MOLECULAR AND CELLULAR BIOLOGY, Oct. 1987, p. 3428–3437
0270-7306/87/103428-10$02.00/0

Vol. 7, No. 10

In Vitro Splicing Pathways of Pre-mRNAs Containing Multiple Intervening Sequences†

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Received 13 February 1987/Accepted 23 June 1987

We analyzed the in vitro splicing pathways of three multi-intervening-sequence (IVS) pre-mRNAs: human β-globin, which contains two IVSs (K. M. Lang, V. L. van Santen, and R. A. Spritz, EMBO J. 4:1991–1996, 1985); rat α-lactalbumin, which contains three IVSs; and murine interleukin-3, which contains four IVSs. We found that there are highly preferred pathways of IVS removal from these multi-IVS pre-mRNAs in vitro. The three IVSs of rat α-lactalbumin pre-mRNA were excised sequentially from 5′ to 3′; in most molecules, IVS1 was removed first, followed by IVS2 and finally by IVS3. The splicing pathway of interleukin-3 pre-mRNA in vitro was more complex. The four IVSs were excised in a highly preferred temporal order, but the order was not strictly sequential or directional. In most molecules, IVS1 and IVS4 were removed first, either simultaneously or in rapid succession. Subsequently, IVS2 was excised, followed by IVS3. The observed splicing pathways apparently resulted from differences in lag times and maximum excision rates of the different IVSs. We detected no exon skipping during splicing of these transcripts in vitro. These observations have implications for proposed models of splice site selection.

Many eucaryotic genes contain intervening sequences (IVSs) that are removed from nuclear mRNA precursors (pre-mRNA) by RNA splicing (for reviews, see references 10 and 26). The sequence requirements for accurate splicing are well known. However, normal pre-mRNAs may contain several IVSs and a number of cryptic splice sites, and it is not known how the splicing machinery selects the correct pairs of splice sites. If no mechanism existed to ensure that only the appropriate 5′ and 3′ splice sites are joined, exons might be omitted from the mRNA (exon skipping). Several different mechanisms could ensure orderly IVS removal: (i) splice sites might be recognized by a linear scanning process initiating at one end of the pre-mRNA; (ii) individual IVSs might be excised at different rates; or (iii) the pre-mRNA might undergo conformational changes during splicing.

A number of laboratories have attempted to define in vivo splicing pathways for pre-mRNAs that contain multiple IVSs. In most studies, only steady-state RNA was analyzed (4, 7, 9, 13, 23, 24, 29, 32, 35), making it impossible to prove that there were precursor-intermediate-product relationships in observed RNA species. However, studies of splicing of two different adenovirus transcripts isolated at various times after in vivo labeling demonstrated that for the major late transcript, IVS1 was removed before IVS2 (14) and that for the E2A transcript, IVS2 was generally removed before IVS1 (8).

To determine whether there is a preferred order of removal of IVSs from pre-mRNAs containing multiple intervening sequences, we had previously analyzed the splicing pathways of human β- and γ-globin pre-mRNAs in vitro (19). Globin genes contain only two IVSs; therefore, their transcripts represent the simplest class of multi-IVS pre-mRNAs. We now report the analysis of splicing pathways of two more complex multi-IVS pre-mRNAs: rat α-lactalbumin (αLA), which contains three IVSs, and murine interleukin 3 (IL3), which contains four IVSs. For both the rat αLA and murine IL3 pre-mRNAs, we observed highly preferred pathways of IVS removal. For αLA pre-mRNA, the three IVSs were excised sequentially from 5′ to 3′. However, for IL3 pre-mRNA, the preferred pathway of IVS excision was more complex. First, IVS1 and IVS4 were removed at approximately the same time, followed by IVS2, and finally by IVS3.

MATERIALS AND METHODS

Materials. Restriction enzymes were obtained from New England BioLabs, Inc., SP6 RNA polymerase was from Promega or New England BioLabs, placental RNase inhibitor was from Promega or Amersham Corp., DNase I was from Worthington Diagnostics, RNase T1 was from Calbiochem-Behring, radiochemicals were from New England Nuclear Corp. and Amersham Corp., GpppG and T4 polynucleotide kinase were from Pharmacia Inc., and DNA polymerase (Klenow fragment) was from New England Nuclear Corp.

Transcription and processing reactions. The human β-globin mRNA transcriptional template (pSP6βc) contained complete human β-globin cDNA inserted into pSP64 (20). The rat αLA transcriptional template (pSP6α-LA; Fig. 1A) contained the rat αLA gene inserted into pSP65 (20). The 5′ end of the gene was from pαLA2.5, the middle section was from pαLA0.9 and pαLA1.1, and the 3′ end of the gene was from the cDNA clone pαLA35 (5, 28). The murine IL3 transcriptional template (pSP6IL3; Fig. 1B) contained the murine IL3 gene inserted into pSP65 and included the 5′ end of the cDNA clone pcDIL3 (33) and the rest of the gene from the genomic clone pλMGM12 (22). The poly(A) tail-encoding portion of pGγ3′c (18) was added to the 3′ end of the β-globin, αLA, and IL3 transcriptional templates.

pSP6βc and pSP6α-LA linearized with *Hin*dIII and

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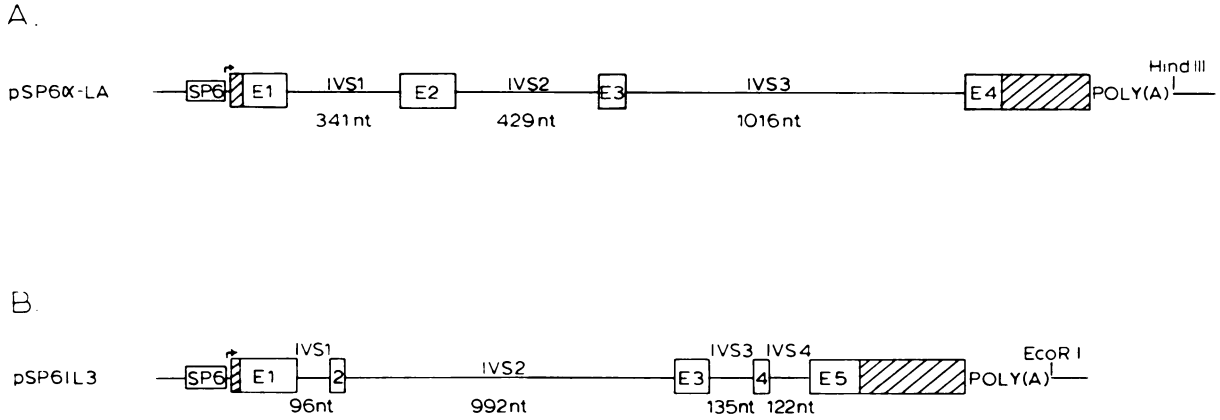


FIG. 1. SP6 αLA and IL3 transcriptional templates. Arrows indicate the site and direction of transcription initiation from the SP6 promoter. (A) pSP6α-LA; (B) pSP6IL3. Hatched boxes here and in other figures represent 5′ and 3′ untranslated sequences. Abbreviations: E1, E2, E3, E4, and E5, exons 1, 2, 3, 4, and 5, respectively. The positions of IVS1, IVS2, IVS3, IVS4, and their corresponding sizes are indicated. In vitro transcripts of linearized templates terminate at the indicated *Hin*dIII site of pSP6α-LA and at the *Eco*RI site of pSP6IL3.

pSP6IL3 linearized with *Eco*RI were transcribed with SP6 RNA polymerase (11). The 50-μl reaction mixture contained 40 mM Tris hydrochloride (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol, 2 μl placental RNase inhibitor (20 to 30 U/μl), 500 μM each ATP and CTP, 100 μM each UTP and GTP, 500 μM GpppG, 31 μCi of [α^{32}P] UTP, 2.5 μg of DNA template, and 15 U of SP6 RNA polymerase. β-Globin mRNA and αLA transcripts were isolated by oligo(dT) cellulose column chromatography (2). αLA and IL3 transcripts were processed in HeLa cell nuclear extracts as described previously (6, 15), except that placental RNase inhibitor was omitted from the reactions.

