mRNA (pre-mRNA) constructs has been examined both in vitro and in vivo. When a κ-immunoglobulin pre-mRNA containing multiple J segment splice sites is processed in vitro, the splice sites are used with equal frequency. The presence of signal exon, S-V intron, or variable (V) region has no effect on splice site selection in vitro. Nuclear extracts prepared from a lymphoid cell line do not restore correct splice site selection. Splice site selection in vitro can be altered by changing the position or sequence of J splice donor sites. These results differ from the processing of similar pre-mRNAs expressed in vivo by transient transfection. The 5′-most J splice donor site was exclusively selected in vivo, even in nonlymphoid cells, and even in transcripts where in vitro splicing favored a 3′ J splice site. The in vitro results are consistent with a model proposing that splice site selection is influenced by splice site strength and proximity; however, our in vivo results demonstrate a number of discrepancies with such a model and suggest that splice site selection may be coupled to transcription or a higher-order nuclear structure.

Most eucaryotic primary mRNA transcripts (pre-mRNAs) contain intervening sequences (introns), which must be spliced from the mRNA to form a mature mRNA (for a review, see reference 27). Although considerable progress has been made on elucidating the mechanism and biochemistry of the splicing process, it remains unclear what factors direct the selection of a splice site in the pre-mRNA. Several different experimental approaches and statistical analyses of pre-mRNA sequences have demonstrated that the primary sequences around the intron-exon boundaries (consensus splice sites) play an important role in the selection of splice sites (1, 12, 23, 29, 32). However, since several consensus splice sites which are not used frequently exist in pre-mRNA, consensus splice site sequences alone are not sufficient to designate a splice site. Because eucaryotic mRNAs may contain many intervening sequences, models of splice selection must account for the correct pairing of multiple 5′ and 3′ splice sites. Also, models must include temporal and tissue-specific use of alternative splice sites, which have been observed in a number of systems (for a review, see reference 8).

Several different factors affecting splice site selection have been proposed, including higher-order structure of the pre-mRNA, either alone or complexed with nuclear proteins or ribonucleoproteins (17, 34), and exonic or intronic sequences other than those at the exon-intron boundary (24, 30). Scanning models propose that the splicing machinery identifies splice sites through a linear search of the pre-mRNA (18, 33). In a "first-come, first-served" model, transcription provides a scanning function; splice sites are recognized on the nascent RNA as it is transcribed, and are assembled into "committed" presplicing complexes (1). The inherent splice site strength (defined as "the affinity of a splice site for splicing factors and/or the ability of a splice site to participate in splicing complex formation"), in combination with the proximity of the splice sites, has also been hypothesized to play a role in splice site selection (30). Interactions of the pre-mRNA with various nuclear proteins and ribonucleoproteins are likely to be involved in splice site selection, especially in the control of alternative splicing (3, 5, 6, 10, 13, 14).

The mouse κ-immunoglobulin gene represents an interesting problem in splice site selection. The primary transcription unit for the κ-immunoglobulin gene is produced after the rearrangement of one of several hundred variable (V) gene segments to one of four functional joining (J) gene segments (designated 5′-J1-J2-J3-J4-3′) located 2.5 to 3.8 kilobases (kb) 5′ of the constant (C) gene segment (for a review, see reference 36). While each J segment contains a functional 5′ splice site, depending on which J segment a V gene has joined, the primary transcript can contain from one to four functional 5′ splice sites. A functional mature mRNA is formed only when splicing involves the J to which the V is joined. Northern blot (RNA blot) hybridization analysis of mRNA from a variety of κ-immunoglobulin-producing plasmacytomas suggests that, in vivo, the splicing machinery chooses only the 5′-most donor splice site (28). However, when some κ-immunoglobulin primary transcripts are spliced in vitro in HeLa nuclear extracts, this selectivity is apparently lost (i.e., all of the J segment splice sites are equally used) (15, 20). Other cases of discrepancies between the in vivo and in vitro splicing behavior of pre-mRNAs containing multiple splice sites have been noted (1).

We have extended the study of κ pre-mRNA processing both in vitro and in vivo. The potential role of the signal exon and intron structures in splice site selection was addressed, and a method was developed to produce lymphoid extracts with splicing activity to examine possible tissue specificity in immunoglobulin mRNA processing. Constructs were also made to address the potential role of splice site strength and proximity in both in vitro and in vivo processing. Our results show that J segment splice site usage in vitro can be altered by changing the position or the sequence of the splice donor. These results differ from processing of pre-mRNA from the same constructs transfected into cells. The 5′-most J segment was exclusively selected in vivo, even in κ transcripts

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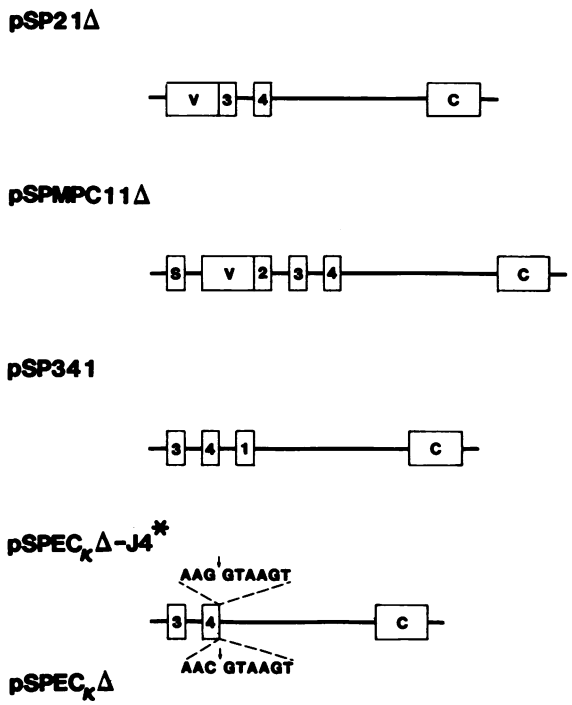

FIG. 1. Schematic structures of κ-immunoglobulin precursor mRNAs used as substrates for in vitro splicing. Open boxes are exon sequences, and solid lines represent intron sequences. For details on construction of the SP6 plasmids used to generate the pre-mRNAs, refer to Materials and Methods. The signal exon, variable region, and constant region are labeled S, V, and C, respectively. Joining (J) segments 1, 3, and 4 are numbered. The sequence above the pSPEC$_κ$Δ is that of the wild-type J4 5′ splice site (the arrow indicates the exon-intron boundary). The lower sequence is the mutated J4 5′ splice site of pSPEC$_κ$Δ-J4*.

where in vitro splicing favored a 3′ J splice site. Possible explanations consistent with the observed results and proposed models of splice site selection are discussed.

