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Order of Intron Removal during Splicing of Endogenous Adenine Phosphoribosyltransferase and Dihydrofolate Reductase Pre-mRNA

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Using a strategy based on reverse transcription and the polymerase chain reaction, we have determined the order of splicing of the four introns of the endogenous adenine phosphoribosyltransferase (*aprt*) gene in Chinese hamster ovary cells. The method involves a pairwise comparison of molecules that retain one intron and have either retained or spliced another intron(s). A highly preferred order of removal was found: intron 3 > 2 > 4 = 1. This order did not represent a linear progression from one end of the transcript to the other, nor did it correlate with the conformity of the splice site sequences to the consensus sequences or to the calculated energy of duplex formation with U1 small nuclear RNA. By using actinomycin D to inhibit RNA synthesis, the in vivo rate of the first step in splicing was estimated for all four introns; a half-life of 6 min was found for introns 2, 3, and 4. Intron 1 was spliced more slowly, with a 12-min half-life. A substantial amount of RNA that retained intron 1 as the sole intron was exported to the cytoplasm. In the course of these experiments, we also determined that intron 3, but not intron 4, is spliced before 3′-end formation is complete, probably on nascent transcripts. This result is consistent with the idea that polyadenylation is required for splicing of the 3′-most intron. We applied a similar strategy to determine the last intron to be spliced in a very large transcript, that of the endogenous dihydrofolate reductase (*dhfr*) gene in Chinese hamster ovary cells (25 kb). Here again, intron 1 was the last intron to be spliced.

Most mammalian genes contain multiple introns. Although great progress has been made in determining the chemistry of splicing, it is still not well understood how the splicing machinery selects the correct pairs of splice sites and how a multi-intron pre-mRNA is accurately spliced without deleting exons. The recognition of correct splice sites could involve specific sequences, preferred higher-order RNA structures, or both. The relative homology of the 5′ and 3′ splice sites to the consensus sequences is critical but insufficient to specify a splice site; many sequences that conform closely to the consensus sequence are not selected (46, 52).

Several models have been proposed to help explain splice site selection. In the scanning model, splice sites might be recognized by a linear scanning process initiating at one end of the pre-mRNA. However, the experimental predictions of this model have not been borne out (26, 50). In the first-come-first-served model, spliceosome assembly may initiate on the nascent transcript as soon as the necessary RNA sequences have been synthesized, with splicing taking place at some later time (2). In the exon definition model, exons are somehow recognized as units of splicing during assembly of the splicing machinery by factors that first bind to the 5′ and 3′ ends (51).

Another mechanism that could contribute to splice site selection is the order of splicing: removal of one intron may facilitate the subsequent splicing of another intron, for example, through a change in secondary structure or the juxtaposition of a protein binding site. For example, such sequential splicing has been invoked to explain the alternative splicing of rat preprotachykinin transcripts (38).

In order to address this idea, it is first necessary to determine if the constitutive splicing of multi-intron transcripts in general occurs in a defined order. Splicing intermediates for most genes exist in very small amounts and so have been difficult to detect, especially for genes specifying nonabundant products such as most housekeeping genes. Thus, only a limited number of mammalian genes have been directly tested in vivo for the order of splicing (19, 21, 27, 39, 44, 53, 55, 60, 61).

The technique of combining reverse transcription with PCR amplification (RT-PCR) provides adequate sensitivity for the detection of low levels of splicing intermediates. In this study, we present a general strategy for using RT-PCR in conjunction with appropriate combinations of intron and exon primers to determine the order of splicing of any small pre-mRNA in vivo. Using this strategy, we have examined the order of splicing of the introns of adenine phosphoribosyltransferase (*aprt*) pre-mRNA in Chinese hamster ovary (CHO) cells. The primary transcript of this gene contains four introns, one of which contains an unconventional GC dinucleotide at its 5′ end (14). The 2-kb size of the transcript facilitates the detection of splicing intermediates by RT-PCR. We found a highly preferred splicing pathway for the four introns. We were also able to measure the in vivo splicing rates for each of the four introns and to ask whether splicing of particular introns is dependent on 3′-end formation.

By a modification of this approach, we were able to determine which intron was spliced last in the much larger (25-kb) transcript of the dihydrofolate reductase (*dhfr*) gene. Our finding that intron 1 is the last to be spliced has implications for a model (57) of how nonsense mutations affect *dhfr* RNA processing.

MATERIALS AND METHODS

Cell culture. To study *aprt* transcripts, we used the D422 line of CHO cells; these cells are hemizygous for the *aprt*

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gene and were kindly supplied by Mark Meuth (5). For the *dhfr* experiments, we used MK42, an amplified CHO cell line having approximately 150 copies of the *dhfr* gene (45).

RNA analysis. Total RNA was extracted from exponentially growing D422 or MK42 CHO cells by a guanidinium thiocyanate method (20). The RNA was treated with DNase and analyzed for different types of splicing intermediates by RT-PCR. DNase treatment was carried out in a 100-μl reaction mixture containing 2 mM $MgCl_2$, 300 U of RNasin (Promega), 26 U of RNase-free DNase I (Boehringer Mannheim), and 10 μg of total RNA. After incubation at 37°C for 75 min, 0.2% sodium dodecyl sulfate was added, the solution was extracted once with an equal volume of phenol-chloroform, and the RNA was precipitated with ethanol. cDNA was synthesized in a 20-μl reaction mixture containing 1 μg of RNA (in water), 25 ng of primer, 10 mM dithiothreitol, 40 U of RNasin, 0.5 mM deoxynucleoside triphosphates (the last four components from Promega), 4 μl of 5× RT buffer, and 200 U of Moloney murine leukemia virus reverse transcriptase (both from Bethesda Research Laboratories). The reaction mixture was incubated at 37°C for 60 min. A portion, usually half, of the cDNA mixture was used directly for PCR. To check for residual DNA in the RNA sample, one RT reaction was carried out without reverse transcriptase.

PCRs were typically carried out as follows. A standard 50-μl mixture contained 10 μl of the cDNA mixture, 4 μl of 10× PCR buffer (Mg^{+2} free), 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 2.5 μCi of [^{32}P]dATP (3,000 Ci/mmol; Amersham), 1.25 U of *Taq* polymerase (Promega), and 350 ng of each of two primers. The different primers used are described in Results and listed in Table 1. PCR was performed for 20 to 30 cycles. Each cycle consisted of denaturation at 94°C for 1 min, primer annealing at 59°C for 75 s, and primer extension at 72°C for 2 min. Five microliters of each PCR sample was electrophoresed in a 5% polyacrylamide gel. The gel was dried, and the radioactive bands were visualized by autoradiography.

RNA decay rates. Actinomycin D (5 μg/ml) was added to exponentially growing CHO D422 cells at time zero, and total or nuclear RNA was extracted from the cells at various times thereafter. Total RNA was purified by a guanidinium thiocyanate method (20). The preparation of nuclei with Triton X-100 and the extraction of nuclear RNA were done by the method of Fu and Manley (16). RNA was treated with DNase I and reverse transcribed with a specific 3′ primer as described above. For each intron examined, a specific RT primer corresponding to a sequence in the next downstream exon was used, so that the splicing rate of the intron being examined was measured in a manner independent of the splicing rate of the other introns. The cDNA was amplified with [^{32}P]dATP in the reaction mixture as described above. In the case of total RNA, the same cDNA was used to amplify a region of the mRNA with two exon primers. Since we found that the half-life of *aprt* mRNA is 16 h, the amount of this RNA should not change appreciably within short time intervals and it can serve as an internal control. After gel electrophoresis, the PCR products were quantified with a PhosphorImager (Molecular Dynamics). In short-term experiments (1 h), each time point was corrected according to the relative intensity of the mRNA band.

Southern blotting. RT-PCR of *aprt* mRNA was carried out as described above except that no radioactive nucleotide was used. One-fifth of each PCR sample was electrophoresed in a 1% agarose gel. Southern blotting to a Zeta-Probe GT membrane (Bio-Rad) was carried out by following the instructions of the manufacturer. A plasmid (pH2) (33) carrying the entire *aprt* gene (2 kb) in pBR322 was used as a hybridization probe. The probe was labeled with ^{32}P by using a random priming kit (Boehringer Mannheim).

