Correct in vivo splicing of the mouse immunoglobulin κ light-chain pre-mRNA is dependent on 5' splice-site position even in the absence of transcription

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In transcripts from the rearranged mouse immunoglobulin κ light-chain locus, the intron separating the variable (V) plus joining (J) exon from the constant (C) exon contains up to three additional J regions, each with a functional 5' splice site. Previously, HeLa cells transfected with DNA encoding κ light chains have been shown to mimic κ -producing lymphocytes in splicing exclusively to the upstream-most 5' splice site, whereas selectivity is lost when κ transcripts containing two more J regions are incubated in HeLa cell or lymphocyte nuclear extracts. Here we demonstrate that the fidelity of in vivo splicing depends on neither V-J rearrangement, the instability of erroneously splicing transcripts, nor a hierarchy of J-region 5' splice site utilization. Analysis of the splicing of presynthesized κ transcripts injected into *Xenopus* oocytes demonstrates that correct 5' splice-site selection is independent of transcription. Implications for in vitro studies of regulated splice-site pairing are discussed.

[**Key Words:** Pre-mRNA; alternative splicing; immunoglobulin light chain; 5' splice site]

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Although the number of characterized mRNAs that arise from alternative splicing continues to increase, the principles governing splice-site selection remain obscure (for reviews, see Green 1986; Leff et al. 1986; Padgett et al. 1986; Breitbart et al. 1987). In some instances, a single 5' splice site is joined to one of several 3' splice sites; conversely, one of a number of 5' splice sites can be joined to a single 3' splice site. A combination of these events can occur, giving rise to exon skipping. Because different patterns prevail in different cell types or stages of differentiation, it is often assumed that interactions of the pre-mRNA with cell-specific components of the splicing apparatus must be responsible. Alternatively, subtle changes in the general splicing machinery could conceivably lead to alternative modes of splice-site pairing. In either case, *cis*-acting elements within the transcript itself will determine the response to *trans*-acting factors and be instrumental in specifying the nature of the final spliced product.

To begin to elucidate the molecular mechanism of alternative splice-site selection, we have chosen to study a comparatively simple system in which the interactions of *cis*-acting elements with the general splicing machinery, rather than cell-specific *trans*-acting factors, seem to govern splice-site selection (Kedes and Steitz 1987). Splicing of the mouse immunoglobulin κ light-chain pre-mRNA is an example of highly specific alter-

native 5' splice-site selection. The κ light-chain locus in pre-B cells comprises several hundred variable protein-coding (V) regions, [each with a promoter and short leader (L) exon upstream]. These are separated by a large distance [as yet undefined, although ~23 kb in humans (Klobeck et al. 1987)], from a 1.5-kbp cluster of four short (36–38 bp), highly homologous (~65%) joining (J) regions (Max et al. 1979; for a review of immunoglobulin structure and expression, see Tonegawa 1983; Honjo and Habu 1985). Each J sequence is followed immediately by a 5' splice site, and the last J (J4) is separated by ~2.5 kbp from a single downstream 3' splice site adjacent to the constant (C) exon (Fig. 2). During B-cell differentiation, DNA recombination converts the germ-line configuration to one in which a single leader-variable unit is joined to the 5' end of one of the J regions. This creates a variable (V) plus J (V + J) exon. Because four J regions exist, the second intron of the κ chain pre-mRNA in mature lymphocytes can include as many as three unused 5' splice sites. These are nearly identical in sequence (each has an eight out of nine fit to the 5' splice-site consensus sequence) (see Fig. 1). Yet, the only κ gene products detected at both the mRNA and protein levels arise from exclusive use of the 5' splice site immediately downstream from the V + J exon (Kabat et al. 1983).

Previously, both we (Kedes and Steitz 1987) and Lowery and Van Ness (1987) attempted to study mouse κ

Figure 1. J regions of the mouse immunoglobulin κ light-chain gene. The J1–J4 regions are aligned at their 5' splice sites, indicated by a downward-pointing arrow. Shaded regions indicate perfect homology. The boxed sequences encompassing the 5' splice site represent the consensus sequence.

light-chain J-region 5' splice-site selection *in vitro*. We observed unexpectedly that in either lymphocyte (Kedes and Steitz 1987; Lowery and Van Ness 1988) or HeLa cell (Kedes and Steitz 1987; Lowery and Van Ness 1987) nuclear extracts, all available J-region 5' splice sites in synthetic transcripts are used equivalently. In contrast, studies in which HeLa cells were transfected with plasmids containing comparable rearranged κ gene segments showed correct utilization of only the upstream-most 5' splice site. Thus, 5' splice-site selection in the κ light chain appears to be neither cell-type- nor species-specific *in vivo* but cannot be studied effectively *in vitro* because of loss of selectivity.

Here we have probed the structure of the κ pre-mRNA and tested several previously suggested mechanisms that might underlie correct *in vivo* 5' splice-site selection. Because others have shown that exon length and sequences can affect the efficiency of splice-site usage (Reed and Maniatis 1986; Furdon and Kole 1988), we have asked whether the V region somehow renders the 5' splice site of the juxtaposed J region most attractive to the splicing machinery. We have also investigated whether, conversely, germ-line sequences of a J region might be inhibitory. Another explanation for 5' splice-site selection in κ transcripts is that a hierarchy of J1 > J2 > J3 > J4 in affinity for either splicing factors or the 3' splice site itself exists. Finally, it has been suggested that selectivity depends on a dynamic interplay between splicing and transcription, thereby somehow ensuring that the first 5' splice site transcribed is the only one paired to a single 3' splice site (Khouri et al. 1979; Osheim et al. 1985; Aebi and Weissmann 1987). The results we have obtained argue that it should be possible to design simple uncoupled *in vitro* splicing systems that will allow further dissection of the molecular mechanisms of alternative splice-site selection.

