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# In Vitro Splicing of Kappa Immunoglobulin Precursor mRNA

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**The in vitro splicing of kappa immunoglobulin precursor mRNA was studied as an example of a naturally occurring mRNA possessing multiple 5′ splice sites. Several kappa mRNAs were generated in vitro by using an SP6 transcription system and were spliced in nuclear extracts derived from HeLa cells. Products and intermediates resulting from in vitro splicing were identified and characterized. In contrast to the in vivo situation, in which apparently only the 5′-most splice donor site is used, all of the 5′ splice sites were used in vitro with equal frequency. Neither the presence or absence of variable region coding sequences nor the deletion of intron sequences had an effect on in vitro splice site selection.**

In the past several years various approaches have been used to examine the processing of eucaryotic mRNA, and they have revealed several sequence requirements for the accurate and efficient removal of intronic sequences (for a review, see reference 19). By manipulating gene structure, sequences important to the 5′ and 3′ splice sites have been identified (18, 23, 30). Many mRNAs contain multiple introns, and thus multiple 5′ and 3′ splice sites, which must be paired correctly to generate functional mature mRNA (2). The question of how the cell determines which of several possible splice sites to use is as yet not answered. Scanning models postulate a mechanism whereby the splicing machinery binds the precursor mRNA and scans it in a linear fashion for splice sites (13, 27). Many models propose that the higher-order structure of a precursor RNA, either alone or in a ribonucleoprotein complex, contributes to the selection of splice sites (11, 22). Additional factors which have been suggested include the proximity of splice sites to one another, the primary sequence of the splice site itself, and sequences (such as coding sequences) further removed from the splice site (25).

Recent studies have made use of in vitro extracts derived from HeLa cells and have shown that these extracts are capable of accurately processing precursor RNA (5, 8, 10, 20). Such in vitro studies have enabled a number of investigators to isolate and identify intermediates and products of mRNA splicing. For the most part, the products obtained in in vitro systems accurately reflect the products observed in in vivo studies, although some differences between in vitro and in vivo splice patterns have recently been noted (1, 28). Moreover, several studies have used artificially constructed RNAs with duplicated donor and/or acceptor sites to study splice site selection (11, 12, 25). Although the primary sequence of the splice sites can be exactly controlled with this approach, it is difficult to predict the effect of duplicating sequences on the secondary structure of the RNA and thus its possible effect on splice site selection. In this context we decided to examine the splicing of mouse kappa immunoglobulin light chain precursor mRNA as an example of a naturally occurring mRNA possessing multiple equivalent splice sites, which in vivo must be selectively used. Kappa immunoglobulin genes are functionally transcribed only after rearrangement of one of several hundred variable (V) gene segments to one of four functional junctional (J) gene segments (designated 5′-J1-J2-J3-J4-3′), which lie between 2.5 and 3.8 kilobases upstream of the constant (C) gene segment (for a review, see reference 29). Each of the four J segments contains a functional splice donor sequence; depending on which J segment a V gene has joined, the primary transcript can contain one (V-J4) to four (V-J1) equivalent splice sites. However, a functional mature mRNA is formed only when splicing involves the J to which the V is joined.

In this study we examined the in vitro splicing of several mouse kappa immunoglobulin precursor mRNAs. In contrast to the in vivo situation, in which only the 5′ splice sequence of the joining segment to which the V gene segment has recombined is utilized, there is no discrimination in vitro among the J segment splice sites. In addition, the presence or absence of a variable region gene segment does not appear to alter splice site selection.

