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Alternative splicing of SV40 early pre-mRNA *in vitro*

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ABSTRACT

Simian virus 40 (SV40) early pre-mRNA is spliced using either of two alternative 5' splice sites and a common 3' splice site to produce two mRNAs that encode the T and t antigens. We have studied alternative splicing of SV40 early pre-mRNA *in vitro* using a HeLa cell nuclear extract. Synthetic SV40 early transcripts are processed to T and t antigen mRNAs *in vitro*. As in SV40-infected cells *in vivo*, cleavage at the T antigen 5' splice site is more efficient than cleavage at the t antigen 5' splice site *in vitro*, although both of these 5' splice sites are utilized relatively inefficiently *in vitro*. The ratio of cleavage at the T and t antigen 5' splice sites is not changed significantly by a number of alterations in the conditions under which the *in vitro* splicing reactions are carried out.

INTRODUCTION

Most higher eukaryotic genes contain intervening sequences (IVSs) that interrupt protein coding regions. During maturation of nuclear mRNA precursor transcripts (pre-mRNAs) to mRNAs the intervening sequences are excised and the exons ligated, a process known as RNA splicing. In some cases, a single transcript can be spliced using different combinations of splice sites, sometimes in a tissue-specific manner, yielding multiple mRNAs that encode different proteins. Thus, regulation of gene expression can occur at the level of alternative pre-mRNA splicing.

Simian virus 40 (SV40) early transcripts are spliced using either of two alternative 5' splice sites and a common 3' splice site (reviewed in 1). The resultant mRNAs encode the T and t antigens (see Figure 1). The t antigen 5' splice site is 269 bases downstream of the T antigen 5' splice site; the T and t antigen polypeptides thus share a common amino-terminus and diverge at their carboxyl-termini. The proportion of T to t antigen mRNAs in SV40 infected cells (approximately 4:1) does not appear to be regulated by viral products and does not change over the course of SV40 lytic infection. However, the ratio of T to t antigen mRNAs is affected by the temperature at which infected or transformed cells are grown (2) and by deletions between the T and t antigen 5' splice sites (3).

We have used a HeLa nuclear extract system (4) to study alternative splicing of SV40 early pre-mRNA *in vitro*. We have found that alternative splicing of SV40 early pre-mRNA occurs *in vitro*. The efficiency of splicing SV40 early pre-mRNA *in vitro* is low, and the ratio of T and t antigen mRNAs produced *in vitro* is somewhat higher than *in vivo*. The efficiencies of cleavage at the T and t antigen 5' splice sites were only partially related to their relative positions in the transcript. The conditions for optimal utilization of the T and t antigen 5' splice sites in vitro are the same; a number of alterations in the conditions at which the *in vitro* splicing reactions are carried out do not significantly alter the ratio of cleavage at the T and t antigen 5' splice sites.

MATERIALS AND METHODS

Transcriptional template plasmids

Plasmid pSP6SVE (see Figure 1) was constructed by ligating a 2.7- kb Xho I-BamH I fragment from pX-8 (5) containing the early region of SV40 to a Sal I-BamH I fragment from pSD346A (P. Good, personal comm.), a derivative of pSP64 (6) containing a 100-base poly(A) tract downstream of the unique BamH I cleavage site. The segment between the Hind III and Pst I sites of the pSP64 polylinker (sequence AGCTTGGGCTGCA) was deleted to reduce the distance between the SP6 transcriptional initiation site and the SV40 sequences.. Plasmid pSP6SVEΔSB (see Figure 1) is analogous to pSP6SVE, but the segment between SV40 nucleotides 4766 and 5192 that includes the T antigen 5' splice site has been deleted.

In vitro transription, splicing, and RNA analyses

Prior to transcription, pSP6SVE and pSP6SVEΔSB DNAs were cleaved at the Bgl II site immediately 3' to the poly(A) tract. Template DNAs were then transcribed with SP6 RNA polymerase (Promega) as previously described (7) in the presence of G(5')ppp(5')G (PL Biochemicals), and full-length transcripts were purified by oligo(dT)cellulose column chromatography. Microinjection of transcripts into *Xenopus* oocytes (8), preparation of HeLa cell nuclear extracts (9), and *in vitro* splicing reactions (4) were exactly as described. *In vitro* splicing was carried out at 30° C in 60 mM KCl, 3.2 mM $MgCl_2$, 500 μM ATP, and 20 mM creatine phosphate for 6 hrs unless otherwise indicated. In reactions that included anti-(U1)RNP antiserum, HeLa nuclear extract and RNAsin (Promega) were added to lyophilized antiserum and incubated for 30 min on ice before addition of the remaining components of the splicing reaction. Control reactions included nuclear extract preincubated with lyophilized antiserum buffer (146 mM NaCl, 14.1 mM H_3BO_3, 2.5 mM NaB_4O_7, pH 8.4). Control RNA was isolated from SV40 transformed monkey (Cos 7) cells (10) as described (11). S1 nuclease and T1 nuclease protection analyses