Results

To define the parameters governing 5' splice-site selection in immunoglobulin κ light-chain transcripts, we first constructed plasmids containing κ gene segments inserted downstream from the powerful human β -actin promoter (Gunning et al. 1987). We concentrated on model transcripts containing only the J3 and J4 5' splice sites so that analysis of the processed RNA would reflect simple binary splicing decisions.

Variable-region sequences are not required for accurate 5' splice-site selection in vivo

The V region could be important for correct 5' splice-site selection in κ transcripts because it either binds an essential factor or confers a necessary structure on the pre-mRNA. We therefore asked whether deletion or replacement of this sequence might lead to loss of selectivity. The relevant constructs and potential splicing patterns of their transcripts are diagrammed in Figure 2 (see first three templates). pV3-4 is the control construct representing a V-J3 rearrangement; transcripts from this template have an intact (307 bp) V region juxtaposed to J3 (37 bp), followed sequentially by the entire sequence between J3 and J4 (301 bp), J4 (37 bp), a truncated J4-C intron (251 bp) and, finally, the first 51 nucleotides of C. pV(Δ BamHI)3-4 is identical to pV3-4, except for a 109-nucleotide deletion from the 3' end of the 307-nucleotide V region, whereas p β G3-4 has the entire V region replaced with most of the second exon (204 bp) of the human β -globin gene (see Materials and methods).

Two days after transfection with each of these plasmids into HeLa cells, cytoplasmic RNA was isolated and subjected to primer extension analysis, using a primer complementary to the C exon (see Fig. 2). [Nuclear RNA was also analyzed and gave qualitatively identical results to those in Figure 2 (data not shown).] Transfection with the parent vector without an insert (see Materials and methods) gives no signal above background (data not shown). Transfection with the control plasmid pV3-4 leads to a primer extension product of 456 nucleotides, consistent with the exclusive use of the J3 5' splice site (Fig. 3, lane 1), as reported previously (Kedes and Steitz 1987). A 794-nucleotide primer extension product, which would indicate use of the J4 5' splice site, is absent. The 109-nucleotide deletion in the V region of the plasmid pV(Δ BamHI)3-4 has no effect on selection of the J3 5' splice site; the predicted 685-nucleotide primer extension product resulting from use of the downstream J4 5' splice site (Fig. 2, follow broken arrows) is noticeably absent, whereas the 347-nucleotide product from the J3-to-C splice can be seen clearly (Fig. 3, lane 3). Likewise, construct p β G3-4, in which the entire V region is replaced with the second exon of the human β -globin gene, gives rise to a processed RNA that arises from exclusive use of the upstream J3 5' splice site. The primer extension product is a distinct band of 358 nucleotides (Fig. 3, lane 2), whereas a primer exten-

Figure 2. Schematic representation of κ constructs transfected into HeLa cells. The pre-mRNAs transcribed from the human β -actin promoter are shown along with the potential splicing patterns. The solid arrows following the transcripts indicate the splicing path observed, and the broken arrows the potential but unobserved alternate (see Fig. 3). The predicted primer extension lengths (in nucleotides) are represented by a left-pointing arrow below each final product. The primer, which hybridizes to the C exon (see Materials and methods), is indicated by the thicker segment of this arrow, and the 5' radiolabeled end by an asterisk (*). (UT) 5'-Untranslated region of the human β -actin gene; (IVS1) first intervening sequence of the human β -actin gene; (PL) polylinker; (V) variable region; (J3) joining region 3; (J4) joining region 4; (C) constant region; (PA) SV40 polyadenylation signal; (β G) β -globin second exon (see Materials and methods). Constructions of pV3-4, pV(Δ BamHI)3-4, p β G3-4, pV3/4, and pV3(Δ 5'ss)-4 are described in Materials and methods.

sion product of 696 nucleotides, which would represent use of the downstream J4 5' splice site, is not seen (see Figs. 2 and 3, lane 2). These data indicate that in transfected HeLa cells the V region can be either deleted or replaced by other exon sequences and still allow accurate selection of the upstream-most J region 5' splice site. Analysis by RNase T1 mapping confirmed these results: Only transcripts containing the J3-C were de-

tected in RNA isolated from transfected HeLa cells (data not shown; see Kedes and Steitz 1987). Because the uniformly labeled complementary RNA probes had specific activities up to two orders of magnitude higher than the 5' end-labeled primer extension products, they would have favored the detection of the longer J4-to-C spliced products from pV3-4, p β G3-4, and pV(Δ BamHI)3-4 (see Fig. 2).

Figure 3. Results of primer extension analyses on cytoplasmic RNA from HeLa cells transfected with the plasmids diagrammed in Fig. 2. HeLa cell transfections and primer extension analyses were conducted as described in Materials and methods, and the products were separated on a 4% polyacrylamide/8 M urea gel. (Lane 1) pV3-4; (lane 2) pG3-4; (lane 3) pV(Δ BamHI)3-4; (lane 4) pV3/4; (lane 5) pV3(Δ 5'ss)-4. Sizes in nucleotides of the primer extension products are indicated by left-pointing arrows on the right, and sizes of DNA markers (in nucleotides) are indicated at the left.

To show that the J4 5' splice site has not somehow been inactivated in our constructs, we also tested a plasmid in which 12 bp from the 5' end of the J3 exon had been fused to last 20 bp of the J4 exon. This J3/J4 chimera deletes the J3 5' splice site while preserving the J4 5' splice site and downstream sequences (see pV3/4 in Fig. 2). When this plasmid was transfected into HeLa cells, the J4 5' splice site of the resultant transcript was efficiently spliced to the 3' splice site of C, giving a primer extension product of 469 nucleotides (see Figs. 2 and 3, lanes 4). This result demonstrates that the J4 5' splice can be used if *cis*-competition from the upstream J3 5' splice site is eliminated.