RNA analyses. Cleavages at the 5′ and 3′ splice sites were analyzed by S1 nuclease protection analysis with ^{32}P-end-labeled double-stranded DNA probes (3, 33). Exon ligation was analyzed by RNase T1 protection assay (19). Processed RNA (12.5 ng) was hybridized to full-length cDNA probes digested with RNase T1 and was electrophoresed in 4% denaturing polyacrylamide gels (19, 27). The RNase T1 analyses with the IL3 half-probes were electrophoresed in 6% denaturing polyacrylamide gels.

RESULTS

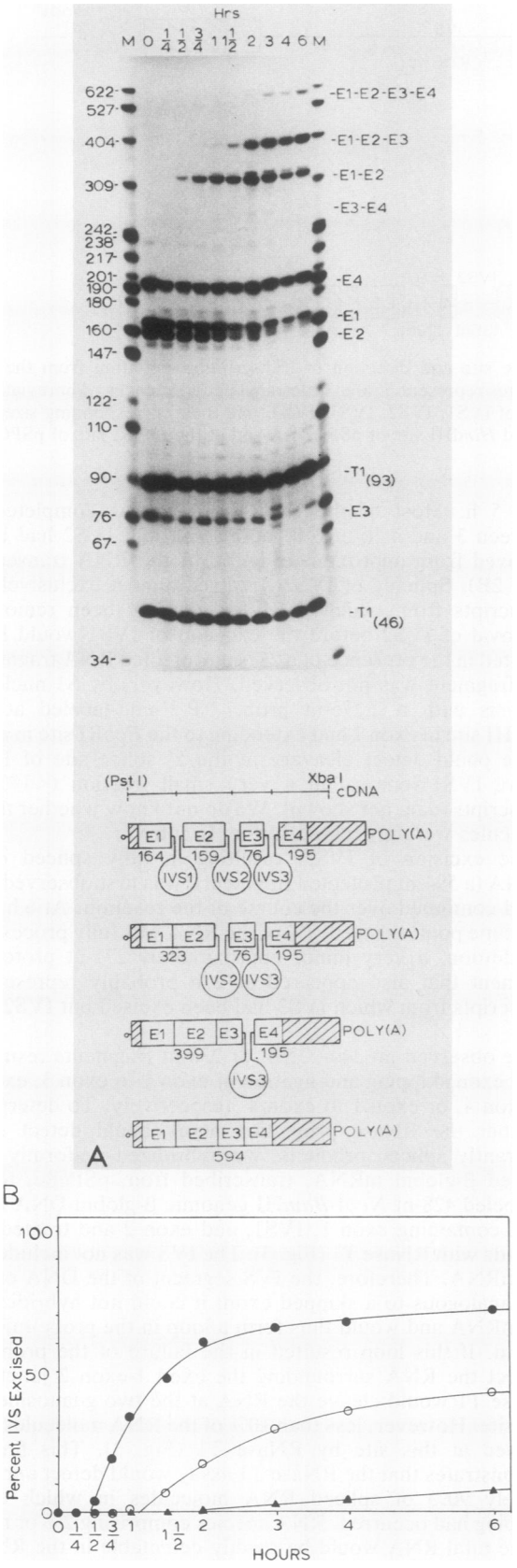
In vitro splicing pathway of αLA pre-mRNA. During processing of rat αLA pre-mRNA in vitro, the three IVSs were excised in a highly preferred order. IVS1 was first, followed by IVS2, and finally by IVS3. In a very small percentage of the molecules (<1%), cleavage occurred at the 5′ splice site of IVS2 before cleavage occurred at the 5′ splice site of IVS1.

The approximately 2.6-kilobase αLA transcripts were processed in vitro and analyzed by the RNAse T1 protection assay (Fig. 2A). The 590-nucleotide (nt) *Pst*I-*Xba* cDNA fragment from pαLA35 used as a probe protected all of exons 1 (164 nt), 2 (159 nt), and 3 (76 nt) and the 5′ portion of exon 4 (195 nt). Excision of IVS1 and ligation of exons 1 and 2 were first observed at 30 min resulting in the appearance of a 323-nt protected RNA fragment. IVS1 was removed from most pre-mRNA molecules by 2 h, although splicing of IVS1 continued slowly for at least 6 h, by which time IVS1 had been removed from 70% of the transcripts (Fig. 2B).

Splicing of IVS2 (producing a 399-nt protected fragment) was first observed at 1 h (Fig. 2A) and continued over the next 5 h. Most of the IVS2 splicing was completed at between 3 and 4 h; at 6 h, both IVS1 and IVS2 had been removed from approximately 40% of the RNA transcripts (Fig. 2B). Splicing of IVS2 occurred almost exclusively in transcripts from which IVS1 had already been removed. Removal of IVS2 before the excision of IVS1 would have resulted in the presence of a 237-nt protected RNA fragment; this fragment was not observed. However, by S1 nuclease analysis with a 2,275-nt probe ^{32}P-3′-end-labeled at the *Bam*HI site in exon 1 and extending to the *Eco*RI site in exon 4, we could detect cleavage at the 5′ splice site of IVS2 before IVS1 removal in a very small fraction (<1%) of transcripts (data not shown). We do not know whether these molecules were processed further in vitro.

The excision of IVS3, resulting in fully spliced αLA mRNA (a 594-nt protected fragment), was first observed at 2 h and continued over the course of the reaction. At 6 h, the final time point assayed, 7% of the RNA was fully processed. In addition, a very minor approximately 271-nt protected fragment that also appeared at 2 h probably represented transcripts from which IVS3 had been excised but IVS2 had not.

We observed no 240-, 359-, or 236-nt fragments resulting from exon skipping and ligation of exon 1 to exon 3, exon 2 to exon 4, or exon 1 to exon 4, respectively. To determine whether the RNase protection assay could detect such aberrantly spliced products, we hybridized uniformly ^{32}P-labeled β-globin mRNA, transcribed from pSP6βc, to an unlabeled 428-nt *Nco*I-*Bam*HI genomic β-globin DNA fragment containing exon 1, IVS1, and exon 2 and treated the hybrids with RNase T1 (Fig. 3). The IVS was not included in the mRNA. Therefore, the IVS segment of the DNA probe was analogous to a skipped exon; it could not hybridize to the mRNA and would thus form a loop in the probe-mRNA hybrid. If this loop resulted in the failure of the probe to protect the RNA surrounding the exon 1-exon 2 junction, RNase T1 would cleave the RNA at the two guanosines at that site. However, less than 10% of the RNA molecules was cleaved at this site by RNase T1 (Fig. 3). This finding demonstrates that the RNase T1 assay would detect approximately 90% of spliced RNA molecules in which exon skipping had occurred. RNA species comprising 2% or more of the total RNA would be readily detectable in the RNase T1 assay; however, we did not detect any apparent products



of exon skipping. Therefore, exon skipping did not occur in a significant fraction of αLA transcripts spliced in vitro.

In vitro splicing pathway of murine IL3 pre-mRNA. Murine IL3 transcripts were processed in vitro via a complex pathway. In most of the transcripts, IVS1 and IVS4 were removed early in the reaction, either simultaneously or in rapid succession. IVS2 was removed next, followed shortly by IVS3. Several other minor splicing pathways also apparently existed.