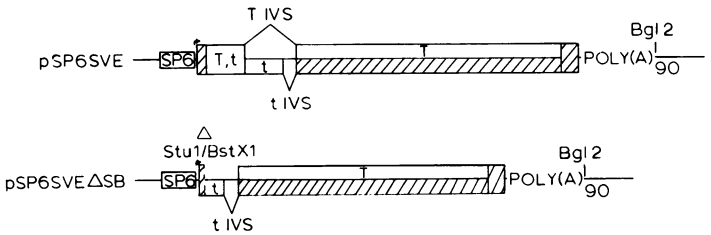

Figure 1. SV40 *in vitro* transcription templates. Details of constructions are presented in the text. Arrows indicate the site and direction of transcriptional initiation adjacent to the indicated SP6 promoter. Boxes above and below the line are T antigen and t antigen specific RNA segments, respectively. Hatched boxes represent 5′ and 3′ untranslated sequences. Open boxes denote exons. The T and t antigen intervening sequences (IVS) and poly (A) tails are indicated. *In vitro* transcripts of linearized templates terminate at the indicated Bgl II site.

were as previously described (7). T antigen and t antigen cDNA plasmids pSVT#5 and pSVt were the kind gifts of Y. Gluzman and J. Manley, respectively. Relative intensies of bands on autoradiograms were determined using a Hoefer GS300 scanning densitometer.

RESULTS

Synthetic SV40 early pre-mRNA can be cleaved at both the T and t antigen 5′ splice sites in *Xenopus* oocytes

Capped and polyadenylated SV40 early pre-mRNA was synthesized using SP6 RNA polymerase with pSP6SVE as template. *In vivo* SV40 early mRNA contains multiple 5′ termini (5,12,13), and the relationship between specific 5′ termini and splicing pattern of SV40 early transcripts has not been established. Therefore, because pSP6SVE transcripts contain a unique 5′ terminus, it was necessary to determine whether pSPSVE transcripts can be cleaved at both the T and t antigen 5′ splice sites. Accordingly, pSP6SVE transcripts were microinjected into *Xenopus* oocyes and RNA was prepared after 6 hrs. To assay for transcript cleavage at the T and t antigen 5′ splice sites, we performed S1 nuclease analysis using as probe a 1732 bp double-stranded 3′ end-labeled Xho I-Pvu II DNA fragment from pX-8 (5). As shown in Figure 2, pSP6SVE transcripts were cleaved at both the T antigen (323-base protected fragment) and t antigen (603-base protected fragment) 5′ splice sites in *Xenopus* oocytes. The ratio of cleavage at the T and t antigen 5′ splice sites was approximately 1:8, similar to that observed when SV40 DNA is introduced into *Xenopus* oocytes (14). These data demonstrate that pSP6SVE transcripts (containing only a single 5′ terminus) can be cleaved at both the T and t antigen 5′ splice sites.

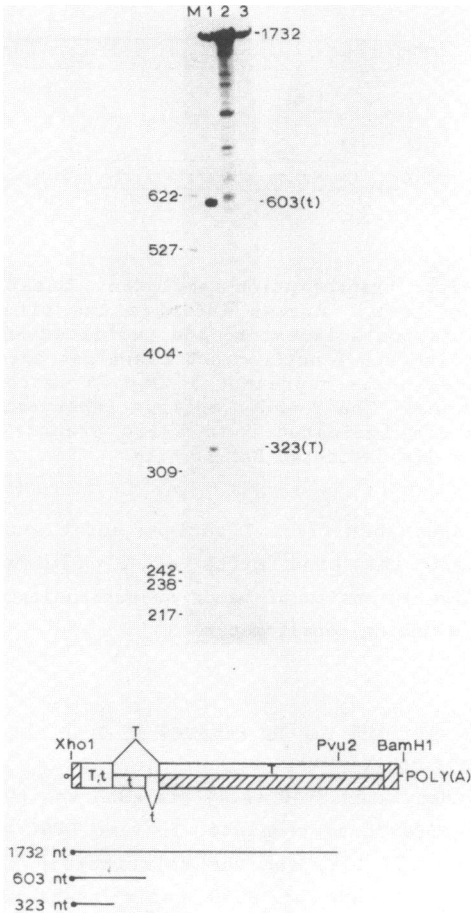

Figure 2. S1 nuclease analyses of cleavage of pSP6SVE transcripts at the T and t antigen 5' splice sites in *Xenopus* oocytes. Details of the probe and protected fragments are presented in the text. Open circle represents the cap structure and filled circle indicates the labeled end of the probe. T and t antigen splices are indicated. Lanes: M, size marker 1 (3' end-labeled Msp I digest of pBR322); 1, pSP6SVE transcripts recovered from oocytes; 2, uninjected pSP6SVE transcripts; 3, mock S1 analysis without pSP6SVE transcripts.

