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# The Simian Virus 40 Small-t Intron, Present in Many Common Expression Vectors, Leads to Aberrant Splicing

MANLEY T. F. HUANG† AND CORNELIA M. GORMAN*

*Department of Cell Genetics, Genentech Inc., 460 Point San Bruno Boulevard, South San Francisco, California 94080*

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**Polymerase chain reaction analysis was used to identify aberrant splicing of the simian virus 40 small-t intron present in pRSVcat. We examined factors governing the selection and relative use of aberrant 5′ splice sites derived from the chloramphenicol acetyltransferase-coding region. Our results indicated that transcripts from virtually any cDNA positioned upstream of the small-t intron could contain alternative 5′ splice sites and therefore be subject to deletions within the protein-coding region.**

On the basis of observations linking splicing to the production of stable cytoplasmic RNA (2, 13–15, 17, 21, 25, 34, 35), the small-t antigen intron of simian virus 40 (SV40) has been incorporated into a wide variety of expression vectors (1, 9, 10, 16, 22, 24, 32). This intron is a primary component of pRSVcat and the entire pSV2 series of vectors, the latter of which carry, among other genes, the *Escherichia coli gpt* (24), neomycin (32), chloramphenicol acetyltransferase (CAT) (10), and beta-galactosidase (16) genes. More generalized expression vectors also contain the small-t intron; these include vectors used for expression cloning (1) as well as the selectable marker vector introduced into infiltrating lymphocytes used in preliminary human clinical trials.

While examining CAT activity in mammalian cells transfected with plasmid pRSVcat (9) or pRSVcat-I (a derivative that lacks the small-t intron), we unexpectedly observed that pRSVcat produced six- to eightfold-lower levels of CAT activity. Even though pRSVcat produced more cytoplasmic RNA, its transcripts were shown by polymerase chain reaction (PCR) analysis to be either unspliced, accurately spliced (small t), or aberrantly spliced. The latter were formed from alternative 5′ splice sites located within the CAT-coding sequence. The functional requirement of these alternative splice sites appears to be their strong homology (seven of nine base pairs [bp]; hereafter referred to as 7/9-bp homology) to the 5′ splice site consensus sequence that is believed to interact with small nuclear RNA (20, 23, 37, 38). Since expression vectors contain the small-t intron usually positioned 3′ of the inserted gene, any cDNA that contains such a functional sequence has the potential for cryptic splicing. We have examined the functional implications of aberrant small-t splicing with respect to intron length, cell-type-specific splicing, and interactions between U1 small nuclear RNA and 5′ splice junctions.

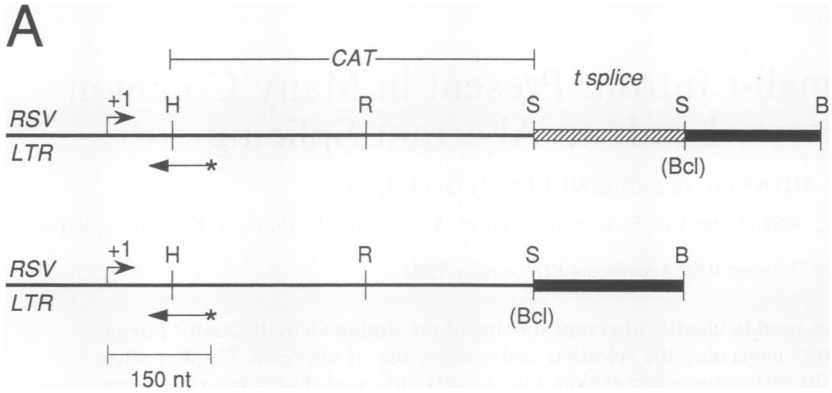
CV-1 and 293 cells were transfected with either pRSVcat or pRSVcat-I (Fig. 1A). Although both vectors initiated transcription at the correct start site within the Rous sarcoma virus long terminal repeat (9), these two vectors directed markedly different amounts of CAT-specific message (Fig. 1B). In both cell types, CAT-specific message was higher from pRSVcat than from pRSVcat-I (Table 1; Fig. 1B, lanes 1 and 3 versus lanes 2 and 4). This result is in agreement with other work demonstrating that mRNA containing an intron is polyadenylated and therefore transported much more efficiently than mRNA without an intron (18a). However, the concentrations of CAT-specific message transcribed from pRSVcat and pRSVcat-I were inversely proportional to CAT activity (Table 1). This discrepancy suggested that not all of the spliced messages produced functional CAT protein.