The approximately 2.2-kilobase IL3 transcripts were processed in vitro and analyzed by the RNase T1 protection assay (Fig. 4B). The 465-nt *Pst*I-*Xba*I cDNA fragment from pcD-MCGF used as a probe protected a 205-nt fragment of exon 1, all of exons 2 (42 nt), 3 (96 nt), and 4 (42 nt), and a 98-nt segment of exon 5. Therefore, exon 3 and exon 5 (96- and 98-nt fragments, respectively) were not resolved by the gel electrophoresis system, and the exon 2 and exon 4 fragments comigrated at 42 nt. Unexpectedly, we observed excision of two IVSs (IVS1 and IVS4) at approximately the same time. A 247-nt exon 1-exon 2 protected RNA fragment (Fig. 4), resulting from excision of IVS1, was first observed at 1 h after the start of the splicing reaction, accumulated rapidly until approximately 3 h, and then accumulated more slowly until IVS1 had been removed from 90% of the transcripts at 8 h (Fig. 4B). We also observed excision of another IVS at approximately the same time, although perhaps beginning slightly earlier than IVS1 excision. This IVS could not be identified from this experiment (Fig. 4A) because the excision of IVS2 alone, IVS3 alone, and IVS4 alone would each have resulted in protected fragments (exon 2-exon 3, exon 3-exon 4, or exon 4-exon 5) of approximately 140 nt. To differentiate among these possibilities, we analyzed spliced IL3 RNA by RNase T1 digestion with two cDNA half-probes (Fig. 5). The 5′ half-probe (Fig. 5A), a 185-nt *Hin*dIII-*Bam*HI cDNA fragment from pcD-MCGF, protected an 86-nt segment of exon 1, all of exon 2 (42 nt), and the 5′ portion of exon 3 (64 nt). Excision of IVS1 was first observed at 0.5 h, resulting in 128- and 134-nt exon 1-exon 2 protected fragments. Excision of IVS2, first detected at 1 h after the start of the splicing reaction, was represented by two RNA species; most of the RNA was in an exon 1-exon 2-exon 3 protected fragment (192 nt); only a small amount was in an exon 2-exon 3 (106 nt) protected fragment. Therefore, the 140-nt (exon 4-exon 5) fragment seen (Fig. 4) could not have resulted from the excision of IVS2 alone because IVS2 splicing followed the excision of IVS1 in most molecules. The 3′ half-probe (Fig. 5B), a 177-nt

FIG. 2. RNase T1 protection analyses of αLA pre-mRNA splicing. (A) Uniformly ^{32}P-labeled pSP6α-LA transcripts were processed in vitro, hybridized to a 594-nt *Pst*I-*Xba*I cDNA fragment, digested with RNase T1, and electrophoresed in a 4% denaturing polyacrylamide gel. Lanes: M, ^{32}P-3′-end-labeled *Msp*I-digested pBR322 as molecular size standards; 0 to 6, 0- to 6-h portions of the splicing reaction. Abbreviations: E1, E2, E3, and E4, exons 1, 2, 3, and 4, respectively; E1-E2, ligated exons 1 and 2; E1-E2-E3, ligated exons 1, 2, and 3; E1-E2-E3-E4 ligated exons 1, 2, 3, and 4; E3-E4, ligated exons 3 and 4; $T1_{(93)}$ and $T1_{(46)}$, complete RNase T1 fragments from IVS1 and IVS3, respectively. $T1_{(93)}$ contains 22 repeats of the sequence CCT, and $T1_{(46)}$ contains 13 repeats of the sequence ATT. (B) Scanning densitometry analysis of the autoradiograph illustrated above. Each point represents the percent total RNA (normalized for the number of uridines in the protected fragments) which has had the IVS excised. Symbols: ●, IVS1 excision from αLA transcripts; ○, IVS2 excision from αLA transcripts; ▲, IVS3 excision from αLA transcripts.

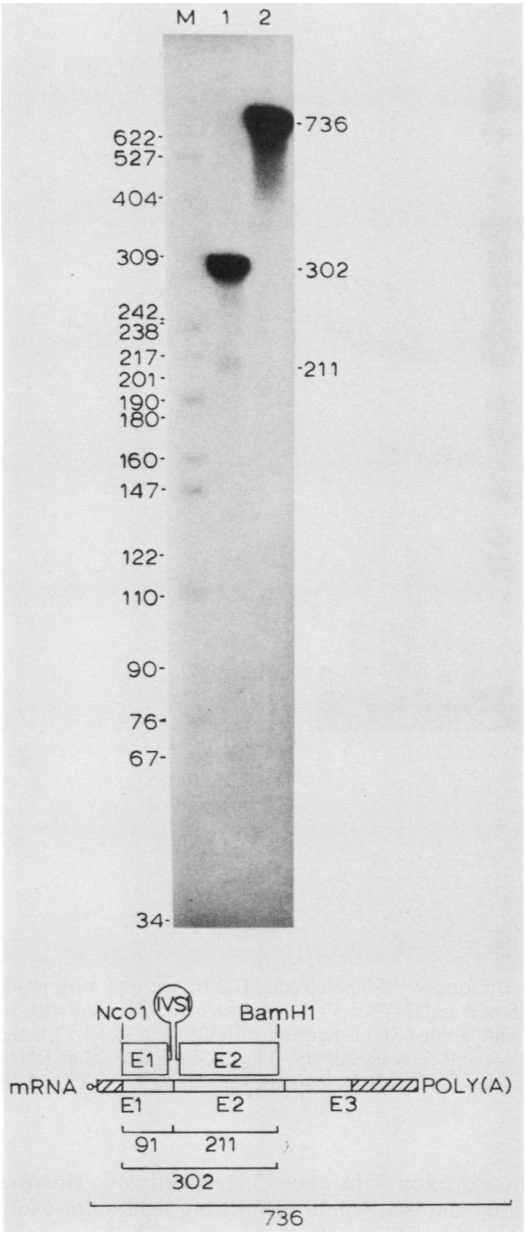


FIG. 3. RNase T1 cleavage of RNA-DNA hybrids at the site of single-stranded DNA loops. Uniformly ^{32}P-labeled β-globin mRNA transcripts of pSP6βc (12.5 ng) were hybridized to an unlabeled 428-nt *Nco*I-*Bam*HI human β-globin genomic DNA fragment, digested with RNase T1, and electrophoresed in a 4% denaturing polyacrylamide gel. Lanes: M, ^{32}P-end-labeled *Msp*I-digested pBR322; 1, RNase T1 digestion; 2, undigested β-globin mRNA. The 91-nt protected fragment was visible on a longer exposure of this gel. Abbreviations: E1, E2, and E3, exons 1, 2, and 3, respectively.

*Bam*HI-*Xba*I cDNA fragment from pcD-MCGF, protected the 3′ portion of exon 3 (38 nt), all of exon 4 (42 nt), and the 5′ portion of exon 5 (97 nt). The excision of IVS4 was first observed at 0.5 h, resulting in 139- and 149-nt exon 4-exon 5 protected fragments. The excision of IVS3 alone, producing an 80-nt exon 3-exon 4 protected fragment, was not observed at all, and exon 3-exon 4-exon 5 protected fragments (177 and 188 nt), were not observed until 1 h after the start of the

