The EMBO Journal vol.14 no.13 pp.3226–3235, 1995

Experimental evidence for RNA *trans*-splicing in mammalian cells

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We present evidence that mammalian cells have the ability to generate functional mRNA molecules by *trans*-splicing. Rat cells, transformed by an early SV40 DNA fragment (*Bst/Bam*) synthesize a truncated T antigen (T1 antigen), although the cells do not have a direct sequence homology for the T1 antigen at the DNA level. The *Bst/Bam* DNA fragment encodes exclusively for the second SV40 T antigen exon (aa 83–708) and contains the entire small t antigen intron. To synthesize the corresponding mRNA (T1 mRNA), the cells utilize a cryptic 5′ splice site within the second exon (codons for aa 131/132) as donor site and the upstream small t antigen 3′ splice site as the acceptor site. Since these sites are in an inverted order on the pre-mRNA, two *Bst/Bam* transcripts are required to generate one T1 mRNA molecule. HeLa cell nuclear extracts also performed the *trans*-splicing reaction *in vitro*.

Key words: mammalian cells/mRNA/SV40 T antigen/ *trans*-splicing

Introduction

Splicing is a complex nuclear RNA processing event, where different exons from pre-mRNA molecules are joined together. Utilizing different alternative 5′ or 3′ splice sites, multiple protein isoforms can be generated from a common primary transcript (van Santen and Spritz, 1986; Adami and Babiss, 1991; Gattoni *et al.*, 1991; Guo *et al.*, 1991; reviewed by Horowitz and Krainer, 1994; Sharp 1994).

New kinds of mRNA molecules and proteins can also be generated by *trans*-splicing. *Trans*-splicing is a post-transcriptional processing event that joins RNA segments of two independent transcripts together to generate new functional mRNA species. This mechanism was first demonstrated in *Trypanosoma brucei* (Murphy *et al.*, 1986; Sutton and Boothroyd, 1986). In these cells, a 39 nt 'mini-exon', derived from 140 nt small leader (SL) RNAs is *trans*-spliced to all pre-mRNA molecules (reviewed by Bonen, 1993; Ullu *et al.*, 1993). SL RNA *trans*-splicing also occurs in nematodes (Krause and Hirsh, 1987; Blumenthal and Thomas, 1988; Bruzik *et al.*, 1988; Nilsen, 1993), trematodes (Rajkovic *et al.*, 1990) and in *Euglena* cells (Tessier *et al.*, 1991). This nuclear RNA processing step resembles *cis*-splicing, with the formation of branched intermediates but without the participation of the U1 and U5 snRNPs, known to be essential for *cis*-splicing (reviewed by Agabian, 1990; Bonen, 1993; Nilsen, 1993; Watkins *et al.*, 1994). Interestingly, SL RNA *trans*-splicing was also demonstrable in a heterologous cell system after transfection of the appropriate nematode DNA constructs into Cos cells (Bruzik and Maniatis, 1992).

Trans-splicing has also been demonstrated in plant cell organelles (Koller *et al.*, 1987; Kück *et al.*, 1987; Chapdelaine and Bonen, 1991; reviewed by Sharp, 1991). In these organelles, intron splicing is self-catalytic: both *cis*- and *trans*-splicing reactions occur without the participation of U snRNPs or other nuclear splicing cofactors (Chapdelaine and Bonen, 1991).

In mammalian cells, RNA processing by *trans*-splicing was also suggested by computer analysis (Dandekar and Sibbald, 1990) and by different *in vitro* and *in vivo* studies. Using HeLa cell nuclear extracts and short synthetic RNA molecules, it was demonstrated that these cells have the ability to join RNA segments from two different precursor molecules together by *trans*-splicing (Konarska *et al.*, 1985; Solnick, 1985). Furthermore, cDNA sequencing experiments supported the proposal that *trans*-splicing might account for mRNA generation *in vivo*, but alternative *cis*-splicing could not be entirely excluded (Joseph *et al.*, 1991; Shimizu *et al.*, 1991; Sullivan *et al.*, 1991).

While analysing the oncogenic potential of the early SV40 DNA region, we obtained conclusive experimental evidence that mammalian cells can generate functional mRNA molecules by *trans*-splicing.

The early SV40 region encodes for two related proteins, the large T antigen and the small t antigen, generated by alternative *cis*-splicing utilizing two different 5′ donor sites and one common 3′ acceptor site. To obtain specific information about which of the multiple T antigen functions are essential for cell transformation, various early SV40 DNA fragments were microinjected into tissue culture cells (Graessmann *et al.*, 1984). Maximally transformed cells were obtained after the transfer of the early SV40 DNA *Bst*XI/*Bam*HI (*Bst/Bam*) fragment into rat cells. This DNA segment encodes exclusively for the second T antigen exon (amino acid 83–708) and contains the entire small t antigen intron while lacking the large T antigen 5′ splice site (Figure 1). Transformed cells synthesize a truncated version of the T antigen, the T1 antigen. The T1 antigen has no direct sequence homology at the DNA level in the transformed cells. Synthesis of the corresponding mRNA (T1 mRNA) occurs by *trans*-splicing. To generate the T1 mRNA, the cells utilize a cryptic 5′ splice site within the second exon of the T antigen (codons for aa 131/132) and the authentic 3′ splice site common to both T and t antigen. Since these two sites are in an inverted order in the pre-mRNA (3′–5′ instead of 5′–3′) two separate *Bst/Bam* transcripts are

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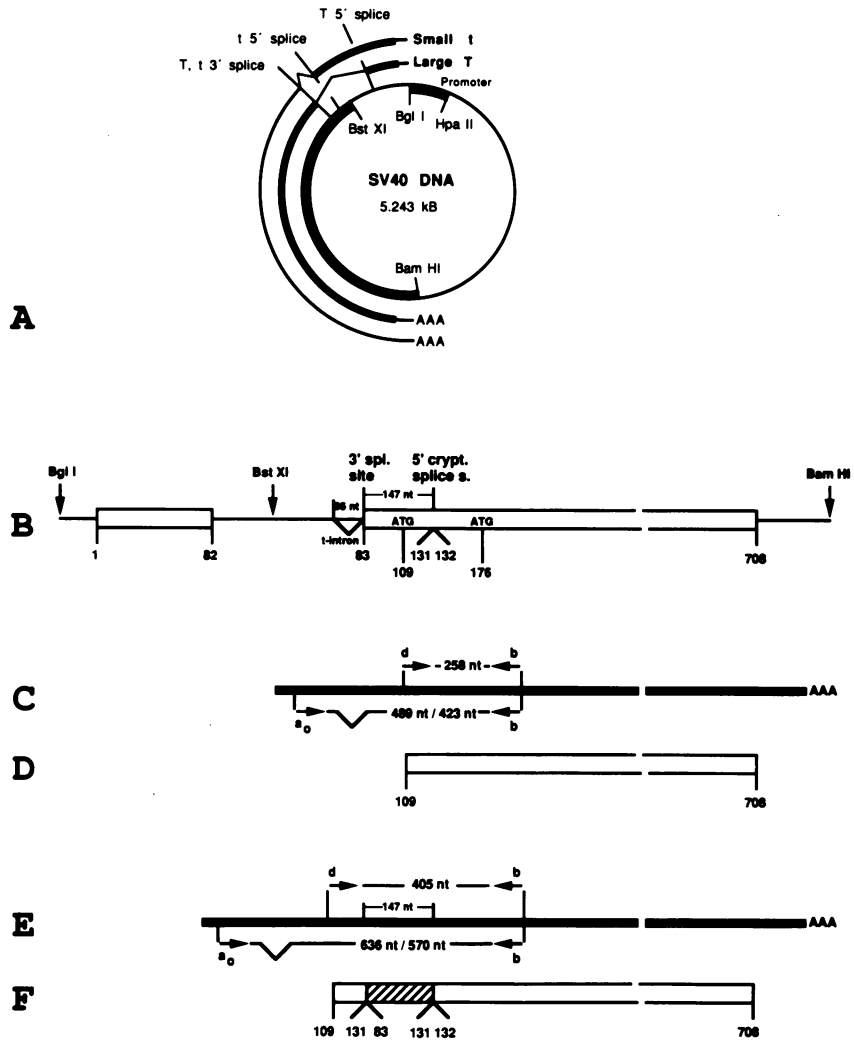