TABLE 1. Primers used in this study

Primer no.	Gene	Orientation	Region	Location (bp)[a,b]
1	*aprt*[a]	5′	Exon 1	37
2	*aprt*	3′	Intron 2	489
3	*aprt*	5′	Intron 1	87
4	*aprt*	3′	Exon 3	1409
5	*aprt*	5′	Exon 2	287
6	*aprt*	3′	Intron 3	1488
7	*aprt*	5′	Intron 2	520
8	*aprt*	3′	Exon 4	1615
9	*aprt*	5′	Exon 3	1400
10	*aprt*	3′	Intron 4	1740
11	*aprt*	5′	Intron 3	1531
12	*aprt*	3′	Exon 5	1833
13	*aprt*	3′	Exon 5	1898
14	*aprt*	3′	>Exon 5	2215
15	*aprt*	3′	>Exon 5	2267
16	*aprt*	3′	Intron 1	203
17	*aprt*	3′	Exon 2	306
18	*aprt*	5′	Exon 4	1618
19	*dhfr*[b]	5′	Exon 1	831
20	*dhfr*	3′	Exon 6	1544
21	*dhfr*	5′	Intron 1	−31 (exon 2)
22	*dhfr*	5′	Intron 2	−73 (exon 3)
23	*dhfr*	5′	Intron 3	−57 (exon 4)
24	*dhfr*	5′	Intron 4	−67 (exon 5)
25	*dhfr*	3′	Intron 5	+55 (exon 5)
26	*dhfr*	3′	Exon 2	1007
27	*dhfr*	3′	Exon 3	1094
28	*dhfr*	3′	Exon 4	1253
29	*dhfr*	3′	Exon 5	1294

[a] Data for primers 1 to 18 from the sequence described in reference 14. The sizes of *aprt* exons 1 to 5 are 80, 106, 134, 78, and 143 bp, respectively. The sizes of *aprt* introns 1 to 4 are 128, 978, 155, and 110 bp, respectively.

[b] Data for primers 19 to 29 from sequences described in references 34 and 6. The sizes of the *dhfr* exons 1 to 6 are 149, 50, 106, 127, 116, and 600 bp, respectively. The sizes of the *dhfr* introns 1 to 5 are 0.3, 2.5, 8.6, 2.6, and 9.4 kb, respectively (7).

RESULTS

In vivo splicing pathway of the *aprt* transcripts. To determine the order of splicing of endogenous *aprt* transcripts, we used RT followed by PCR of total CHO cell RNA. We used two sets of primers to determine the order of splicing of two adjacent introns; each set included an intron and an exon primer. By extending this comparison to overlapping pairs, we could deduce an overall order of splicing for all four introns. The use of one intron primer ensured the detection of pre-mRNA molecules without interference from the predominant mature mRNA.

We will present the experimental rationale by discussing one example in detail: whether intron 3 is spliced before intron 4 or vice versa (Fig. 1). If intron 4 is spliced before intron 3, primers that extend from exon 3 and intron 4 (primer set 1 in Fig. 1B) will yield a molecule that includes both introns, because when intron 4 is present, intron 3 is present as well. In contrast, primers that extend from intron 3 and exon 5 will give two types of molecules: one that includes both introns and one that has only intron 3, intron 4

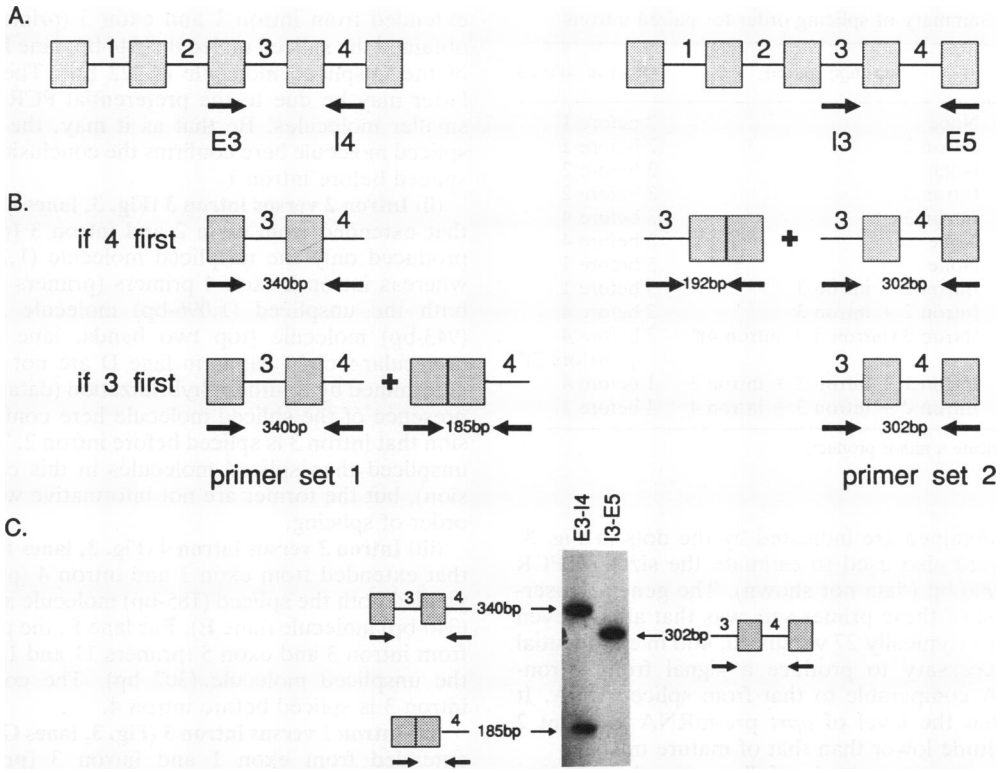

FIG. 1. Relative splicing order of introns 3 and 4. Boxes and lines designate, respectively, the exons and introns of the CHO *aprt* transcript (not to scale). Arrows represent the PCR primers. (A) The two different sets of intron (I) and exon (E) primers used to determine the splicing order of introns 3 and 4. (B) The different types of molecules expected if intron 3 is spliced before intron 4, or vice versa. (C) The RT-PCR products obtained. Total RNA was extracted, treated with DNase, reverse transcribed, amplified in the presence of radioactive dATP, and separated by polyacrylamide gel electrophoresis. Twenty-seven cycles of amplification were used. The sizes indicated correspond to those predicted by the structures shown.

having already been spliced (Fig. 1B, primer set 2). If intron 3 is spliced before intron 4, on the other hand, the first pair of primers will yield the spliced and unspliced molecules and the second pair will yield only the unspliced molecule (Fig. 1B). If there is no preferred order, both sets of primers will yield the spliced and unspliced molecules. As shown in Fig. 1C, primers that extended from exon 3 and intron 4 produced both the spliced and unspliced molecules and the intron 3-exon 5 pair yielded only the unspliced molecule. We conclude that in *aprt* transcripts, intron 3 is spliced before intron 4. In this type of analysis, it is the presence of the partially spliced molecule that is instructive, as totally unspliced molecules could originate from the primary transcript and so be uninformative.