Synthetic SV40 early pre-mRNA can be spliced to both T and t antigen mRNA's *in vitro*

Capped and polyadenylated pSP6SVE transcripts were incubated in HeLa cell nuclear extracts and aliquots of the splicing reactions were removed at time intervals. Transcript cleavage at the T and t antigen 5' splice sites was assayed by S1 nuclease anlaysis using the probe described above. As

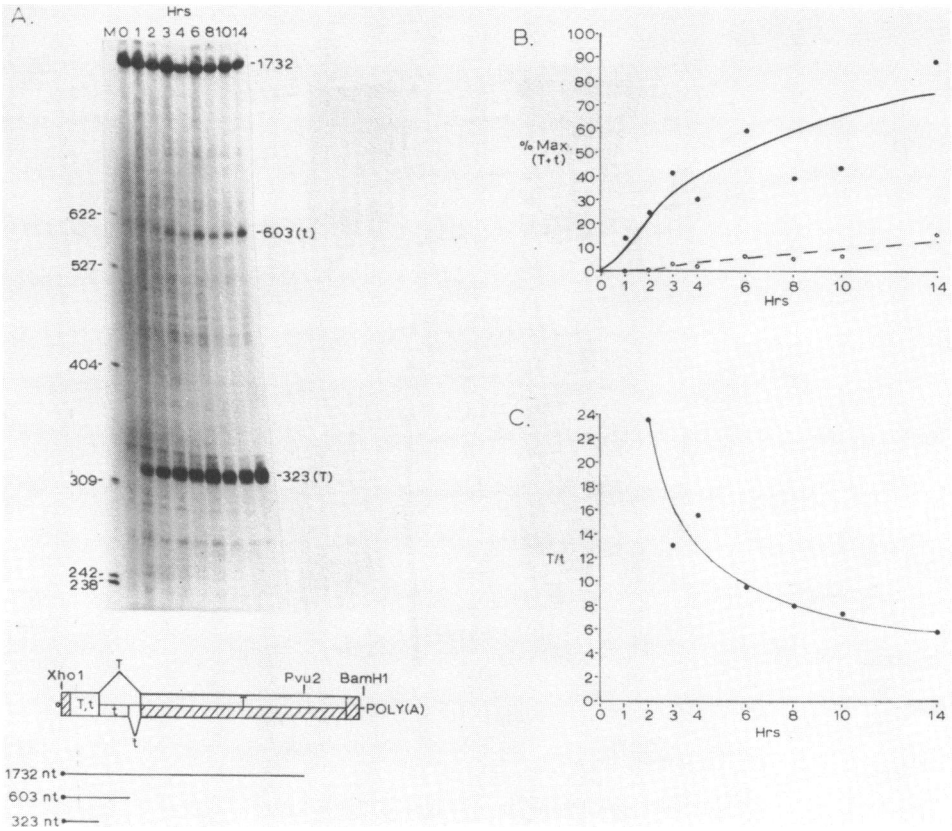

Figure 3. S1 nuclease analyses of cleavage of pSP6SVE transcripts at the T and t antigen 5′ splice sites in a HeLa cell nuclear extract *in vitro*. Details of the S1 probe and protected fragments are presented in the text and Figure 2. A. Lanes: M, size marker 1; 0-14, 0-14 hr aliquots of the splicing reaction. B. Quantitation of cleavage at the T and t antigen 5′ splice sites over time. The intensities of the bands shown in Fig. 3A were determined by scanning densitometry. Solid line, cleavage at the T antigen 5′ splice site; broken line, cleavage at the t angigen 5′ splice site. C. Ratio of cleavage at the T and t antigen 5′ splice sites over time.

shown in Figure 3A, pSP6SVE transcripts were cleaved at both the T and t antigen 5′ splice sites *in vitro*. The efficiency of cleavage and ratio of transcripts cleaved at the T antigen versus t antigen 5′ splice sites varied somewhat between experiments. In the experiment illustrated in Figure 3A, which was representative, the final ratio of T to t cleavage was approximately 6:1, similar to that observed in SV40-infected and SV40 transformed cells under standard cell culture conditions (2). As shown in