The splicing pattern of cytoplasmic RNA was analyzed by PCR (18). In CV-1 cells three prominent bands were detected, whereas four bands dominated the 293 cell pattern (Fig. 2). DNA sequencing determined that band 1 contained unspliced sequences containing the entire small-t intron and that band 2 contained RNA that correctly processed the small-t splice. Therefore, both bands 1 and 2 contained message that encoded full-length, active CAT protein. Bands 3 and 4 resulted from cryptic splicing events between the small-t 3′ splice site and new 5′ splice sequences, which shared a 7/9-bp homology (indicated by dots in Fig. 2) to the 5′ splice site consensus sequence, (CA)AG/GT(AG)AGT (23). Band 3 contained a cryptic splice site derived from the novel 5′ splice sequence, TGG/GTGAGT, located 570 bp upstream of the native small-t 5′ splice site. Band 4 mapped to another newly created 5′ splice site located 600 bp upstream of the bona fide small-t 5′ site. Another, less prominent transcript mapped to a region containing a 6/9-bp homology to the 5′ consensus (* in Fig. 2). The shortened transcripts represented by bands 3, 4, and * did not encode an active CAT protein (data not shown). Therefore, only a fraction of the correctly initiated transcripts produced by pRSVcat were full length and yielded active protein.

Aberrant splicing of pRSV cat apparently involves complementarity of the novel splice sites to U1 small nuclear RNA and is reminiscent of the pattern of alternative splicing exhibited by SV40 (5, 26–28). First, U1 is involved in pre-mRNA lariat formation and 5′ exon cleavage by specific interaction with the 5′ splice site consensus sequence (3, 4, 36–38). Since the cryptic 5′ sites within the CAT-coding region have a 7/9-bp match with this sequence, they apparently fulfill a sequence-specific criterion for use as a 5′ splice junction.

Second, the aberrant splices occurred even though the small-t 5′ splice site (AAG/GTAAAT) is already highly complementary to U1 (8/9-bp homology) and is closest to the 3′ splice sequence. This result supports the hypothesis that other constraints, such as position or size, prevent the efficient use of the small-t intron (3, 12, 27). It has been shown that during infection, SV40 preferentially uses a

* Corresponding author.
† Present address: GenPharm US, Inc., South San Francisco, CA 94080.