Using the above strategy, we designed sets of primers to determine the splicing pathway of the four introns of *aprt* pre-mRNA. Figure 2 shows the *aprt* gene and the different sets of primers used. Total CHO cell RNA was extracted, treated with DNase, reverse transcribed with a 3′ primer, and then PCR amplified with the primers described in Table 1. The incorporation [α-^{32}P]dATP during the PCR allowed the detection of products by autoradiography after native gel electrophoresis. Figure 3 shows the different types of molecules that were obtained with each set of primers, and Table 2 summarizes these results. To be sure the expected PCR products were *aprt* specific, a similar experiment in which the unlabeled products were separated by agarose gel electrophoresis and detected by Southern hybridization with a probe consisting of the 2-kb *aprt* gene was carried out. The

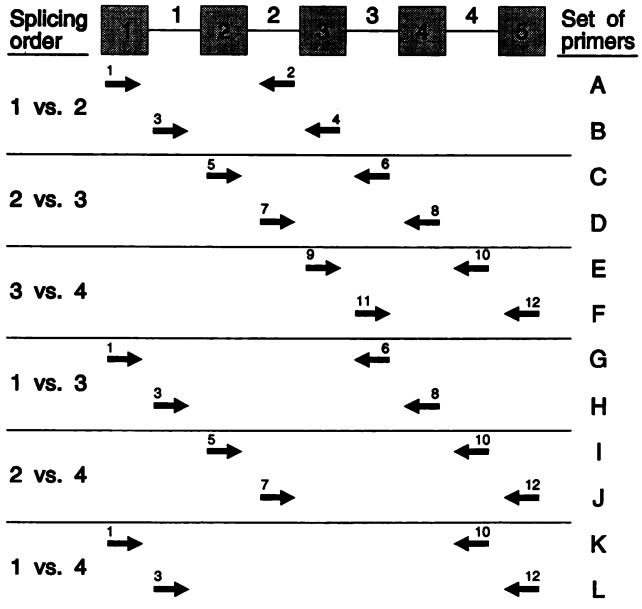

FIG. 2. PCR primers used for the pairwise comparisons of adjacent intron splicing.

TABLE 2. Summary of splicing order for paired introns

Intron pair	Primer set	Intron(s) spliced	Order of splicing
1 vs 2	A	None	2 before 1
	B	Intron 2	2 before 1
2 vs 3	C	None	3 before 2
	D	Intron 3	3 before 2
3 vs 4	E	Intron 3	3 before 4
	F	None	3 before 4
1 vs 3	G	None	3 before 1
	H	Intron 2 + intron 3	3 before 1
2 vs 4	I	Intron 2 + intron 3	2 before 4
	J	Intron 3 (intron 3 + intron 4)[a]	2 before 4 (4 before 2)[a]
1 vs 4	K	Intron 1 + intron 2 + intron 3	1 before 4
	L	Intron 2 + intron 3 + intron 4	4 before 1

[a] Parentheses indicate a minor product.

specific bands obtained are indicated by the dots in Fig. 3. Agarose gels were also used to estimate the sizes of PCR products over 900 bp (data not shown). The general observation with most of these primer sets was that about seven more PCR cycles (typically 27 versus 20, still in exponential phase) were necessary to produce a signal from intron-containing RNA comparable to that from spliced RNA. It thus appears that the level of *aprt* pre-mRNA is about 2 orders of magnitude lower than that of mature mRNA.

These results are summarized as follows (numbered primers are defined in Table 1 and illustrated in Fig. 2).

(i) Intron 1 versus intron 2 (Fig. 3, lanes A and B). Primers that extended from exon 1 and intron 2 (primers 1 and 2) yield mainly the unspliced molecule (452 bp; lane A), with just a trace of the spliced species (325 bp; lane A, lower dot). The conclusion reached is that intron 2 is usually spliced before intron 1. To confirm this order, we used primers that extended from intron 1 and exon 3 (primers 3 and 4) and obtained the spliced molecule (344 bp; lane B) but little if any of the unspliced molecule (1,322 bp). The absence of the latter may be due to the preferential PCR amplification of smaller molecules. Be that as it may, the presence of the spliced molecule here confirms the conclusion that intron 2 is spliced before intron 1.

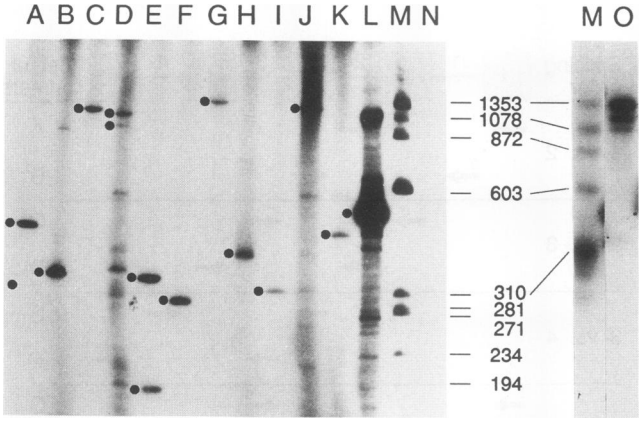

FIG. 3. Splicing intermediates of the *aprt* gene as detected by RT-PCR analysis. Total RNA was analyzed as described in the legend to Fig. 1. The PCR primers used for lanes A to L are shown in Fig. 2, where the same designations are used. Lane M contains molecular size markers (end-labeled *Hae*III fragments of φX174); numbers indicate size in base pairs. Lane N is a control without reverse transcriptase with primer set C. The dots indicate bands that hybridized to an *aprt*-specific probe upon Southern hybridization of identically produced nonlabeled PCR products. Lane O represents a Southern blot of a 1% agarose gel containing nonradioactive PCR products generated as for lane J; the probe was a plasmid carrying the entire *aprt* gene.

(ii) Intron 2 versus intron 3 (Fig. 3, lanes C and D). Primers that extended from exon 2 and intron 3 (primers 5 and 6) produced only the unspliced molecule (1,202 bp; lane C), whereas intron 2-exon 4 primers (primers 7 and 8) yielded both the unspliced (1,096-bp) molecule and the spliced (943-bp) molecule (top two bands, lane D). The lower-molecular-weight bands in lane D are not *aprt* specific, as determined by Southern hybridization (data not shown). The presence of the spliced molecule here confirms the conclusion that intron 3 is spliced before intron 2. There were more unspliced than spliced molecules in this case (see Discussion), but the former are not informative with regard to the order of splicing.

(iii) Intron 3 versus intron 4 (Fig. 3, lanes E and F). Primers that extended from exon 3 and intron 4 (primers 9 and 10) yielded both the spliced (185-bp) molecule and the unspliced (340-bp) molecule (lane E). For lane F, the primers extended from intron 3 and exon 5 (primers 11 and 12) and gave only the unspliced molecule (302 bp). The conclusion is that intron 3 is spliced before intron 4.

(iv) Intron 1 versus intron 3 (Fig. 3, lanes G and H). Primers extended from exon 1 and intron 3 (primers 1 and 6) produced the unspliced molecule (1450 bp; lane G). For lane H, the primers extended from intron 1 and exon 4 (primers 3 and 8) and the main product was the fully spliced molecule without intron 2 and intron 3 (396 bp). The conclusion is that intron 3 (as intron 2) is spliced before intron 1.

(v) Intron 2 versus intron 4 (Fig. 3, lanes I and J). Primers extended from exon 2 and intron 4 (primers 5 and 10) yielded the spliced molecule without introns 2 and 3 (324 bp; lane I). Therefore, intron 2 and intron 3 are spliced before intron 4. Primers extended from intron 2 and exon 5 (primers 7 and 12) produced mainly a band corresponding to the unspliced molecule (1,313 bp; lane J). The high background in this lane prompted us to repeat this separation in an agarose gel, with detection of the amplified bands by Southern hybridization. Lane O in Fig. 3 shows, in addition to the 1,313-bp unspliced molecule, a band corresponding to a molecule from which intron 3 had been spliced but which retains intron 4 (1,160 bp). We conclude that intron 2 is spliced before intron 4. A lesser amount of a molecule without introns 3 and 4 (1,052 bp) is also evident, indicating that it is possible for intron 4 to be spliced before intron 2. Densitometric analysis of these bands showed that the splicing of intron 4 before intron 2 is a minor pathway (<25%).

(vi) Intron 1 versus intron 4 (Fig. 3, lanes K and L). Primers extended from exon 1 and intron 4 (primers 1 and 10) produced the spliced molecule without introns 1, 2, and 3 (443 bp; lane K). For lane L, the primers extended from intron 1 and exon 5 (primers 3 and 12) and also yielded the spliced molecule without introns 2, 3, and 4 (503 bp; lane L). Thus, either intron 4 or intron 1 can be the last to be spliced. It is also evident that the molecule retaining intron 1 generates a much more intense signal than molecules containing only intron 4. In general, intron 1-containing PCR products were present as relatively intense bands, suggesting that such a molecule may accumulate as a predominant unspliced species (see below).