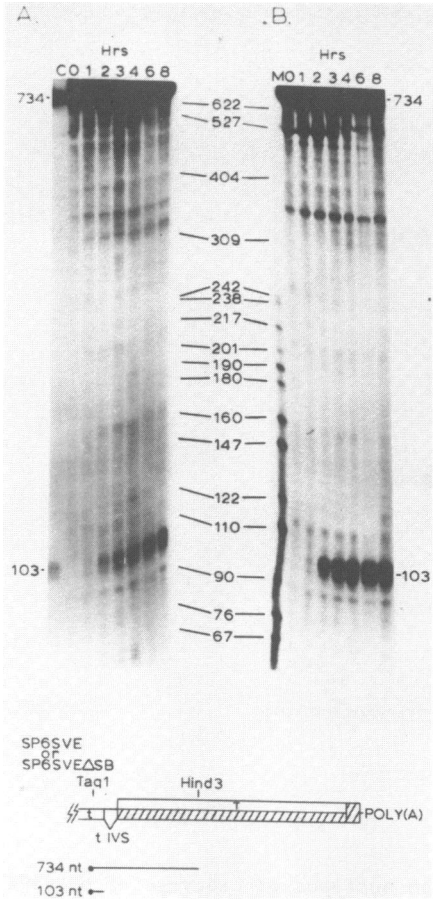

Figure 4. S1 nuclease analyses of cleavage of pSP6SVE and pSP6SVEΔSB transcripts at the t antigen 5′ splice site *in vitro*. Details of the probe and protected fragments are presented in the text. (A) pSP6SVE transcripts; Lanes: C, Polyadenylated Cos 7 cell RNA; 0-8, 0-8 hr aliquots of the splicing reaction. (B) pSP6SVEΔSB transcripts; Lanes: M, size marker 1; 0-8, 0-8 hr aliquots of the splicing reaction.

Figure 3B, cleavage at the T and t antigen 5′ splice sites continued for at least 14 hrs *in vitro*, and the T:t ratio decreased over time (Figure 3C).

In other experiments (data not shown), use of the Xho I-Pvu II S1 probe described above led to the appearance of a somewhat diffuse band several bases larger than the 603-nt band produced by *bona fide* cleavage at the t antigen 5′ splice site. This diffuse band was typically seen when splicing was inhibited by manipulation of experimental conditions (see below). It probably resulted from nonspecific nucleolytic cleavages at multiple sites

just downstream from the t antigen 5' splice site in transcripts that were not protected by assembly into splicing ribonucleoprotein complexes. To distinguish between these nonspecific cleavages and *bona fide* cleavage at the t antigen 5' splice site, we also assayed spliced pSP6SVE transcripts by S1 analysis using as probe a 734-bp double-stranded 3' end-labeled Taq I-Hind III DNA fragment from pSP6SVE. As shown in Figure 4A, transcripts cleaved at the t antigen 5' splice site protect a 103-base fragment of this probe; this protected fragment comigrated with that protected by RNA isolated from SV40-transformed monkey cells. The bands resulting from nonspecific cleavages just downstream from the t antigen 5' splice site were considerably less prominent using this probe (see for example Figure 7, lane 10), probably because they were spread over a relatively large area of the gel and thus did not appear as a single band. The time course of cleavage at the t antigen 5' splice site was similar when assayed using either the Taq I-Hind III (Figure 4A) or Xho I-Pvu II (Figure 3) S1 probes. These data thus verify specific cleavage of pSP6SVE transcripts at the t antigen 5' splice site *in vitro*.

To assay cleavage of pSP6SVE transcripts at the common SV40 early 3' splice site, we conducted S1 nuclease analysis using as probe a 1116-bp double-stranded 5' end-labeled Ava II-Hind III DNA fragment from pSP6SVE. As shown in Figure 5A, transcript cleavage at the SV40 early 3' splice site (569-base protected fragment) was readily observed by 1 hr. This fragment comigrated with that protected by RNA isolated from SV40-transformed monkey cells. In addition, a 915-base protected fragment was observed that results from transcripts cleaved at the T antigen 5' splice site but not at the 3' splice site (the presumed T antigen lariat splicing intermediate). This protected fragment is most abundant at 1 hr, and disappears over time. Surprisingly, we detected no 635-base protected fragment representing RNAs cleaved only at the t antigen lariat 5' splice site (the presumed t antigen lariat splicing intermediate), even by prolonged autoradiographic exposure (see Discussion).

Two unpredicted low abundance bands of approximately 590 and 825 bases are also visible in Figure 5A. These products were also identified by primer extension analysis of spliced pSP6SVE transcripts using a 40-bp double-stranded Hinf I-Dde I fragment from downstream of the SV40 early 3' splice site as primer (data not shown), but were never observed in S1 nuclease or primer extension analyses of unspliced pSP6SVE transcripts or of transcripts incubated under conditions in which splicing did not occur. In the experiments reported here, only approximately 85 percent of pSP6SVE transcripts are capped; the remainder are uncapped. Krainer et al. (4) have previously shown that uncapped β-globin transcripts undergo specific nucleolytic cleavages in the HeLa nuclear extract that are unrelated to