## MATERIALS AND METHODS

**Construction of plasmid vectors.** Construction of the plasmid vectors used in this study is depicted in Fig. 1. The kappa sequence in all construct descriptions is numbered on the basis of the system of Max et al. (14). Cloning of the *Bam*HI fragment containing a rearranged kappa (V-J3) allele from the mouse plasmacytoma 2154 has been previously described (6). To construct the plasmid pSp21, the 5.0-kilobase *Eco*RI-*Bam*HI fragment, containing the variable region, J segments 3 and 4, and the constant region, was ligated into the plasmid pSP65. The plasmid pSp21Δ was constructed by removing the *Bgl*II-*Bam*HI segment (bases 2355 to 6248) containing 2.3 kilobases of the J-C intron and all of the constant region and by replacing it with a *Sau*3AI fragment (bases 3976 to 4790) containing 643 bases of the J-C intron and the first 171 bases of the constant region. The plasmid pSpECκΔ was constructed by first inserting the *Avr*II-*Bam*HI fragment (bases 1356 to 6248) containing joining segments 3 and 4 and the constant region into pSP65 and deleting intron sequences exactly as described above for pSp21. For in vitro transcription, pSp21Δ and pSpECκΔ were linearized with *Xba*I and with *Hpa*I, respectively.

**In vitro splicing and RNA analysis.** RNAs were prepared from the SP6 vector constructs essentially as described by Melton et al. (17). The RNAs were capped by the inclusion of 500 μM $m^7$GpppG in the reaction (9) and were radioactively labeled with [α-$^{32}$P]GTP. Nuclear extracts were prepared from HeLa cells by the method of Dignam et al. (3). In vitro splicing was carried out as described by Krainer et al. (10). Precursor RNA concentration in the splicing reaction

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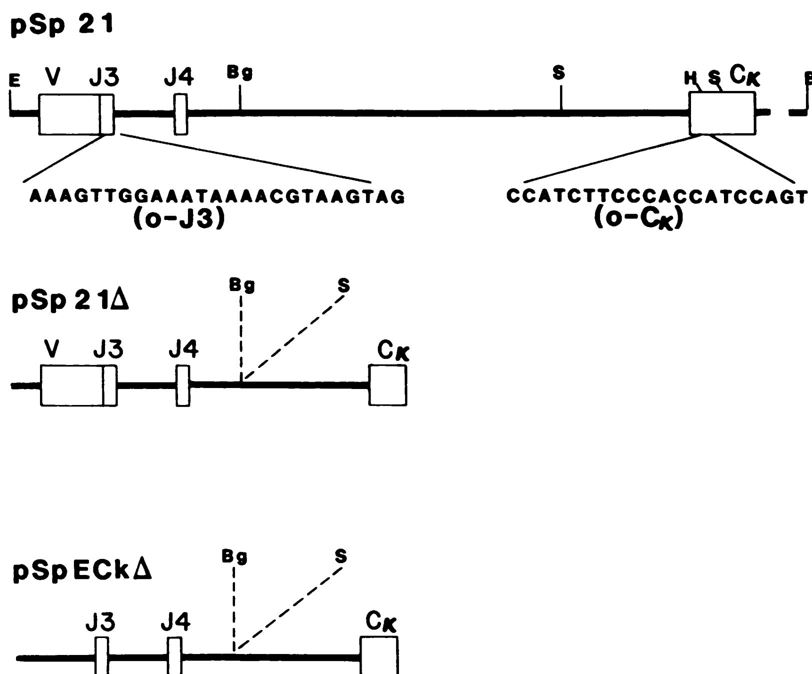


FIG. 1. Structures of κ immunoglobulin inserts in SP6 plasmids used to generate precursor mRNAs. Open boxes represent exon sequences, and solid lines represent intron sequences. For details on construction of the plasmids, refer to Materials and Methods. The V-J3 clone used to construct pSp21 and pSp21Δ was isolated from the plasmacytoma PC2154 (6). The region between the *Bgl*II and the *Sau*3A1 sites, along with sequences 3′ of the *Sau*3A1 site in the constant region, were deleted in the construction of pSp21Δ. pSpECκΔ contains J segments 3 and 4, no variable region, and the same deletions of intron and constant region sequences as pSp21Δ. The location of o-J3 and o-Cκ, the deoxyoligonucleotides used for primer extension analysis (Fig. 3), is shown beneath pSp21. Restriction sites are E (*Eco*RI), S (*Sau*3AI), H (*Hpa*I), Bg (*Bgl*II), and B (*Bam*HI).