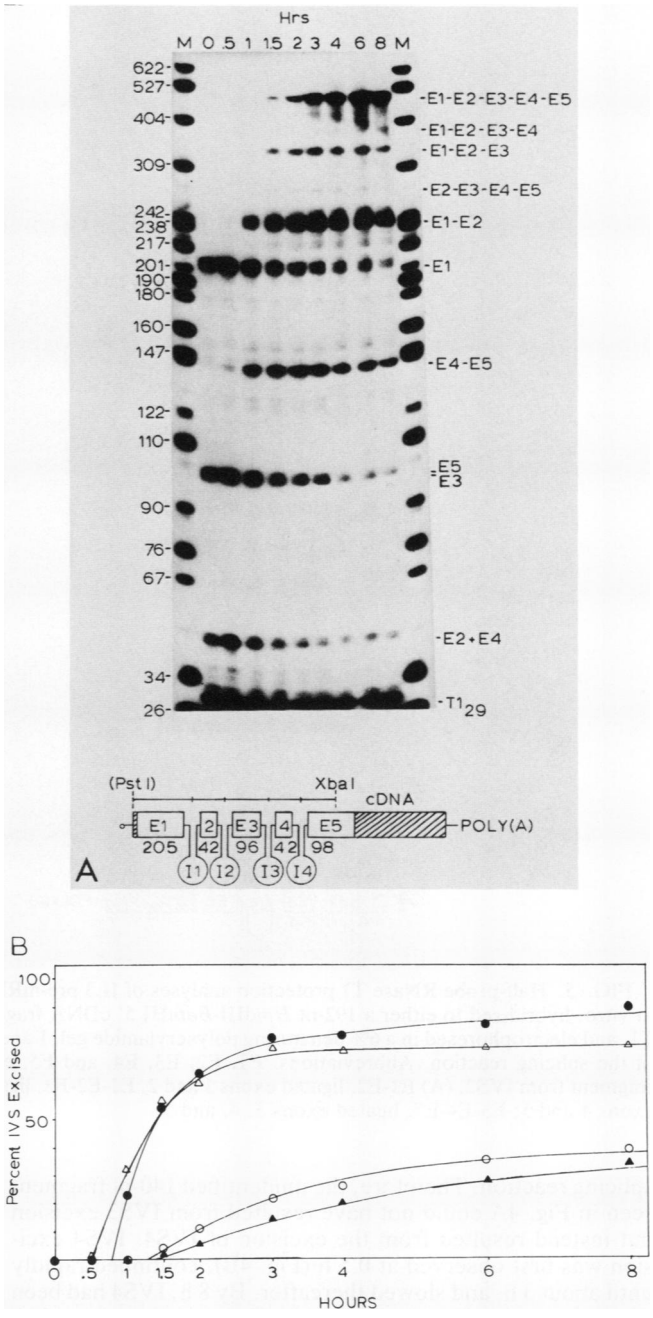


FIG. 4. RNase T1 protection analyses of IL3 pre-mRNA splicing. (A) Uniformly ^{32}P-labeled pSP6IL3 transcripts were processed in vitro, hybridized to a 483-nt *Pst*I-*Xba*I cDNA fragment, digested with RNase T1, and electrophoresed in a 4% denaturing polyacrylamide gel. Lanes: M, ^{32}P-3′-end-labeled *Msp*I-digested pBR322; 0 to 8, 0- to 8-h aliquots of the splicing reaction. Abbreviations: E1, E2, E3, E4, and E5, exons 1, 2, 3, 4, and 5, respectively; E1-E2, ligated exons 1 and 2; E4-E5, ligated exons 4 and 5, E1-E2-E3, ligated exons 1, 2, and 3; E1-E2-E3-E4 ligated exons 1, 2, 3, and 4; E1-E2-E3-E4-E5, ligated exons 1, 2, 3, 4, and 5; E2-E3-E4-E5, ligated exons 2, 3, 4, and 5; E1-E2-E3-E4, ligated exons 1, 2, 3, and 4; $T1_{29}$, a complete 29-nt RNase T1 fragment from IVS2. (B) Scanning densitometry analysis of the autoradiograph illustrated above. Each point represents the percent total RNA (normalized for the number of uridines in the protected fragments) which has had the IVS excised. Symbols: ●, IVS1 excision from IL3 transcripts; ○, IVS2 excision from IL3 transcripts; ▲, IVS3 excision from IL3 transcripts; △, IVS4 excision from IL3 transcripts.

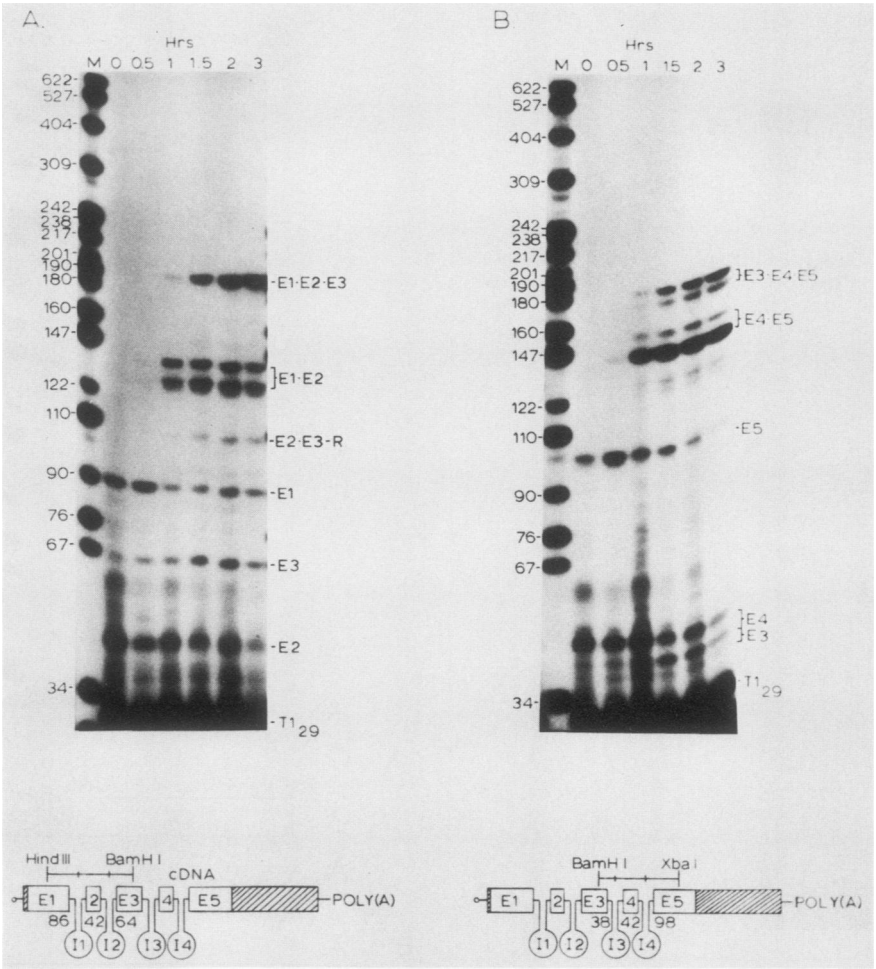


FIG. 5. Half-probe RNase T1 protection analyses of IL3 pre-mRNA splicing. Uniformly ^{32}P-labeled pSP6IL3 transcripts were processed in vitro, hybridized to either a 192-nt *Hin*dIII-*Bam*HI 5′ cDNA fragment or a 178-nt *Bam*HI-*Xba*I 3′ cDNA fragment, digested with RNase T1, and electrophoresed in a 6% denaturing polyacrylamide gel. Lanes: M, ^{32}P-3′-end-labeled *Msp*I-digested pBR322; 0 to 3, 0- to 3-h aliquots of the splicing reaction. Abbreviations: E1, E2, E3, E4, and E5, exons 1, 2, 3, 4, and 5, respectively; $T1_{29}$, a complete 29-nt RNase T1 fragment from IVS2. (A) E1-E2, ligated exons 1 and 2; E1-E2-E3, ligated exons 1, 2, and 3; E2-E3-R, ligated exons 2 and 3. (B) E4-E5, ligated exons 4 and 5; E3-E4-E5, ligated exons 3, 4, and 5.

splicing reaction. Therefore, the unidentified 140-nt fragment seen in Fig. 4A could not have resulted from IVS3 excision but instead resulted from the excision of IVS4. IVS4 excision was first observed at 0.5 h (Fig. 4B), continued rapidly until about 3 h, and slowed thereafter. By 8 h, IVS4 had been removed from 75% of IL3 transcripts. These data thus demonstrate that IVS1 and IVS4 are removed from IL3 pre-mRNA in vitro at approximately the same time, beginning 0.5 h after the start of the splicing reaction.

The excision of IVS2, producing a 343-nt exon 1-exon 2-exon 3 fragment (Fig. 4A), was first observed at 1.0 h and continued until at least 8 h after the start of the splicing reaction, at which time IVS2 had been removed from approximately 40% of the transcripts. IVS3 excision (fully spliced mRNA, 483 nt) was first observed at 1.5 h (Fig. 4A), when 1% of transcripts were fully spliced (Fig. 4B), and at 8 h, approximately 35% of the RNA was fully spliced.

There apparently was not a significant amount of exon skipping during splicing of IL3 transcripts in vitro. We observed no 301-, 303-, 84-, 140-, or 194-nt fragments that would have resulted from exon skipping and ligation of exon 1 to exon 3, exon 1 to exon 5, exon 2 to exon 4, exon 2 to exon 5, and exon 3 to exon 5, respectively. However, a 247-nt fragment that would result from ligation of exon 1 to exon 4 could not be resolved from the 247-nt fragment that resulted from the appropriate ligation of exon 1 to exon 2.