The combination of these data yielded the preferred

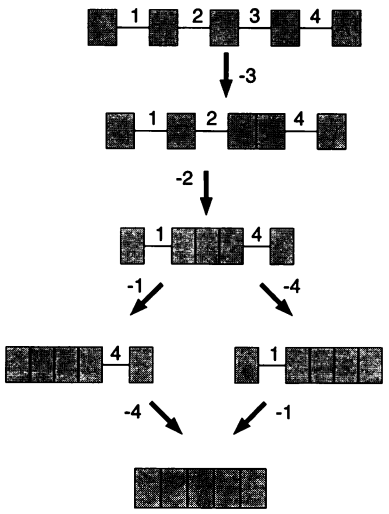

FIG. 4. The preferred splicing pathway of the four introns (lines between boxes denoting exons) of the *aprt* gene in CHO cells.

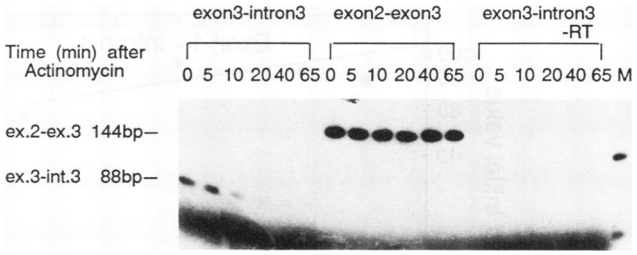

FIG. 5. Decay of an exon-intron junction after inhibition of RNA synthesis. CHO D422 cells were treated with actinomycin D, and at the indicated times, total RNA was extracted, treated with DNase, and reverse transcribed with an exon 4 primer (primer 8 listed in Table 1). PCR of the resulting cDNA amplified either the exon 3-intron 3 junction (lanes 1 to 6) (primers 9 and 6 listed in Table 1) or exon 2-exon 3, representing mainly mature mRNA as a relatively stable mRNA control (lanes 7 to 12) (primers 5 and 4 listed in Table 1). Lanes 12 to 18 are controls without reverse transcriptase (−RT), with which primers 9 and 6 were used.

splicing pathway of the *aprt* gene shown in Fig. 4: intron 3 > 2 > 1 = 4.

In vivo splicing rates. The preferred order of splicing depicted in Fig. 4 may reflect an intrinsically different splicing rate for each intron. To test the idea that differences in splicing rates could explain the splicing order, we measured the decay rates of the various intron-exon joints after treatment with actinomycin D.

Actinomycin D was added to exponentially growing CHO D422 cells; at various times thereafter, either total or nuclear RNA was extracted. To detect specific exon-intron regions, RT-PCR was carried out with a 5′ primer in the exon and a 3′ primer in the adjacent downstream intron. This amplification strategy thus detects cleavage at the donor site. In each case, the RT primer corresponded to a sequence in the next downstream exon, so that the splicing rate of the intron in question was measured in a manner independent of the splicing of other introns. The cDNA was amplified with [α-^{32}P]dATP in the reaction mixture, and PCR products were quantified with a PhosphorImager. When total RNA was analyzed, the amounts of pre-mRNA so detected were normalized to the amount of mRNA present as detected with two exon primers in a parallel amplification of the same cDNA. We found that the half-life of *aprt* mRNA was 16 h (data not shown), so this internal control does not change appreciably over the 65-min time course examined.

An autoradiogram showing the results of an experiment measuring the decay rate of the exon 3-intron 3 region is shown in Fig. 5, and the normalized quantitative results are plotted in Fig. 6C. The plotted line represents an exponential decay curve with no lag and a half-life of 6 min. Also present was a stable component that constituted about 15% of the initial signal. A residual stable component was seen for all exon-intron junctions examined (Fig. 6A through E) and was seen in a similar examination of several exon-intron junctions in *dhfr* gene transcripts (data not shown). In one case (intron 3-intron 4 primers), actinomycin D treatment was extended and the residual RNA was found to be undiminished after 15 h. Controls omitting reverse transcriptase indicated that this signal was not derived from DNA. The source of this stable RNA is not clear. It is not likely to originate from an incomplete inhibition of RNA synthesis, as the rate of tritiated uridine incorporation is reduced to less than 1% of the control level by 15 min after the addition of 5 μg of actinomycin D per ml (data not shown). It is possible that a fraction of the RNA is stabilized by actinomycin D.

The decay rate curves for the four different exon-intron junctions are shown in Fig. 6A through E. Whereas there was little difference in the decay rates of introns 2, 3, and 4, intron 1 appeared stable. The half-lives of introns 2, 3, and 4 were 6.2, 6.0, and 5.6 min, respectively, not significantly different. Total or nuclear RNA yielded essentially the same results for these introns. In contrast, intron 1 in total RNA was much more stable, showing little decay in 65 min (Fig. 6A). A slower splicing rate for intron 1 was also suggested by the pairwise comparisons described above (Fig. 3).

We further examined the behavior of this last intermediate by measuring its level after longer times in actinomycin D. As shown in Fig. 6F, in total cellular RNA a molecule containing intron 1 as its only intron (primers in intron 1 and exon 5) had a decay rate of about 4.6 h. Thus it appears that this intermediate is an additional final product of the *aprt* gene. We next determined the nuclear-cytoplasmic distribution of this molecule. More than 65% was present in the cytoplasmic fraction; in comparison, only 5 to 10% of molecules containing intron 3 or intron 4 were found in the cytoplasmic fraction. Intron 1 must be spliced in order to generate a mature mRNA molecule. By examining nuclear RNA, we were able to detect a fraction of intron 1-containing molecules that was spliced with a half-life of 12 min (Fig. 6E). Finally, we asked whether the presence of high levels of intron 1-containing *aprt* RNA was peculiar to CHO cells. Using the same RT-PCR analysis (intron 1 and exon 5 primers), we found similar high levels of this molecule in total cellular RNA from mouse L cells (data not shown).

Splicing of nascent transcripts: effect of polyadenylation on last intron splicing. One general factor that could influence the order of splicing is transcription itself. One can imagine that the splicing of an intron could either require a complete polyadenylated transcript or be inhibited by the presence of downstream transcript sequences. There is evidence for the splicing of nascent RNA (4, 30) as well as evidence that all introns can be spliced from a completed transcript (19, 24, 42, 55). Indeed, these two mechanisms are not mutually exclusive. Having established the order of splicing, we wished to address two questions about the role of transcrip-