was approximately 0.1 nM. Spliced products were analyzed on denaturing polyacrylamide gels. For analysis by primer extension, spliced products were separated on a denaturing polyacrylamide gel, located by autoradiography, cut out of the gel, and eluted by soaking the gel slice overnight in 10 mM Tris (pH 7.5)–1 mM EDTA–0.1% sodium dodecyl sulfate–0.3 M sodium acetate. One pmol of $^{32}$P-end-labeled oligonucleotide primer plus the RNA sample were coprecipitated and redissolved in 20 μl of 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA–0.25 mM NaCl. Samples were heated for 5 min at 90°C and allowed to anneal for 2 h at 62°C. Primer extension reactions were then carried out as described previously (16). Dideoxy sequencing of RNA was performed by including the appropriate dideoxynucleotide in primer extension reactions as described above. Sequences across splice junctions were obtained by primer extension of the gel-purified spliced products by using $^{32}$P-end-labeled o-Cκ primer and by separating the primer-extended products on a denaturing polyacrylamide gel. The primer-extended products were eluted from the gel and sequenced by the method of Maxam and Gilbert (15).

## RESULTS

**In vitro splicing of pSp21Δ RNA.** The plasmid pSp21Δ (Fig. 1) produces an RNA transcript with several features convenient for studying the splicing of kappa immunoglobulin mRNA. The transcript begins in the first intron, between the signal exon and variable region exon of a natural immunoglobulin mRNA, eliminating the signal exon along with its 5′ splice site. This simplifies analysis of the splicing intermediates and products by eliminating splicing of this first exon. Analysis of splicing intermediates and products is also simplified but relevant in the mouse plasmacytoma 2154 kappa gene because the variable region is recombined to J3, thus producing an mRNA with two duplicated donor splice sites (J3 and J4). Intron sequences and the 3′ portion of the constant region were deleted (see Materials and Methods) to reduce the size of the transcript and thus make possible analysis of the splicing intermediates and products on denaturing polyacrylamide gels. A time course analysis of the products produced when pSp21Δ RNA is spliced in the HeLa nuclear extract is shown in Fig. 2. Several RNA species are visible; they were tentatively assigned the identities shown schematically in Fig. 1 on the basis of several criteria. First, the 30-min lag in the appearance of the bands is characteristic of splicing intermediates and products in the HeLa nuclear extract (10). Four bands appear below the precursor RNA at the sizes expected for the 5′-exon intermediates (F and H) and final spliced products (E and G) if splicing had occurred at both J3 and J4. The bands migrating above the precursor (A to D) show the anomalous migration characteristic of the lariat intron–3′-exon intermediate and the excised lariat intron (4, 26). The presence of four lariat structures is also consistent with splicing to both J3 and J4. If the amount of label present in bands E and G (e.g., an 800-nucleotide RNA should contain twice as much label as a 400-nucleotide RNA) is taken into account, it appears that both joining segments are used with approximately equal frequency. This frequency of usage was observed with several different extract preparations and dilutions. The

FIG. 2. Analysis of the in vitro splicing of pSp21Δ RNA. (A) Time course. In vitro splicing reaction mixtures (10 μl) containing pSp21Δ RNA (1 fmol) were allowed to proceed for the times (in minutes) shown. Reactions were terminated by placing mixtures on dry ice. RNA was isolated and analyzed by electrophoresis on 3.5% polyacrylamide gels containing 8 M urea followed by autoradiography. Schematic structures of the splicing intermediates and products are shown. The sequence around the branch point is shown beneath the schematic structure of band A. The branch nucleotide is marked with an asterisk. Below the schematic structures for bands E and G are shown the sequences around the splice junctions (marked by arrows). The numbers to the left are molecular weight standards in bases. (B and C) Sequencing gel showing the

appearance of all of the products described above is dependent on the presence of $Mg^{2+}$ and ATP, both of which have been demonstrated to be required for in vitro splicing (10). The additional bands present in the lanes are probably a combination of premature termination of the SP6 polymerase in the RNA synthesis reaction and of degradation of the precursor (see time zero lane). In particular, the band migrating at approximately 830 nucleotides has been identified as a premature termination product, terminating just 3′ of J4. To be sure that the premature termination product was not generating any of the processing products, we gel purified the full-length transcript, and the same processing products were observed (data not shown).