In most transcripts, IVS1 and IVS4 appeared to be removed simultaneously in vitro. However, S1 nuclease analyses demonstrated that in one small fraction of transcripts, IVS1 was removed first and that in another small fraction, IVS4 was removed first. To analyze cleavage at 5′ splice sites, we used a 1,813-nt probe 3′ end-labeled at the *Hin*dIII site in exon 1 and extending to the *Nco*I site in exon 5 (Fig. 6). By 30 min after the start of the splicing reaction, we observed fragments protected by transcripts cleaved only at the 5′ splice sites of IVS1 (85 nt), IVS4 (1,488 nt), and (to a much lesser extent) IVS3 (1,311 nt). The 85-nt fragment protected by transcripts cleaved at the IVS1 5′ splice site continued to accumulate over 3 h. The 223-nt fragment protected by transcripts cleaved only at the IVS2 5′ splice site (i.e., without cleavage at IVS1 splice sites), a very minor component of the RNA, was first observed at 1 h and accumulated slowly. The 1,311-nt fragment protected by transcripts cleaved only at the 5′ splice site of IVS3 (but not

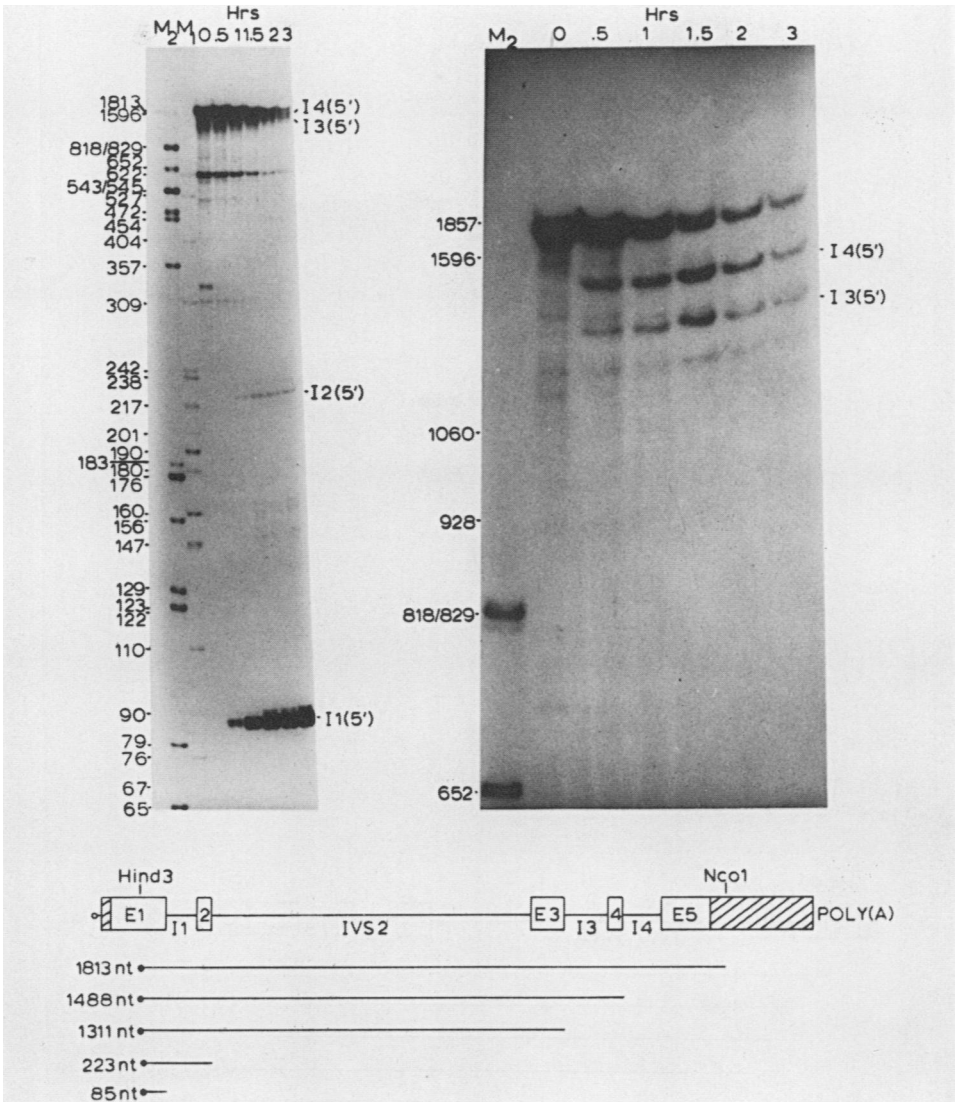


FIG. 6. S1 nuclease analysis of cleavage at the 5′ splice sites of IL3 pre-mRNA. pSP6IL3 transcripts were processed in vitro, hybridized to a ^{32}P-3′-end-labeled 1,813-nt *Hin*dIII-*Nco*I DNA fragment, digested with S1 nuclease, and electrophoresed in a 4% denaturing polyacrylamide gel. The gel in the left panel was electrophoresed for 1.5 h, and the gel in the right panel was electrophoresed for 6 h. Lanes: M_1, ^{32}P-3′-end-labeled *Msp*I-digested pBR322; M_2, ^{32}P-3′-end-labeled *Msp*I-digested M13mp2; 0 to 3, 0- to 3-h aliquots of the splicing reaction. Abbreviations: I1(5′), cleavage at the 5′ splice site of IVS1 (85 nt); I2(5′), cleavage at the 5′ splice site of IVS2 (223 nt); I3(5′), cleavage at the 5′ splice site of IVS3 (1,311 nt); I4(5′), cleavage at the 5′ splice site of IVS4 (1,488 nt); 1,813 nt, full-length probe. Symbols: ○, the cap structure; ●, the labeled end of the probe.

within IVS1 or IVS2) was first observed at 0.5 h, accumulated until 1.5 h, and then slowly disappeared. The 1,488-nt fragment, protected by transcripts cleaved only at the 5′ splice site of IVS 4, generally followed the same pattern as the 1,311-nt fragment described above, but was present in slightly larger amounts. The amount of full-length probe (1,813 nt), protected by uncleaved pre-mRNA, decreased over time.

To analyze cleavage at both the 5′ and 3′ splice sites of processed IL3 RNA, we used as an S1 probe the same 1,813-nt *Hin*dIII-*Nco*I 5′ fragment described above, but it was 5′ end-labeled at the *Nco*I site in exon 5 (Fig. 7). The 325-nt fragment protected by transcripts cleaved at the 5′ splice site (but not the 3′ splice site) of IVS4 was first observed at 0.5 h, accumulated until 1 h, and then gradually disappeared. Cleavage at the 3′ splice site of IVS4 (resulting in a 203-nt protected fragment) was first observed at 1 h after the start of the splicing reaction, and the amount of this fragment increased substantially over the next 2 h. A very small amount of a 502-nt fragment, protected by transcripts cleaved at the 5′ splice site of IVS3 (without cleavage at IVS4 splice sites or at the IVS3 5′ splice site), was also observed at 0.5 h. The amount of this fragment was greatest at 1 h, and it disappeared by 2 h. A 367-nt fragment, protected by transcripts cleaved at the 3′ splice site of IVS3 (without prior cleavage at IVS4 splice sites), was first seen at 1 h and subsequently gradually disappeared. A very small amount of a 598-nt fragment, protected by transcripts cleaved at the 3′ splice site of IVS2 (without prior cleavage in IVS3 or IVS4), was seen at 1 h, and it remained constant for the next 2 h. A small amount of a 1,632-nt fragment, protected by transcripts cleaved only at the 3′ splice site of