MATERIALS AND METHODS

Construction of plasmid vectors. Figure 1 shows the SP6 vector constructs used in this study. The numbering of kappa DNA sequence is as described by Max et al. (21). pSP21Δ and pSPEC$_κ$Δ have been previously described (20). pSPMPC11Δ is derived from the productively rearranged κ allele of the mouse myeloma MPC11 (16). MPC11 sequences beginning 5′ at the *Pvu*II site −35 bases from the mRNA start site to the *Bam*HI site 3′ of the constant region were inserted into pSP64. The same deletion of intron sequences and sequences 3′ of the constant region made in pSP21Δ and pSPEC$_κ$Δ (20) was then performed. The vector pSP341 was constructed by inserting a *Hin*dIII-*Sty*I fragment (bases 659 to 882), containing the J1 segment and surrounding sequences, into the *Bst*EII site 205 bases 3′ of the J4 segment of pSPEC$_κ$Δ. The vector pSPEC$_κ$Δ-J4* was made by changing the C residue at position 2100 of the J4 segment splice donor sequence in pSPEC$_κ$Δ to a G residue. Site-specific mutagenesis was performed as described by Taylor et al. (35).

The plasmid vectors used in the transfection studies are depicted in Fig. 6. All vectors are based on pAT153, a derivative of pBR322 lacking sequences "poisoning" replication in COS cells (37). A 342-base *Hin*dIII-*Pvu*II simian virus 40 (SV40) fragment, containing enhancer sequences, origin of replication, and the early promoter, was used to drive transcription of the immunoglobulin sequences (9). The 3′ end of all constructs is the *Bam*HI site 3′ of the immunoglobulin constant region, retaining sequences necessary for polyadenylation. The vector pATSV21 contains the same *Eco*RI-*Bam*HI immunoglobulin sequences as pSP21 (20). The vector pATSV21Δ has the same deletion of intron sequences as pSP21Δ. The vector pATSVEC$_κ$ is derived from germ line sequences (bases 1357 to 6230, *Avr*II-*Bam*HI) and resembles pSPEC$_κ$Δ, except that pATSVEC$_κ$ retains the full J-C intron. The vector pATSVEC$_κ$-J4* contains the same mutation of the J4 splice donor described for pSPEC$_κ$Δ-J4*. The vector pATSV341 has the same manipulation of the J1 segment described for pSP341 but was constructed in the pATSVEC$_κ$ vector. Insertion of the J1 segment in the exact location as in pSP341 necessitated removal of 794 bases of intron sequence (bases 2305 to 3099).

In vitro splicing and preparation of nuclear extracts. Preparation of SP6 transcripts, preparation of HeLa nuclear extracts, in vitro splicing reactions, and analysis of spliced products were performed as previously described (20). Nuclear extracts from Ramos (ATCC CRL 1596) cells were prepared by the method of Shapiro et al. (31), with the following modifications. Ramos cells were maintained at 0.5 × 10^6 to 1.0 × 10^6 cells per ml in RPMI 1640 (supplemented with glutamine, penicillin-streptomycin, and 10% heat-inactivated fetal calf serum). After isolation, a sample of nuclei from 10^9 cells (initial cell count) was suspended in 2 ml of NR (nuclear resuspension) buffer (31) containing 0.33 M NaCl. Conductivity of the nuclei plus NR buffer was then adjusted to that of NR buffer containing 0.38 M NaCl. The nuclei were extracted for 30 min at 4°C and removed by centrifugation for 30 min at 12,000 × *g* at 4°C. The supernatant was then dialyzed as described elsewhere (31) and used for in vitro splicing.

Transfections. Transfection of COS cells was performed by the DEAE-dextran method as described by Lopata et al. (19). Nuclear RNA was isolated 48 h posttransfection, and the poly(A)$^+$ fraction was isolated. RNAs were glyoxylated prior to separation on agarose gels and transferred to diazobenzyloxymethyl-paper (2). Blots were hybridized with nick-translated probes. RNA molecular weight markers (Bethesda Research Laboratories, Inc.; 0.16- to 1.77-kb and 0.24- to 9.5-kb ladders) were used to calculate the size of RNAs.

RESULTS

Splice site selection in vitro not influenced by the signal exon-intron. The plasmid construct pSP21Δ (Fig. 1) can produce an mRNA transcript containing two J segment 5′ donor splice sequences, which we previously reported were both used in vitro with about equal frequency in extracts derived from HeLa cells (20). However, the mRNA generated from this construct lacked the 5′-untranslated region, the signal exon, and the small intron between the signal and V region found in naturally occurring κ mRNA. The possibility that these sequences, or the splicing of the S-V intron, might play a role in selective J segment splicing could not be ruled out. The pSPMPC11Δ SP6 vector produces a pre-mRNA designed to address this possibility. Figure 2 shows a time course analysis of the in vitro splicing of the pSPMPC11Δ pre-mRNA in HeLa nuclear extract. Several bands can be seen appearing with the ~30-min lag charac-