Fig. 1. (**A**) Diagram of the SV40 genome; the positions of the early SV40 promoter fragment (*Hpa*II/*Bgl*I) and of the *Bst*XI/*Bam*HI DNA fragment used for generation of the p14 construct are indicated by the bold segments. (**B**) Diagram of the large T antigen coding region: the first exon contains aa 1–82 and the second exon aa 83–708. The cryptic 5′ splice site (codons for aa 131/132) is located 147 nt downstream from the 3′ splice site used for excision of the large T and the small t antigen introns. (**C**) Represents the *Bst*XI/*Bam*HI transcript with the 66 nt small t antigen intron; the position and the genomic distance of the primers d–b and a_0–b are indicated (after excision of the small t antigen intron the distance between the primers a_0 and b is 423 nt). (**D**) Shows the T2 antigen with amino acid sequence 109–708. (**E**) Represents the T1 mRNA with the 147 nt insertion encoding for the 49 amino acids 83–131 (see F). The distance between the primers d and b is 405 nt; the distance between the primers a_0 and b is 636 nt and, after excision of the small t antigen intron, it is 570 nt. Figure 7 shows how the T1 mRNA is generated by *trans*-splicing. (**F**) Shows the T1 antigen with the amino acid sequence 109–131 followed by the sequence 83–708.

required to generate one T1 mRNA molecule by *trans*-splicing.

To confirm further that mammalian cells have the capability to perform the *trans*-splicing reaction, the *Bst*/*Bam* RNA (cRNA) was synthesized *in vitro*. Using HeLa cell nuclear extracts for *in vitro* RNA processing, the same *trans*-splice product was obtained as in the transformed cells.

Results

The second exon of the early SV40 large T antigen can generate two truncated T antigens, the T2 and T1 antigen

To analyse the transformation potential of the second exon of the SV40 T antigen, the plasmid p14 was constructed. This plasmid contains the early SV40 promoter (*Hpa*II/*Bgl*I segment), and the *Bst*XI/*Bam*HI (*Bst*/*Bam*) segment of the SV40 DNA (Figure 1A). This *Bst*/*Bam* fragment contains the distal half of the large T antigen intron, the entire small t antigen intron and the coding sequence for the second exon of the T antigen. Following microinjection of the p14 DNA into the nuclei of rat 2 cells, two categories of T antigen-positive cell clones were obtained. Cells of the first category (e.g. clone p14/5) synthesized a truncated T antigen of ~76 kDa (T2 antigen) (Figure 2) and exhibited the morphology and growth characteristics of the parental rat 2 cells. These cells did not grow in soft agar and did not form tumours after injection into nude mice. Cells of the second category (e.g. clone p14/2) synthesized, in addition to the T2 antigen, a second truncated T antigen (T1 antigen) of ~82 kDa (Figure 2). These cells exhibited all the characteristics of transformed cells, including tumour formation in nude mice. Since only those cells that synthesized the T1 antigen were malignantly transformed, the question of interest was:

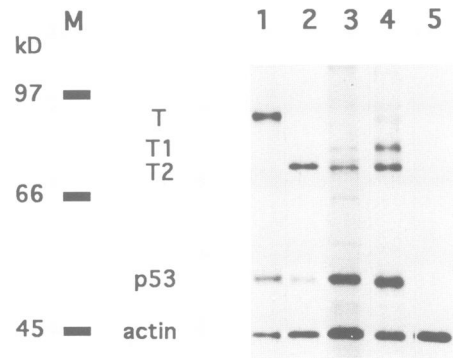
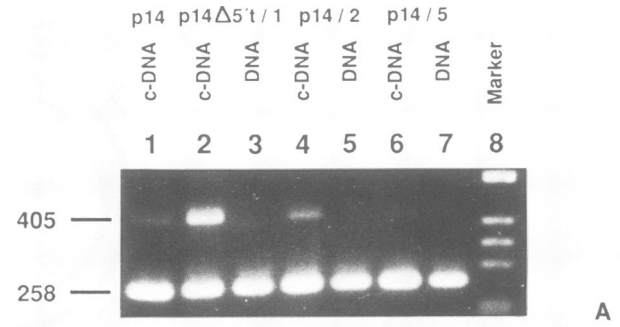


Fig. 2. Electrophoretic mobility of large T, T1 and T2 antigens. (1) T antigen isolated from SV40-transformed rat 2 cells; (2) T2 antigen isolated from the clone p14/5 cells; (3) T1 and T2 antigen isolated from clone p14/2 cells; (4) T1 and T2 antigens isolated from clone p14TΔ-5′t/1 cells; (5) control: rat 2 cells. The T antigens were immunoprecipitated with hamster anti-T serum and separated on a 7.5% polyacrylamide–SDS gel. The positions of the Coomassie Blue-stained size markers (M) are shown, as are the positions of the proteins p53 and actin which are co-precipitated with the T antigen.



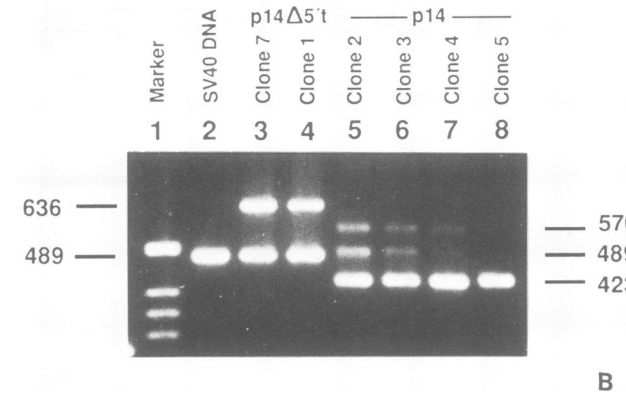


Fig. 3. (**A**) Agarose gel electrophoresis of PCR products generated with the primers d and b from cDNAs and genomic DNAs from the different cell lines. (1) cDNA PCR from rat 2 cells 48 h after transfection of p14 DNA; (2) cDNA and (3) genomic DNA PCRs from p14Δ-5′t/1 cells; (4) cDNA and (5) genomic DNA PCRs from p14/2 cells; (6) cDNA and (7) genomic DNA PCRs from p14/5 cells; (8) DNA size marker (BRL), the top fragment has 513 bp. The 405 bp cDNA PCR fragment is derived from the T1 mRNA, the 258 bp cDNA PCR fragment is generated from the T2 mRNA and the precursor RNA. The exact sizes of the PCR products were determined by DNA sequencing (Figure 4A and B). (**B**) PCR products generated with the primers a_0 and b from cDNAs of different cell lines. (1) DNA size marker; (2) PCR from SV40 DNA; (3) and (4) cDNA PCRs from p14Δ-5′t/7 and p14Δ-5′t//1 cells; (5) – (8) cDNA PCRs from cell clones p14/2, p14/3, p14/4 and p14/5. The size of the 489 bp fragment corresponds to the genomic distance between the primers a_0 and b; this fragment is derived from the unspliced precursor RNA. The 423 bp PCR segment is derived from the T2 mRNA after splicing of the proximal 66 nt small t antigen intron. The 570 bp cDNA PCR segment is generated from the T1 mRNA after *trans*-splicing and subsequent *cis*-splicing of the proximal small t antigen intron in the p14 cells. The 636 bp cDNA PCR fragment is derived from the T1 mRNA in the p14Δ-5′t cells. This PCR product still contains the proximal small t antigen intron, which cannot be removed in these cells, the fragment therefore is 66 nt larger than the PCR product derived from p14 cell clones (lanes 5–7).

what is the difference between the two kinds of truncated T antigen molecules? The size difference of ~6 kDa indicated that the T1 antigen is ~40–60 amino acids larger than the T2 antigen. Since it was possible to precipitate both the T1 antigen and the T2 antigen with antibodies that selectively recognized the C-terminal part of the large T antigen, we assumed that they would differ in their N-terminal portion, and that translation of the corresponding mRNA would start with different AUGs. As shown in Figure 1B, amino acid 109 is the first and amino acid 176 is the second methionine in the N-terminal part of the second T antigen exon. Our initial assumption was that the T1 antigen contains the wild-type T antigen amino acid sequence 109–708 and the T2 antigen the amino acid sequence 176–708 (Figure 1B). To test this hypothesis, the T1 and T2 antigens were isolated and subjected to N-terminal protein microsequencing. By this method, we were able to determine the first 12 amino acids of both truncated T antigen molecules. These experiments revealed that the N-terminal part of the T1 and T2 antigens were identical. This identical sequence comprised the amino acids 109–120 of the wild-type SV40 T antigen (Figure 1D and F, Figure 4A and B). This meant that the size difference between T1 and T2 antigen (Figure 2) was not due to the use of an alternative translation initiation signal.