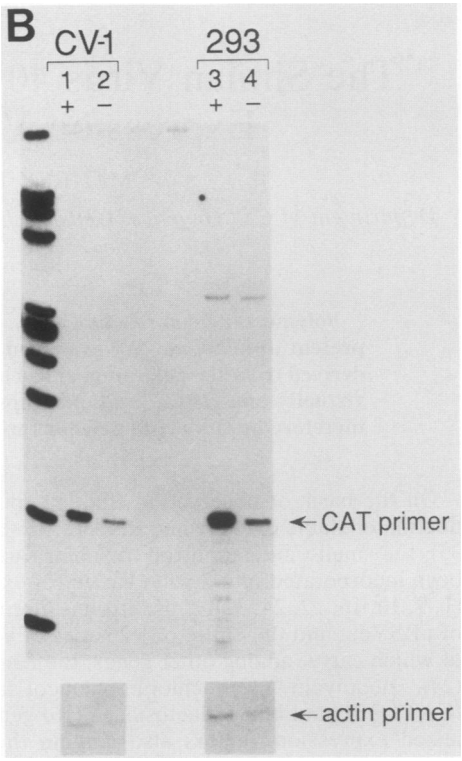




FIG. 1. Comparison of RNA produced from vectors either containing or lacking the SV40 small-t antigen splice site. (A) Linear maps of pRSVcat (top) and pRSVcat-I (bottom). Restriction sites: H, *Hin*dIII; R, *Eco*RI; S, *Sau*3A; B, *Bam*HI; Bcl, *Bcl*I. The transcriptional start site of the Rous sarcoma virus long terminal repeat (RSV LTR) is indicated by +1 and the rightward arrows. The CAT gene is flanked by *Hin*dIII and *Sau*3A sites. Symbols: ▨, small-t intron in pRSVcat (nt 4710 to 4100 of SV40 [31]); SV40 early polyadenylation sequence (nt 2772 to 2535 of SV40). pRSVcat-I was constructed by first modifying a derivative of pSV2cat (10) that contains two *Bam*HI sites (C. M. Gorman and B. H. Howard, unpublished results). This derivative contains a second *Bam*HI site immediately 3′ of the CAT gene, located at the position of the most 5′ *Sau*3A site shown in pRSVcat. Digestion with *Bam*HI removed the small-t intron and SV40 polyadenylation site. Next, the early SV40 polyadenylation site was reisolated from SV40 DNA as a *Bcl*I-*Bam*HI fragment (■) and ligated to the *Bam*HI-restricted pSV2cat derivative to create pSV2cat-I. The *Bcl*I site from the SV40 polyadenylation fragment generates a *Sau*3A site upon religation to the *Bam*HI site. This CAT transcription unit, lacking an intron, was then isolated from pSV2cat-I as a *Hin*dIII-*Bam*HI fragment and placed into the corresponding sites of pRSVcat to generate pRSVcat-I. (B) Quantitative analysis of cytoplasmic RNA. CV-1 (lanes 1 and 2) and 293 (lanes 3 and 4) cells were transfected (7, 11) with either pRSVcat, containing the small-t splice site (+ lanes), or pRSVcat-I, lacking the small-t splice site (− lanes). Cells were cotransfected with a human growth hormone plasmid (8) as an internal control (see footnote to Table 1). Total cytoplasmic RNA was harvested approximately 24 h after transfection. Primer extension reactions were performed by using $^{32}$P-labeled synthetic oligonucleotide primers with homologies to the 5′ portion of either CAT or beta-actin RNA (8, 19). The primer extension products were electrophoresed through a 6% polyacrylamide–8 M urea gel alongside $^{32}$P-labeled *Hae*III φX174 DNA markers. Primer extension products representing accurately initiated CAT or beta-actin transcription are marked. The location of CAT primer binding is indicated in the linear diagram below. Since this primer anneals to a region 20 bp downstream of the ATG for CAT, the primer extension products reflect the level of amino-terminal RNA. Relative CAT expression and human growth hormone values for this experiment are shown in Table 1.

5/9-bp-match 5′ splice site in producing large-T antigen mRNA rather than the 8/9-bp match found in the small-t 5′ site. Zhuang et al. (37, 38) showed that by changing the large-T 5′ splice site to more closely match the U1 sequence, use of the large-T site was further increased. Alternatively, if the small-t 5′ site was placed upstream of the large-T site, the small-t sequence was used very efficiently. Noble et al. (27) have shown that in vitro, SV40 strongly preferred the large-T over the small-t 5′ splice site, although some aberrant splicing occurred when the large-T site was removed (28). However, in vivo, when the large-T 5′ site was deleted, the small-t site was used and there was little evidence for the use of a new 5′ splice site. In our experiments, the small-t 3′ splice site was used efficiently; however, the choice of 5′ splice sites occurred upstream of the bona fide small-t site at two sites that have 7/9-bp complementarities with U1. Indeed, by mapping the PCR products (data not shown), a 6/9-bp site has also been identified (Fig. 2, *). We conclude that U1 sequence complementarities, as well as the position of the small-t 5′ splice site, were critical factors in the selection of the cryptic splices.

The relative utilization of 5′ splice sites in SV40 large-T antigen can be influenced by cellular factors. Fu et al. (3) demonstrated that the relative use of the small-t versus large-T 5′ splice sites varied from 10- to 15-fold between HeLa and 293 cells. The small-t intron was removed more efficiently from 293 cells than from HeLa cells. This relative difference may have been caused by the preference of cellular splicing factors in 293 cells for the small-t versus large-T lariat branch site (3, 4, 26). A similar pattern was observed with our CAT constructs: although CAT activity was always greater from cells transfected with pRSVcat-I than with pRSVcat, this difference was less marked in 293 cells (Table 1). Since PCR mapping showed correctly spliced small-t transcripts in 293 cells but not in CV-1 cells (Fig. 2), we conclude that 293 cells utilized the small-t 5′ splice site more efficiently. This effect was not due to the presence of adenovirus transforming proteins expressed in 293 cells, since the same pattern of splicing occurred in other human embryonic kidney (HEK) cell lines that do not express E1a or E1b such as primary HEK cells and a T-antigen-transformed HEK cell line (data not shown).