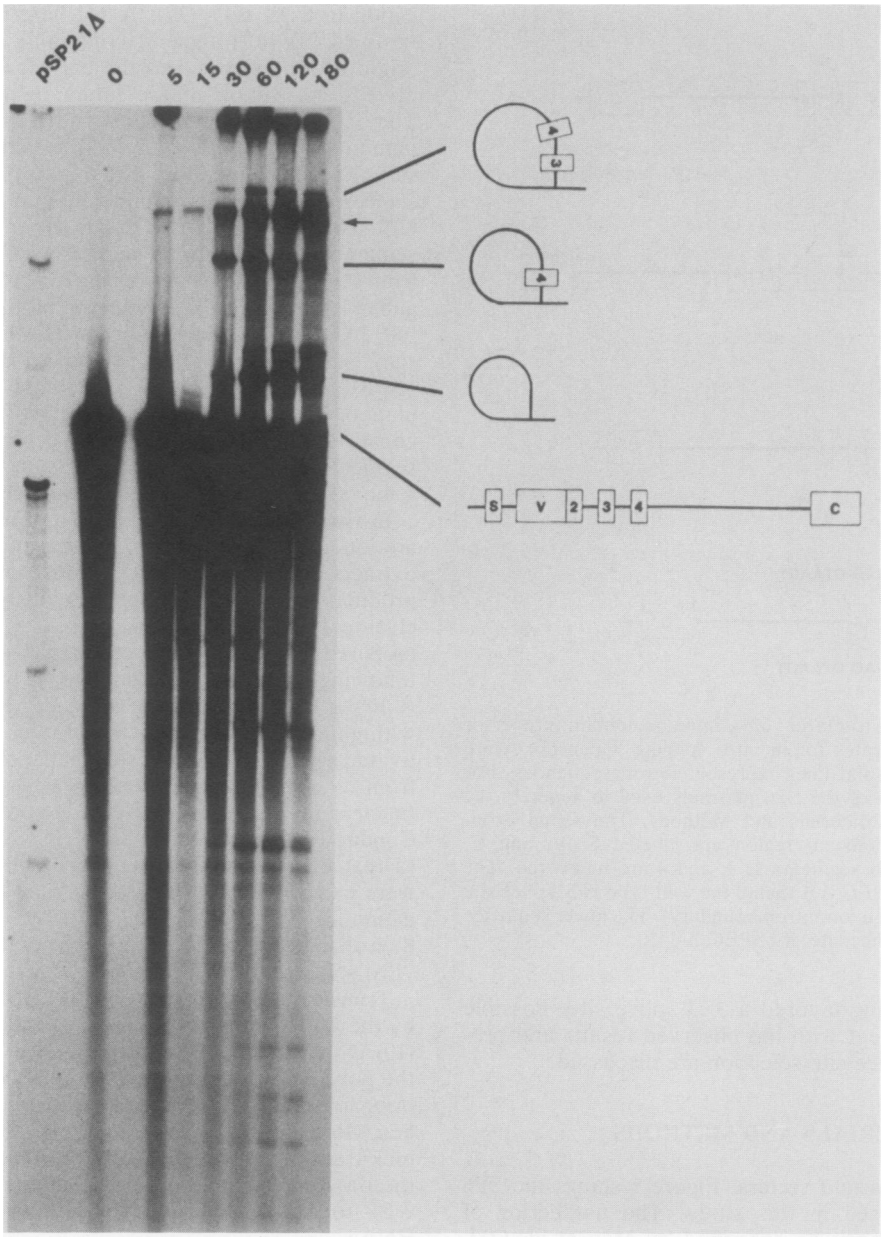

FIG. 2. In vitro splicing of pSPMPC11Δ pre-mRNA. In vitro splicing reaction mixtures containing radiolabeled pSPMPC11Δ pre-mRNA were allowed to proceed for the times (in minutes) shown. RNA was isolated and analyzed on 3.5% (1/40 bis) polyacrylamide gels containing 8 M urea, followed by autoradiography. Bands corresponding to the precursor mRNA and lariat structures are labeled with their corresponding schematic structures. The band indicated by the arrow has not been characterized but is present at time 0 and is unlikely to represent a product of splicing. Also shown is a time point at 120 min of the in vitro processing of pSP21Δ pre-mRNA, the products of which have been described in detail previously (20).

teristic of in vitro splicing. The large number of bands appearing is consistent with splicing occurring to all three J segments in the pSPMPC11Δ mRNA. Taking into account the splicing event removing the S-V intron and splicing to all three J segments, a total of 29 splicing products and intermediates can be predicted. Analysis of all of these products and intermediates was unmanageable; however, the appearance of the lariat structures alone can be used as an assay for splicing to the three J segments. Lariat structures resulting from splicing to all three J segments are clearly observed in Fig. 2. These lariat structures were unambiguously assigned their identity on the basis of their anomalous migration, the time lag and ATP dependence of their appearance, their comigration with lariat structures produced from the splicing of pSP21Δ (lariats from J3 and J4 splicing), and their size relative to size markers following debranching of lariat structures to their linear forms (results not shown). On gels with higher percentage of acrylamide, the 5′ exon (signal sequence) splicing intermediate and the small (175-base) lariat product resulting from the splicing of signal exon to variable region segment were detectable (data not shown). The kinetics of appearance and intensity of these bands