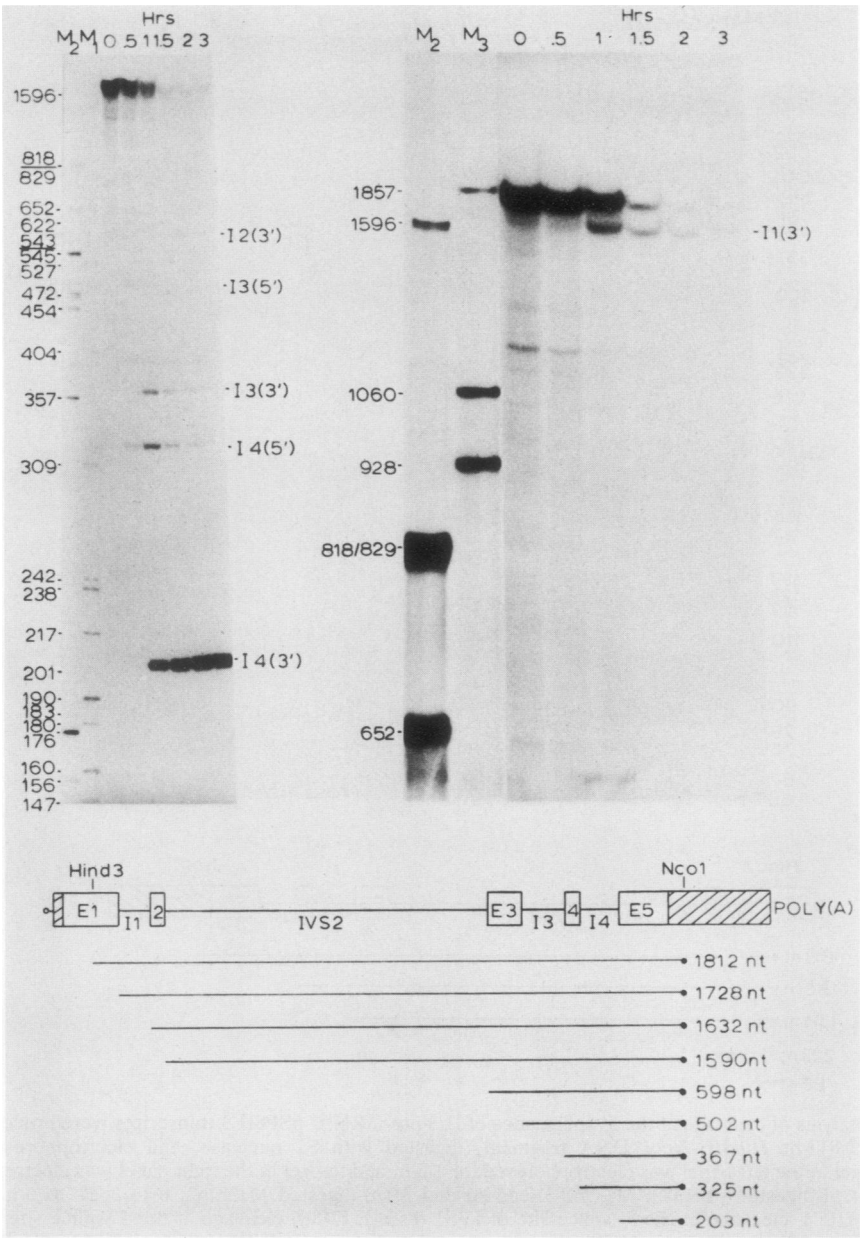


FIG. 7. S1 nuclease analysis of cleavage at the 5′ and 3′ splice sites of IL3 pre-mRNA. pSP6IL3 transcripts were processed in vitro, hybridized to a ^{32}P-5′-end-labeled 1,812-nt *Hin*dIII-*Nco*I DNA fragment, digested with S1 nuclease, and electrophoresed in a 4% denaturing polyacrylamide gel. The gel in the left panel was electrophoresed for 2 h and the one in the right panel was electrophoresed for 6 h. Lanes: M_1, ^{32}P-3′-end-labeled *Msp*I-digested pBR322; M_2, ^{32}P-3′-end-labeled *Msp*I-digested M13mp2; M_3, ^{32}P-3′-end-labeled *Bst*NI-digested pBR322 as size markers; 0 to 3, 0- to 3-h aliquots of the splicing reaction. Abbreviations: I4(3′), cleavage at the 3′ splice site of IVS4 (203 nt); I4(5′), cleavage at the 5′ splice site of IVS4 (325 nt); I3(3′), cleavage at the 3′ splice site of IVS3 (367 nt); I3(5′), cleavage at the 5′ splice site of IVS3 (502 nt); I2(3′), cleavage at the 3′ splice site of IVS2 (598 nt); 1,590 nt, unobserved cleavage at the 5′ splice site of IVS2; I1(3′), cleavage at the 3′ splice site of IVS1 (1,632 nt); 1,728 nt, unobserved cleavage at the 5′ splice site of IVS1; 1,812 nt, full-length probe. Symbols: ○, the cap structure; ●, the labeled end of the probe.

IVS1, was detected at 1 h and gradually disappeared over the 3-h time course. We were unable to detect a 1,728-nt fragment protected by transcripts cleaved only at the IVS1 5′ splice site. However, by S1 analysis with a 210-nt *Hin*dIII-*Ava*II probe ^{32}P-5′-end-labeled in exon 2 (data not shown), we demonstrated that cleavage at the 5′ splice site of IVS1 does precede cleavage at the IVS1 3′ splice site.

Two other very minor (<1% of total RNA) intermediates were also detected by the RNase T1 protection analyses (Fig. 5): a 278-nt exon 2-exon 3-exon 4-exon 5 fragment and a 385-nt exon 1-exon 2-exon 3-exon 4 fragment. These fragments might represent either intermediates in minor splicing pathways or terminal nonproductive reaction products. Because their component parts and final reaction products were represented in other RNA species, it was not possible to distinguish between these two explanations.

It is also likely that in another small fraction of transcripts (<1% of total RNA), IVS2 was excised first, and in yet

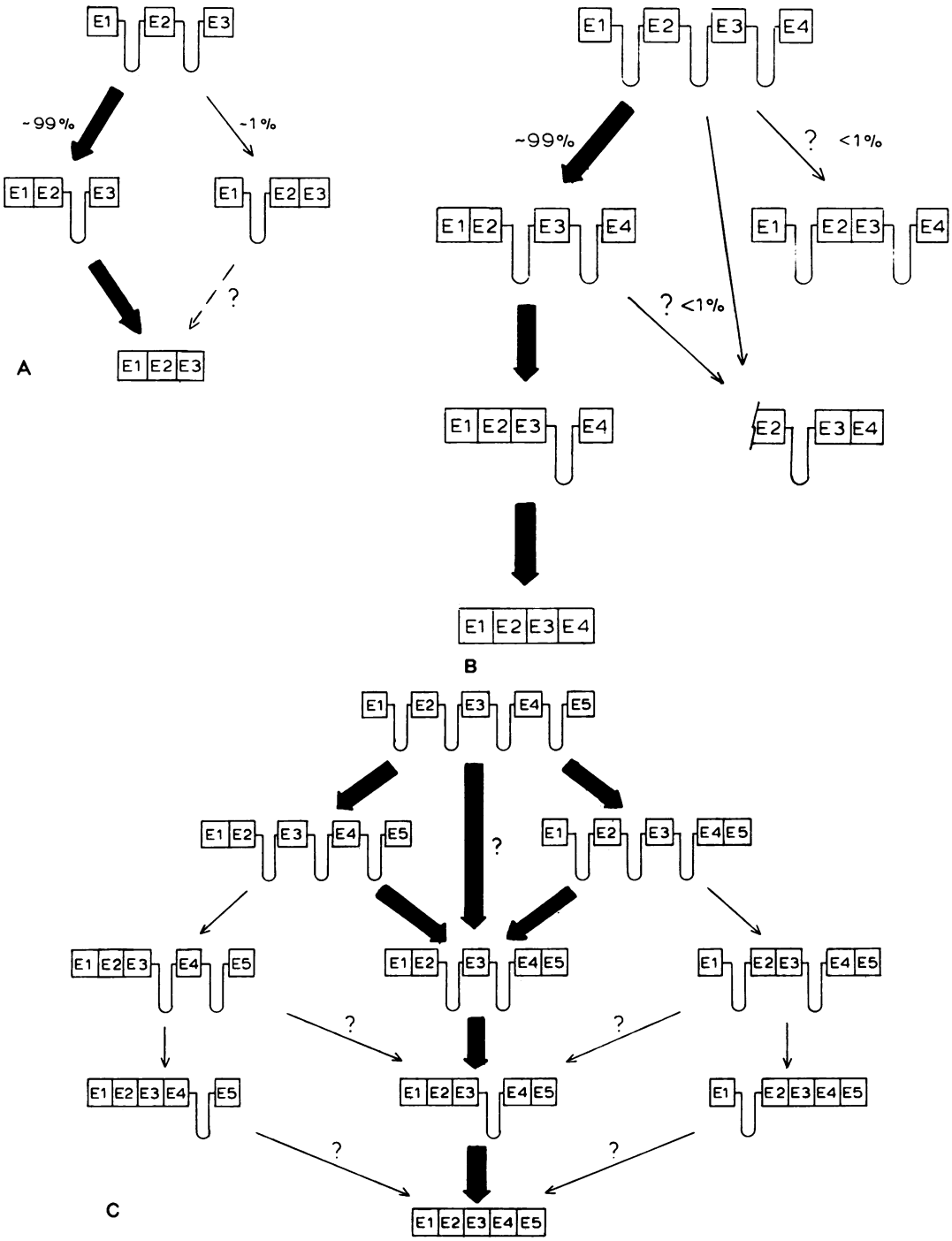


FIG. 8. In vitro splicing pathways of three multi-IVS pre-mRNAs. (A) Human β-globin pre-mRNA (19); (B) Rat αLA pre-mRNA; (C) Murine IL3 pre-mRNA. Abbreviations: E1, E2, E3, E4, and E5, exons 1, 2, 3, 4, and 5, respectively. Symbols: □, exons; ∪ IVSs; ➡, major pathways; →, minor pathways; and ?, possible but unproven pathways.