**Analysis of pSp21Δ RNA spliced products.** Primer extension analysis was performed to further confirm the identities of the bands and to determine branch points. Preparative scale splicing reactions were performed, the products were separated on a denaturing polyacrylamide gel, and the bands corresponding to the spliced products were cut out and eluted. The o-Cκ oligonucleotide (Fig. 1) was used to primer extend bands A to H; o-J3 (Fig. 1) was used to primer extend bands E through H. Primer extension of RNA from bands E and G, tentatively identified as products corresponding to splicing of the C region to J4 and J3, respectively, gave the predicted primer-extended products of 874 and 536 nucleotides, whereas primer extension of RNA from bands E, F, G, and H with o-J3 gave the expected 498-nucleotide products (data not shown).

To confirm accurate splicing, the spliced products (bands E and G) were sequenced. Bands E and G were primer extended with o-Cκ as described above and run on a denaturing polyacrylamide gel, and the full-length transcripts (874 and 536 nucleotides) were cut out, eluted from the gel slice, and sequenced by the method of Maxam and Gilbert (15). The sequencing ladder is shown in Fig. 2. This sequence agrees with the predicted sequence for accurate splicing of the C region to both J3 (Fig. 2B) and J4 (Fig. 2C), demonstrating that splicing had indeed occurred to both J3 and J4 and that the same 5′ and 3′ splice boundaries used in vivo are used in vitro.

Previous studies have shown that reverse transcriptase is unable to read past the branchpoint nucleotide found in lariat structures; therefore, primer extension of the lariat intron–3′-exon intermediate with o-Cκ should yield a stop at the branchpoint nucleotide (26). Fig. 2D shows the results of primer extension of RNA from bands A, B, C, and D (Fig. 2A) with o-Cκ. A prominent primer extension stop can be seen with RNA from bands A and C. The observed doublet (at and just before the branchpoint nucleotide) has been noted in other studies (7, 26). Comparison of these stops with a dideoxy sequencing ladder of unspliced pSp21Δ RNA allows precise mapping of the branchpoint (Fig. 2D). The location of the branchpoint is 28 nucleotides 5′ of the C region and appears to be the same whether splicing is to J3 or to J4. This is consistent with previous observations that the branchpoint nucleotide is an adenosine located 18 to 50 nucleotides from the 3′ splice site (24). In addition, the lack of any significant stops in bands B and D is consistent with their assignment as lariat introns since they should contain no constant region sequences from which to primer extend.

**In vitro splicing of pSp21 RNA.** To check whether the removal of intron sequences might have had an effect on the splicing of pSp21Δ RNA, we examined pSp21 RNA, which contains the full genomic intron sequences that are deleted in pSp21Δ RNA. A 180-min time point of the splicing of pSp21 RNA in vitro in the same nuclear extract is shown in Fig. 3A. Four bands can be seen below the precursor with the lag period characteristic of in vitro splicing (time course not shown). The appearance of these bands is dependent on the presence of ATP and $Mg^{2+}$. The sizes of the bands are consistent with their identification as the 5′-exon intermediates and the final spliced products of splicing to both J3 and J4. Although the lariat structures formed by splicing of pSp21 RNA are not apparent, we believe that the anomalous migration of these much larger structures prevented them from entering the gel. Similar to the case of pSp21Δ RNA, it appears that both the J3 and the J4 5′ splice sites were used with equal frequency, although under equivalent conditions the presence of the larger intron significantly decreased the efficiency of processing.