We therefore postulated that the T1 and T2 antigens in clone p14/2 cells were generated from two different mRNA species (termed here T1 mRNA and T2 mRNA). Northern blot analysis of the clone p14/2 mRNA showed an SV40-specific mRNA band of ~2.2–2.3 kb, but discrimination between T1 mRNA and T2 mRNA was not possible at this point (data not shown). In order to distinguish between T1 and T2 mRNA, the mRNA was isolated from clone p14/2 and clone p14/5 cells and converted into cDNA. Using different sets of SV40-specific primers, the entire cDNA of the p14/2 and the p14/5 cells was characterized by PCR analysis and DNA sequencing. With the p14/5 cDNA as template, all PCR products obtained were identical in size and sequence, as predicted from the wild-type SV40 DNA as shown for the primer combination d and b (Figure 1E, Figure 3A, lane 6, Figure 4A). Therefore, the clone p14/5 cells contained only one SV40-specific mRNA, the T2 mRNA. The translation product of this mRNA, the T2 antigen (Figure 2, lane 2), contained the wild-type T antigen amino acid sequence 109–708 (Figure 1D). The T2 antigen lacked the first 27 amino acids of the second T antigen exon, which are proximal to the translation initiation AUG (methionine 109).

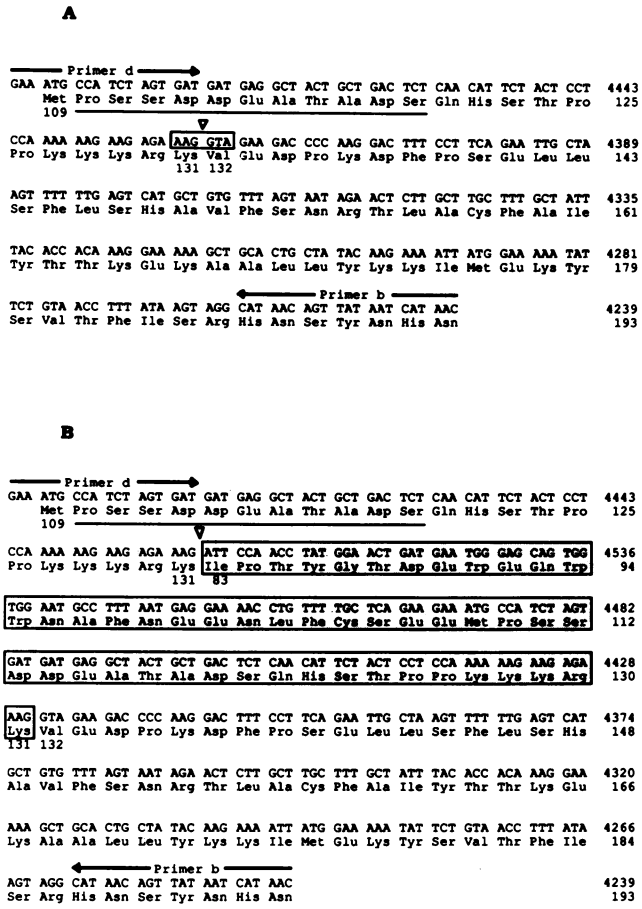


Fig. 4. Sequence analysis of (**A**) the T2 cDNA 258 bp PCR product and (**B**) the T1 cDNA 405 bp PCR product. The cDNAs were PCR amplified between the primers d and b (horizontal arrows), then cloned and sequenced. The upper numbers in the right-hand margin are the nucleotide numbers, the lower ones are the amino acid numbers, in accordance with the SV40 nomenclature. The underlined amino acids 109–120 of the T2 (A) and T1 antigen (B) were confirmed by protein microsequencing. (A) The cryptic 5′ splice site between the nucleotides coding for Lys131 and Val132 (AAG/GTA) is indicated; the triangle shows the cleavage site. (B) The framed area contains the 147 nt long stretch (4571–4425) coding in the T1 antigen for the wild-type T antigen aa 83–131.

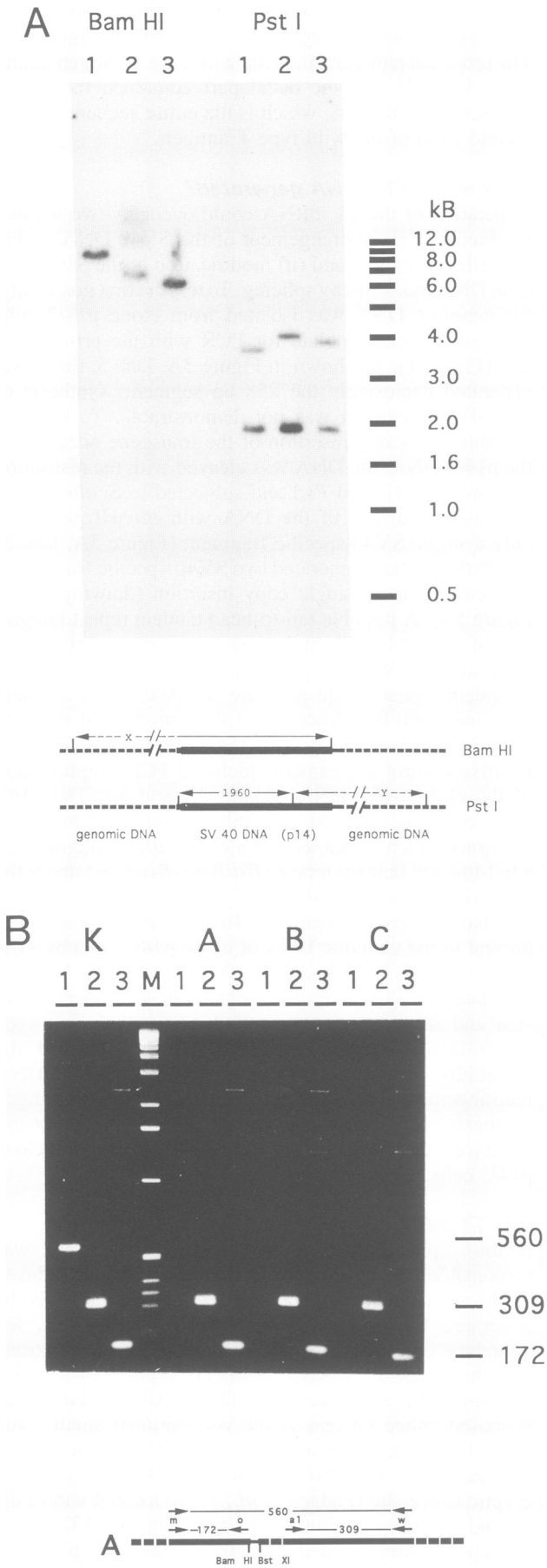
However, with the p14/2 cDNA as template and the primers d and b (see Table I), two SV40-specific fragments were obtained. As indicated in Figure 1C, the primers d and b are specific for the proximal part of the *Bst/Bam* segment and have a genomic distance of 258 bp. With these primers, PCR generated not only the expected 258 bp but an additional 405 bp segment as well (Figure 3A, lane 4). DNA sequencing experiments confirmed that the 258 bp segment was identical to the SV40 wild-type DNA sequence 4496–4239 (Figure 4A). We also cloned and sequenced the 405 bp segment. These experiments revealed that this DNA fragment contained the same 258 bp sequence but included, in addition, a 147 bp insertion between nucleotides 4425 and 4424 (T antigen aa 131/132). This 147 bp insertion corresponded exactly to the wild-type SV40 nucleotide sequence 4571–4425, encoding the 49 wild-type T antigen amino acids 83–131 (Figure 1B). Therefore, the clone p14/2 cells had synthesized two SV40-specific mRNA species: the T2 mRNA and the 147 nucleotide larger T1 mRNA. The translation product of the T1 mRNA, the T1 antigen, consisted of two distinct wild-type T antigen segments, as shown in Figure 7B. The proximal part contained the wild-type T antigen amino acids 109–131 and the distal part contained the amino acid sequence 83–708, which is the entire sequence of the second exon of the wild-type T antigen.