Selection is not due to instability of incorrectly spliced transcripts

We have also examined the possibility that splicing does occur at the downstream J4 5' splice site but that the

resulting products are inherently unstable and therefore not detected (see Fig. 2, spliced products indicated by broken arrows). If this were the case, correct 5' splice-site selection seen in the J3/J4 fusion described above would arise merely because elimination of 'poison' sequences contained in the intervening sequences between the J regions allowed detection of a stable J4-to-C spliced RNA. We devised a construct in which all but the first 7 bp of the intervening sequence between the two J regions remained intact [pV3(Δ 5'ss)-4; Fig. 2 and Materials and methods] and transfected it into HeLa cells. To our surprise, this plasmid produced a stable RNA spliced at the J4 5' splice site (see Fig. 2), as indicated by a primer extension product of 726 nucleotides (see Fig. 3, lane 5). (Note that the lower intensity of this band relative to that of others is not reproducible.) We conclude that the transcript is stable, despite the presence of the normally excised intervening sequences (except for the 7 nucleotides at the J3 5' splice site itself) between J3 and J4.

We also confirmed that the presence of intron sequences upstream of the J3 region do not preclude use of its 5' splice site or lead to instability of resulting products. Downstream from the human β -actin promoter [in a plasmid without the β -actin first intron (Gunning et al. 1987)], we inserted the unrearranged κ gene segment J3 with its germ-line 5'-flanking (intron) sequence (no upstream V region or other exon sequence), the J3-J4 intron, followed by J4, the foreshortened intron and, finally, the first 51 nucleotides of C (see Fig. 4A, p3-4). Figure 4A outlines schematically the two possible splicing patterns for the resultant transcript. A J3-to-C splice would lead to a primer extension product of 157 nucleotides, whereas the product of a J4-to-C splice would be 495 nucleotides. Figure 4B, lane 3, shows analysis of the RNA from HeLa cells transfected with this plasmid. A single band migrating at 157 nucleotides, indicating use of the J3 5' splice site, is observed despite both the lack of any exon sequence adjacent to J3 and the presence of the naturally occurring J3 5'-flanking sequence. A 495-nucleotide product, representing use of the downstream J4 5' splice site is absent even on long exposures. Note that the parent vector without an insert gives no signal over background (Fig. 4B, lane 2). The plasmid pV3-4, tested in parallel as a control for primer extension efficiency, leads to a distinct band migrating at 456 nucleotides (see Fig. 4B, lane 5). Again, RNase mapping confirmed the patterns of splice-site selection deduced from Figure 4 (data not shown).

A J-region hierarchy does not explain 5' splice-site selection

The 5' splice-site selection observed in all of the above transfection experiments, as well as in κ -producing lymphocytes, could reflect an inherently greater affinity of the splicing machinery for the J3 relative to the J4 site. Because this explanation predicts that use of a 5' splice site will be dependent on its rank in this hierarchy of affinity rather than its position in the transcript, we

Figure 4. Analysis of germ-line configuration and J-region transposition on the splicing of κ transcripts in HeLa cells. (A) p3-4, a κ construct in the germ-line configuration and p4-3, an analogous construct, but with its J regions transposed. Their respective pre-mRNAs are shown schematically, along with their potential splicing patterns following solid and broken arrows as in Fig. 2. The lengths of the predicted primer extension products (in nucleotides) are indicated as in Fig. 2. (B) The results of primer extension analyses on cytoplasmic RNA from HeLa cells transfected with the plasmids diagrammed in A. Transfections, primer extension analysis, and gel electrophoresis were conducted as described in Fig. 3. (Lane 1) DNA markers (M); (lane 2) parent plasmid pH β Pr-2-neo without a κ gene insert (see Materials and methods); (lane 3) p3-4; (lane 4) p4-3; (lane 5) pV3-4 (see Fig. 2). pV3-4 was included as a control for primer extension efficiency. Sizes of the primer extension products (in nucleotides) are indicated by left-pointing arrows on the right and sizes of the DNA markers (in nucleotides) are indicated on the left.

tested it directly with construct p4-3 (see Fig. 4A). Here the positions of the J regions are switched (see Materials and methods), but the distances between J4 and J3 and J3 and C are approximately the same as in the p3-4 control (see Fig. 4A). The two potential splices in the p4-3 transcript are diagrammed in Figure 4A (follow arrows): use of the upstream J4 or downstream J3 5' splice site would give rise to a primer extension product of 231 or 536 nucleotides, respectively. Analysis of transcripts produced in HeLa cells after transfection with this plasmid is shown in lane 4 of Figure 4B: a single band migrating at 231 nucleotides indicates exclusive use of the upstream J4 5' splice site. Again, RNase mapping data confirmed this finding (not shown). We conclude that splicing is confined to use of the upstream-most 5' splice site (in this case, that of J4) without regard to its original position in the gene. A J hierarchy model, therefore, seems untenable.

*κ transcripts are spliced correctly in *Xenopus laevis* oocytes*

Indiscriminate use of all available 5' splice sites in vitro compared to correct selection of the upstream-most 5' splice site of κ transcripts in transfected HeLa cells is consistent with models proposing that accurate splicing is dependent upon transcriptional coupling (Aebi and Weissmann 1987). To examine this possibility, we turned to the *X. laevis* oocyte system, where splicing

can be analyzed either in the presence (upon injection of the DNA) or absence (upon injection of the pre-mRNA) of transcription. We first injected presynthesized, gel-purified SP6-generated κ transcripts into the nuclei of oocytes, collected total RNA after incubation times of 0, 10, 30, and 90 min, and analyzed the spliced products. Because the amount of injected RNA recovered from oocytes is significantly lower than that produced in HeLa transfection experiments, we employed the highly sensitive RNase T1 mapping technique and confirmed the appearance of spliced products by primer extension analyses (not shown). Altogether, we tested three transcripts, which are diagrammed schematically in Figure 5, along with their potential spliced products.