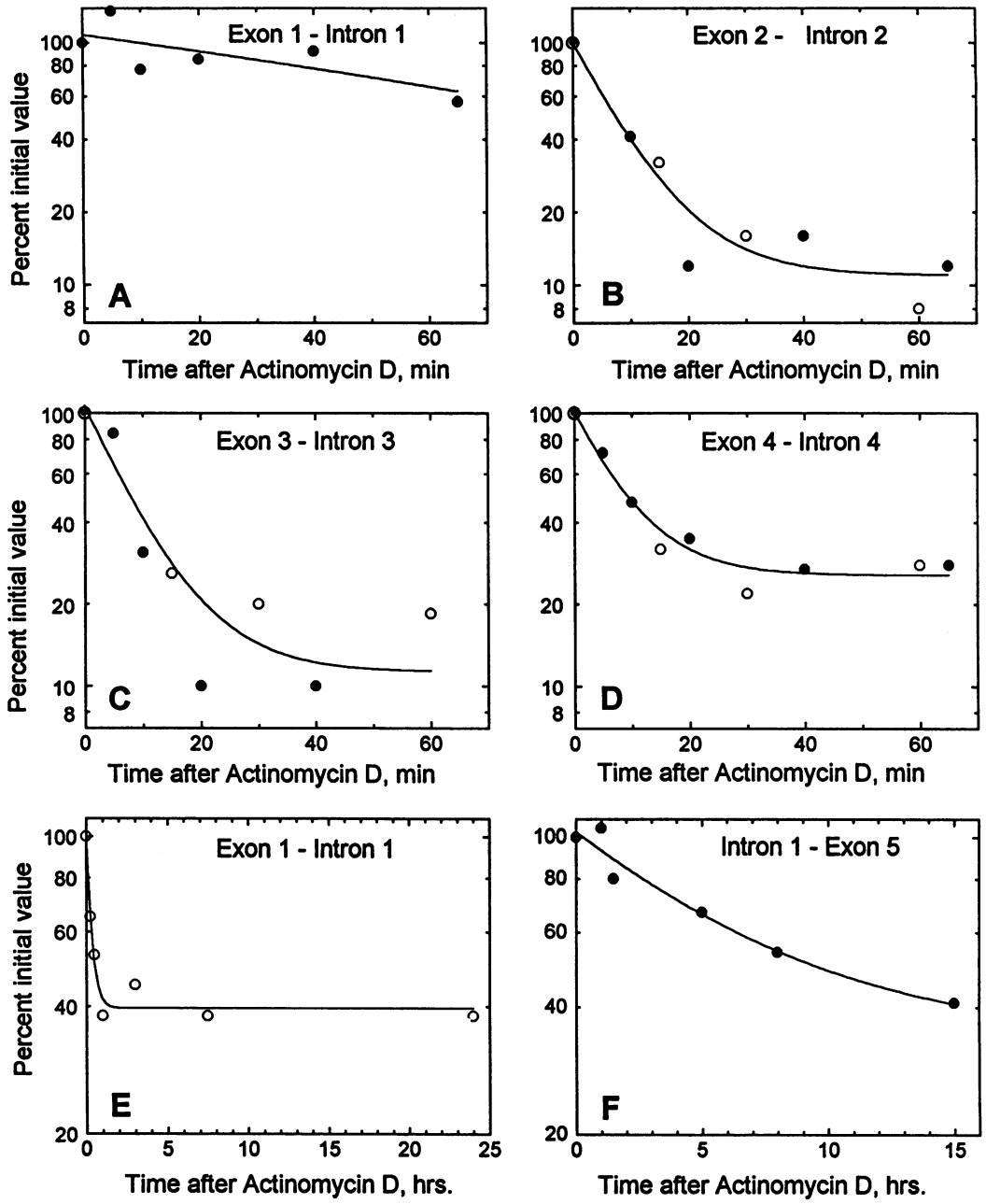

FIG. 6. Decay rates for various exon-intron joints in *aprt* RNA molecules. Total cellular (solid circles) or total nuclear (open circles) RNA was analyzed by RT-PCR following the addition of actinomycin D to inhibit new RNA synthesis. PCR products were separated as described in the legend to Fig. 5 and quantified with a PhosphorImager. Primers described in Table 1 were used to amplify the following regions (the primer numbers listed in Table 1 are shown in parentheses): (A) exon 1-intron 1 (primers 1 and 16), (B) exon 2-intron 2 (primers 5 and 2), (C) exon 3-intron 3 (primers 9 and 6), and (D) exon 4-intron 4 (primers 18 and 10). Panels E and F show long-term (16- to 24-h) decay rates for exon 1-intron 1 (primers 1 and 16) and intron 1-exon 5 (primers 3 and 12), respectively. RT primers were from the immediate downstream exon in each case, except for the experiment presented in panel F, for which random hexamers were used; amplification was for 26 cycles.

tion in this process. First, can the splicing of an intron take place before 3′-end formation is complete? Second, is polyadenylation necessary for the splicing of the 3′-most intron, as has been suggested by in vitro experiments (43, 58)?

To test for the presence of nascent transcripts that have undergone at least some splicing, we used primer combinations that would detect molecules whose synthesis had proceeded beyond the polyadenylation site but that had not yet undergone the cleavage reaction. If cleavage occurs rapidly once the polyadenylation site becomes available, then the uncleaved transcripts that are detected are likely to be still nascent. Two 3′ primers that are located downstream of the *aprt* polyadenylation signal were chosen (32). The 3′-most primer, with its 5′ end 120 bases downstream of the

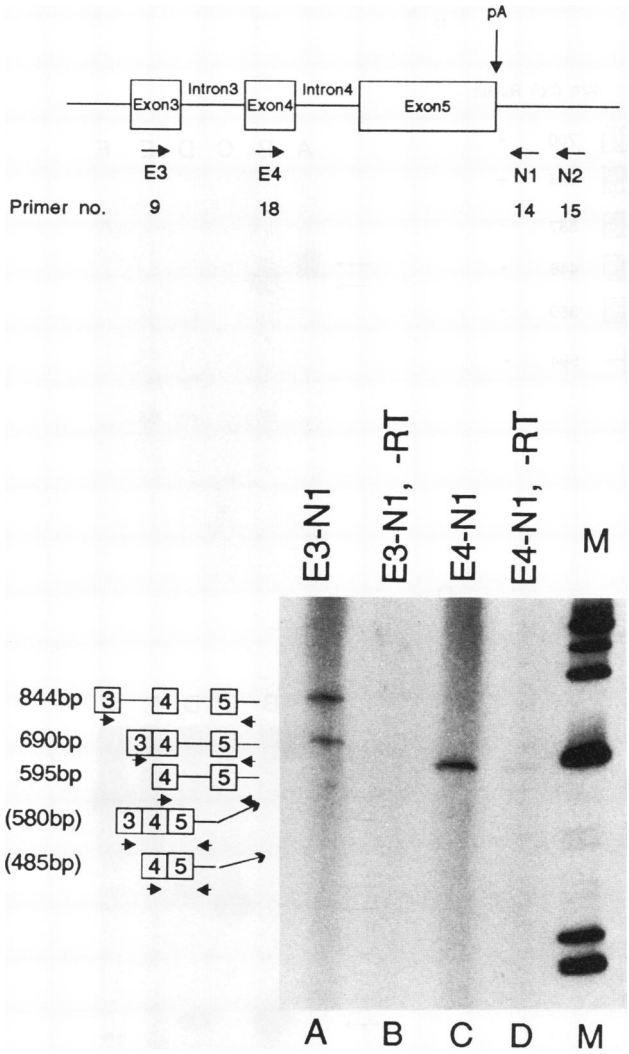

FIG. 7. Splicing of nascent *aprt* transcripts. Total cellular RNA was treated with DNase and subjected to RT-PCR using RT and PCR 3′ primers (N2 and N1, respectively) that originate downstream of the polyadenylation site and a 5′ primer either in exon 3 (E3) or in exon 4 (E4), as shown at the top of the figure. RT-PCR products were labeled with [^{32}P]dATP for 28 cycles and were separated by polyacrylamide gel electrophoresis. Possible products are depicted at their expected migration positions. The arrows indicate the positions of products that might have been expected but were not actually observed. Lane M contains molecular size markers.

polyadenylation signal (32, 37), was used in the RT. A nested adjacent 3′ primer was used for the PCR step, together with a 5′ primer either in exon 3 or in exon 4. The amplified molecules were then examined for the retention or splicing of introns 3 or 4.

The results of this experiment are shown in Fig. 7. The 5′ primer from exon 3 produced molecules of two sizes, one corresponding to unspliced RNA and one corresponding to molecules that had intron 3 spliced but not intron 4 (Fig. 7, lane A). The presence of sequences downstream of the polyadenylation site (from the 3′ primers) in a molecule that lacked intron 3 suggested that at least some splicing takes place on nonpolyadenylated, probably nascent, transcripts. In contrast, no such spliced molecules were seen when the 5′ primer originated in exon 4 (Fig. 7, lane C). This result is consistent with the idea that splicing of the 3′-most intron of the *aprt* transcript is dependent on its polyadenylation.