How is the T1 mRNA generated?

Generation of the T1 mRNA could occur by two distinct mechanisms: (i) rearrangement of the SV40 DNA within the cellular genome and (ii) modification of the SV40 *Bst/Bam* DNA transcript by splicing. To test the first possibility, the genomic DNA was isolated from clone p14/2 cells and used as the template for PCR with the primer pair d–b (Figure 1). As shown in Figure 3A, lane 5, this DNA generated exclusively the 258 bp segment; synthesis of the 405 bp segment was not demonstrable. To test if a possible multicopy insertion of the transgene occurred in the p14/2 DNA, the DNA was cleaved with the restriction enzymes *Bam*HI and *Pst*I and subjected to Southern blot analysis. Treatment of the DNA with *Bam*HI generated only a single SV40-specific fragment (Figure 5A, lane 3) and *Pst*I treatment generated two SV40-specific fragments, as predicted for a single copy insertion (drawing below Figure 5A). A possible tail-to-head tandem repeat integration of two p14 DNA molecules was further excluded by PCR analysis. With the primer pair m and w (Figure 5B), a tandem repeat would generate an SV40 DNA segment of at least 560 bp. However no PCR product of this size or larger was obtained with the genomic p14/2 DNA. Positive control experiments included PCR amplification of the clone p16T DNA (Figure 5B and K). The p16T cell clone was obtained after microinjection of the p16T construct which contained the SV40 *Bst/Bam* fragment as a tail-to-head tandem repeat (*Bst/Bam–Bst/Bam*) under the transcriptional control of the early SV40 promoter. Finally, we characterized the entire SV40 DNA *Bst/Bam* segment present in the genomic DNA of clone p14/2 cells by PCR analysis and DNA sequencing. With the cellular DNA as template, all PCR fragments obtained were identical in size and in sequence, as predicted from the wild-type SV40 DNA. These experiments clearly excluded the possibility of a direct 147 bp insertion by SV40 DNA rearrangement as the reason for T1 mRNA and T1 antigen synthesis. This meant that the T1 antigen transcript did not have a direct equivalent at the DNA level in the clone p14/2 cells.

The T1 mRNA is generated by trans-splicing

It thus appears that an RNA processing event was responsible for the formation of the T1 mRNA. As deduced from our sequencing data, the T1 mRNA can only be generated by splicing. As shown in Figure 4B, the boundary sequence (.AAG/AUU.) between the proximal and the distal T1 mRNA segments represents a splice junction. The 3′ splice site (.UAG/AUU.) used in the proposed splice process is the conventional small t and large T antigen splice acceptor site. The 5′ splice donor site sequence (.AAG/GUAGAA.), however, is a potential cryptic splice site (Padgett *et al.*, 1986) located within the second T antigen exon (codons for aa 131/132). This sequence is similar to the small t antigen 5′ splice site (.AAG/GUAAAU.).



Since this cryptic 5′ splice donor site is not upstream but 147 nucleotides downstream from the 3′ splice acceptor site, generation of the T1 mRNA by splicing requires two separate 2.2 kb SV40 *Bst*/*Bam* transcripts. Therefore, in the clone p14/2 cells, the T1 mRNA can only be generated by *trans*-splicing. Synthesis of the T1 mRNA was also demonstrable in rat 2 cells 48 h after transfection of the p14 DNA (Figure 3A, lane 1).

Our *trans*-splice model (Figure 7) proposes that two identical pre-mRNA molecules are used to generate one T1 mRNA molecule. In this *trans*-splice process, the first pre-mRNA molecule (pre-mRNA A) provides the cryptic 5′ splice site and the T antigen amino acid sequence 109–131 and the second pre-mRNA molecule (pre-mRNA B) provides the 3′ splice site and the amino acid sequence 83–708.

Trans-splicing is in competition with excision of the small t antigen intron

Although cells of both clones p14/2 and p14/5 contained one intact copy of the p14 DNA, clone p14/5 cells synthesized only the T2 mRNA and the T2 antigen, while clone p14/2 cells produced both the T1 and T2 mRNAs and the corresponding truncated T antigens. Since the 3′ splice acceptor site of the small t antigen intron is also used for *trans*-splicing, excision of this intron prevents subsequent T1 mRNA formation by *trans*-splicing. Our hypothesis was that clone p14/5 cells remove the 66 nt t antigen intron more efficiently than do clone p14/2 cells. To test this hypothesis, we analysed t antigen splicing in clone p14/2 and clone p14/5 cells. Small t antigen splicing and *trans*-splicing can be followed by cDNA PCR with the primers a_o and b (see Table I), which bind upstream and downstream respectively of the small t antigen intron (Figure 1C, E). We deduced from the SV40 DNA sequence that the PCR would generate a 489 bp fragment from unspliced precursor RNA, a 423 bp fragment when the 66 nt small t antigen intron had been removed (Figure 1C) and a 570 bp fragment from the *trans*-spliced T1 mRNA (Figure1 E). As shown in Figure 3B lane 8, with the clone p14/5 cDNA as template only the 423 bp DNA fragment was generated in detectable amounts, while all

Fig. 5. (**A**) Southern blot analysis of cellular DNA. The genomic DNA was isolated from (1) p14Δ-5′t/1 cells, (2) p14Δ-5′t/7 cells and (3) p14/2 cells. The DNA was restricted either with *Bam*HI or *Pst*I endonuclease. The blot was hybridized with ^{32}P-labelled SV40 DNA. The drawing below indicates the cleavage sites of *Bam*HI or *Pst*I within the p14 DNA and the genomic DNA. As shown in the drawing, a single copy of the transfected DNA within the genomic DNA generated one fragment of variable size after *Bam*HI digestion and two distinct fragments, one of 1960 bp and another of variable size, after treatment with the *Pst*I endonuclease. (**B**) PCR analysis of p14/2, p14Δ-5′t/1 and p14Δ-5′t/7 genomic DNAs to exclude tandem integrations. With the primer combination m and w, an SV40 DNA tandem integration can be detected by PCR analysis, as shown for the p16T genomic DNA as a positive control in the PCR (K, lane 1): a 560 bp fragment was obtained. In contrast, clone p14Δ-5′t/1 DNA (A, lane 1), clone p14Δ-5′t/7 DNA (B, lane 1) and clone p14/2 DNA (C, lane1) did not generate a PCR fragment with the primers m and w, excluding SV40 DNA tandem integrations up to 5–10 kb distance in these cell lines. The primers m–o and a_1–w generated PCR products of 172 and 309 bp. These pairs were used as internal control (K, A, B and C, lanes 2 and 3), confirming binding of the primers m and w to the cellular SV40 DNA. The diagram below (A) represents a part of the SV40 DNA tandem repeat in the clone p16T cells showing the binding sites of the primers used in the PCR.