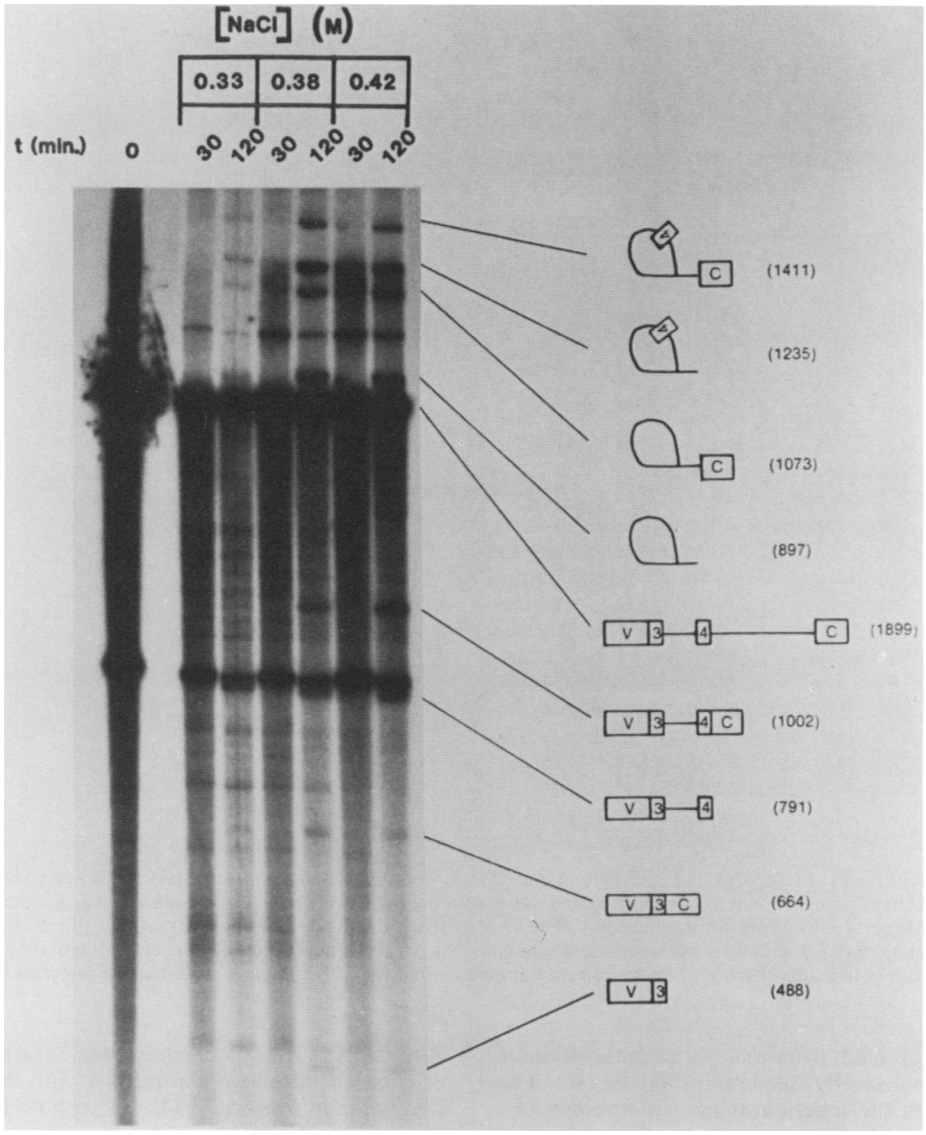

FIG. 3. In vitro splicing in nuclear extracts derived from a lymphoid cell line. Radiolabeled pSP21Δ pre-mRNA was incubated for the times shown in splicing reaction mixtures containing nuclear extracts prepared from Ramos cells. The salt concentration used to extract the nuclei is indicated above the incubation times. Products and intermediates of splicing are labeled with their respective schematic structures. The size (in nucleotides) of the band is in parentheses to the right of the gel.

suggest that the S-V splicing event occurs slightly earlier and more efficiently than the J-C splice in this transcript. We conclude from these results that neither the presence of the signal exon and S-V intron, nor the splicing event removing the S-V intron, restores correct splice site selection in vitro.

In vitro splicing using a lymphoid nuclear extract. All of our previous in vitro splicing had made use of nuclear extracts derived from HeLa cells, a nonlymphoid cell line. To ascertain whether a nuclear extract prepared from a lymphoid cell line might correctly process an immunoglobulin pre-mRNA, a protocol was developed for the preparation of nuclear extracts from lymphoid cells. Attempts to use the standard protocol for preparation of nuclear extracts (11) on a number of lymphoid cell lines were unsuccessful. The protocol described (see Materials and Methods) is adapted from a procedure used to make a high-efficiency HeLa cell nuclear transcription extract and is especially designed to minimize leakage of soluble factors from the nuclei during isolation (31). Figure 3 depicts two time points from the splicing of pSP21Δ pre-mRNA in Ramos (a human Burkitt lymphoma cell line expressing κ-immunoglobulin) nuclear extracts prepared using three different salt concentrations for extraction of nuclei. A critical parameter in the preparation of nuclear extracts for splicing was the salt concentration used for extraction of the nuclei; too little salt resulted in low splicing activity, while too much appeared to result in a high level of nuclease activity. It is apparent from the analysis that the lymphoid extracts behaved exactly as did the HeLa extracts, i.e., both J segment splice donor sites were used with equal frequency. Additionally, supplementing HeLa nuclear splicing extracts with various fractions from the preparation of lymphoid extracts from a variety of cell lines had no effect on splice site selection (results not shown). This result is analogous to results reported by Kedes and Steitz (15).

Alterations in splice site usage in vitro caused by position or sequence changes. The logic behind the design of pSP341 and