TABLE 1. Relative CAT activity in CV-1 and 293 cells after transfection with pRSVcat and pRSVcat-I[a]

| Cell type | CAT activity | | Ratio, −intron/+intron | |
|---|---|---|---|---|
| | pRSVcat | pRSVcat-I | CAT activity | RNA |
| CV-1 | 7,324 | 63,153 | 8.6/1 | 1/5.6 |
| 293 | 168,359 | 620,913 | 3.6/1 | 1/2.6 |

[a] CAT assays were performed as described previously (29). A 5-μl sample of a 100-μl extract was assayed for 15 min. The RNA ratios are based on densitometry scans of Fig. 1B. The intensity of the CAT-specific bands was adjusted for differential amounts of RNA, using the actin-specific bands. The values for RNA produced by pRSVcat are minimal estimates because of the intensity of the bands. As an internal control for transfection efficiency, a human growth hormone plasmid was cotransfected (8). Human growth hormone values (in nanograms per milliliter) were as follows: in CV-1 cells: 53 and 50 for pRSVcat and pRSVcat-I respectively; in 293 cells, 3,120 and 3,347 for pRSVcat and pRSVcat-I, respectively.

The small-t intron (66 nucleotides [nt]) appears to be of the minimum length necessary to form the lariat branch site complex (5, 26–28). Since increasing the length of the small-t intron from 66 nt to either 77 or 117 nt also increases the efficiency of splicing (5), we increased the length of the small-t intron in pRSVcat to test whether its size interferes with correct splicing. An *Spe*I site was introduced by point mutation into the small-t intron at position −26 with respect to the 3′ splice junction (Fig. 3A). The intron was then increased from 66 to 142 nt by inserting 76 nt into the *Spe*I site. 293 cells were transfected with either pRSVcatSpe (parental vector) or pRSVcatSpe+76 (enlarged small-t intron), followed by PCR analysis of cytoplasmic RNA (Fig. 3B). Transcripts from pRSVcatSpe yield a pattern of aberrant splicing similar to that of pRSVcat (Fig. 2 and 3C, lane 1). Still, it is possible that introduction of the point mutation that is adjacent to the branch point used for the small-t intron influences the efficiency of splicing of the small-t intron. There was no unspliced message apparent in the analysis of CAT-specific RNA after transfection of 293 cells with pRSVcatSpe (Fig. 3B, lane 1). Analysis of cells transfected with pRSVcatSpe+76 showed only one major transcript (Fig. 3B, band d). This PCR product was slightly larger than the spliced transcript (Fig. 2, band 2; Fig. 3B, band a) because of the use of a novel 3′ splice site inadvertently introduced by expansion of the small-t intron. Sequence analysis confirmed that the 3′ splice site used in this transcript arose from the sequence AG/GAA shown in Fig. 3A, creating an intron of 105 nt. Whereas the natural length of the small-t intron restricted usage of its 5′ splice site and allowed cryptic upstream sequences within CAT to substitute as 5′ splice junctions, expansion of the small-t intron eliminated aberrant splicing at the 5 junction. Fidelity of the 5′ splice site occurred even though the positions of the cryptic sites within the CAT gene remained the same relative to the small-t 5′ splice sequence.

The proportion of full-length mRNA that actually yielded an active CAT protein transcribed from pRSVcatSpe and from the vector with the enlarged intron, pRSVcatSpe+76, was analyzed. Since PCR might favor the smaller transcripts, we determined the relative abundance of the aberrant transcripts by Northern (RNA) analysis of mRNA isolated from CV-1 cells transfected with pRSVcatSpe and pRSVcatSpe+76 (Fig. 3C). Hybridization using the CAT probe identified all CAT transcripts regardless of the observed cryptic splicing events. Hybridization with the intron probe identified transcripts that either correctly spliced the small-t intron or were unspliced, and therefore coded for active protein, but did not hybridize to the aberrant CAT transcripts identified by PCR analysis in Fig. 3B (lane 1). One species of RNA (* in Fig. 3C, lane 1) migrated at a position expected for CAT transcripts that are correctly initiated, utilize the native 5′ and 3′ small-t splice junctions, and contain approximately 200 bases of poly(A). This RNA was recognized by both the CAT and intron probes. Another species of RNA (** in Fig. 3C, lane 1) migrated at a position expected of RNA that is cryptically spliced, using either of the two 5′ splice sites identified in Fig. 2 and 3B, lane 1. Cells transfected with pRSVcatSpe produced predominantly

