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Trans-splicing and alternative-tandem-*cis*-splicing: two ways by which mammalian cells generate a truncated SV40 T-antigen

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ABSTRACT

The early SV40 *Bst*XI–*Bam*HI (*Bst/Bam*) DNA fragment encodes exclusively for the second exon of the large T-antigen and contains the intact small t-antigen intron. Rat cells transformed by the p14T, a construct that carries the *Bst/Bam* DNA fragment as a tail-to-head tandem duplication, synthesize a truncated T-antigen (T1-antigen) without having a direct equivalent at the DNA level. Formation of the T1-mRNA occurs by means of two distinct mechanisms: alternative-tandem-*cis*-splicing and *trans*-splicing. To generate the T1-mRNA the cells utilize a cryptic 5′ splice site, located within the second exon of the large T-antigen and the regular small t-antigen 3′ splice site. Since these splice sites are in an inverted order two *Bst/Bam* transcripts are required to generate one T1-mRNA molecule. For alternative-tandem-*cis*-splicing the cells utilize a 4.4 kb pre-mRNA that contains the sequence of the entire *Bst/Bam* tandem repeat. The proximal *Bst/Bam* segment provides the 5′ donor splice site and the distal segment the 3′ acceptor site. This requires that the pre-mRNA not be cleaved after the RNA polymerase II has passed the polyadenylation signal of the proximal *Bst/Bam* DNA segment. Synthesis of the 4.4 kb pre-mRNA was demonstrable by RT–PCR but not by Northern blot analysis. For *trans*-splicing, the cells utilize two separate pre-mRNA molecules. One transcript provides the cryptic 5′ splice donor site and the other the 3′ splice acceptor site. To demonstrate this a three base pair deletion was introduced into the proximal *Bst/Bam* segment of the p14T DNA (p14TΔ-3) as a marker, destroying the recognition site for *Pfl*MI restriction enzyme. This deletion allowed the differentiation between the proximal and distal *Bst/Bam* segment. RT–PCR analysis and DNA sequencing confirmed that the p14TΔ-3 transformed cells generate the T1-mRNA by intra- and inter-molecular RNA splicing.

INTRODUCTION

In eukaryotic cells several processing steps are necessary to convert pre-mRNA molecules into mature mRNAs. This includes the formation of the cap structure at the 5′-end, addition of a poly(A) tail at the 3′ part and RNA splicing. The biological significance of these modification steps is not entirely clear, but there is experimental evidence that they have multiple functions and that they are fundamental for the regulation of gene expression. The cap structure, added during transcript elongation, is involved in nuclear mRNA export (1) and increases the translation efficiency (2–5). Polyadenylation, which protects the mRNA from 3′-end degradation, requires various structural elements, including the conserved hexanucleotide sequence AAUAAA (6–9). The polyadenylation signal mediates cleavage of the precursor mRNA during transcript elongation 10–30 nucleotides (nt) downstream from the AAUAAA site (9,10). Through splicing, different exons from one pre-mRNA molecule are joined together in order to create functional mRNA molecules. Utilizing different alternative 5′ or 3′ splice sites, multiple distinct protein isoforms can be generated from a primary transcript (11–15).

New kinds of mRNA molecules are also generated by *trans*-splicing (16–18). In lower eukaryotes such as trypanosomes, nematodes, trematodes and Euglena a small leader sequence (SL) RNA is added to the 5′ region of pre-mRNA molecules, providing the cap structure for all mRNAs. Addition of SL segments is further involved in the processing of polycistronic transcripts into monocistronic mRNA molecules. In nematodes the SL1 is preferentially attached to the free 5′-end of the pre-mRNA and internal cistrons receive mainly the SL2 segment, suggesting further selection mechanisms that distinguish between the external and internal acceptor splice sites (19). Trypanosomes do not contain introns and perform only SL-RNA *trans*-splicing. In nematodes *cis*-splicing and SL-RNA *trans*-splicing occur in parallel on the same pre-mRNA molecules (19). *Cis*-splicing and *trans*-splicing sites have the same splice site consensus sequences and utilize a similar splicing machinery. However, SL-RNA *trans*-splicing occurs without participation of the U1 and U5 spliceosomes, known to be essential for *cis*-splicing in nematodes and in mammalian cells (16–18,20). *Trans*-splicing has been further demonstrated in plant organelles as an autocatalytic process without detectable participation of any U snRNP (21–24).