The first transcript pSP-3-4, is the SP6 homolog of p3-4 (cf., Fig. 5 with Fig. 4A) and contains 48 nucleotides of J3 5'-flanking sequence, J3, the sequence between the J regions, J4, and the same foreshortened J-C intron as described above for the transfection plasmids. Two RNA probes and their regions of complementarity to pSP-3-4, its splicing intermediates, and final product are diagrammed in Figure 6A (top). With probe 3-4 *Ban*I (691 nucleotides), a protected fragment of 555 nucleotides would represent the precursor; a 108-nucleotide fragment would arise from both the J3-to-C spliced product and the J3 5' cutoff exon; and the 458-nucleotide fragment would correspond to the J3 lariat. (Note that although we use the term 'lariat,' the labeled bands in Figure 6 could represent either the linearized or the

Figure 5. Schematic representation of SP6-generated κ transcripts for *Xenopus* oocyte injections. pSP-3-4 and pSP-4-3 are analogous to p3-4 and p4-3, respectively (see Fig. 4A). pSP-4 is identical to pSP-3-4 but codes for a transcript that begins 8 nucleotides downstream of the J3 5' splice site. Potential splicing patterns are indicated following solid and broken arrows, as in Figs. 1 and 3A.

lariat form of the intron and that the analysis does not differentiate between the lariat intermediate with an attached C exon and the intron lariat product.) The results of an injection experiment with pSP-3-4 are shown in Figure 6A, panel 1. A 108-nucleotide fragment and a

458-nucleotide fragment, indicative of splicing to the J3 5' splice site, appear with time and reach a maximum after ~ 30 min (see Fig. 6A, lanes 6–8). Levels of splicing intermediates and products are barely detectable at time '0' (Fig. 6A, lane 6), because in actuality 2–4 min have

Figure 6. (See following page for legend.)

elapsed before the first aliquot of RNA can be harvested. In contrast, RNA from uninjected oocytes gives no signal above background (Fig. 6A, lane 5). Use of the downstream J4 5' splice site would be indicated by protected fragments of 444 nucleotides (the final product and J4 5' cutoff exon) and of 120 nucleotides (the corresponding J4 lariat). The J4 lariat is noticeably absent even after 90 min (Fig. 6A, lanes 6–8); but because the 444-nucleotide fragment from the J4-to-C splice would be difficult to distinguish from the 458-nucleotide fragment corresponding to the J3 lariat, we also hybridized the recovered RNA to a second probe, 3-4 *HhaI* [Fig. 6A, top (2)], which is shorter than probe 3-4 *BanI* in the region complementary to the 5' end of the pSP-3-4 transcript. Thus, all protected fragments, except the ones corresponding to lariats, should be shorter (Fig. 6A, top). In Figure 6A, lane 10, we see that the pattern of protected fragments with probe 3-4 *HhaI* changes as predicted for exclusive use of the upstream J3 5' splice site: a 517-nucleotide (rather than a 555-nucleotide) fragment

from the precursor, a 68-nucleotide (rather than a 108-nucleotide) fragment representing both the J3 final product and 5' cutoff exon, and an unchanged 458-nucleotide for the J3 lariat (rather than a 404-nucleotide fragment corresponding to both the J4-to-C spliced final product and its 5' cutoff exon). These data demonstrate that correct 5' splice-site selection in the pSP-3-4 transcript does not require concomitant transcription in the oocyte.

To ensure that the sequence upstream from J4 neither inhibits use of the J4 5' splice site nor destabilizes a final product and that the J4 splice site remains functional, we also tested a transcript in *Xenopus* oocytes that begins 8 nucleotides downstream of the J3 5' splice site (see Fig. 5, pSP-4). The RNA probe 3-4 *HhaI* and its complementarity to the transcript, splicing intermediates, and final product are diagrammed in Figure 6B (top). After incubation for 30 min in the oocyte, the truncated pSP-4 transcript is spliced successfully at the J4 5' splice site. We observe the expected 457-nucleotide fragment corre-

Figure 6. RNase T1 mapping of *Xenopus* oocyte RNA after injection with transcripts from pSP-3-4, pSP-4, and pSP-4-3. RNA from a one-half oocyte equivalent for each incubation time (see Materials and methods) was hybridized with at least a 10-fold molar excess of the indicated complementary (and uniformly radiolabeled) RNA probe and digested with RNase T1, as described in Materials and methods. The protected fragments were separated by electrophoresis on 6% polyacrylamide/8 M urea gels. The top of each panel depicts the complementarity between each probe and the transcript, splicing intermediates, and final product. The sizes of the predicted T1 fragments are indicated in parentheses. For reasons of clarity, only the protected products that are detected are included in the diagrams. (A) The results from a 90-min incubation of the pSP-3-4 transcript using probes 3-4 *BanI* (1) and 3-4 *HhaI* (2). (Only the 90-min time point is shown for the latter.) (Lane 1) DNA markers (M); (lane 2) 100 cpm of purified 3-4 *BanI* probe alone and without RNase; (lane 3) purified transcript hybridized with the probe 3-4 *BanI* and then digested with RNase; (lane 4) probe 3-4 *BanI* alone with RNase; (lane 5) total RNA from uninjected oocytes; (lanes 6–8) the RNA after 0 (see Materials and methods), 30, and 90 min of incubation of the transcript, respectively; (lane 9) 100 cpm of purified probe 3-4 *HhaI* alone and without RNase; (lane 10) the RNA from the 90-min sample hybridized with probe 3-4 *HhaI*. The protected fragments representing the probes, precursor, intron-containing intermediates (lariat), and final product (and 5' cutoff exon) are indicated along with their sizes (in nucleotides) in the center. (Note that lanes 1–10 are from a single gel.) (B) The results from a 30-min incubation of the pSP-4 transcript using the complementary RNA probe 3-4 *HhaI*. (Lane 1) DNA markers (M) as in A; (lane 2) purified pSP-4 transcript alone hybridized with the probe; (lanes 3–5) the RNA after 0 (see Materials and methods), 10, and 30 min of incubation, respectively, each hybridized with probe 4-3 *BanI*; (lane 6) the RNA after 30 min of incubation but hybridized to probe 4-3 *AvaII*; (lane 7) 100 cpm of purified probe 4-3 *AvaII* without RNase; (lane 8) DNA markers (M), as above. (Note that lanes 1–5 and 6–8 are from two separate gels.)