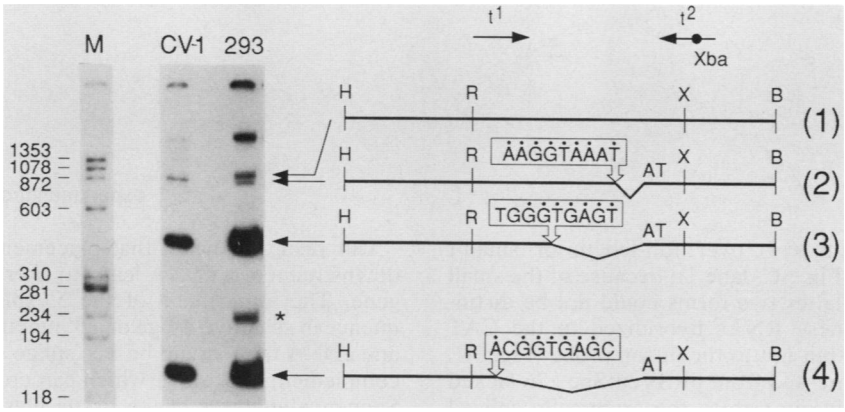
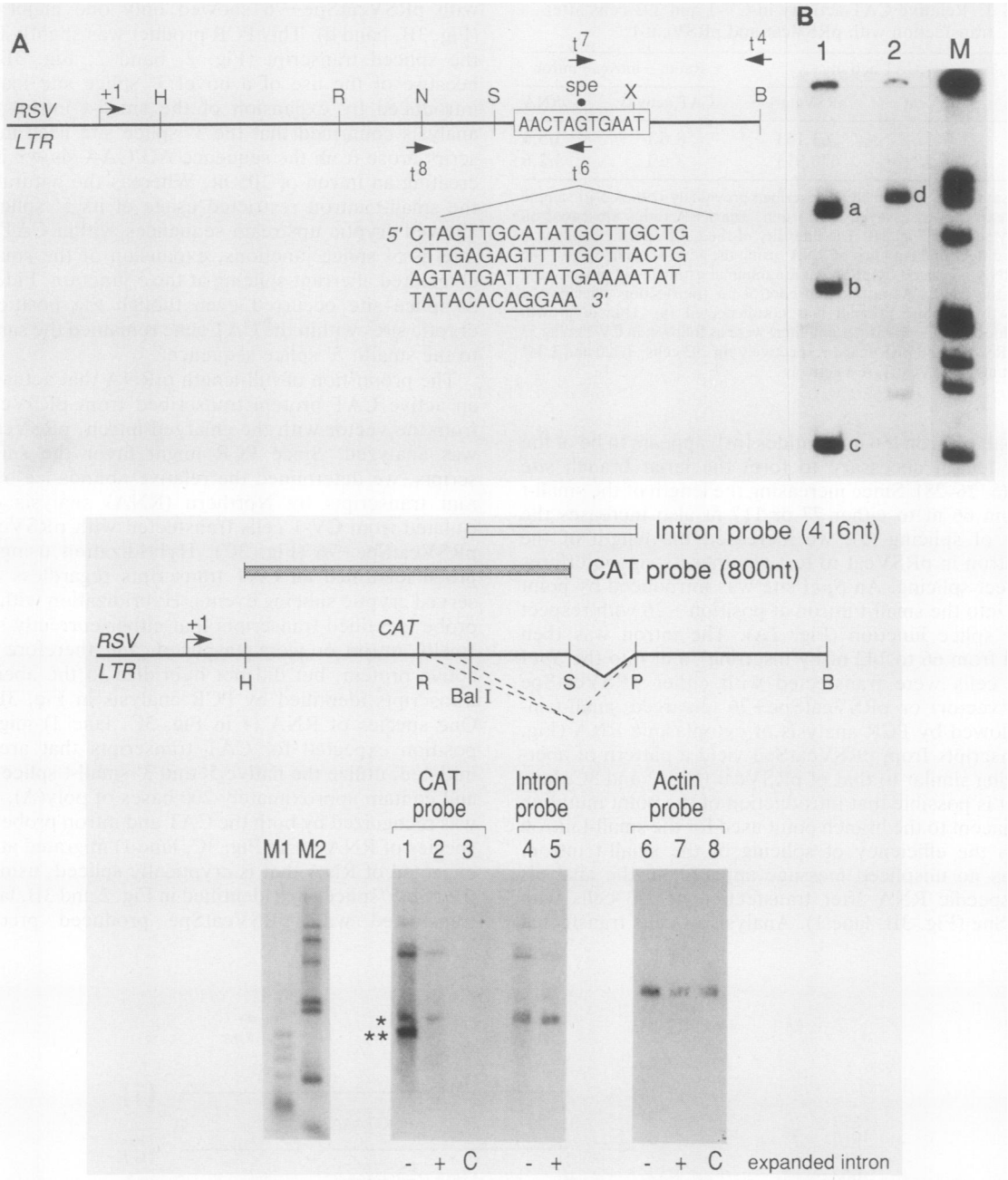