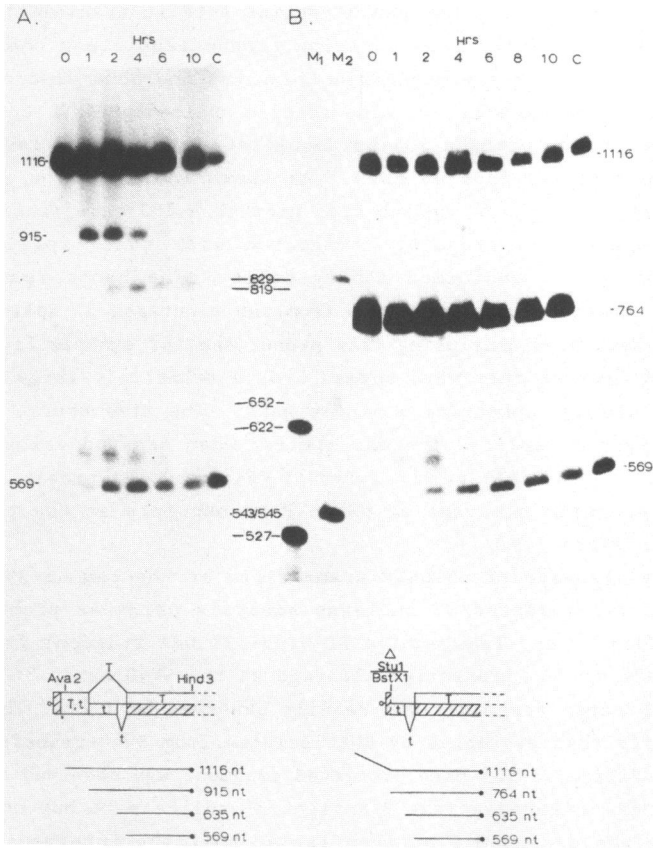

Figure 5. S1 nuclease analyses of cleavage of pSP6SVE and pSP6SVEΔSB transcripts at the common SV40 early 3′ splice site *in vitro*. Details of the probe and protected fragments are presented in the text. (A) pSP6SVE transcripts. Lanes: 0-10, 0-10 hr aliquots of the splicing reaction; C, Polyadenylated Cos 7 cell NA. (B) pSP6SVEΔ transcripts. Lanes: M_1, size marker 1; M_2, size marker 2 (3; end-labeled Msp I digest of M13mp2); 0-10, 0-10 hr aliquots of the splicing reaction; C, Polyadenylated Cos 7 cell RNA.

splicing. We therefore suspect that the 590- and 825-base S1 bands result from analogous nucleolytic cleavages of the uncapped fraction of pSP6SVE transcripts.

Altogether, our results demonstrate that pSP6SVE transcripts are cleaved at the SV40 early 3′ splice site *in vitro*, and the data are consistent with splicing of at least T antigen mRNA via a typical lariat splicing intermediate.

To determine whether the T and t antigen 5′ exons are ligated to the common SV40 early 3′ exon *in vitro*, we conducted T1 nuclease protection

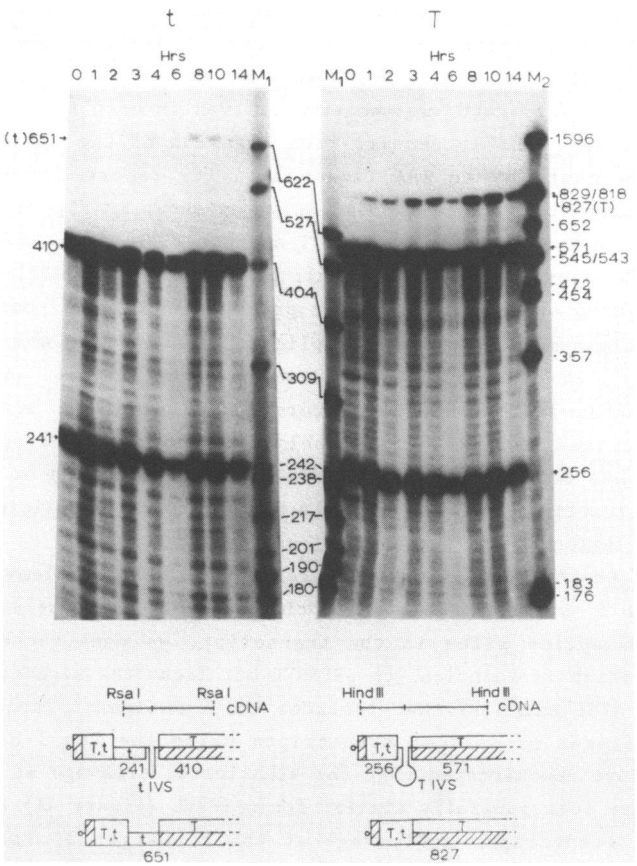

Figure 6. T1 nuclease protection analyses of production of fully spliced t and T antigen mRNAs from pSP6SVE transcripts *in vitro*. α^{32}[P] radiolabeled transcripts were spliced, hybridized to t and T antigen cDNA fragments, and digested with T1 ribonuclease. Details of the probe and protected fragments are presented in the text. (A) Production of t antigen mRNA. Lanes: 0-14, 0-14 hr aliquots of the splicing reaction; M_1, size marker 1. (B) Production of T antigen mRNA. Lanes: M_1, size marker 1; 0-14, 0-14 hr aliquots of the splicing reaction; M_2, size marker 2.