Table I. SV40 DNA-specific primers used in the PCRs

Index	Nucleotide sequence (5′–3′)	5′ end of primer on SV40	Orientation
g	GCAAAGATGGATAAAGTTT	5169	sense
a_o	TGTGGTTTGGACTTGATC	4727	sense
a_1	GACAAACTACCTACAGAG	4670	sense
x (Δt)	TCTAAGCGCAATATAAAATTTTTAAGTG	4643	sense
d	GAAATGCCATCTAGTGAT	4496	sense
m	CCTGAACCTGAAACATAA	2708	sense
r	GCTTTAAATCTCTGTAGG	4644	antisense
c	ATCACTAGATGGCATTTC	4479	antisense
w	ACTAAACACAGCATGACT	4362	antisense
b	GTTATGATTATAACTGTTATG	4239	antisense
k	CTCTGCTTCTTCTGGGTT	4029	antisense
s	AATGTTGTACACCATGCA	3570	antisense
l	CATGGTGACTATTCCAGG	3234	antisense
o	CCAGACATGATAAGATAC	2537	antisense

Orientation: sense = sequence analogous to RNA sequence, antisense = sequence complementary to RNA sequence; the underlined sequence is a mutated SV40 triplet.

the three 423, 489 and 570 bp PCR fragments were obtained with the p14/2 cDNA (lane 5). The p14/3 (lane 6) and p14/4 (lane 7) are two further T1 antigen-positive cell lines. DNA sequencing confirmed that the 489 bp fragment was derived from unspliced precursor RNA, the 423 bp fragment from spliced T2 mRNA and the 570 bp cDNA PCR fragment from the T1 mRNA after small t antigen splicing (Figure 1E). These results strongly indicate that efficient excision of the small t antigen intron is in competition with *trans*-splicing.

Consequently, the *trans*-splice efficiency should increase when small t antigen *cis*-splicing is inhibited. To investigate this possibility, we destroyed the small t antigen 5′ splice site within the p14 DNA by site-directed mutagenesis (GTA was converted to CGC, Figure 7A), generating the plasmid p14Δ-5′t. The p14Δ-5′t DNA was then co-transfected with the pHSV106 DNA (which contains the HSV *tk* gene) into thymidine kinase-negative rat 2 cells, and HAT medium-resistant, T1 antigen-positive cell clones were isolated (e.g. clones p14Δ-5′t/1 and p14Δ-5′t/7). As described for the genomic DNA of the p14/2 cell clone, restriction enzyme and PCR analysis of the p14Δ-5′t/1 DNA excluded a possible multiple copy insertion (Figure 5A, lane 1) or a tandem integration of the p14Δ-5′t DNA (Figure 5B, lane A1). For PCR analysis, the RNA was isolated from the p14Δ-5′t/1 and p14Δ-5′t/7 cells, converted into cDNA and subjected to PCR using the primers a_o and b. The experiments confirmed that the mutation of the small t antigen 5′ splice site entirely abolished small t antigen *cis*-splicing and significantly increased the *trans*-splicing efficiency. As shown for both cell lines (Figure 3B, lanes 3 and 4), the specific 423 bp cDNA PCR product was not generated, verifying that small t antigen *cis*-splicing did not occur in these cells. In contrast, the T1 mRNA was synthesized in these clones in large amounts, as shown by efficient synthesis of the 636 bp cDNA PCR segment. DNA sequencing confirmed that the 636 bp PCR segment was generated from the T1 mRNA, which still contained the small t antigen intron at the proximal part (Figure 1E). Utilizing the primer pair d and b for cDNA PCR analysis, we were able to demonstrate that the *trans*-splice efficiency increased ~5-fold in all p14Δ-5′t cell lines analysed when compared with the

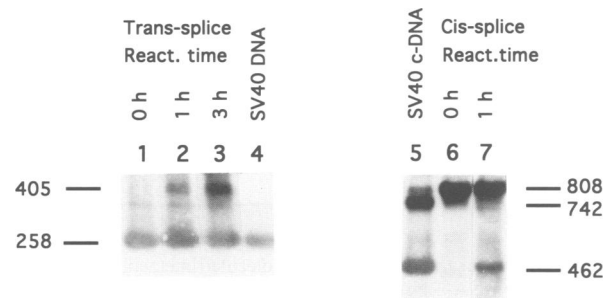


Fig. 6. *In vitro trans*-splicing of the pT7-14Δ-5′t cRNA with HeLa cell nuclear extracts. Lanes 1–3: cDNA PCR products obtained 0–3 h after incubation of the pT7-14Δ-5′t cRNA in HeLa cell extract; T1 mRNA synthesis is indicated by generation of the 405 bp DNA fragment in the PCR. Lane 4: PCR product obtained with SV40 DNA as template (control). For these PCR amplifications the primers d and b (Figures 1 and 7B) were used. The right side of the figure shows *in vitro cis*-splicing of large T antigen in HeLa cell nuclear extracts using pT7-SVT/t cRNA as substrate. Lanes 6–7: cDNA PCR products obtained 0 and 1 h after incubation of the pT7-SVT/t cRNA in HeLa cell extract. Lane 5: PCR products of SV40 cDNA; total RNA was isolated from wild-type SV40 DNA transformed cells, converted into cDNA and amplified using the primer pair g and w. The blot was hybridized with [^{32}P]SV40 DNA.

trans-splice efficiency of the clone p14/2 cells. This was indicated by the amount of the 405 bp product synthesized (Figure 3A, lanes 2 and 4). The same increase in the *trans*-splice efficiency was also demonstrable when T1 antigen synthesis was analysed by gel electrophoresis (Figure 2, lanes 3 and 4).

In vitro synthesized RNA is trans-spliced in HeLa cell extracts

To determine whether *in vitro* synthesized *Bst/Bam* RNA (cRNA) can be *trans*-spliced by HeLa cell nuclear extracts, the early SV40 promoter of the p14Δ-5′t DNA was replaced by the T7 promoter, generating pT7-14Δ-5′t, and this construct was transcribed *in vitro* by means of the T7 RNA polymerase. The purified 2.2 kb T7-14Δ5′t cRNA (50 ng) was added to the nuclear extracts and the splicing reaction was terminated after 0, 1 and 3 h by phenol extraction. The extracted RNA was then converted into cDNA and analysed by PCR. As shown in Figure 6, lanes