after 0 (see Materials and methods), 10, and 30 min of incubation, respectively. The protected fragments representing the probe, precursor, intron-containing fragments (lariat), and the final product (and 5' cutoff exon), along with their sizes (in nucleotides), are indicated at the right. (C) The results from a 30-min incubation of the pSP-4-3 transcript, using the complementary RNA probes 4-3 *BanI* (1) and 4-3 *AvaII* (2). (Lane 1) DNA markers (M) as in A; (lane 2) purified pSP-4-3 transcript hybridized with probe 4-3 *BanI* (lanes 3–5) the RNA after 0 (see Materials and methods), 10, and 30 min of incubation, respectively, each hybridized with probe 4-3 *BanI*; (lane 6) the RNA after 30 min of incubation but hybridized to probe 4-3 *AvaII*; (lane 7) 100 cpm of purified probe 4-3 *AvaII* without RNase; (lane 8) DNA markers (M), as above. (Note that lanes 1–5 and 6–8 are from two separate gels.)

sponding to the precursor, the 345-nucleotide fragment for both the J4 splice product and 5' cutoff exon, as well as the 125-nucleotide fragment for the J4 lariat (Fig. 6B, lane 5). (Note that the fragment migrating just below the indicated 125-nucleotide fragment is an artifact arising from additional T1 RNase cutting.) We conclude that *Xenopus* oocytes can utilize the J4 5' splice site when not in *cis*-competition with the upstream J3 5' splice site and that the product is not destabilized by the presence of the J4 upstream flanking sequence.

Although we detected no hierarchy of J-region splice-site utilization in the HeLa cell system, it was necessary to test the splicing apparatus of the *Xenopus* oocyte for a preference for the J3- over the J4-region 5' splice site. We therefore constructed the plasmid pSP-4-3, in which the positions of the two J segments are switched. [This construct is analogous to p4-3, described above for the HeLa cell transfection experiments except that the κ gene segments are inserted downstream from the SP6 promoter rather than the human β -actin promoter (cf., Figs. 3A and 4).] As with the pSP-3-4 transcript, two probes differing in length at their 3' ends were used to ascertain the identity of the RNA species arising from the splicing of the pSP-4-3 transcript. The RNA probes and their complementarity to the transcript, splicing intermediates, and final product are shown in Figure 6C (top). The 739-nucleotide probe, 4-3 *Ban*I, would lead to a 632-nucleotide fragment for the precursor, a 132-nucleotide fragment for both the J4 spliced product and the J4 5' cutoff exon, and a 494-nucleotide fragment for the corresponding J4 lariat. After 10 min, bands of 132 and 494 nucleotides appear, and at 30 min, reach maximum intensity (Fig. 6C, lanes 4 and 5). However, a protected RNA fragment of about 500 nucleotides could also represent both the final product and J3 5' cutoff exon arising from use of the downstream J3 5' splice site. These two possibilities were distinguished using a shorter probe, 4-3 *Ava*II [see Fig. 6C, top (2)], which should shift the precursor and both the final product and 5' cutoff exon-protected fragments, whereas the corresponding J4 lariat fragment (494 nucleotides) would remain unchanged. On the other hand, the 494-nucleotide fragment should shift to 394 nucleotides if, in fact, it represents the J3 final product and 5' cutoff exon. As can be seen in Figure 6C, lane 6, with the 4-3 *Ava*II probe, the precursor fragment does shift from 632 to 524 nucleotides, the final product and the 5' cutoff exon both shift from 132 to 30 nucleotides (the latter runs off the gel in this experiment), and no 394-nucleotide fragment is generated. Thus, the unchanged 494-nucleotide fragment must represent the J4 lariat. Furthermore, a 136-nucleotide fragment, which would correspond to the J3 lariat using either probe, is conspicuously absent (see Fig. 6C, lanes 4–6). Therefore, despite transposition of the J regions, the upstream-most 5' splice site is again selected in pre-synthesized κ transcripts injected into the *Xenopus* oocyte.

Discussion

A problem currently impeding the elucidation of molecular mechanisms underlying alternate splicing is the in-

ability to reproduce, *in vitro*, splice-site selection observed *in vivo*. Consistently, splice sites that are ignored or less favored *in vivo* are used constitutively in equivalent transcripts *in vitro* (Noble et al. 1986; Van Santen and Spritz 1986; Bovenber et al. 1987; Norton and Hynes 1988). Only in one case (Schmitt et al. 1987) has the altering of *in vitro* conditions (lowering the ionic strength of the splicing extract) progressively favored the pairing of two downstream 5' splice sites relative to an upstream site with a single 3' splice site; however, whether such ionic changes are relevant to the *in vivo* situation is unclear. This disappointing *in vitro* versus *in vivo* discrepancy is also observed in the selection of J-region 5' splice site in κ pre-mRNA: The exclusive choice of the upstream-most 5' splice site in lymphocytes can be reproduced in transfected HeLa cells (Kedes and Steitz 1987; Lowery and Van Ness 1988) but is lost in HeLa (Kedes and Steitz 1987; Lowery and Van Ness 1987) or lymphocyte (Kedes and Steitz 1987; Lowery and Van Ness 1988) nuclear extracts in which splicing occurs at all available J-region 5' splice sites.