**In vitro splicing of pSpECκΔ RNA.** To check whether the absence of variable region sequences might affect the selection of J segment splice sites, we examined the splicing of the RNA transcript from pSpECκΔ. This transcript contains J segments 3 and 4 in a germline configuration, i.e., with no variable region. It also has the same intron deletion as pSp21Δ RNA. A 120-min time point of the processing of pSpECκΔ RNA is shown in Fig. 3B. The appearance of the bands marked by arrows is dependent on $Mg^{2+}$ and ATP and occurs with the lag characteristic of in vitro splicing. The four bands below the precursor mRNA migrate at the positions predicted for 5′-exon intermediates and spliced products arising from splicing to both J3 and J4. The four bands above the precursor are the corresponding lariat intron–3′-exon intermediates and lariat intron products; these bands comigrate with the lariat structures generated from the splicing of pSp21Δ mRNA. The relative intensities of the bands arising from the splicing of pSpECκΔ suggest that both the J3 and the J4 splice sites are equally utilized; thus, the absence of variable region sequences appears to have no effect on the selection of joining segment 5′ splice sites.

## DISCUSSION

Although kappa immunoglobulin precursor mRNA can contain up to four functional 5′ splice sites (J1 to J4), in vivo the only mature mRNA products observed are generated by splicing involving the J to which a V gene segment has joined (21). We examined the in vitro processing of a kappa transcript containing two J sequences (and thus a duplication of two 5′ splice sites) and found no distinction of J usage. It is possible that the in vivo processing of κ transcripts does

splice junctions of the spliced products resulting from in vitro processing of pSp21δ RNA. Bands E and G (Fig. 2A) were gel purified and primer extended with end-labeled o-Cκ, and the gel-purified primer-extended products were sequenced by the method of Maxam and Gilbert (15). (B) Sequence of the 536-base extended product (band G-J3 to the Cκ splice). The sequence read from the gel is complementary to the predicted RNA sequence shown beneath the schematic structure for band G (Fig. 2A). (C) Sequence of the 874-base primer-extended product (band E-J4 to the Cκ splice). The predicted sequence is shown below the schematic structure for band E (Fig. 2A). (D) Determination of the branchpoint. RNAs corresponding to bands A to D (Fig. 2A) were gel purified and analyzed by primer extension with $^{32}P$-end-labeled oCκ (as described in Materials and Methods). The sequencing ladder was generated by primer extension of unspliced pSp21Δ RNA (10 fmol per reaction) with o-Cκ in the presence of dideoxynucleotides. It may be noted that the sequence obtained is complementary to the sense strand. Comparison of the primer extension stop in lanes A and C with the sequencing ladder allows mapping of the branchpoint nucleotide (asterisk).