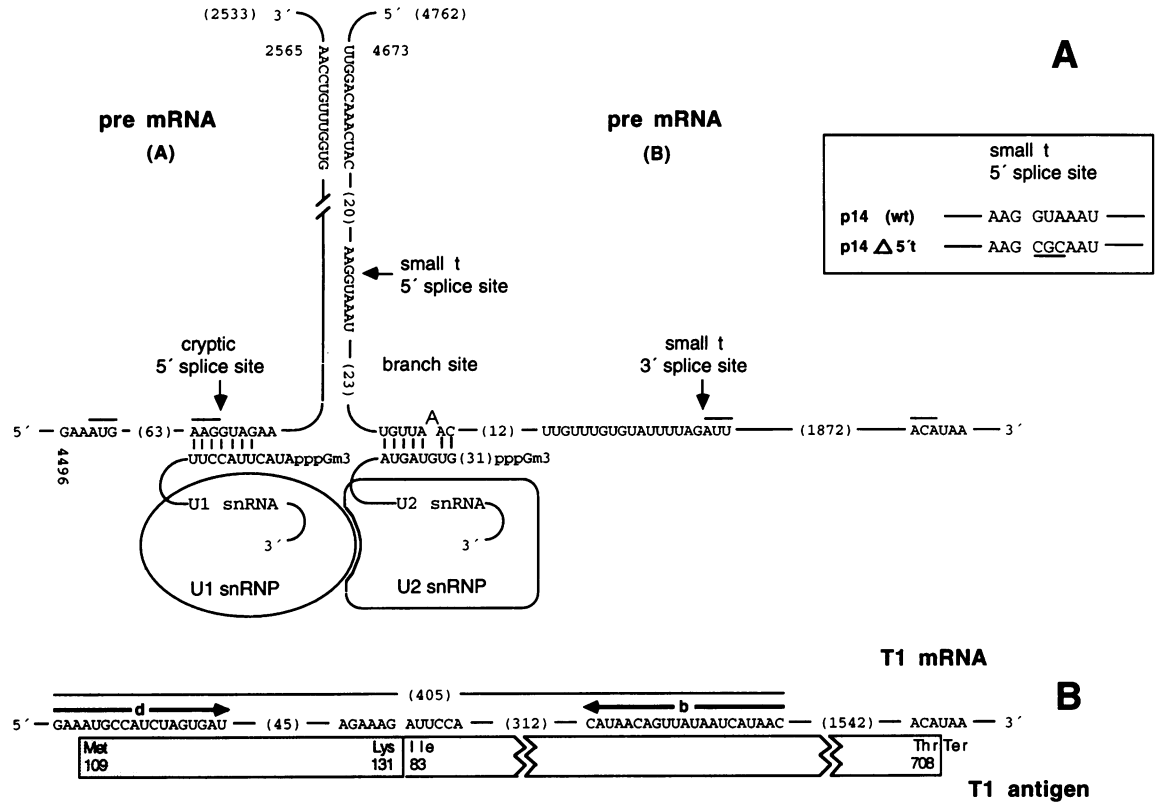


Fig. 7. Model for *trans*-splicing and molecular interactions between two pre-mRNA molecules in the *trans*-splicing pre-spliceosome complex. (**A**) Possible RNA–RNA interactions either by a direct base pairing or indirect base pairing at the stage of the (pre)spliceosome complex. The figure also shows the small t antigen 5′ splice site; utilization of this site prevents the use of the cryptic 5′ splice site on the other RNA molecule by *trans*-splicing. One of the three possible branch sites in the early SV40 pre-mRNA is shown (Noble *et al.*, 1987). The framed area on the right side of the picture shows the sequence of the small t 5′ splice site mutated in the pre-mRNA of p14Δ-5′t cells. (**B**) The T1 mRNA produced after *trans*-splicing and the binding sites of the primers d and b, which generate a 405 bp PCR product using the T1 cDNA as template. The translation start codon (Met109) and stop codon (after Thr708) for T1 antigen synthesis are also shown. The proximal part of the T1 antigen (aa 109–131) had been derived from the pre-mRNA molecule A, the distal part (aa 83–708) had been derived from the second pre-mRNA molecule B.

2 and 3, formation of T1 mRNA by *trans*-splicing *in vitro* was demonstrated by generation of the expected 405 bp PCR product using the primers d and b.

Control experiments included *cis*-splicing of *in vitro* synthesized wild-type early SV40 cRNA by HeLa cell nuclear extracts (Figure 6, lanes 6 and 7). For PCR amplification, the primers g and w were used. The 808 bp segment was derived from the unspliced RNA, the 742 bp segment was generated from the small t antigen-spliced RNA and the 462 bp segment from the large T antigen-spliced RNA. As shown in Figure 6, lane 7, HeLa cell extracts only performed large T, but not small t antigen *cis*-splicing, thus the 742 bp segment was not generated in the *in vitro* experiment. These results are in accordance with earlier studies from Noble *et al.* (1986).

Discussion

In this investigation, we have demonstrated that mammalian cells have the potential to generate functional mRNA molecules by *trans*-splicing. Rat cells transformed by the early SV40 *Bst/Bam* DNA fragment (e.g. clone p14/2) synthesize a truncated T antigen molecule, the T1 antigen (T1 mRNA), which does not have a direct sequence homology at the DNA level. This is shown by Southern blot, PCR analysis and by DNA sequencing. The *Bst/Bam* fragment encodes the second T antigen exon (aa 83–708) and contains the entire small t antigen intron (Figure 1).

To generate the T1 mRNA, the cells utilize a cryptic 5′ splice site within the second exon and the conventional 3′ splice site used for large T antigen and small t antigen *cis*-splicing. Since this 3′ splice acceptor site is not downstream but upstream from the cryptic 5′ splice site, generation of the T1 mRNA requires two unique *Bst/Bam* 2.2 kb pre-mRNA molecules. The cell lines used in the present investigation (e.g. clone p14/2) contained exclusively one copy of the transgene, a multicopy insertion or a direct tandem repeat of the p14 DNA was excluded by Southern blotting and PCR analysis (Figure 5A and B); generation of the T1 mRNA in the clone p14/2 was only possible by *trans*-splicing. For the *trans*-splice reaction, the donor and the acceptor splice sites are located on two separate 2.2 kb pre-mRNA molecules. Cells which contained a p14 DNA tandem repeat in a direct tail-to-head orientation (*Bst/Bam–Bst/Bam*) generated the T1 mRNA also by *cis*-splicing (e.g. 16T cell clone, Eul *et al.*, in preparation). To generate the T1 mRNA by this mechanism, the cells have to utilize the cryptic 5′ splice site from the first *Bst/Bam* segment and the 3′ splice acceptor site from the second segment of the same pre-mRNA molecule.

Trans-splicing was further demonstrated in *in vitro*

experiments with HeLa cell nuclear extracts (Figure 6). *In vitro* synthesized *Bst/Bam* RNA was also *trans*-spliced after microinjection into the nuclei of TC7 cells (Graessmann *et al.*, manuscript in preparation).

As a consequence of T1 mRNA formation by *trans*-splicing, the cells synthesize the T1 antigen and are converted into maximally transformed cells that grow in soft agar and form tumours after injection into nude mice. This observation again raises the question of why T1 antigen-positive cells are fully transformed while those cells which synthesize only the T2 antigen are not. One important difference between the two truncated T antigen molecules is that the T1 antigen contains the wild-type T antigen amino acid sequence 83–109 not present in the T2 antigen (Figure 1D and F). One property attributed to this T antigen domain is a high binding affinity for the tumour repressor protein Rb110 (De Caprio *et al.*, 1988; Ewen *et al.*, 1989). Further experiments will have to be conducted to evaluate whether this T antigen function is crucial for the maximal transformation mediated by the T1 antigen.

Since the *Bst/Bam* transcript also contains the intact small t antigen intron, excision of this intron is in competition with *trans*-splicing. The small t *cis*-spliced RNA molecules lack the 3′ splice acceptor site and therefore are unsuitable for *trans*-splicing. Interestingly, cells of some lines (e.g. p14/5 cells) performed small t antigen *cis*-splicing with a high efficiency and failed to synthesize the T1 mRNA and T1 antigen (Figures 2 and 3) in detectable amounts. However, at a high passage number (40–50), cells of these lines consistently started to synthesize the T1 antigen as well (data not shown). It has been demonstrated that small t antigen splicing efficiency depends upon the relative concentration of different splicing cofactors, such as the ASF/SF2 protein (Ge and Manley, 1990; Krainer *et al.*, 1990; Mayeda and Krainer, 1992). The expression rate of these factors is cell type-dependent (Ge and Manley, 1990) and we may assume that fluctuations of the expression rate may also occur within cells of established cell lines (e.g. rat 2 cells). It is tempting to speculate that clone p14/5 cells exhibit a higher expression rate of these proteins than clone p14/2 cells. To further demonstrate the competition between excision of the small t antigen intron and *trans*-splicing, the small t antigen 5′ splice site was destroyed by site-directed mutagenesis (p14Δ-5′t). As a consequence of this mutation, there was an ~5-fold increase in *trans*-splicing. (e.g. clone 14Δ-5′t/1 cells, Figures 2 and 3) .

Competition between *cis*- and *trans*-splicing is presumably the main reason why *trans*-splicing has not been reported in wild-type SV40-infected or SV40-transformed cells so far. In these cells, excision of the large T antigen intron is the dominant splicing process (van Santen and Spritz, 1986) which deletes the common small t and large T antigen 3′ splice site that is essential for *trans*-splicing. However, with our cDNA PCR experiments, we have been able to demonstrate that *trans*-splicing also occurs in wild-type SV40-transformed cells, although at a very low level (Eul *et al.*, in preparation).