The last intron to be spliced in the CHO *dhfr* gene. Having developed a general method for determining the relative order of splicing of two adjacent introns, we applied it to test a model we had proposed to explain the effect of nonsense mutations in reducing mRNA levels in mammalian cells. We had previously shown that nonsense codons in the *dhfr* gene in CHO cells reduced mRNA levels by an order of magnitude. There was a marked polarity for this effect: nonsense codons in exons 2 through 5 of this 6-exon gene reduced mRNA, whereas translational stop codons in the sixth exon had no effect (57). This reduction was due neither to reduced transcription rates nor to an effect on the stability of mature mRNA, leaving RNA processing as the remaining target. The observed polarity provoked the hypothesis that translation may facilitate the splicing as well as the coordinate transport out of the nucleus of an RNA molecule. When ribosomes are arrested at the nonsense codon, splicing and transport stop and the partially processed RNA is subsequently degraded. If splicing is the rate-limiting step in RNA transport, then nonsense mutations in the last exon would not reduce RNA levels. This form of the translational translocation model (57) implies that the last intron to be spliced is the 3′-most intron: for a nonsense mutation in the penultimate exon to have an effect, a ribosome must reach that exon through spliced mRNA, yet the last intron target must persist unspliced. Therefore, we wished to test the prediction that intron 5 is the last intron to be spliced in *dhfr* pre-mRNA.

The primary transcript of the *dhfr* gene is 25 kb long and contains five introns, all but one of which are large (>2.5 kb) (7). Our strategy was to detect those molecules that had a solitary intron, by definition, the last intron to be spliced; such molecules would be capable of being amplified with primers from that intron and a terminal exon. We first used a common 3′ primer from exon 6 and a series of 5′ primers from introns 1 to 4. As outlined schematically in Fig. 8A, the appearance of a small RT-PCR product with a particular intron primer would maintain the candidacy of that intron as the last to be spliced; the absence of this molecule would eliminate it as a possibility. The effectiveness of each primer was verified by using genomic DNA (data not shown). The results are shown in Fig. 8B (lanes A through E) and summarized in Fig. 8A. Only in the case of intron 1 was an RT-PCR product obtained. That is, intron 1 was present in a molecule from which all downstream introns had been spliced. This result argued against intron 2, 3, or 4 occurring as the last intron. To test intron 5, primers from intron 5 and exon 1 were used (Fig. 8A, set F). No PCR product of the expected size was found, leaving intron 1 as the only intron to be spliced last. To confirm the conclusion that intron 1 was the last intron to be spliced, we performed a second experiment using a common primer in intron 1 and varying its partner among exons 2 to 6 (Fig. 8C). All primer pairs yielded a product, as expected if the last intermediate had all introns spliced except intron 1 (Fig. 8C and D).

DISCUSSION

Splicing of *aprt* transcripts occurs in a highly preferred order. The strategy described here should be useful for determining the in vivo order of splicing of any small transcript. By performing RT-PCR with different exon and intron primers, each partially spliced intermediate can be

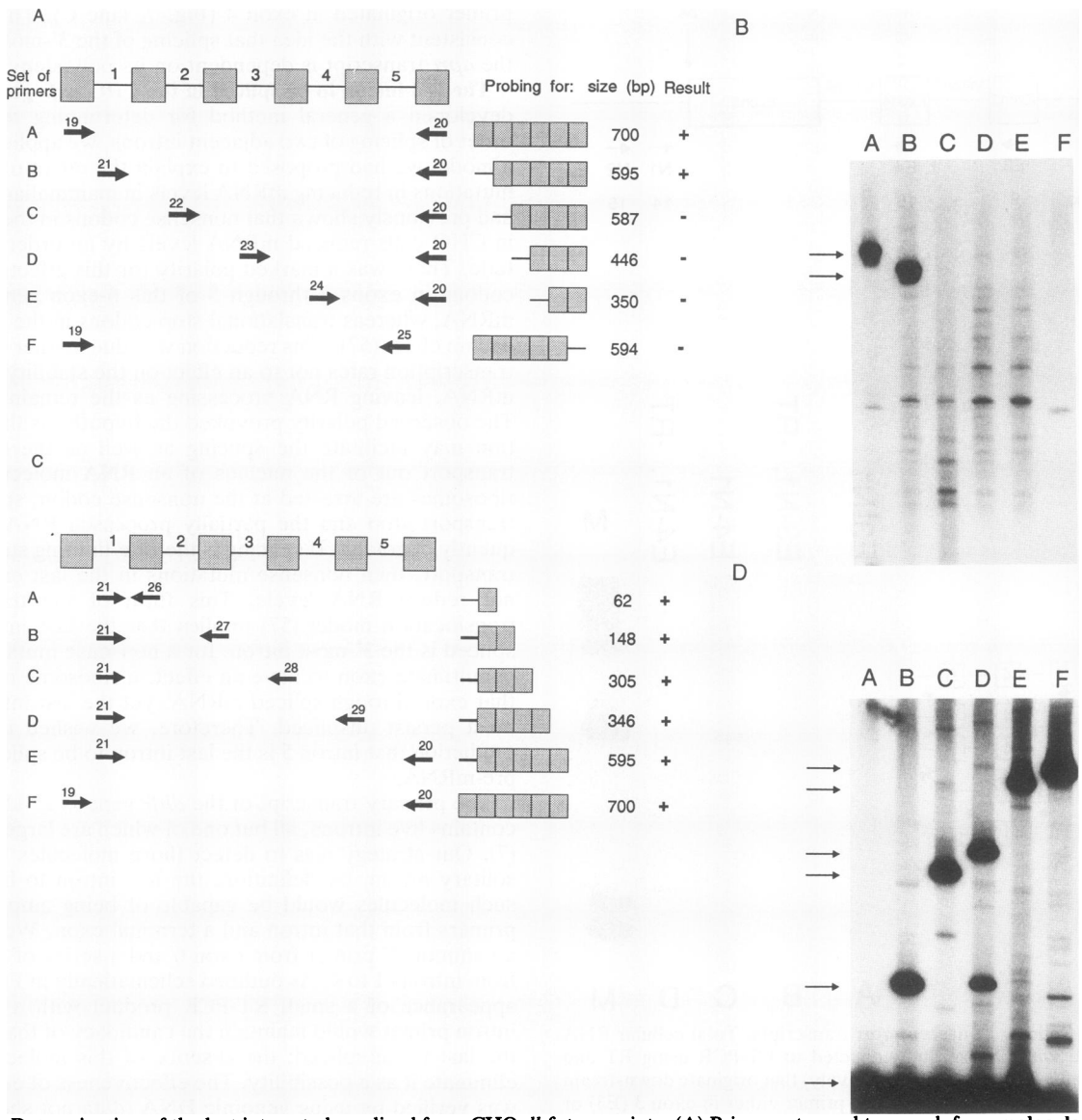

FIG. 8. Determination of the last intron to be spliced from CHO *dhfr* transcripts. (A) Primer sets used to search for a molecule containing a solitary intron (numbered lines). The PCR product sought is depicted at the right in each case. (B) Autoradiogram of a polyacrylamide gel showing the PCR products for the reactions indicated in panel A. Random hexamers were used to prime the RT reaction. Thirty PCR cycles were used. In lane A, only one-fifth as much of the PCR product was loaded. The arrows indicate the presence of bands of the predicted sizes. (C) Primer sets used to confirm the presence of RNA molecules containing intron 1 as the sole intron. (D) Gel electrophoresis results for the primer sets indicated in panel C. The conditions were the same as those described for panel B. Fragment sizes for intron 1-containing molecules were confirmed by comparison with PCR products from a plasmid minigene containing intron 1 as the sole intron (pDCH1P) (11).

readily detected and the preferred order of splicing can be deduced. Since the method relies on the analysis of steady-state RNA, it does not provide direct evidence of precursor-product relationships. However, several considerations in our experiments argue against the idea that the RT-PCR products we analyzed originated from side products or dead-end molecules rather than true intermediates in the splicing pathway. First, for the RT step, we used a specific primer from the last exon in *aprt* mRNA and not random hexamers. Thus, all amplified molecules examined originated from a larger transcript that included the 3′ end of the primary transcript. Second, we measured the rate of disappearance of exon-intron joints in *aprt* transcripts after treating cells with actinomycin to stop new RNA synthesis. The lability of these regions rules out their origin from stable dead-end products that may accumulate as splicing by-products. The 6-min half-lives observed for most of the joints are consistent with what might be expected for true splicing intermediates, on the basis of the low (less than 1%) steady-state levels of unspliced RNA relative to those of stable (16-h half-life) mature mRNA.