analyses using both t antigen and T antigen cDNA probes on aliquots of a single splicing reaction. In this assay IVS RNA segments are digested, but exon segments are protected. Exon ligation results in protected fragments equal in size to the sum of the individual protected exon segments. As shown in Figure 6A, a 642-bp double-stranded Rsa I fragment of t antigen cDNA, isolated from the plasmid pSVt (kindly provided by J. Manley), protects 241- and 410-base segments of the unligated exons of SV40 early pre-

mRNA and a 651-base segment of spliced t antigen mRNA. Completely spliced t antigen mRNA was detectable in this assay by 3-4 hrs and increased in abundance over at least a ten hr period. We note that a trace amount of a 651-base protected fragment was observable even in unspliced pre-mRNA; this trace background probably results from ligation of the 241- and 410-base protected fragments by an RNA ligase activity reportedly present in T1 nuclease preparations (15,16). Similarly, as shown in Figure 6B, an 823-bp double-stranded Hind III fragment of T antigen cDNA, isolated from plasmid pSVT#5 (kindly provided by Y. Gluzman), protects 256- and 571-base segments of the unligated exons of SV40 early pre-mRNA and an 827-base segment of spliced T antigen mRNA. Completely spliced T antigen mRNA was detectable by this assay by 1 hr and increased in abundance over at least an 8 hr period. A trace background of an 827-base protected fragment was also observable even in unspliced pre-mRNA, presumably for the reason discussed above. These data thus demonstrate that pSP6SVE transcripts can be processed to mature T and t antigen mRNAs in the HeLa nuclear *in vitro* splicing system.

Effects of deletion of the T antigen 5' splice site

To test the hypothesis that the relative levels of cleavages at the T and t antigen 5' splice sites result principally from the relative positions of these two splice sites in the transcript, we constructed pSP6SVEΔSB (Figure 1), which is identical to pSP6SVE but lacks the segment between SV40 nucleotides 4766 and 5192 that includes the T antigen 5' splice site. S1 nuclease analyses of spliced transcripts using the Taq I-Hind III probe described above demonstrated that the kinetics of cleavage at the t antigen 5' splice site were generally similar for pSP6SVE (Figure 4A) and pSP6SVEΔSB (Figure 4B) transcripts. Cleavage at the t antigen 5' splice site was approximately three times more efficient with pSP6SVEΔSB transcripts than with pSP6SVE transcripts. However, S1 nuclease analysis of the same spliced pSP6SVE RNA used to generate Figure 4, using the Xho I-Pvu II probe described above, demonstrated that in this experiment cleavage at the T antigen 5' splice site was at least twelve times more efficient than was cleavage at the t antigen 5' splice site (data not shown). Thus, deletion of the T antigen 5' splice site does increase the efficiency of cleavage at the t antigen 5' splice site, but not nearly to the efficiency of cleavage at the T antigen 5' splice site.

To assess cleavage of pSP6SVEΔSB transcripts at the SV40 early 3' splice site, we conducted S1 nuclease analysis using the Ava II-Hind III probe described above. As shown in Figure 5B, uncleaved pSP6SVEΔSB transcripts protect a 764-base segment of this probe. The rate of cleavage of pSP6SVEΔSB transcripts at the SV40 early 3' splice site was somewhat less than the rate of cleavage of pSP6SVE transcripts at this site, reflecting the lack of a component of transcripts cleaved at the T antigen 5' splice

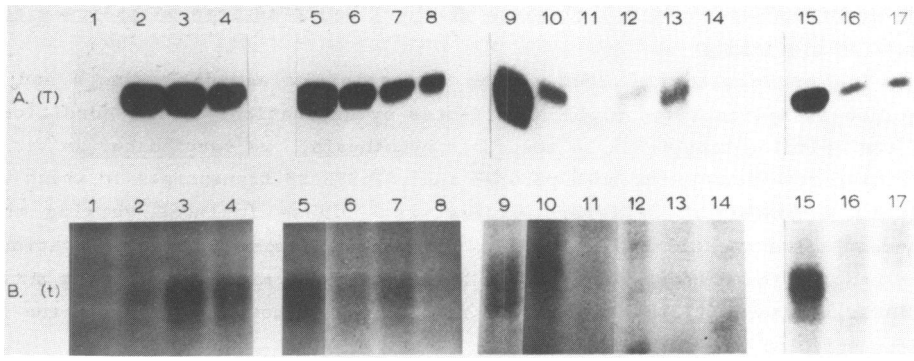

Figure 7. Effects of modifications of conditions on cleavage of pSP6SVE transcripts at the T and t antigen 5′ splice sites *in vitro*. Details of the probes and protected fragments are presented in the text and in Figures 2 and 4. Only the protected fragments are shown. (A) Cleavage at the T antigen 5′ splice site. (B) Cleavage at the t antigen 5′ splice site. Standard reactions were carried out at 30°C for 6 hrs in 60 mM KCl, 3.2 mM $MgCl_2$, 500 μM ATP, 20 mM creatine phosphate. Lanes 1-4, effect of temperature [lanes: 1, 4°C; 2, 25°C; 3, 30°C (standard reaction); 4, 35°C]. Lanes 5-8, effect of K^+ concentration [lanes: 5, 60 mM (standard reaction); 6, 80 mM; 7, 100 mM; 8, 120 mM]. Lane 9, standard reaction. Lane 10: No added ATP. Lanes 11 and 12, effect of Mg^{++} concentration (lanes: 11, 0 mM; 12, 9.6 mM). Lane 13, 1 mM vanadyl ribonucleoside complex added. Lane 14, HeLa nuclear extract preincubated at 45°C for 30 min. Lanes 15-17, effect of anti-(U1)RNP antiserum (lanes: 15, antiserum buffer only; 16, 2 μl antiserum; 17, 4 μl antiserum).

site. Furthermore, it can be seen in Figure 5B that the efficiency of cleavage of pSP6SVEΔSB transcripts is quite low; the majority of transcripts are completely uncleaved *in vitro*.