To probe the role of *cis*-acting signals in the selection of J-region 5' splice sites, we first introduced a variety of modified κ -expressing plasmids into HeLa cells. These constructs contained only the κ light-chain J3 and J4 regions and a deletion in the J4/C intron, but we had ascertained previously that exclusive utilization of the 5'-most splice site occurs when all four J segments or the complete intron are present in transfected HeLa cells (Kedes and Steitz 1987). Because HeLa cells thus mimic κ -producing lymphocytes in their splicing behavior, we believe that our conclusions in this model system can be extrapolated to the *in vivo* situation.

We are surprised to learn that the J regions in κ transcripts are able to direct proper 5' splice-site selection without being joined to additional exon sequences. Each J region (36–38 nucleotides), although longer than the 20 nucleotides found to be a minimum exon length for efficient *in vitro* splicing of monointronic pre-mRNA (Parent et al. 1987), is shorter than the 90 nucleotides required to bias splicing in *cis*-competition between 5' splice sites *in vitro* (Reed and Maniatis 1986). In the latter experiments, Reed and Maniatis found that when a fragment containing the last 16 nucleotides of exon 1 of the human β -globin was placed downstream from the full-length exon 1 (155 nucleotides), splicing occurred exclusively at the upstream 5' splice site. The splice-site choice, however, was reversed when the downstream site was preceded by 90 nucleotides of exon sequence, even in the presence of a full-length exon 1 (with its 5' splice site) upstream. (Note that this study did not examine intermediate exon lengths between 16 and 90 nucleotides.) Similar conclusions were reported recently by Lowery and Van Ness (1988).

Our observation that use of either the J3 or the J4 5' splice site is unperturbed by the presence of retained upstream germ-line sequences leads to two conclusions: (1) The choice of the upstream-most 5' splice site does not depend on inhibitory intron sequences to inactivate all downstream, unrearranged J-region 5' splice sites; and (2) the J-J intervening sequences that would be retained

in any product spliced at a downstream J region do not, in themselves, lead to rapid degradation. The only sequences we have not ruled out as destabilizing are the 8 nucleotides at the upstream 5' splice site that were deleted in pV3(Δ 5'ss)-4 (Fig. 3). However, the possibility that an upstream 5' splice-site consensus sequence alone leads to rapid degradation is made unlikely by naturally occurring counter examples: The γ B γ -fibrinogen mRNA of both rat (Crabtree and Kant 1982) and human (Fornace et al. 1984), the 13S and 12S E1A mRNAs of adenovirus (Berk and Sharp 1978; Chow et al. 1977), the small-t mRNA in SV40 (Khoury et al. 1979), and the P element mRNA in *Drosophila* somatic cells (Laski et al. 1986) all retain an unused 5' splice site(s) and yet are stable.

We also found no evidence of a J3-J4 hierarchy, although the κ -chain J regions do differ slightly in their sequences (see Fig. 1). Earlier work had suggested that certain examples of alternative splicing might reflect different competition efficiencies among splice sites, even if all conform to the consensus (Breathnach et al. 1978; Seif et al. 1979; Lerner et al. 1980; Rogers et al. 1980; Sharp 1981; Mount 1982). Such effects on splice-site selection have been observed in vitro, as well as in the in vivo splicing patterns of late SV40 RNAs (Somashekhar and Mertz 1985), human β -globin mRNAs with duplicated 5' splice sites (Cunningham et al. 1988), and human fibronectin RNA (Mardon et al. 1987). Moreover, certain 5'/3' splice-site pairs can have a particular intrinsic affinity even when a competing splice site is interposed between them (Eperon et al. 1986; Furdon and Kole 1988), leading to exon skipping. Yet the spliced products of constructs in which the J regions were switched demonstrated that the splicing of κ transcripts in HeLa cells follows a strict rule of selecting the upstream-most 5' splice site. Similar observations were reported recently by Lowery and Van Ness (1988).

Next, we addressed the possibility that correct splicing requires coupling to transcription, which would explain the loss of selectivity in vitro. We observed that *Xenopus* oocytes injected with presynthesized κ transcripts with J3 and J4 in either order gave splicing patterns identical to those in HeLa cells. Thus, even in the absence of transcription, the upstream-most 5' splice site is consistently chosen. That oocytes do not exhibit an intrinsic bias in always choosing the first of alternative 5' splice sites has been demonstrated by Fradin et al. (1984); they reported that oocytes synthesize significantly more SV40 small-t mRNA relative to large-T mRNA, meaning a preference for the downstream 5' splice site in that case. The oocyte data are also consistent with our conclusion from the HeLa cell experiments that differential transcript stability is not a major factor in determining which product is seen. Specifically, the pSP-3-4 and pSP-4-3 splicing intermediates, which contain the entire sequence between the upstream J-region 5' splice site and the C exon, survive in the oocytes for ≥ 30 min (Fig. 6); because they include all intron sequences that would be present in a spliced RNA arising from use of the downstream 5' splice site, such products should therefore be readily detectable if they are produced. Moreover, if RNAs were spliced at the

downstream 5' splice site but rapidly degraded, we would expect approximately half the amount of final product from pSP-3-4 or pSP-4-3 compared to pSP-4 (assuming that splicing occurs at the two sites with similar frequencies); yet we find that the yield of spliced product from the pSP-3-4 or pSP-4-3 transcript is approximately equal to that for the pSP-4 transcript. Taken together, these data argue strongly that the splicing machinery acts at only a single 5' splice site in each of the three transcripts tested in the oocyte.