Our results suggest that the processing of intron sequences from the *aprt* primary transcript is via the preferred order $3 > 2 > 4 = 1$. Splicing here does not occur as a linear process starting at either end of the primary transcript. Similar results have been obtained for the few transcripts examined

TABLE 3. Appraisal of *aprt* splice sites

Intron	5′ splice donor sequence and score[a]	3′ splice acceptor sequence and score	Sum of 5′ and 3′ scores	$\Delta G°_{37}$[b] (kcal/mol)
1	UUAG/gugagau, 88	cuccuuguucccag/G, 87	174	−8.9
2	CGAG/gcgagug, 76(93)[c]	ucucauccuaacag/G, 82	158 (175)[c]	−8.8
3	CAAG/guaagca, 94	cuuccugucugcag/G, 87	181	−9.2
4	GGAG/guaagag, 91	ucccuucaccccag/G, 85	176	−9.3
Consensus	CAG/guaagu, 100	uuuuuuuuuuuuag/G, 100	200	−13.4

[a] Score of the best fit to the rodent consensus sequences (52); for donor sequences, positions −3 to +6 were included. For acceptors, positions −14 to +1 were scored. The minimum scores for sequences that retain the well-conserved GU of donors or the AG of acceptors are 34 and 27.2, respectively.

[b] Standard free-energy change of duplex formation with the 9-base U1 small nuclear RNA sequence complementary to the splice donor consensus sequence. Thermodynamic parameters were from the work of Freier et al. (15). Calculations excluded isolated base pairs and bulges and included GU base pairs, loops, and terminal base pairs or mismatches. The terminus for a helix that extended to the end of the 9-base consensus sequence was considered to be at the next base beyond this boundary (56). 1 kcal = 4.184 kJ.

[c] Scores in parentheses are from treatment of the unorthodox GC as GT.

for splicing order in vivo. For the chick ovomucoid pre-mRNA (55) and mouse and Chinese hamster thymidine kinase pre-mRNA (19), intron excision occurs via a preferred pathway and intron 1 is one of the last to be spliced. The processing of α1-acid glycoprotein pre-mRNA also does not take place in a linear sequence from the 5′ end (53). In contrast, in the rat phosphoenolpyruvate carboxylase gene (21), the mouse immunoglobulin heavy chain locus (39), the rabbit β-globin gene (61), and the mouse tumor necrosis factor β gene (60), a 5′-to-3′ order was proposed. A preferred order of splicing has also been demonstrated in cell extracts for three mammalian transcripts studied by Lang and Spritz (28), only one exhibited a 5′-to-3′ order. This variety of intron removal orders argues against the idea that the correct mating of exons is ensured by a linear splicing mechanism (27). It should be pointed out that our results as well as these earlier studies address the order of actual splicing at a site and not the order of commitment of that site to splicing. In particular, these results do not speak against a first-come-first-served model for intron identification (2), since the formation of committed presplicing complexes on introns (or exons) may occur as they are synthesized, with splicing taking place later. On the other hand, it is clear that accurate splicing of multi-intron pre-mRNA can be initiated and completed in the absence of transcription (24).

What determines the order of splicing of *aprt* pre-mRNA? Many different factors can influence the efficiency of splicing, including sequences at splice junctions (1, 29, 40, 41, 48), the lengths and sequences of introns (18, 49), the sequences of adjacent exons (50, 51, 59), and RNA secondary structure (12, 31, 54). Any or all of these factors could also play a role in the order of splicing, and some are discussed below.

An examination of the *aprt* donor and acceptor splice site sequences showed only small differences in scores for agreement with the consensus sequences (35), as shown in Table 3. The exception is a notable one: intron 2 has a GC dinucleotide at its 5′ end. This intron is thus one of the few exceptions (23) to the nearly universal conservation of GU at this position in splice donor sites. A GC at the 5′ end of intron 2 has been conserved among *aprt* genes of humans, mice, and hamsters, consistent with the notion that this sequence plays a functional role. Despite its unorthodoxy, intron 2 is neither the first nor the last to be spliced. A calculation of energies of duplex formation between the four donor sites and U1 small nuclear RNA similarly failed to reveal a correlation with splicing order (Table 3).

We looked for a feature in intron 3 that could distinguish it as the first intron to be spliced. Dot-matrix profiles of similarity between the mouse and hamster, human and hamster, and human and mouse *aprt* gene pairs revealed conservation in a 65-bp region in intron 3 (bases +21 to +85 of the hamster intron) (32). The homologous sequences in intron 3 do not appear in a fixed position relative to the splice junctions and do not appear in any gene other than *aprt*. The functional significance of this conserved sequence is unknown. It may contribute unique secondary structural features or a binding site that favors interactions with splicing factors. The absence of the third intron from minigene constructs did not markedly affect the transfection efficiency of a recombinant *aprt* gene (32); therefore, the presence of intron 3 is not an obligatory requirement for the initiation of splicing.

What is the significance of a preferred order of pre-mRNA splicing? On one hand, it may simply reflect the relative propensities of the individual splice sites to participate in splicing. On the other hand, it may result from a fundamental mechanism in splicing, that the splicing of a given intron is required for the subsequent splicing of another. Hoffman and Grabowski (22) and Nasim et al. (38) have convincingly demonstrated the role of such a mechanism in the alternative splicing of preprotachykinin pre-mRNA. In the results reported here, the different intensities of some of the RT-PCR products are consistent with the idea of an obligatory requirement. When two RT-PCR products derived from the same primer set were of similar size, we had some confidence that RT-PCR was reflecting the true ratio of intermediates. In these cases, the unspliced species was always more prominent than the corresponding spliced molecule. For instance, the paucity of molecules that had intron 3 spliced but intron 2 retained suggests that the splicing of intron 2 ensues rapidly once intron 3 has been spliced, consistent with the idea that the splicing of intron 2 may be dependent upon the prior splicing of intron 3 (Fig. 3, lanes D and O). Thus, early splicing events could produce superior substrates for subsequent splices. We are testing this idea by the in vivo analysis of splicing intermediates produced by a series of *aprt* splicing mutants.

A cytoplasmic *aprt* RNA retaining an intron. Two unexpected findings were that intron 1 is not spliced efficiently and that molecules containing intron 1 as the sole intron are exported to the cytoplasm. About 60% of these molecules are in the cytoplasm (data not shown), and they are relatively stable, with a half-life of 4.5 h compared with the 16-h half-life of *aprt* mRNA. Intron 1-containing RNA molecules accumulate to a level that is about 5% that of *aprt* mRNA, but this number must be considered only an estimate, as it is based on RT-PCR measurements made with different prim-

ers sets (data not shown). A stop codon in intron 1 precludes the translation of a protein related to APRT from this RNA, and only short reading frames otherwise exist. Thus, there is no reason to think that this RNA is an mRNA. More than 60% of the *aprt* RNA molecules containing intron 1 are also present in the cytoplasm of mouse L cells (data not shown). The splice site sequences of intron 1 are not exceptionally poor in fitting the consensus (Table 3), arguing against an intrinsic deficiency in the splice site sequences themselves.

In some molecules, intron 1 is spliced before intron 4, making intron 4 the last intron to be spliced. It is possible that intron 1 is spliced more efficiently from a splicing intermediate that contains both introns than from a molecule in which it is the sole remaining intron. This mechanism would be similar to that proposed above in which prior splicing influenced subsequent splicing, but in the opposite direction. To investigate further the inefficient splicing of intron 1, we are comparing the in vivo splicing efficiency of this intron by using *aprt* constructs that differ in the number and quality of introns present.