another small fraction, IVS3 was removed first. As described above, fragments representing these minor splicing intermediates (exon 2-exon 3 and exon 3-exon 4 fragments) would have comigrated with (and thus not be distinguishable from) the 140-nt exon 4-exon 5 RNase T1-resistant fragment seen in Fig. 4A. The existence of these minor splicing intermediates was supported by the detection of a minor exon 2-exon 3 fragment (106 nt) at 1 h in the 5′ cDNA half-probe experiment (Fig. 5A), in which the excision of IVS2 was not masked by the excision of IVS4. In addition, S1 nuclease analysis using a probe 3′-end-labeled in exon 1 (Fig. 6) demonstrated that a small amount of transcripts cleaved at the 5′ splice site of IVS3 (but not at the IVS1 or IVS2 splice sites) at 0.5 h and also some transcripts cleaved at the 5′ splice site of IVS2 (but not at the IVS1 splice sites) at 1 h. Cleavages at the 3′ splice sites of both IVS2 and IVS3

were observed at 1 h with an S1 probe 5'-end-labeled in exon 5 (Fig. 7). Together, these observations indicated that IVS2 and IVS3 were each excised first from a small fraction of IL3 transcripts.

DISCUSSION

To determine whether pre-mRNAs with multiple IVSs are processed by preferred pathways, we analyzed the in vitro splicing pathways of four such pre-mRNAs: human β- and γ-globin pre-mRNAs (each containing two IVSs; 19), rat αLA pre-mRNA (containing three IVSs), and murine IL3 pre-mRNA (containing four IVSs). In each case, the pre-mRNA is processed in vitro via a highly preferred, although not necessarily exclusive, pathway.

These pathways are summarized in Fig. 8. The two IVSs of human β- and γ-globin pre-mRNAs were excised sequentially, IVS1 before IVS2 (Fig. 8A) (19). Similarly, the three IVSs of rat αLA pre-mRNA were also excised sequentially, IVS1 first, then IVS2, and finally IVS3 (Fig. 8B). However, the processing of murine IL3 pre-mRNA (containing four IVSs) in vitro followed a more complex pathway (Fig. 8C). IVS1 and IVS4 apparently were removed at approximately the same time, followed by IVS2 and finally by IVS3. IVS1 and IVS4 might be removed simultaneously, in rapid succession (IVS4 preceding IVS1 or vice versa), or both. S1 nuclease analyses demonstrated that IVS1 can be removed from transcripts containing IVS4 and vice versa. However, splicing of IVS1 and IVS4 occurs so rapidly that it is impossible to distinguish between simultaneous and successive excision of IVS1 and IVS4 in the majority of molecules. For all three of the pre-mRNAs studied, we also observed a number of minor (<1% of total RNA) splicing products that might either be bona fide intermediates in minor splicing pathways or might be dead-end splicing products that are not processed to mRNA. In no case did we ever detect exon skipping in vitro. Therefore, our observations demonstrate that there are highly preferred, but not necessarily obligatory, pathways of IVS removal from multi-IVS pre-mRNAs in vitro. The virtually simultaneous excision of IL3 IVS1 and IVS4 in vitro suggests that more than one spliceosome can assemble on that pre-mRNA molecule, consistent with the observation of double splicing complexes during splicing of the two IVS adenovirus type 2 major late pre-mRNAs in vitro (4a).

Analyses of the kinetics of the splicing reactions suggest that the preferred splicing pathways that we observed resulted from two variables for each IVS: the lag time before IVS excision begins and the maximum excision rate. We have previously found (19) that for human β-globin pre-mRNA, the lag time for IVS1 (130 nt) excision in vitro is approximately 0.25 to 0.5 h; the lag time for IVS2 (850 nt) excision is approximately 0.75 to 1 h; and the maximum rate of IVS1 excision is approximately three times that of IVS2. In the present study, we found that for rat αLA pre-mRNA, the lag time for IVS1 (341 nt) excision in vitro was approximately 0.5 h; for IVS2 (429 nt), it was 1 h; and for IVS3 (1,016 nt), it was 2 h. The maximum rate of IVS1 excision was 3 times that of IVS2 and was 30 times that of IVS3. For murine IL3 pre-mRNA, the lag times for excision of IVS1 (96 nt) and IVS4 (122 nt) were approximately 0.5 h, and those for IVS2 (992 nt) and IVS3 (135 nt) were both approximately 1.25 h. The maximum rates of IVS1 and IVS4 excision were the same and were approximately 4.5 times that of IVS2 and 7 times that of IVS3. The lag time tended to correlate inversely with the maximum excision rate, but we had insufficient data to determine whether this correlation was significant. Furthermore, neither the lag time nor the maximum excision rate related simply to the length of the IVS, the length of either the 5' or 3' exons, or the position of the IVS in the transcript. We have previously shown that at least the maximum excision rate of an IVS in vitro can be greatly affected by the presence or absence of other IVSs in the pre-mRNA (19). In addition, the position of the IVS in the transcript can greatly affect its ability to be spliced in vivo (12). Therefore, it seems likely that both the lag time and the maximum excision rate of an IVS are complex functions of splice site accessibility, (related to secondary and tertiary structures in the pre-mRNA), affinity of the splice sites for nuclear splicing factors, and perhaps IVS size.

Proposed models of splice site recognition must be reconsidered in light of recent data on splicing pathways of multi-IVS pre-mRNAs. In simple directional scanning models, splice site recognition initiates at one end of the transcript and proceeds linearly along the pre-mRNA (17, 30). However, scanning models are inconsistent with the results of experiments in which splice sites were duplicated (16), with the differences between the pathways of adenovirus type 2 major late transcripts (IVSs removed sequentially from 5' to 3') and E2A transcripts (IVSs removed from 3' to 5)' in vivo (8, 14) and with the in vitro pathway of IL3 pre-mRNA splicing described here. Aebi et al. (1) have proposed a different type of directional (first come, first served) model in which the splice sites of each IVS are recognized and committed in the nascent transcript, thus preventing subsequent interactions between inappropriate pairs of splice sites (exon skipping). This proposal is consistent with the observation of ribonucleoprotein particles near intron-exon junctions in nascent transcripts in vivo (25). The first come, first served model might be modified to accommodate differential rates of splice site recognition on the nascent transcript (instead of immediate recognition of splice sites). Differential rates of splice site recognition might result from different affinities of splice sites for splicing factors, different availabilities of splice sites as a result of the secondary and tertiary structure of the nascent transcript (which may change as IVSs are excised), and possibly steric effects that might prevent simultaneous assembly of neighboring spliceosomes within a certain distance. This modified scheme would still tend to suppress exon skipping in vivo but could accommodate preferred, but not strictly directional, orders of IVS excision and even alternative splicing. Models linking splice site recognition and transcription are therefore attractive. However, it is difficult to reconcile such models with rare (1) or absent (4a, 19) exon skipping when multi-IVS pre-mRNAs are spliced in vitro. Furthermore, mutations at 5' splice sites of distal IVSs can cause exon skipping in vivo (21, 31), whereas by the first come, first served model, the splice sites of the promoter-proximal IVS should already be committed and exon skipping should not occur.