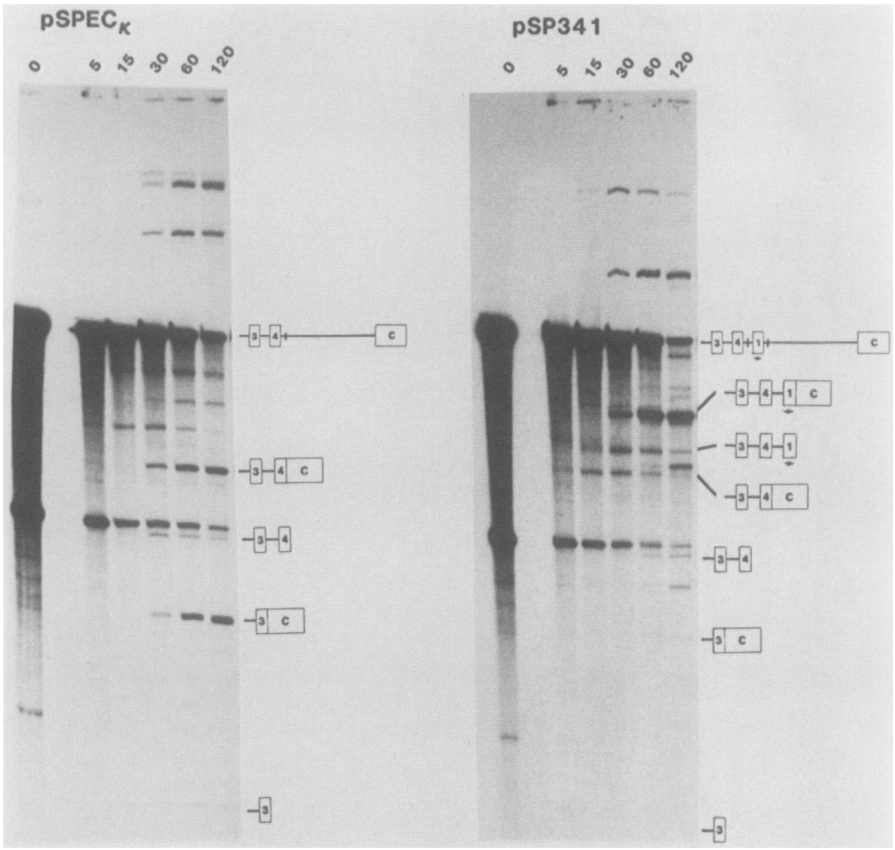

FIG. 4. Comparison of the in vitro processing of pSPEC$_\kappa\Delta$ and pSP341 pre-mRNAs. Radiolabeled pSPEC$_\kappa\Delta$ and pSP341 pre-mRNAs were incubated for the times (in minutes) shown in nuclear extracts under splicing conditions. RNA was isolated and analyzed on a denaturing 5% (1/33 bis) polyacrylamide gel, followed by autoradiography. The bands corresponding to the precursor mRNA, the 5′-exon intermediate, and the final spliced product are labeled with their corresponding schematic structures. The J segments are labeled with their respective numbers. The constant region exon is indicated by a C. The small arrow indicates that the J1 segment is in the sense orientation.

pSPEC$_\kappa\Delta$-J4* was derived from the splice site strength-proximity model proposed by Reed and Maniatis (30). This model describes both the inherent splice site strength of a potential splice site and the proximity of the 5′ and 3′ splice sites as being two important parameters in splice site selection. The balanced splice site selection observed in the in vitro splicing behavior of immunoglobulin pre-mRNAs could be explained in the context of this model by proposing that these two parameters are in balance. The more-proximal (to the constant region 3′ splice site) J segment (J4) would have a weaker inherent splice site strength than the J segment farthest from the constant region (J1). Furthermore, the model predicts that a perturbation in either the splice site strength of the J segment or the proximity of the J segments (relative to one another) should have an effect on this balance.

The pre-mRNA produced by pSP341 was designed to test a perturbation of the proximity parameter. In this construct, the J1 segment has been moved 3′ of the J4 segment, making J1 now the most-proximal J segment to the acceptor site. The spacing between the J segments is approximately the same. Figure 4 shows a time course analysis of the in vitro splicing of pSPEC$_\kappa\Delta$ and pSP341 pre-mRNAs. Splicing of the pSPEC$_\kappa\Delta$ pre-mRNA shows approximately equal splicing to both J3 and J4. The pSP341 pre-mRNA, however, shows a marked preference (greater than 80%) to select the J1 splice donor, consistent with the prediction of the model. As a control, the J1 segment was inserted in the opposite orientation, producing an mRNA with the same size insertion in the intron as pSP341 but with no functional J1 splice donor. The J3 and J4 splice sites were once again used in a balanced manner (results not shown).

The SP6 plasmid pSPEC$_\kappa\Delta$-J4* was designated to change the splice site strength parameter of one of the J segments. Site-specific mutagenesis was used to change the J4 splice donor sequence from an 8 of 9 match of the consensus splice donor sequence to a 9 of 9 match. Several lines of evidence suggest that this should result in a "stronger" splice site (25, 37). An analysis of the in vitro splicing of pSPEC$_\kappa\Delta$ and pSPEC$_\kappa\Delta$-J4* is shown in Fig. 5. This mutation resulted in a significant shift in the use of the J4 splice donor in pSPEC$_\kappa\Delta$-J4* compared with pSPEC$_\kappa\Delta$, as evidenced by a greater accumulation of the final spliced product resulting from splicing to J4.

In vivo analyses of immunoglobulin splicing by transfection. To study the splicing of immunoglobulin pre-mRNAs in vivo, immunoglobulin sequences were inserted into vectors appropriate for transient expression in COS cells (Fig. 6). The vectors were designed to produce pre-mRNAs in vivo which resemble the pre-mRNAs produced in vitro by the SP6-based vectors. Northern analyses of transfected, intact κ genes have demonstrated that the processed product is identical in size (1.2 kb) to naturally occurring transcripts (Brian Van Ness, unpublished observations), consistent with