Competition between *trans*- and *cis*-splicing has also been demonstrated in nematodes, which perform both RNA splice patterns simultaneously (Blumenthal and Thomas, 1988; Conrad *et al.*, 1993a,b; Nilsen, 1993). In these cells, *cis*-splicing is dominant when a protein coding precursor RNA contains a valid 5′ and 3′ splice site at its most proximal part. When, however, the 5′ splice site is deleted, *cis*-splicing is prevented and the remaining 3′ splice site combines with the 5′ splice site of an SL RNA molecule to generate a new mRNA molecule by *trans*-splicing (Conrad *et al.*, 1991, 1993a).

In nematodes (Blumenthal and Thomas, 1988; Bruzik *et al.*, 1988; Nilsen, 1993) and trypanosomes (Agabian, 1990; Bonen, 1993), *trans*-splicing differs from *cis*-splicing in several aspects. The SL RNA 'mini-exons' do not contain any protein coding sequences but provide a methylated cap structure for the protein coding acceptor RNA; the SL RNA–protein complex also replaces the U1 snRNP, which is known to be an absolute prerequisite for *cis*-splicing (Bruzik *et al.*, 1988; Bonen, 1993; Nilsen, 1993; Ullu *et al.*, 1993).

For *trans*-splicing in plant mitochondria and plastids, extensive base pairing within the introns of the two pre-mRNAs seems to be essential (Chapdelaine and Bonen, 1991; Knoop *et al.*, 1991; Sharp, 1991). These organelles do not contain U snRNAs and RNPs (Cech, 1986; Saldanha *et al.*, 1993) and the functions of the different snRNAs are replaced by a highly conserved RNA secondary structure specific for all group II introns (Sharp, 1991; Suchy and Schmelzer, 1991; Saldanha *et al.*, 1993).

In our model (Figure 7A) we propose that, in mammalian cells, generation of the T1 mRNA by *trans*-splicing differs from the *trans*-splice process in trypanosomes, nematodes and plant cell organelles. The mechanism of the *trans*-splice process presumably is similar to *cis*-splicing, requiring the formation of an equivalent pre-spliceosomal complex. Formation of the pre-spliceosomal complex may be facilitated by a direct base pairing between the two precursor mRNA molecules, as indicated in Figure 7A. We have not tested so far whether the base pairing is a prerequisite for T1 mRNA *trans*-splicing. It was demonstrated in *in vitro* experiments that intermolecular RNA–RNA hybridization increased the *trans*-splicing efficiency significantly (Konarska *et al.*, 1985). RNA–RNA association might be promoted by interaction of the pre-mRNA molecules with different hnRNP proteins (Burd and Dreyfuss, 1994; Portman and Dreyfuss, 1994) or it may occur during an early step in the assembly of the pre-spliceosome complex (Sharp, 1987). In analogy to *cis*-splicing, the U1 snRNP could bind via its snRNA to the cryptic 5′ splice site on the first pre-mRNA (molecule A) and the U2 snRNP to the branch site of the second pre-mRNA (molecule B). The donor 5′ splice site and the branch site of the two RNA molecules are brought together by a U1/U2 snRNP interaction/association (Mattaj *et al.*, 1986; Chabot and Steitz, 1987; Lutz and Alwine, 1994).

Although *trans*-splicing has been suggested as being a widespread RNA processing system among eukaryotic cells (Konarska *et al.*, 1985; Solnick, 1985; Sharp, 1987; Dandekar and Sibbald, 1990; Joseph *et al.*, 1991; Nigro *et al.*, 1991; Shimizu *et al.*, 1991; Sullivan *et al.*, 1991), no clear experimental evidence has been obtained so far that authentic mRNA molecules and functional proteins are generated in mammalian cells by *trans*-splicing. In our investigations, we have demonstrated that rat cells are able to generate the T1 antigen by means of *trans*-splicing. Our results further support the hypothesis that *trans*-

splicing is a regular RNA processing mechanism in mammalian cells. The best candidates for the *trans*-splice reaction are those donor pre-mRNA molecules, where the 5′ splice donor site is not followed by a functional 3′ splice site, which would favour a *cis*-splice reaction. Any candidate for an RNA acceptor molecule must still contain a functional 3′ splice acceptor site. In the case of the T1 mRNA, donor and acceptor RNA molecules are the same primary transcripts; but it may also be that independent transcripts, derived from different gene loci, are joined together to generate hybrid protein molecules through *trans*-splicing.

Materials and methods

Plasmid constructs

The pSVT/t plasmid contains the entire early SV40 region from the *Hpa*II to the *Bam*HI site inserted into the *Acc*I/*Bam*HI sites of pSPT 19 DNA (Pharmacia). The pSVTΔ-5′t contains the same SV40 region with the small t antigen 5′ splice site consensus sequence mutated (GTA→CGC). To inactivate the small t antigen 5′ splice site by primer-directed mutagenesis, the SV40 wild-type sequence 5169–4644 was amplified by PCR with the primers g and r (yielding a 526 bp segment) and the sequence 4643–4029 was amplified with the primer pair x and k (yielding a 615 bp segment) using the Vent polymerase (Biolabs). The primer x contained the mutated (3 nt) sequence. The purified PCR products were phosphorylated with ATP and T4 polynucleotide kinase. The 526 bp DNA was cleaved with *Bst*XI at nucleotide 4766, generating a 404 and a 122 bp fragment; the 615 bp DNA was cleaved with the endonuclease *Pfl*MI at nucleotide 4563, yielding a 80 bp and a 535 bp fragment. The 122 and 80 bp fragments were ligated and inserted into the *Bst*XI/*Pfl*MI site of the pSVT/t DNA, from which the *Bst*XI–*Pfl*MI segment (202 bp) had been removed previously by gel separation. Through this cloning strategy, the small t antigen 5′ splice site wild-type G/GTA sequence was converted into the G/CGC sequence, which created a new *Hha*I site that allowed the identification of positive pSVTΔ-5′t clones. DNA sequencing experiments confirmed that the small t antigen 5′ splice site was mutated in the pSVTΔ-5″t DNA and that the other nucleotides of the new inserted 202 bp *Bst*XI–*Pfl*MI segment were identical to the wild-type SV40 DNA sequence.

The p14 plasmid contains the SV40 promoter (*Hpa*II/*Bgl*I) and the *Bst*XI/*Bam*HI early SV40 DNA fragment (see Figure 1). For preparation of this construct, the pSVT/t DNA was cleaved with the *Sfi*I (*Bgl*I) and the *Bst*XI enzymes. After gel separation, the nucleotide overhangs of the plasmid DNA were removed by T4 polymerase in the presence of all four dNTPs and the blunt ends were ligated.

The p14Δ-5′t contains the same SV40 DNA segments as the p14 DNA but with the small t antigen 5′ splice site mutated; to obtain the p14Δ-5′t DNA, the pSVTΔ-5′t DNA was cleaved with the *Sfi*I (*Bgl*I) and the *Bst*XI enzyme and religated as described above for preparation of the p14 DNA.

The pT7-SVT/t contains the entire early SV40 coding region (*Bgl*I/*Bam*HI) under the transcriptional control of the T7 promoter. To obtain this plasmid, the SV40 promoter was removed from the pSVT/t DNA by *Sfi*I and *Nco*I digestion; the restriction sites of the plasmid DNA were converted into blunt ends by T4 polymerase in the presence of the dNTPs and the blunt ends were then ligated.

The pT7-14Δ-5′t contains the *Bst*XI/*Bam*HI early SV40 DNA fragment with the small t 5′ splice site deleted under the transcriptional control of the T7 promoter. To obtain the pT7-14Δ-5′t DNA, the pSVTΔ-5′t plasmid was cleaved with *Nco*I and *Bst*XI. After removal of the 820 bp *Nco*I/*Bst*XI SV40 DNA fragment, which contains the SV40 promoter, the first T antigen exon and the proximal part of the large T intron, the two restriction sites of the plasmid DNA were converted to blunt ends and re-ligated as described above.

The constructs were propagated in *Escherichai coli* cells (NM 522) and the crucial modifications were confirmed by DNA sequencing.