It will ultimately be necessary to determine whether the splicing pathways that we have defined in vitro accurately reflect the processing pathways of these pre-mRNAs in vivo. The splicing pathway of normal rabbit β-globin pre-mRNA in vivo is the same as that which we observed for normal human β- and γ-globin pre-mRNA in vitro (19). Similarly, the normal preferred splicing pathway of the leader of the adenovirus type 2 major late transcript appears to be the same in vivo (14) and in vitro (4a). However, some splice site mutations can result in different patterns of aberrant splicing

in vitro versus in vivo (1). Therefore, we are currently studying the splicing pathways of the αLA and IL3 pre-mRNAs in vivo.

ACKNOWLEDGMENTS

We thank P. Qasba for the αLA plasmids pαLA2.5, pαLA0.9, pαLA1.1, and pαLA35; F. Lee for the IL3 plasmids pcD-MCGF and pλMGM-12; J. Ross for S1 nuclease; and R. Rueckert for HeLa cells.

This work was supported by grant DMB-8505902 from the National Science Foundation.

LITERATURE CITED

1. **Aebi, M., H. Hornig, R. A. Padgett, J. Reiser, and C. Weissmann.** 1986. Sequence requirements for splicing of higher eukaryotic nuclear pre-mRNA. Cell **46**:555–565.
2. **Aviv, H., and P. Leder.** 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci. USA **69**:1408–1412.
3. **Berk, A. J., and P. A. Sharp.** 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. Cell **12**:721–732.
4. **Bovenberg, R. A. L., W. P. M. van de Meerendonk, P. D. Baas, P. H. Steenbergh, C. J. M. Lips, and H. S. Jansz.** 1986. Model for alternative RNA processing in human calcitonin gene expression. Nucleic Acids Res. **14**:8785–8803.

4a. **Christofori, G., D. Frendewey, and W. Keller.** 1987. Two spliceosomes can form simultaneously and independently on synthetic double-intron messenger RNA precursors. EMBO J. **6**:1747–1755.

5. **Dandekar, A. M., and P. K. Qasba.** 1981. Rat α-lactalbumin has a 17-residue-long COOH-terminal hydrophobic extension as judged by sequence analysis of the cDNA clones. Proc. Natl. Acad. Sci. USA **78**:4853–4857.
6. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11**:1475–1489.
7. **Donaldson, D. S., A. R. McNab, G. Rovera, and P. J. Curtis.** 1982. Nuclear precursor molecules of the two β-globin mRNAs in Friend erythroleukemia cells. J. Biol. Chem. **257**:8655–8660.
8. **Gattoni, R., P. Keohavong, and J. Stévenin.** 1986. Splicing of the E2A premessenger RNA of adenovirus serotype 2: multiple pathways in spite of excision of the entire large intron. J. Mol. Biol. **187**:379–397.
9. **Goldenberg, C. J., and H. J. Raskas.** 1979. Splicing patterns of nuclear precursors to the mRNA for adenovirus 2 DNA binding protein. Cell **16**:131–138.
10. **Green, M. R.** 1986. Pre-mRNA splicing. Annu. Rev. Genet. **20**:671–708.
11. **Green, M. R., T. Maniatis, and D. A. Melton.** 1983. Human β-globin pre-mRNA synthesized *in vitro* is accurately spliced in *Xenopus* oocyte nuclei. Cell **32**:681–694.
12. **Greenspan, D. S., and S. M. Weissman.** 1985. Synthesis of predominantly unspliced cytoplasmic RNAs by chimeric herpes simplex virus type 1 thymidine kinase-human β-globin genes. Mol. Cell. Biol. **5**:1894–1900.
13. **Hatzoglou, M., C. E. Sekeris, and R. W. Hanson.** 1985. Processing of phospho*enol*pyruvate carboxykinase (GTP) RNA *in vivo*. Proc. Natl. Acad. Sci. USA **82**:4346–4350.
14. **Keohavong, P., R. Gattoni, J. M. LeMoullec. M. Jacob, and J. Stévenin.** 1982. The orderly splicing of the first three leaders of the adenovirus-2 major late transcript. Nucleic Acids Res. **10**:1215–1229.
15. **Krainer, A. R., T. Maniatis, B. Ruskin, and M. R. Green.** 1984. Normal and mutant human β-globin pre-mRNAs are faithfully and efficiently spliced *in vitro*. Cell **36**:993–1005.
16. **Kühne, T., B. Wieringa, J. Reiser, and C. Weissmann.** 1983. Evidence against a scanning model of RNA splicing. EMBO J. **2**:727–733.
17. **Lang, K. M., and R. A. Spritz.** 1983. RNA splice site selection: evidence for a 5′→3′ scanning model. Science **220**:1351–1355.
18. **Lang, K. M., and R. A. Spritz.** 1985. Cloning specific complete polyadenylylated 3′-terminal cDNA segments. Gene **33**:191–196.
19. **Lang, K. M., V. L. van Santen, and R. A. Spritz.** 1985. The two intervening sequences of human β- and γ-globin pre-mRNAs are excised in a preferred temporal order *in vitro*. EMBO J. **4**:1991–1996.
20. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12**:7035–7056.
21. **Mitchell, P. J., G. Urlaub, and L. Chasin.** 1986. Spontaneous splicing mutations at the dihydrofolate reductase locus in Chinese hamster ovary cells. Mol. Cell. Biol. **6**:1926–1935.
22. **Miyatake, S., T. Yokota, F. Lee, and K.-I. Arai.** 1985. Structure of the chromosomal gene for murine interleukin 3. Proc. Natl. Acad. Sci. USA **82**:316–320.
23. **Nelson, K. J., J. Haimovich, and R. P. Perry.** 1983. Characterization of productive and sterile transcripts from the immunoglobulin heavy-chain locus: processing of μ_m and μ_s mRNA. Mol. Cell. Biol. **3**:1317–1332.
24. **Noteborn, M., A. Arnberg, M. de Jonge, A. B. Geert, and M. Gruber.** 1986. Splicing pathways of the chicken apo very low density lipoprotein II (pre)messenger RNA. FEBS Lett. **194**:151–156.
25. **Osheim, Y. N., O. L. Miller, and A. L. Beyer.** 1985. RNP particles at splice junction sequences on Drosophila chorion transcripts. Cell **443**:143–151.
26. **Padgett, R. A., P. J. Grabowski, M. M. Konarska, S. Seiler, and P. A. Sharp.** 1986. Splicing of messenger RNA precursors. Annu. Rev. Biochem. **55**:1119–1150.
27. **Padgett, R. A., S. F. Hardy, and P. A. Sharp.** 1983. Splicing of adenovirus RNA in a cell-free transcription system. Proc. Natl. Acad. Sci. USA **80**:5230–5234.
28. **Qasba, P. K., and S. K. Safaya.** 1984. Similarity of the nucleotide sequences of rat α-lactalbumin and chicken lysozyme genes. Nature (London) **308**:377–380.
29. **Ryffel, G. U., T. Wyler, D. B. Muellener, and R. Weber.** 1980. Identification, organization and processing intermediates of the putative precursors of *Xenopus* vitellogenin messenger RNA. Cell **19**:53–61.
30. **Sharp, P. A.** 1981. Speculations on RNA splicing. Cell **23**:643–646.
31. **Treisman, R., S. H. Orkin, and T. Maniatis.** 1983. Specific transcription and RNA splicing defects in five cloned β-thalassaemia genes. Nature (London) **302**:591–596.
32. **Tsai, M.-J., A. C. Ting, J. L. Nordstrom, W. Zimmer, and B. W. O'Malley.** 1980. Processing of high molecular weight ovalbumin and ovomucoid precursor RNAs to messenger RNA. Cell **22**:219–230.
33. **Weaver, R. F., and C. Weissmann.** 1979. Mapping of RNA by a modification of the Berk-Sharp procedure: the 5′ termini of 15S β-globin mRNA precursor and mature 10S β-globin mRNA have identical map coordinates. Nucleic Acids Res. **7**:1175–1193.
34. **Yokota, T., F. Lee, D. Rennick, C. Hall, N. Arai, T. Mosmann, G. Nabel, H. Cantor, and K.-I. Arai.** 1984. Isolation and characterization of a mouse cDNA clone that expresses mast-cell growth-factor activity in mònkey cells. Proc. Natl. Acad. Sci. USA **81**:1070–1074.
35. **Zeitlin, S., and A. Efstratiadis.** 1984. *In vivo* splicing products of the rabbit β-globin pre-mRNA. Cell **39**:589–602.