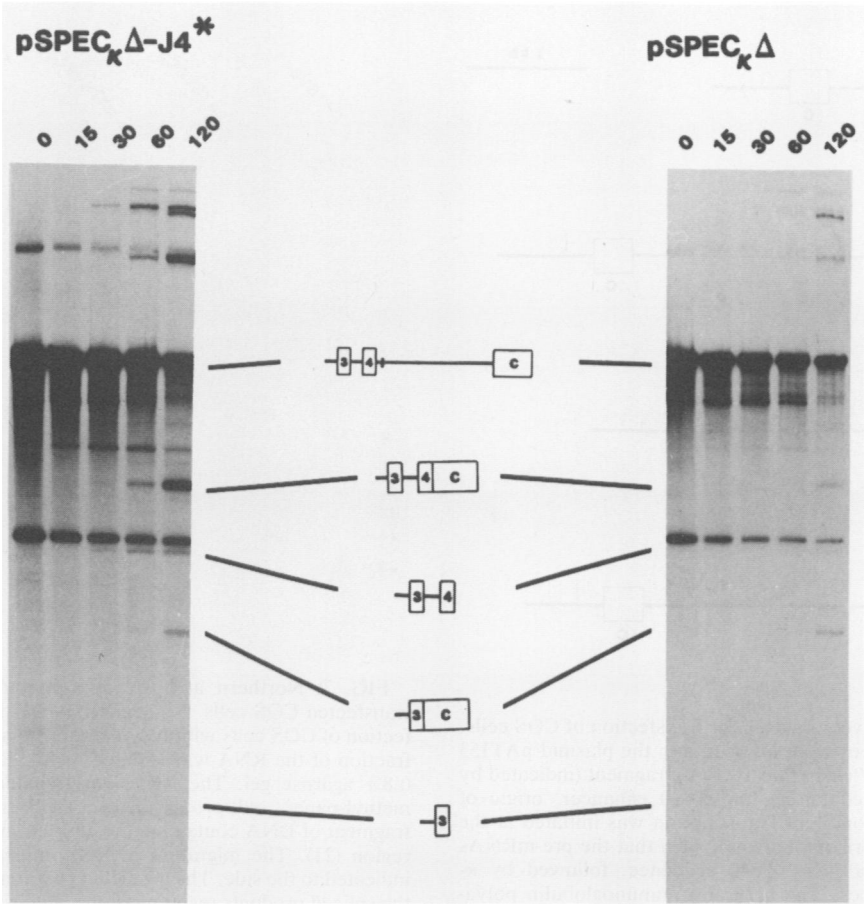

FIG. 5. Analysis of the in vitro splicing of pSPEC$_\kappa$Δ-J4* pre-mRNA. Radiolabeled pSPEC$_\kappa$Δ-J4* was incubated under splicing conditions for the times (in minutes) shown. The pSPEC$_\kappa$Δ-J4* pre-mRNA is identical to the pSPEC$_\kappa$Δ pre-mRNA, except for a mutation in the 5′ splice sequence of the J4 segment (Fig. 1). RNA was isolated and analyzed on a denaturing 5% (1/30 bis) polyacrylamide gel. A similar analysis of pSPEC$_\kappa$Δ pre-mRNA is shown for comparison. Bands corresponding to the precursor mRNA, the 5′-exon intermediate, and the final spliced product are indicated by their corresponding schematic structures. The J segments are labeled with their respective numbers, and the constant region is labeled with a C.

the observation that the 3′ end of the mature transcript generated from the κ vector is also composed of 220 bases of untranslated RNA and about 150 bases of poly(A). Primer extension analysis of pATSV21 demonstrated accurate initiation at the SV40 start site (results not shown). Since the 3′ end of these constructs is also identical to the natural κ gene, we have assumed an equivalent 3′ sequence in the processed mRNA. Northern analysis of poly(A)$^+$ nuclear mRNA from the transfected COS cells is shown in Fig. 7 and provides sufficient resolution to distinguish among possible products of splicing. The most striking result from the analysis of the in vivo splicing of the immunoglobulin mRNAs is that in all cases splicing appears to occur exclusively to the 5′-most J segment splice site. On the basis of the expected transcript size and polyadenylation for each construct, the size of the mature product is as expected for splicing to J3 (sizes given in the legend to Fig. 7). Even in the case of the pre-mRNAs generated by pATSV341 and pATSVEC$_\kappa$-J4*, where in vitro splicing favored the 3′-most J segment, only the 5′-most J segment splice site was selected in vivo. Thus, a nonlymphoid (COS) cell line is capable of correctly processing an immunoglobulin transcript, suggesting that immunoglobulin splicing is not a tissue-specific phenomenon. This is consistent with the conclusion drawn from in vitro experiments with the lymphoid nuclear splicing extracts. Accurate 5′ splice selection in κ pre-mRNA has also recently been reported in HeLa cells (15). We have been unable to detect in vivo any products resulting from splicing to a J segment other than the 5′-most. We cannot, however, formally rule out the possibility that inappropriate splice products are very unstable in vivo and not detectable by our assay.

Several other observations can be made by comparing the in vivo results with those obtained in vitro. The pre-mRNAs produced by pATSV21 and pATSV21Δ are identical, except for the deletion in the intron of pATSV21Δ. Analysis of the mRNAs produced upon transfection of these two vectors shows that both splice to J3. Therefore, intron size does not affect immunoglobulin splice site selection in vivo, in agreement with the results obtained from in vitro analysis of pSP21 and pSP21Δ pre-mRNA. In vitro, however, the longer intron was spliced much less efficiently (20). There is no apparent difference in vivo between the ratio of spliced product to precursor. Similar to the results obtained with pSP21Δ and pSPEC$_\kappa$Δ in vitro, comparison of the mRNA obtained from transfection of pATSV21 and pATSVEC$_\kappa$ suggests that the presence of the variable region or signal exon is not required in correct κ-immunoglobulin splice site selection in vivo.

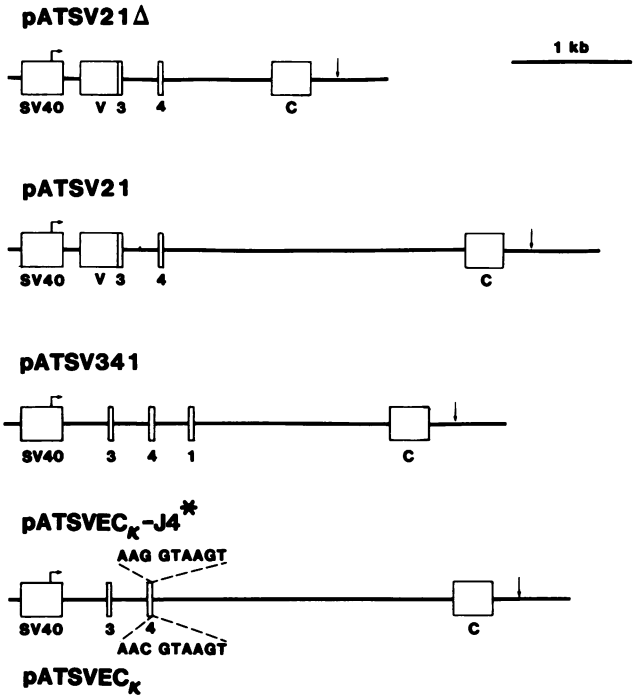

FIG. 6. Structures of vectors used for transfection of COS cells. Immunoglobulin sequences were inserted into the plasmid pAT153 directly 3′ of a 342-base *Pvu*II-*Hin*dIII SV40 fragment (indicated by the box labeled SV40) containing the SV40 enhancer, origin of replication, and early promoter. Transcription was initiated at the SV40 early promoter (horizontal arrow), such that the pre-mRNAs generated contain 98 bases of SV40 sequence, followed by κ-immunoglobulin sequences. The natural κ-immunoglobulin polyadenylation site was used (indicated by the vertical arrow). Variable and constant regions are labeled with a V and C, respectively. The J segments are labeled with their respective numbers. For details on construction of the vectors, refer to Materials and Methods.