There are two parameters known to affect 5' splice-site choice that we have not tested with the κ transcript. (1) In vivo studies of pre-mRNA constructs in which 5' splice-site regions were duplicated have shown that the upstream-most site is usually preferred when a single 3' splice site exists (Kuhne et al. 1983; Lang and Spritz 1983; Eperon et al. 1986). Alternatively, the downstream copy may be favored, but this preference can be lost if the sites are brought very close together (Cunningham et al. 1988). Because the J-region 5' splice sites are separated from their neighbors by 273 to 630 bp, selectivity seems unlikely to result from splice-site interference. (2) In some cases, splice-site selection in vivo reflects preferential intron removal: Very short introns are less likely to be spliced out than longer ones. A striking example is the favoring of the large-T over small-t splice in SV40 transcripts (Fu and Manley 1987), which can be eliminated by increasing the length of the small-t intron (66 nucleotides) with filler sequences. Because the lengths of introns in our constructs range from 244 to 582 nucleotides, well over the minimum length of ~ 60 nucleotides (Wieringa et al. 1984) required for efficient splicing, differences in intron length are unlikely to explain the complete exclusion of the downstream J-region 5' splice site(s) in κ transcripts.

A model that invokes simple proximity to the cap structure (Konarska et al. 1984) in the absence of other *cis*-acting signals could explain 5' splice-site selection among the mouse κ light-chain J regions. How such a mechanism operates on the molecular level remains to be deciphered. Although the 5' splice site was no more than 422 nucleotides from the 5' cap in the constructs used here, the distance was greater than 1000 nucleotides in previously examined constructs and still the upstream-most site was selected exclusively (Kedes and Steitz 1987). Because correct splicing is seen in two different cell types (HeLa cells and *Xenopus* oocytes), which normally do not produce κ mRNA, the *trans*-acting factors involved must be of general character. Known components of the splicing machinery include snRNPs, factors not (tightly) associated with snRNPs (Furneaux et al. 1985; for reviews, see Green 1986; Padgett et al. 1986; Steitz et al. 1987), and hnRNP proteins (Choi et al. 1986; for reviews, see Dreyfuss 1986; Dreyfuss et al. 1988). Although only U1 snRNPs have been shown to be directly involved in recognition, any of these other factors could also contribute to fidelity in 5' splice-site choice. For instance, isolated U1 snRNPs bind to mutant 5' splice sites in model human β -globin transcripts, whereas these sites are not used in complete splicing extracts (Chabot and Steitz 1987). The existence

of other factors conferring discrimination is also consistent with the observations of Mayeda et al. (1986), who demonstrated that the binding of U1 snRNPs to a 5' splice site in vitro was enhanced by addition of a nuclear extract DEAE–Sephadex fraction lacking U1 and U2 RNA. One or more such factors directing proper splice-site selection could be lost or destroyed during the extraction procedure currently used to prepare active nuclear splicing systems. The resulting imbalance could lead to improper spliceosome assembly, allowing sites discriminated against in vivo to be used in vitro. Because we have demonstrated that accurate 5' splice-site selection in the κ pre-mRNA is independent of transcription, future studies aimed at dissecting the molecular basis of alternative splicing should not need to pursue the difficult task of coupling transcription and splicing in an in vitro system.

Materials and methods

Materials

All enzymes and labeled nucleotides were purchased from New England Biolabs, Boehringer–Mannheim, Promega Biotec, New England Nuclear, and Amersham. *X. laevis* mature females were purchased from *Xenopus* I (Madison, Wisconsin).

SP6 transcription

Linearized plasmid templates were transcribed with SP6 RNA polymerase (Melton et al. 1984), and the transcripts were purified by denaturing polyacrylamide gel electrophoresis as described (Black et al. 1985).

Recombinant DNA

λ Phage clones of the immunoglobulin κ light-chain genes (gifts from S. Lewis and D. Baltimore), containing the variable region V_{κ} as well as the J and C regions, have been described (Lewis et al. 1984). Standard cloning techniques (Maniatis et al. 1982) were employed to generate the pH β APr-1 and pH β Pr-2-neo (Gunning et al. 1987) and pSP6 (Melton et al. 1984) constructs. The vector pH β APr-1 contains the human β -actin promoter, 78 bp of 5' untranslated region (UT), 832 bp of the first intervening sequence (IVS1) of the β -actin gene fused at its 3' splice site to a short polylinker (PL), followed by a simian virus 40 poly(A)-addition site (PA). pV3-4, a derivative of pH β APr-1, possesses an intact V region rearranged to J3, followed by J4 and the first 51 nucleotides of the C region, and has been described (plasmid A in Kedes and Steitz 1987). pV(Δ BamHI)3-4 is identical to pV3-4, except that a 109-bp BamHI fragment (from –2 to –110 bp upstream of the V-J3 junction) is deleted from the V region. p β G3-4 differs from pV3-4 in that the first 204 bp of the second exon of the human β -globin gene and 54 bp of its 5'-flanking sequence replace the entire V region (except for the last A-T bp) plus 269 bp of its 5'-flanking sequence. pV3/4 is also derived from pV3-4 by the ligation of the first 12 bp of J3 to 13 bp of pSP64 PL sequence and this, in turn, to the last 20 bp of J4, thereby deleting the J3 5' splice site and the J-J intervening sequence, as well as creating a 45-bp J3/4 fusion. pV3(Δ 5'ss)-4, similarly derived from pV3-4, results from the deletion of the last 22 bp of J3 plus the first 7 bp of the J-J intervening sequence.