FIG. 2. PCR mapping of CAT transcripts. CV-1 and 293 cells were transfected with pRSVcat. Twelve hours after glycerol shock, cells were harvested for RNA isolation. cDNA was prepared from 50 μg of total RNA by primer extension, using the $t^2$ primer (5′-GCCTCATCATCTCTAGA-3′). $t^2$ hybridizes 110 nt downstream of the 3′ splice site of the small-t intron and includes a single point mutation that introduces a unique *Xba*I site into the PCR products. After primer extension, samples were treated with RNase, and the DNA was precipitated in ethanol, using 4 M ammonium acetate. Samples were suspended in water for the PCR reaction (18). Thirty cycles were performed at 65°C for 4.5 min, using primers $t^1$ (nonradioactive) and $t^2$ (mixture of radioactive and nonradioactive). Primer $t^1$ (5′-GCTCATCCGGAATTCCGTATG-3′) overlaps the *Eco*RI site within the CAT gene. Samples were phenol-chloroform extracted, ethanol precipitated, and suspended in 100 μl of Tris-EDTA (10 mM, pH 7.5). A 5-μl sample was electrophoresed through a 6% polyacrylamide gel. After electrophoresis, bands 1 and 3 from CV-1 cells were isolated from the gel, subcloned, and sequenced. The AT in bands 2 to 4 indicates the correct usage of the small-t 3′ site. Restriction sites: H, *Hin*dIII; R, *Eco*RI; X, *Xba*I; B, *Bam*HI. Lane M, Size markers, indicated in nucleotides on the left.




shorter CAT transcripts (**) over full-length or small-t spliced transcripts (*) (Fig. 3C, lane 1); because of the small size of the intron, the latter two forms could not be distinguished in this gel. These RNAs hybridized to the CAT probe (Fig. 3C, lane 1) but not to the intron probe (Fig. 3C, lane 4). CAT RNA expressed from pRSVcatSpe+76 lacked transcripts corresponding in size to the cryptically spliced transcripts seen in Fig. 3C, lane 1 (*). Therefore, RNA with the expanded small-t intron (Fig. 3C, lanes 2 and 5) did not utilize any 5′ cryptic splice sites identified by PCR and DNA sequence analysis. In this experiment, the amounts of unspliced or small-t spliced RNA (*) made from these vectors corresponded directly to CAT activity (Fig. 3C, lanes 4 and 5 and legend). Thus, both PCR and Northern blot analyses show that expansion of the small-t intron is sufficient to eliminate aberrant splicing.