Because the efficiency of transcript cleavage at the t antigen 5′ splice site was increased approximately three-fold by the ΔSB deletion, one might expect a 635-base fragment protected by pSP6SVEΔSB transcripts cleaved only at the t antigen 5′ splice site (corresponding to a t antigen lariat splicing intermediate) to have been detectable in this experiment. However, we were again unable to detect this fragment (see Discussion), although we did observe the 590-base band discussed above that is seen on analysis of pSP6SVE transcripts.

Altogether, these data confirm that processing of SV40 early transcripts to t antigen mRNA can occur *in vitro* and demonstrate that the efficiency of cleavage at the t antigen 5′ splice site is not solely determined by its position 3′ to the T antigen 5′ splice site.

The conditions for optimal cleavage at the T and t antigen 5' splice sites *in vitro* are similar

The proportions of SV40 early transcripts cleaved at the T and t antigen 5' splice sites might be affected by alterations in the conditions of the splicing reaction. To test this hypothesis, we carried out *in vitro* splicing reactions using both pSP6SVE and pSP6SVEΔSB transcripts in which we varied a number of different parameters. These included varying the concentration of KCl, $MgCl_2$, or ATP, varying the temperature of incubation, pre-heating the HeLa nuclear extract at 45° C for 30 min, or including anti-(U1)RNP antisera (17,18) or vanadyl ribonucleoside complexes (19) in the *in vitro* splicing reactions.

As shown in Figure 7, each of these manipulations affected *in vitro* cleavage of pSP6SVE transcripts at the T antigen 5' splice site similarly to cleavage at the t antigen 5' splice site. Cleavages at the T and t antigen 5' splice sites were assayed by S1 nuclease analysis using the Xho I-Pvu II and Taq I-Hind III probes described above. Cleavage at both the T antigen (Figure 6A) and t antigen (Figure 6B) 5' splice sites requires ATP (lanes 9 versus 10), is maximal at 3.2 mM $MgCl_2$ (lanes 9,11, and 12), 60 mM KCl (lanes 5 to 8), and 30°C (lanes 1 to 4), and is inhibited by pre-treatment of the HeLa cell nuclear extract at 45°C for 30 min (lanes 9 versus 14), the inclusion of 1 mM vanadyl ribonucleoside complexes (lanes 9 versus 13), or anti-(U1)RNP antiserum (lanes 15 to 17). Preincubation of the HeLa nuclear extract with anti-(U1)RNP antisera for longer than 30 min also failed to completely abolish splicing activity for SV40 early and also human β- and γ-globin pre-mRNAs (data not shown). We also observed essentially identical effects of all of these manipulations on cleavage of pSP6SVEΔSB transcripts at the t antigen 5' splice site (data not shown). Thus, these results indicate that cleavages at the T antigen and t antigen 5' splice sites occur under similar optimal conditions *in vitro* for those parameters that we tested.

DISCUSSION

We have demonstrated alternative splicing of SV40 early pre-mRNA *in vitro* in the HeLa cell nuclear extract system. Patterns of alternative splicing can be influenced *in vivo* by sequence changes distant from the splice sites themselves (3,20-24). Therefore, we constructed pSP6SVE such that the synthetic SV40 transcripts would be as similar to authentic SV40 early pre-mRNA as feasible. The capped, polyadenylated pSP6SVE transcripts include all of the sequences of the major SV40 early transcripts, with only 21 additional vector (plus two SV40)-derived bases at the 5' end and 53 extra SV40 (plus two vector)-derived bases at the 3' end preceding the poly(A) tail.

When pSP6SVE transcripts are injected into *Xenopus* oocytes, they are cleaved at the T and t antigen 5′ splice sites at a ratio of 1 to 8, similar to transcripts of microinjected SV40 DNA (14). In contrast, in a cell-free extract derived from HeLa cells, pSP6SVE transcripts are preferentially cleaved at the T antigen 5′ splice site. The ratio of T antigen to t antigen mRNAs produced *in vitro* was approximately 6:1 to 17:1. Although this is somewhat greater than the approximately 4:1 ratio observed in mammalian cells *in vivo* (2), in most cases the *in vitro* splicing reactions were only incubated for 6 hrs, at which time the T:t ratio is still decreasing (see Figure 3C).