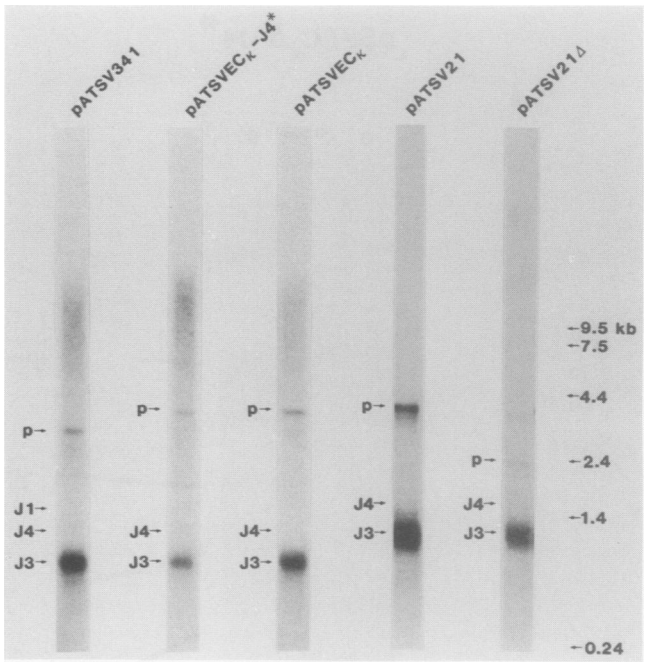

FIG. 7. Northern analysis of κ-immunoglobulin mRNA from transfected COS cells. Nuclear RNA was isolated 48 h after transfection of COS cells with the vectors shown in Fig. 6. The poly(A)$^+$ fraction of the RNA was isolated, glyoxylated, and separated on a 0.8% agarose gel. The RNA was transferred to diazobenzyloxymethyl-paper and probed with a nick-translated *Eco*RI-*Bam*HI fragment of DNA containing the entire κ-immunoglobulin germ line region (21). The migration of RNA molecular weight markers is indicated to the side. The predicted positions of the pre-RNA (p) and the spliced products resulting from splicing to each of the J segments (J3, J4, or J1) are indicated for each vector. Assuming 150 bases of poly(A), the predicted sizes for J3 splice products are 1,130, 1,195, and 1,290 bases for pATSV341, pATSVEC$_K$, and pATSV21, respectively; J4 splice products would be 338 bases longer.

DISCUSSION

Figure 8 summarizes the results obtained, both in vitro and in vivo, for the κ-immunoglobulin pre-mRNA constructs we have examined thus far. When a κ-immunoglobulin pre-mRNA containing multiple J segment 5′ donor splice sites is processed in vitro, the splice sites are used with approximately equal frequency. The presence of the signal exon, S-V intron, or variable region has no effect on splice site selection. Removal of intronic sequences increases the efficiency of splicing in vitro but does not affect splice selection. Taken together, our results now demonstrate that the primary or potential secondary structure of a κ pre-mRNA itself does not allow for selective splice site usage in the in vitro system. Nuclear extracts prepared from a lymphoid cell line do not restore correct splice site selection in vitro. While this manuscript was in preparation a similar observation was reported (15), although it is noteworthy that our method of extract preparation differs significantly in an attempt to minimize losses from nuclear preparations (see Materials and Methods).

Contrary to the in vitro situation, when the splicing of κ-immunoglobulin transcripts is examined in vivo (by transient transfection into COS cells), only the 5′-most J segment is selected. Whereas the presence of the signal or V region exons had no positive effect on splice site selection in vitro, the absence of these sequences did not alter the splice site selectivity observed in vivo. These results clearly demonstrate that the exclusive use of the 5′-most J splice donor site is not directed by sequences brought to the locus from the DNA rearrangement event, as previously suggested (28). Moreover, since COS cells are a nonlymphoid cell line, and are capable of selective J splice site usage, we conclude that κ-immunoglobulin splicing is not a tissue-specific phenomenon. This is in agreement with Kedes and Steitz (15), who obtained similar results upon transfection of κ-immunoglobulin constructs into HeLa cells. We note, however, in our experimental system, unlike that reported by Kedes and Steitz, we can detect a stable spliced product with a κ-immunoglobulin pre-mRNA lacking a variable region, perhaps due to differences in the 5′ and 3′ ends of our pre-mRNA or to the different cell line transfected.

The κ-immunoglobulin system, with its balanced splice site usage in vitro, is a good model system in which to test the splice site strength-proximity model proposed by Reed and Maniatis (30). Perturbing either the location of the J segments relative to one another (proximity, pSP341) or the sequence of one of the J segment splice sites (splice site strength, pSPEC$_K$Δ-J4*) affected the balanced J segment splice site usage observed in vitro. These observations are consistent with the model. Our observation (20) that a construct with a deletion of intron sequences (pSP21Δ) splices more efficiently (yet retains the balanced J segment usage) than an identical construct with the full intron (pSP21)