p3-4 and p4-3 are derivatives of the parent plasmid pH β Pr-2-neo, which is similar to pH β APr-1 (above), except that it contains only the first 48 bp of the human β -actin 5' UT and lacks

the human β -actin IVS1 (while additionally containing the SV2-neomycin resistance gene). Downstream from the 48 bp human β -actin 5' UT p3-4 has inserted a 795-bp fragment containing at its 5' end 39 bp of upstream flanking sequence, followed by J3, the J3-J4 intervening sequence, J4, and the same foreshortened 244 bp J-C intron and first 51 nucleotides of C found in the pH β APr-1 constructs. Downstream from the 48 bp human β -actin 5' UT p4-3 has inserted the following fragments (from 5' to 3'): 43 bp of PL, 69 bp of J4 5'-flanking sequence, J4, 110 bp of J4 3'-flanking sequence, 215 bp of J3 5'-flanking sequence, J3, 117 bp of J3 3'-flanking sequence, 21 bp of SP6 PL, the 135 bp immediately upstream of C and, finally, the first 51 bp of C.

pSP-3-4 has been described before (J3-J4-C in Kedes and Steitz 1987). Its κ configuration, inserted downstream from the SP6 promoter, is identical to that of p3-4 (above), except that the 5' end of the SP6 runoff transcript contains 14 nucleotides of SP6 PL plus 48 nucleotides of J3 5'-flanking sequence. pSP4 is similar to pSP-3-4, except that the κ insert begins 7 nucleotides downstream from the J3 5' splice site, and the 5' end of the SP6 transcript has 25 nucleotides of SP6 PL sequence. The κ configuration of the pSP-4-3 insert is identical to that of p4-3 (above) but has 8 nucleotides of SP6 PL at the 5' end of its SP6 runoff transcript and only 17 nucleotides of pH β Pr-2-neo PL preceding the J4 5'-flanking sequence.

Probe 3-4 *Ban*I is an SP6 runoff transcript complementary to the pSP-3-4 precursor, beginning 61 nucleotides upstream of J3 and ending 113 nucleotides into the J4-C intron. In addition, the probe has noncomplementary tails of 28 nucleotides at its 5' end and 21 nucleotides at its 3' end. Probe 3-4 *Hha*I is identical to 3-4 *Ban*I, except that its complementarity to the pSP-3-4 precursor begins 21 nucleotides before J3 and it has no noncomplementary 3' tail. Probe 4-3 *Ban*I is an SP6 runoff transcript complementary to the pSP-4-3 precursor, beginning 69 nucleotides upstream of J4 and ending 133 nucleotides into the J3-C intron. This probe also has noncomplementary tails of 21 nucleotides at its 5' end and 100 nucleotides at its 3' end. Probe 4-3 *Av*aII is identical to 4-3 *Ban*I, except that its complementarity to the pSP-4-3 precursor begins 18 nucleotides into J4 and it has no noncomplementary 3' tail.

Transfection of HeLa cells

Transfections and immunofluorescence assays to monitor transfection efficiency were carried out as described (Weber et al. 1984). Transfection efficiencies ranged between 30% and 40%. Cytoplasmic RNA was isolated according to Berk and Sharp (1978).

Microinjections of *X. laevis* oocytes

X. laevis oocytes were surgically removed from adult *X. laevis* females and defolliculated by treatment with collagenase (type IA, Sigma) at 1 mg/ml overnight, or 2 mg/ml for 5 hr, both at 18°C. Oocytes were stored, microinjected, and incubated in modified Barth's saline (MBS) (Gurdon 1977). Approximately 40 nl of 0.1 mg/ml gel-purified SP6-transcribed RNA resuspended in distilled H₂O was microinjected into the germinal vesicle of stage VI (Dumont 1972) oocytes, using 8 μ m-outer-diameter glass needles pulled on a Brown–Flaming model P-80 pipette–puller (Sutter Instruments, San Rafael, California). Injection volume was determined independently by microinjection of isotope-labeled marker. After incubation at 20°C, samples of 10 oocytes free of visible mechanical damage were taken from sets of injected oocytes and frozen on dry ice. The first samples (labeled time 0) were actually taken 2–4 min after injection.

X. laevis oocyte RNA purification

RNA was extracted from oocytes as follows: Ten frozen oocytes were lysed, without thawing, by addition of 250 μ l of guanidinium extraction buffer [4.2 M guanidine isothiocyanate; 0.5% sarkosyl; 25 mM Tris (pH 8.0); 0.7% 2-mercaptoethanol], vortexed briefly, and transferred to a new microfuge tube containing 250 μ l phenol, 250 μ l chloroform, and 250 μ l extraction buffer [1% SDS; 0.1 M Tris (pH 8.0); 10 mM EDTA]. The samples were then vortexed for ~20 sec, spun in a tabletop microfuge for 2 min, and extracted once with 500 μ l chloroform. The RNA was precipitated from the aqueous layer by freezing the sample plus 500 μ l of isopropanol on solid CO₂. After spinning in a microfuge for 7 min, the samples were washed twice with 75% ethanol and dried under vacuum. The pellets were resuspended in 6 μ l of 90% formamide with ~1 mg/ml each of xylene cyanol and bromphenol blue dyes. Three microliters were loaded per lane onto polyacrylamide gels.

Primer extension and RNase mapping

Typically, 10% of the RNA collected from a confluent 6-cm (diam.) tissue culture dish, or the equivalent of one-tenth to one-half *Xenopus* oocyte, was used for each assay. The 25-nucleotide oligodeoxynucleotide complementary to nucleotides 9–33 of the C exon was 5' end labeled using [γ -³²P]ATP and polynucleotide kinase (Maniatis et al. 1982). Hybridization and primer extension was then performed as described (Solnick 1985). RNase T1 mapping was performed according to Melton et al. (1984), except that RNase A was omitted. RNA and DNA samples were analyzed on either 4% or 6% polyacrylamide gels with 8 M urea, as indicated in the figure legends.

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Correct in vivo splicing of the mouse immunoglobulin kappa light-chain pre-mRNA is dependent on 5' splice-site position even in the absence of transcription.

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