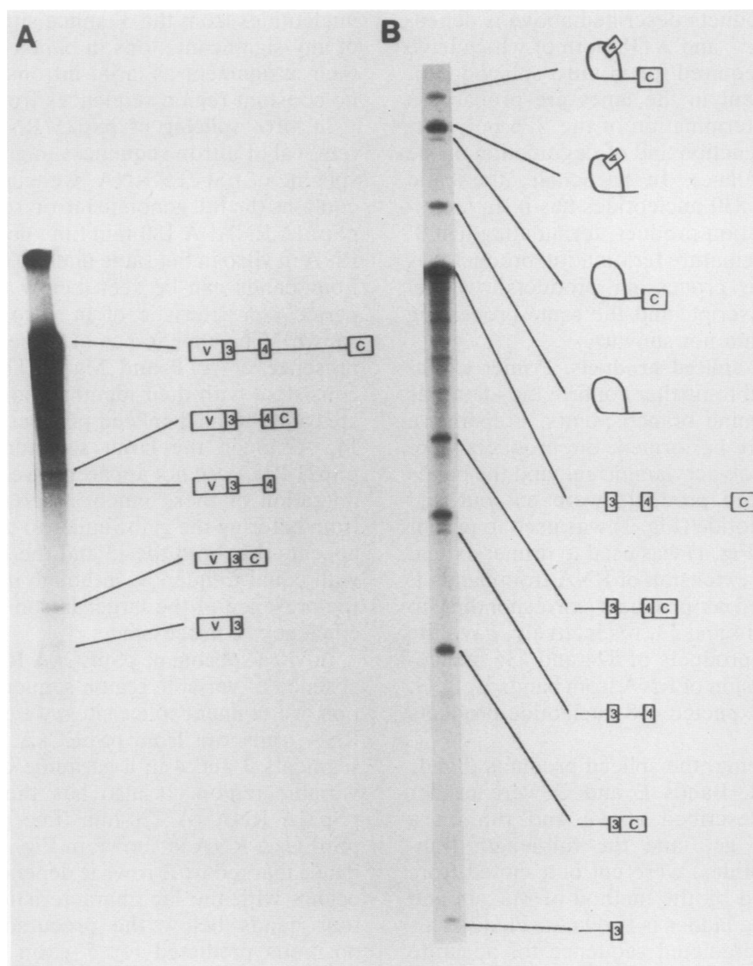


FIG. 3. In vitro processing of pSp21 and pSpECκΔ precursor RNAs. (A) A 180-min time point of the in vitro splicing of pSp21 RNA analyzed on a 3.5% polyacrylamide–8 M urea gel. The bands identified as spliced products and 5'-exon intermediates are marked with a corresponding schematic diagram. No lariat-containing intermediates or products are visible (see text). (B) A 120-min time point of the in vitro processing of pSpECκΔ RNA. Radiolabeled pSpECκΔ RNA was processed and analyzed as described above. Schematic diagrams mark the bands assigned as products and intermediates of splicing.

not distinguish among multiple J sequences and that the aberrant products have not been detected. This seems unlikely in light of the results of Perry et al. (21), in which nuclear RNA from a variety of kappa-producing plasmacytomas was analyzed by Northern blot hybridizations. Only products and intermediates arising from the splicing to the 5'-most J segment (the one to which the V gene segment is joined) were observed. It was suggested that the preferential, or even exclusive, use of the 5'-most splice site is due to some secondary structure of the primary transcript and that it involves the V gene coding sequence. Although our initial construct was lacking a portion of the intron, which may have eliminated imporant secondary structure, we obtained the same products when the entire intron remained intact (Fig. 3A). When RNA from a construct which contained the same two J sequences and no V coding gene sequences was examined, we also showed that both J splice sites were used in vitro (Fig. 3B). Moreover, we examined RNA from a construct containing all four J segments and obtained evidence that in vitro all four J splice sites are used (data not shown). Our results suggest that 5' splice site selection cannot be properly directed simply by secondary structures within the κ transcripts used in our in vitro assays. Our results also suggest that, for κ immunoglobulin RNA, selective splice donor usage in vitro is not influenced by the V gene coding sequence or intron length.

There may be several possible explanations for the apparent inconsistencies that we observed between the in vitro and in vivo processing of κ precursor mRNA. In an effort to simplify the analysis, the in vitro-generated transcripts lacked the 5' signal exon and a short 5' intron found in the in vivo transcript. Given the considerable sequence heterogeneity of the hundreds of V gene segments and 5' introns, the primary sequence itself probably does not play a role in splice site selection; however, by using these constructs, we cannot rule out the effect of the S-V splicing event on J splice site selection. It seems more likely that there are some limitations in the in vitro system. The extracts that we generated may be deficient in some factors which ensure selective splice site usage. There is the remote possibility that lymphoid cells produce specific factors for selective J splicing which are not present in our HeLa extracts. However, when we supplemented HeLa extracts with similarly prepared lymphoid extracts we did not see any changes in the splicing pattern. Certainly, in vivo RNA processing is probably a highly organized process within the nucleus and

occurs with a much higher efficiency than is observed in vitro. This could have an effect on the efficiency of splice site selection. Transcription and splice site recognition may be a coupled process in vivo, such that the first 5′ splice site transcribed is recognized by a component of the splicing machinery and subsequent recognition of additional donor sequences is sequestered until a 3′ acceptor site is reached. Indeed, Aebi et al. (1) have recently proposed that differences that they observed between in vitro and in vivo splice patterns may be explained by a "first come, first served" principle. Scanning models of splice site selection have been proposed (13, 27). On the basis of our results, a simple 5′ to 3′ scanning model does not explain the process in the in vitro system. The apparent contradiction could be resolved, however, if the scanning was coupled to transcription itself or to some higher-ordered nuclear organization.

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