In vivo splicing rates. We have estimated the in vivo rate of splicing at particular donor splice sites by following the disappearance of the 5′ exon-intron joints after treatment with actinomycin D. Since we could not directly follow the appearance of newly spliced exons in mRNA (as in a pulse-chase experiment), we cannot formally rule out the possibility that RNA degradation as well as splicing contributed to the decay rates we measured. Degradation is probably not a major contribution, however, since it has been shown that most transcribed mRNA sequences do appear as cytoplasmic mRNA (10, 36). Weil et al. (60) also concluded that splicing rather than degradation was the principal fate of primary transcripts. Since we followed 5′ donor site joint disappearance, we examined only the first step of splicing: cleavage at the 5′ splice site. The half-lives of introns 2, 3, and 4 splicing were similar, about 5 to 6 min. As we did not observe large differences between the three introns, we can conclude that the first step in splicing is not a limiting one that determines the order of splicing. It should be noted that the half-life of the intron 2 donor site is not different from that of intron 3 or 4, despite the unorthodox GC instead of the usual GU at the splice site. The half-life of intron 1 splicing was somewhat longer, 12 min.

The splicing rates of 5 to 6 min found here could be overestimations, as in this time frame the kinetics of actinomycin D entry and binding to DNA may be contributing to the measurement. The rates are in good agreement with those estimated previously by various methods. Beyer and Osheim (4) examined the splicing of nascent transcripts in *Drosophila* embryos by ultrastructural analysis. For introns removed cotranscriptionally, intron loop formation took place within 2 min and splicing took place within 3 min (4). A half-life of less than 10 min was also estimated for mouse tumor necrosis factor β transcript splicing on the basis of decay in actinomycin D (60). Pulse-labeling was used to measure splicing of adenovirus E2A pre-mRNA; rates of under 2 min were found (17).

Although intron 3 is the first intron to be spliced, the rate of splicing of this intron was not noticeably faster than those of other introns. However, only the first step in splicing was measured here; it is possible that a subsequent step is rate limiting. Splicing in cell extracts is characterized by a long (15- to 60-min) lag during which spliceosomes are assembled (47). In vivo, this assembly must take place much more rapidly, as we saw an exponential decay of exon-intron joints without delay after the addition of actinomycin.

A substantial residual level of intron-containing RNA was found for each intron examined (Fig. 6). The origin of these stable signals is not clear. One possibility is that they represent nascent transcripts frozen by the actinomycin D treatment. Alternatively, actinomycin D may stabilize a fraction of the RNA molecules.

Splicing of transcripts prior to 3′-end formation. By using 3′ primers located downstream of the presumed polyadenylation site, we were able to examine molecules that had not yet undergone 3′-end processing (cleavage and polyadenylation). Having established the order of splicing, we asked whether specific introns were ever spliced prior to 3′-end formation. There is evidence in the literature both for splicing of nascent transcripts and for splicing of completed transcripts. Evidence for the former in *Drosophila melanogaster* comes from the electron microscopic studies of Beyer and Osheim (4) mentioned above and from an analysis of the kinetics of splicing after the induction of transcription by ecdysone (30), but there are also several examples of splicing of polyadenylated RNA (19, 24, 42, 55). Whether an intron will be spliced while still on a nascent RNA may simply depend on how fast it is spliced and how far it is located from the 3′ end of a transcript.

With respect to *aprt* intron 3, the first intron to be spliced, we found both spliced and unspliced transcripts among molecules that had not yet undergone 3′-end processing, indicating that splicing of this intron is not dependent on 3′-end formation. These results are in agreement with the idea that splicing can occur both on nascent transcripts as well as on completed molecules. If indeed these molecules represent nascent transcripts and if transcription proceeds at a constant rate of about 1 kb/min through the *aprt* gene, then spliceosome assembly and splicing can occur very rapidly. The distance between the 5′ splice site of intron 3 and the primer sites downstream of the polyadenylation site is only 838 bp. If we assume there are no blocks to transcription in this region, then both spliceosome formation and splicing at this intron can take place in the time it takes for RNA polymerase to transcribe 838 nucleotides (less than 1 min) plus the time it takes to cleave at a newly formed polyadenylation site.

In contrast to what was observed with intron 3, no splicing of the 3′-most intron, intron 4, was detected in molecules that had not undergone 3′-end formation. This result is consistent with the idea that the polyadenylation process plays a role in the splicing of the 3′-most intron, as has been shown in in vitro studies of adenovirus RNA (43). However, it is also conceivable that there is simply too little time between the completion of intron 4 and the cleavage reaction to allow even partial splicing to take place.

It is interesting to note that the highest level of residual actinomycin-resistant RNA was seen in the case of intron 4, for which about 30% of the initial RNA was still present after 1 h (Fig. 6D). Perhaps the origin of this stable RNA lies in molecules that have been prevented from reaching the site of 3′-end formation and so cannot splice intron 4. If only excised introns are degraded in the nucleus, then such molecules would be neither spliced nor degraded.

Splicing order and the connection between translation and RNA processing. We used a similar but more limited strategy to examine the order of splicing of a very large pre-mRNA molecule, the 25-kb primary transcript of the hamster *dhfr* gene. Since all but one of the introns in this transcript are large (2 to 9 kb) (7), the RT-PCR technique is unable to detect the same array of spliced versus unspliced molecules as seen in the small *aprt* transcript. Nevertheless, it should

be possible, with somewhat less confidence, to determine the order of splicing in these large molecules as well. For example, the absence of an RT-PCR product with primers from the 3′ end of intron 1 and from exon 3 would imply that intron 1 is spliced before intron 2; the presence of a band would imply the opposite. In the present study, our objective was more modest: to determine which intron is the last to be spliced. This question was readily approached by using RT-PCR, as we were seeking molecules that retained only a single intron. These PCR products would all be less than 1 kb long, since they lack all introns except the one corresponding to the primer, and this primer location was chosen to be adjacent to the downstream exon.

We were interested in determining the last intron to be spliced in *dhfr* transcripts in order to test a prediction of a model for how nonsense mutations reduce *dhfr* mRNA levels. Since neither the stability of mature mRNA nor the rate of transcription is affected by these mutations, we had proposed a connection between translation and RNA processing (8, 57). In this basic model, translation facilitates RNA processing by pulling an emerging RNA molecule through a nuclear pore into the cytoplasm. However, in its simplest form, this model did not explain many exceptional nonsense mutations that escaped this low-mRNA-level phenotype. In particular, all mutations in the last exon were immune, an effect seen not only for *dhfr* but also for human triosephosphate isomerase (13), human β-globin (3), and hamster *aprt* (25). The translational translocation model was therefore embellished by the proposal that translation facilitates splicing as well, pulling the RNA not only out of the nucleus but concomitantly through the splicing machinery. In this version of the model, splicing is the rate-limiting factor in RNA export. When a ribosome encounters a nonsense codon, translation ceases, downstream splicing and export are inhibited, and the pre-mRNA is degraded. However, when a ribosome reaches a nonsense codon in the last exon, all splicing must have been completed and so no effect is seen. This model predicts that the last intron to be spliced is the 3′-most intron. Otherwise, nonsense mutations in the penultimate exon would also be immune, and they are not (at least not those at the proximal end of the penultimate exon (8, 13). The result found here, that intron 1 and not intron 5 is the last intron to be spliced in the *dhfr* gene, argues strongly against the possibility that translation affects RNA splicing. This conclusion is supported by our finding that there is no accumulation of splicing intermediates in *aprt* nonsense mutants (25); the latter result has also recently been reported for the triosephosphate isomerase gene (9).

Summary. The mechanism by which multiexon transcripts exhibit a highly preferred order of splicing may be intimately tied to the larger problem of intron recognition. The consensus sequences for splice donor and splice acceptor sites have neither the length nor the exactness to ensure distinction from the many pseudosites that are present in most pre-mRNA molecules. While RNA secondary and tertiary structures are likely to play a role in the avoidance of these false sites, the physical elimination of potential competitors for a particular splice by a requisite prior splicing event may also turn out to be part of this selection process. The methods described here should allow this idea to be tested by determining the effect of mutations on the order of splicing.

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