Cells and DNA transfer

TK-negative rat 2 cells were cultivated in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal calf serum (Graessmann *et al.*, 1984). Microinjection and DNA transfection were performed as described elsewhere (Graessmann *et al.*, 1981; Graessmann and Graessmann, 1983). The plasmids were co-transfected with the pHSV106 DNA, which contains the thymidine kinase gene. Positive rat 2 cell clones were selected in HAT medium (hypoxanthine, aminopterin and thymidine).

T, T1 and T2 antigen preparation

About 10^7 cells were incubated for 1 h starvation at 37°C in a methionine-free DMEM, then labelled for 2 h in the same medium with 20 μCi of [^{35}S]methionine. The cells were washed with phosphate-buffered saline (PBS) and lysed in 1% Nonidet P40 (NP-40), 150 mM NaCl, 10 mM β-mercaptoethanol and 20 mM Tris–HCl, pH 8.6 at 4°C for 30 min. For immunoprecipitation, either hamster anti-T serum or monoclonal antibodies which recognize exclusively the last 11 amino acids of the C-terminal part of the T antigen were used as described previously (Graessmann *et al.*, 1984). For N-terminal protein microsequencing, the antigens T1 and T2 were isolated from 10^8 cells as described above, separated on a 7.5% polyacrylamide–SDS gel and transferred to a membrane (Immobilon P, Millipore) by Western blotting. Areas containing the antigens were excised from the filter; the proteins were eluted and sequenced as described elsewhere (Hewick *et al.*, 1981).

Isolation of cellular DNA and RNA

The genomic DNA from 1–5×10^7 cells was isolated as described elsewhere (Graessmann *et al.*, 1979). Total cellular RNA was extracted from 10^7 cells and purified with the TRISOLV reagent (includes a guanidinium thiocyanate buffer and phenol) using the protocol recommended by the supplier (AGS). The RNA was further purified from remaining DNAs by two subsequent digests with RQ I DNase (RNase free, 10 U/ml) (Promega) in 50 mM Tris, 10 mM NaCl, 6 mM $MgCl_2$ and 10 mM $CaCl_2$, pH 7.9 at 37°C for 30 min.

Southern blot analysis

Genomic DNA (10 μg) was digested with 2 U restriction enzymes/μg DNA at 37°C for 4 h. The fragments were separated on a 1% agarose gel, transferred to a nylon filter (Hybond, Amersham) and UV-crosslinked. The hybridization was performed overnight in a buffer containing 1% BSA, 7% SDS, 0.5 M sodium phosphate, pH 7.3 and ^{32}P-labelled SV40 DNA at 65°C. The filter was washed with 1% SDS, 40 mM sodium phosphate, pH 7.3, at 65°C three times for 30 min. The blots were exposed for 1–7 days at –70°C (XAR-film Kodak). The PCR DNA fragments (see Table I) were separated on a 2% agarose gel, transferred to a nylon membrane and, after hybridization with ^{32}P-labelled SV40 DNA, exposed to a film for 15 min–6 h.

cDNA synthesis and PCR analysis

Total cellular RNA (5 μg) was converted into single-stranded cDNA using either 0.5 μg oligo(dT- 15) primer (Pharmacia) or 0.1 μg of SV40 DNA-specific primers (antisense orientation), 0.5 mM of each dNTP and 200 U MuMLV reverse transcriptase in 20 μl enzyme buffer (BRL) at 37°C (dT-15 primer) or 45°C (SV40-specific primer, see below) for 2 h.

The PCRs were performed as described in standard protocols either with 1/10 of the volume (2 μl) of the cDNA preparation or 1 μg of isolated genomic DNA, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 0.5 μg of each primer and 2.5 U *Taq*I DNA polymerase in 100 μl PCR buffer (Cetus/Perklin Elmer). The temperature profile used in 35 cycles of amplification (hot start PCR) was 1 min at 94°C, 1 min at 53°C and 2 min at 74°C per cycle. To analyse a possible tandem integration of the p14 DNA, the time for the amplification reaction at 74°C was increased to 10 min, so that it would be possible to detect a PCR product with a length up to 10 kb, as described elsewhere (Cheng *et al.*, 1994). The absence of detectable amounts of DNA in the total RNA preparation was always verified by PCRs without reverse transcriptase.

Cloning and sequencing of PCR products

PCR products of interest were purified by the Qiagen PCR purification method, phosphorylated at the 5′ ends with ATP and T4 polynucleotide kinase (BRL) in kinase buffer and cloned either in the dephosphorylated blunt end *Eco*RV site of pBluescript SK (+) (Stratagene) or in the *Sma*I site of pUC18 (Pharmacia). After ligation at 14°C overnight, transfection into competent *E.coli* cells (NM 522) and selection on LB ampicillin media, the isolated plasmid DNAs of ampicillin-resistant *E.coli* clones were analysed by restriction digests and analytical PCRs. Positive plasmid DNAs, containing the PCR product of interest, were sequenced directly by the dideoxy nucleotide method using the *Taq* Terminator Cycle Sequencing Kit from Applied Biosystems. The dye terminators

were used to label 0.5–1 μg of double-stranded DNA for sequencing analysis in the Applied Biosytems Model 373 A.

In vitro transcription

The pT7-SVT/t and the pT7-14Δ-5't plasmid DNA were linearized with *Sma*I, which cuts once in the multiple cloning site of the pSPT19 DNA 6 nt downstream of the *Bam*HI site. Linearized DNA (1 μg) was *in vitro* transcribed at 37°C for 2 h with 20 U of T7 RNA polymerase, the cap nucleotide [m7G(5')ppp(5')G] and the 4× NTPs in 20 μl buffer by the method of the supplier (Boehringer Mannheim, T7 Cap-Scribe). The reaction was terminated by EDTA (20 mM final concentration); the RNA was ethanol precipitated and purified from the plasmid DNA by two subsequent digests with 20 U RQ DNase I (RNase free, 10 U/ml) (Promega) in 50 mM Tris, 10 mM NaCl, 6 mM $MgCl_2$ and 10 mM $CaCl_2$, pH 7.9 in 25 μl at 37°C for 30 min, followed by phenol/chloroform extraction and ethanol precipitation. The RNA was redissolved in 10 mM Tris (pH 7.5) at a final concentration of 1 μg/μl. The quality and the correct size of the *in vitro* transcripts were controlled by agarose gel electrophoresis under denaturating conditions (formamide/formaldehyde buffer; Maniatis *et al.*, 1989) and by Northern blot analysis. The pT7-SVT/t DNA generated a 2.7 kb and the pT7-14Δ-5't DNA a 2.2 kb transcript as expected.

In vitro RNA splicing

In vitro transcribed RNA (5–50 ng) was used for the *in vitro* splicing experiments. The reaction mixture contained 0.5 mM ATP, 4 mM $MgCl_2$, 20 mM creatine phosphate, 2 U RNase inhibitor (BRL), 2 μg tRNA, 15 μl HeLa cells nuclear extract in 25 μl total volume, as described in other protocols (Noble *et al.*, 1986, 1987). The nuclear cell extract had been prepared as described by Dignam *et al.* (1983). The splicing reactions were performed at 30°C for 0–3 h. The splicing reaction was terminated by addition of 200 μl of TRISOLV reagent (AGS) and the RNA was further purified as described above. The isolated RNA was then converted into cDNA with 100 U MuMLV reverse transcriptase (BRL) using the SV40-specific primer l at 48°C for 1 h. The pT7-14Δ-5't cDNA was amplified by PCR with the primer pair d and b and the pT7-SVT/t cDNA with the primer pair g and w as described above. The blot was hybridized with the ^{32}P-labelled 2.2 kb *Bst*/*Bam* SV40 DNA fragment.

Acknowledgements

We thank E.Guhl for excellent technical assistance and V.Kruft for microsequencing of the T1 and T2 proteins. The HeLa cell nuclear extracts were a kind gift of A.Bindereif. This work was supported by the DFG, Gr 384/13–1.

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Received on August 29, 1994; revised on February 9, 1995