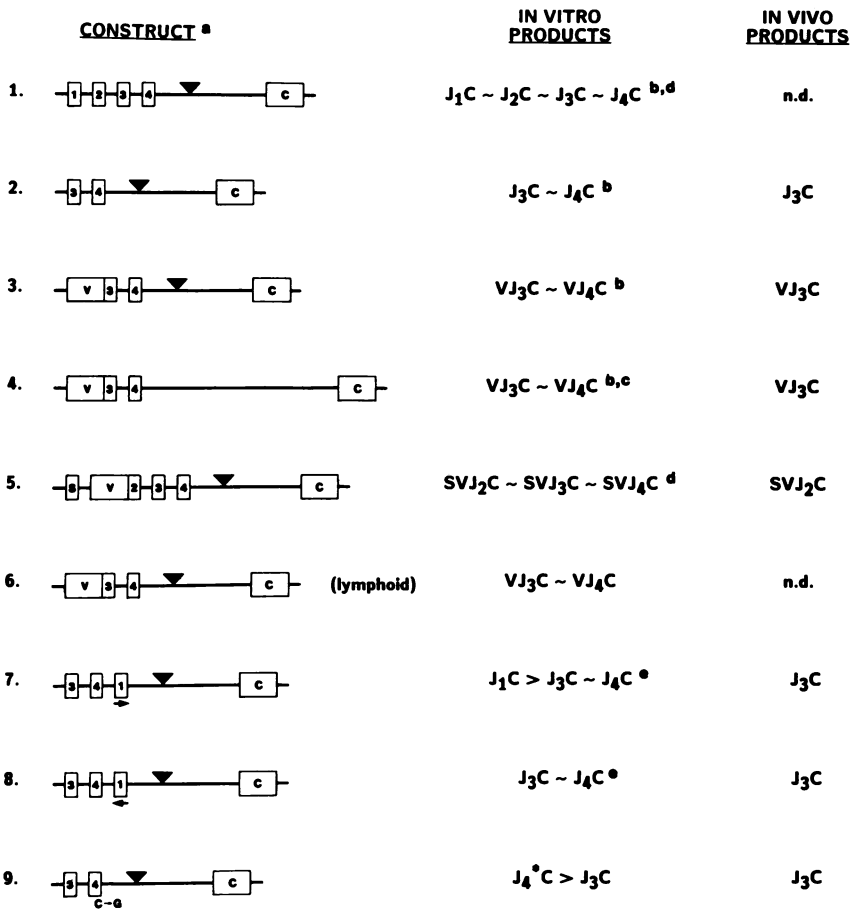

FIG. 8. Summary of the in vitro and in vivo splicing of the κ-immunoglobulin constructs. Superscripts: a, portion of intron deleted (▼); b, data from reference 20; c, product formation less efficient than that observed with construct 3 in vitro; d, products inferred from lariat ratios; e, in vivo and in vitro pre-mRNAs having different deletions of intron sequences. n.d., Not determined.

is also consistent with the model. The deletion of intron sequences would decrease the proximity (to the constant region 3′ acceptor splice site) of both J segments equally, preserving the balanced splice site usage while increasing efficiency. It is interesting to note that all of the κ-immunoglobulin J segment splice sites have identical consensus splice sites (21); differences in the splice site strengths of the J segment donor splice sites might come from surrounding sequences or from differences in splice site accessibility.

In contrast to the results obtained in vitro, neither a perturbation of splice site strength nor of proximity had an effect on in vivo splice site selection. All of the pre-mRNAs tested used only the 5′-most J segment donor splice site. Even for pre-mRNAs (pATSV341 and pATSVEC$_\kappa$-J4*) in which in vitro splicing favors the selection of the 3′-most J segment donor splice site, the 5′-most donor splice site is exclusively used. Additionally, there is no apparent difference in the ratio of spliced to unspliced mRNA between a construct with a deletion of intron sequences (pATSV21Δ) and its counterpart retaining the full intron (pATSV21). Thus, the splice site strength-proximity model needs some modification to explain the discrepancy between the in vivo and in vitro results.

We conclude that an in vitro splicing system is lacking something that allows the in vivo splicing machinery to select the correct J segment donor splice site when processing a κ-immunoglobulin pre-mRNA. One possibility is that some accessory factor(s) necessary for correct splice site selection is lost in the preparation of the nuclear extracts used for in vitro splicing. Since the method used for preparation of the nuclear extracts in this study was designed to minimize leakage during preparation of the nuclei, it is possible such a factor would be retained with the nuclei during the extraction. *Trans*-acting factors affecting splice site selection have been implicated in the alternative selection of splice sites (8, 10). However, if a factor exists which confers correct splice site selection on the splicing of κ-immunoglobulin pre-mRNA, it seems likely that it would not be a specialized component of the lymphoid splicing machinery, since immunoglobulin splicing is not a case of alternative splicing and nonlymphoid cells correctly splice immunoglobulin pre-mRNA. An explanation we favor is that the loss of selectivity observed in vitro in the case of κ-immunoglobulin splicing is a result of isolating the splicing process from transcription. Models coupling splice site selection to transcription have been proposed (1). A first-come, first-served model, whereby a 5′ splice site would be sequentially recognized and sequestered, would explain the in vivo splicing behavior of κ-immunoglobulins. Evidence exists which indicates recognition of the 5′ splice site of a pre-mRNA may be a very early event in splicing (4, 26). A first-come, first-served model postulating cotranscriptional recognition and sequestering of pairs (5′ and 3′) of splice sites (1) would also fit our observations but is inconsistent

with results obtained from some other systems (7, 22). In the absence of transcription, as would exist in an in vitro splicing situation, the splicing machinery may select splice sites without the constraints imposed in vivo and be subject to alterations in proximity and splice site strength. The sequestering of a 5′ donor splice site in vivo might also explain the apparent loss of the proximity effect observed in vitro. The proximity effect observed in our in vitro experiments may be a result of the random collision of splicing factors bound to the 5′ and 3′ splice sites to form a splicing complex (30). In vivo, the donor splice site might be positioned in a constant proximity relative to the location where a 3′ splice site is recognized, eliminating the effect of intron length on splicing. This would be an attractive proposition in light of the extremely long (>60 kb) introns found in some eucaryotic pre-mRNAs and would also have implications for the role of the nuclear matrix in eucaryotic pre-mRNA splicing.

ACKNOWLEDGMENTS

This work was supported by a Public Health Service grant from the National Institutes of Health, with additional support through the Diabetes, Endocrinology Research Core Grant to the University of Iowa. D.E.L. is supported by a predoctoral Cell and Molecular Biology training grant from the National Institutes of Health; B.V.N. receives support from the Searle Scholar Program.

We thank Joe Walder for oligonucleotide synthesis, Larry Solomon for advice of transfection on COS cells, and Leslie Gay for excellent technical assistance.

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