Our results indicate that placement of the small-t intron downstream of a cDNA leads to aberrant splicing within the gene. The importance of the 5′ splice site consensus sequence in selective usage of a 5′ junction suggests that within any cDNA there could be a 5′ splice site with at least 7/9-bp complementarity to U1 which can create an aberrant splice. Sequence analyses of the tissue plasminogen activator and factor VIII cDNAs (6, 30) reveal 17 and 29 potential alternative sites, respectively. Upstream sequences have the potential for being used as alternative splice donors, similar to the manner in which SV40 large-T splice site is used more efficiently than the native small-t splice site. The presence of sequences bearing such a high degree of homology to the 5′ consensus could explain the observation that introns placed 3′ of cDNAs lead to less efficient expression of protein (12), particularly when the intron placed 3′ is the small-t intron

FIG. 3. Enlarging the small-t intron within pRSVcat. (A) Plasmid constructs. A *Spe*I site was created in the small-t intron, using PCR to exchange a G for the C located 27 nt upstream of the 3′ splice site. Primers pairs used in the PCR mutagenesis were $t^4$ (TTCCTGGGGATC CAGAC) and $t^7$ (TGTTAAACTAGTGATTCTAA) (top) and $t^6$ (TTAGAATCACTAGTTTAACA) and $t^8$ (GCCCCCGTTTTCACCATGGG) (bottom). pRSVcatSpe was constructed by ligating the two PCR fragments into pRSVcat at the *Nco*I site within the CAT gene and the *Bam*HI site downstream of the poly(A) addition; 76 bp of synthetic DNA was inserted into this unique *Spe*I site to form pRSVcatSpe+76 (only the 72 bp of the sense-strand sequence is shown). The antisense strand has an additional 4 nt (CTAG) at the 5′ end to recreate the *Spe*I site. Restriction sites: H, *Hin*dIII; R, *Eco*RI; N, *Nco*I; S, *Sau*3A; S, *Spe*I; X, *Xba*I; B, *Bam*HI. RSV LTR, Rous sarcoma virus long terminal repeat. (B) Mapping of RNA transcripts. CV-1 cells were transfected with either pRSVcatSpe (point mutation; lane 1) or pRSVcatSpe+76 (elongated small-t intron; lane 2), along with pCIS.hGH (7a) as an internal control marker. The CAT-specific transcripts were mapped by primer extension and PCR as described in the legend to Fig. 2. Band d (lane 2) was sequenced and shown to contain spliced RNA that utilized the small-t 5′ splice site. The 3′ splice site, however, derived from the last 5 bases of the 72-nt stuffer fragment (AG/GAA; underlined in Fig. 3A). Lane M contains *Hae*III digested ϕX174 DNA markers. (C) Northern blot analysis. Duplicate dishes of CV-1 cells, transfected as described in above, were either assayed for CAT activity or harvested for poly(A)$^+$ cytoplasmic RNA (2). RNA was denatured by glyoxal at 50°C for 1 h and electrophoresed in a 1.2% agarose gel. Lanes: 1, 4, and 6, 1.0 μg of poly(A)$^+$ RNA from pRSVcatSpe-transfected cells; 2, 5, and 7, 1.0 μg of poly(A)$^+$ RNA from pRSVcatSpe+76-transfected cells; 3 and 8, 0.7 μg of poly(A)$^+$ RNA from untransfected CV-1 cells. Presence (+) or absence (−) of the expanded small-t intron is indicated below each lane. After transfer to nitrocellulose paper, separate lanes were cut and hybridized (33) with the DNA probes indicated at the top. Lanes 1 to 3 were hybridized to a DNA fragment spanning the entire CAT-coding region (CAT probe); lanes 4 and 4 were hybridized to a DNA fragment spanning the smaller of the two cryptic introns from the *Bal*I site to the *Pst*I site (intron probe); and lanes 6 to 8 were hybridized to a DNA fragment representing the third exon of human beta-actin. $^{32}$P-end-labeled *Hae*III ϕX174 DNA (lane M1) and *Hin*dIII lambda DNA (lane M2) are shown to the left. Relative CAT activities: pRSVcatSpe, 530,040 cpm; pRSVcatSpe+76, 520,183 cpm. The transfection efficiency monitored by human growth hormone showed that in this experiment, the transfection efficiency of pRSVcatSpe+76 (532 ng/ml) was approximately fivefold lower than that of pRSVcatSpe (2,581 ng/ml). P, *Pst*I; other abbreviations for the diagram are as in Fig. 1.

(16). This finding is of particular interest because of widespread usage of the small-t intron vectors, including expression vectors currently introduced into humans through gene therapy.

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