During the preparation of this manuscript, Noble and coworkers (25) reported a similar study in which they observed splicing of SV40 early pre-mRNA to produce T antigen but not t antigen mRNA *in vitro*. However, their study differed from ours in several important respects. First, as discussed above, pSP6SVE transcripts are very similar in structure to *bona fide* SV40 early pre-mRNA, whereas Noble *et al.* used as substrate a synthetic transcript containing only part of the SV40 early gene region. Second, Noble and colleagues carried out splicing reactions at 20 mM KCl, without polyvinyl alcohol, and with only one-third the usual amount of HeLa cell nuclear extract. Each of these differences from previously-defined optimal *in vitro* splicing conditions (4,26,27) significantly decreases the efficiency of splicing *in vitro*. Our data demonstrate that cleavage at the t antigen 5′ splice site *in vitro* is 6 to 17-fold less efficient than is cleavage at the T antigen 5′ splice site; thus, in the study of Noble *et al.* (25) cleavage at the t antigen 5′ splice site may have been too inefficient to be detected. In subsequent studies, Noble and coworkers have observed splicing to produce both T and t antigen mRNA *in vitro* (J. Noble and J. Manley, personal communication).

The splicing mechanism by which T antigen mRNA is produced appears to be identical to those observed for other pre-mRNAs (28,29). Molecules cleaved at the 5′ splice site but not at the 3′ splice site (corresponding to the "lariat" splicing intermediate) are readily detected. However, we cannot account for our repeated inability to detect the analogous processing intermediate for t antigen mRNA. We have also been unable to detect the excised t antigen IVS by direct electrophoresis of total radiolabeled pSP6SVE or pSP6SVEΔSB splicing products on both one-and two-dimensional denaturing gels (data not shown). It may be that the t antigen lariat splicing intermediate and excised IVS turn over rapidly in the HeLa nuclear extract and are therefore difficult to detect. However, J. Noble and J. Manley (personal communication) have recently characterized the excised t antigen IVS by RNA fingerprint analysis and have shown it to have a lariat structure. Therefore, processing of SV40 early transcripts to both T and t

antigen mRNAs apparently occurs via typical lariat splicing intermediates.

The mechanism by which alternative splice sites are selected to produce multiple mRNAs from a single pre-mRNA species is not known. In the case of SV40 early transcripts, the proportions of stable T and t antigen mRNAs can be influenced by various aspects of the cellular milieu, including the cell type (14 and this report) and growth temperature (2), and also by deletions between the T and t antigen 5' splice sites (3). Furthermore, Reddy et al. (30) have noted that the 5' and 3' halves of the t antigen IVS are self-complementary, and Solnick (31) has observed that patterns of alternative splice site utilization can be influenced by secondary structures in the RNA. Together, these observations suggest that utilization of the T antigen versus t antigen 5' splice sites might be influenced by alternative secondary structures in SV40 early pre-mRNA. Therefore, the proportions of SV40 pre-mRNA cleaved at the T and t antigen 5' splice sites *in vitro* might be altered by modifications of conditions of the splicing reaction that affect RNA secondary structure.

Accordingly, we tested the effects on splicing of pSP6SVE transcripts *in vitro* of several changes of experimental conditions that might affect RNA secondary structure, including altered concentrations of KCl or $MgCl_2$ and altered temperature. We also tested several manipulations that inhibit 5' splice site cleavage *in vitro*, including omission of ATP, pre-treatment of the HeLa cell nuclear extract at 45°C for 30 min, and the inclusion of vanadyl ribonucleoside complexes or anti-(U1)RNP antisera in the splicing reaction, to determine whether production of T and t antigen mRNAs might be affected differentially. None of the manipulations that we tested differentially affected cleavage at the two SV40 early 5' splice sites. In each case, departure from our standard *in vitro* splicing conditions (4,7,26,27) decreased cleavage at the T and t antigen 5' splice sites proportionally. Similar effects were observed on cleavage of pSP6SVEΔSB transcripts at the t antigen 5' splice site. Although we cannot be certain that any of the manipulations tested actually affect putative alternative secondary structures in SV40 early pre-mRNA under the conditions of the *in vitro* splicing reaction, the ranges of KCl or $MgCl_2$ concentrations or temperatures tested would seem likely to exert effects if such structures exist. Therefore, we suggest that selection of the two SV40 early 5' splice sites is probably not mediated by alternative secondary structures in SV40 early pre-mRNA, at least *in vitro*.

The efficiency at which SV40 early transcripts are spliced *in vitro* is very low. We have previously shown that the efficiency of excision of IVS1 from human β-globin pre-mRNA *in vitro* approaches 100 percent by 6-8 hrs (7). In contrast, only a few percent of pSP6SVE transcripts are processed to T and t antigen mRNA over a comparable period. Thus, the majority of pSP6SVE

transcripts are unspliced *in vitro*, possibly because the sequences at the T and t antigen 5′ splice sites are poor substrates for the splicing reaction. In transcripts that lack a "good" 5′ splice site, utilization of several "poor" sites may then occur, and various splices that occur at a low rate may represent a significant fraction of the total. Therefore, utilization of the T and t antigen 5′ splice sites may occur by an essentially stochastic process that can be modified by cellular factors.

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