Recently we demonstrated that *trans*-splicing occurs in mammalian cells as well (25). Rat cells transformed by the early SV40 *Bst*XI–*Bam*HI (*Bst/Bam*) DNA fragment (p14) that encodes exclusively for the second exon of the large T-antigen [wild-type (wt) T-antigen amino acid (aa) 83–708] synthesize a truncated

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T-antigen (T1-antigen) without having a direct equivalent at the DNA level. In order to generate the T1-mRNA, the cells utilize a cryptic 5′ splice site within the second T-antigen exon (nucleotides encoding for aa 131/132) and the small t-antigen 3′ splice site. Since these splice sites are in an inverted order (3′→5′ instead of 5′→3′) on the SV40 DNA level, two *Bst/Bam* transcripts are required to generate one T1-mRNA molecule. One pre-mRNA molecule provides the 5′ splice site and another the 3′ acceptor splice site.

As an alternative to *trans*-splicing formation of the T1-mRNA might also be accessible by alternative-tandem-*cis*-splicing, given the condition that the cells contain the p14 DNA as a tandem repeat. To test this we produced transformed rat cells that carry one copy of the *Bst/Bam* SV40 DNA fragment as a tail-to-head tandem repeat (p14T). These cells generate the T1-mRNA by two distinct mechanisms: by alternative-tandem-*cis*-splicing and by *trans*-splicing. Alternative-tandem-*cis*-splicing requires pre-mRNA molecules that contain the entire sequence of the *Bst/Bam* tandem repeat as a 'polycistronic' 4.4 kb transcript. To generate the T1-mRNA from this pre-mRNA, the cells utilize the cryptic 5′ splice site within the first *Bst/Bam* segment and the small t-antigen 3′ splice site of the second *Bst/Bam* segment. To generate the T1-mRNA by *trans*-splicing the cells use the same splice sites but these sites are located on two distinct pre-mRNA molecules.

MATERIALS AND METHODS

Plasmid constructs

The p14 DNA contains the SV40 promoter (*Hpa*II–*Bgl*I fragment) and the *Bst/Bam*–*Bam*HI early SV40 DNA fragment; the p14T construct contains two *Bst/Bam* DNA fragments as a tandem tail-to-head duplication (*Bst/Bam*-A–*Bst/Bam*-B). To obtain the p14T construct, the p14 DNA was linearized by *Sma*I enzyme treatment (cuts 6 bp downstream from the *Bam*HI site), dephosphorylated by calf intestine phosphatase (CIP) treatment and was blunt-end ligated to the purified and T4 polymerase treated *Bst/Bam* DNA fragment. The p14TΔ-3 DNA contains the 3 base pair (bp) deletion at the *Pfl*MI restriction site of the *Bst/Bam*-A segment. To obtain this construct, the p14 DNA was linearized by *Pfl*MI treatment, the three 3 nt overhangs of the restriction sites were removed by T4 polymerase. The religated p14Δ-3 DNA was then linearized by *Sma*I treatment and the purified 2.2 kb *Bst/Bam* DNA fragment was inserted. The p14TΔ-5′t contains the small t-antigen 5′ splice site mutated at the *Bst/Bam*-Bam-B segment. For preparation of this construct, the *Bst/Bam* fragment was isolated from the pSVΔ-5′t DNA (25) and ligated into the *Sma*I site of the p14 DNA.

Cells and DNA transfer

REF 52 and TK-negative rat 2 cells were cultivated in Dulbecco's modified Eagle medium, supplemented with 5% fetal calf serum (26). The HAT medium contains hypoxanthine, aminopterin and thymidine (BRL). Microinjection and DNA transfection were performed as described elsewhere (27).

T-antigen preparation

About 10^7 cells were incubated for 1 h at 37°C in a methionine-free DME medium, then labelled for 2 h in the same medium with 20 µCi [^{35}S]methionine. The cells were washed with PBS and lysed in 1% Nonidet (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 hamster anti-T-serum was used as described previously (26). The immunoprecipitates were separated by 7.5% SDS–PAGE and exposed for 5 days (Kodak XAR 5).

Isolation of cellular DNA and RNA

The genomic DNA was isolated from $1–5\times10^6$ cells as described elsewhere (28); total cellular RNA was extracted and separated by the guanidinium thiocyanate–cesium chloride density gradient method (28). The RNA was further purified of remaining DNA by two treatments with RQ I DNase (RNase free, 10 U/ml; Pharmacia) 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 8 h. The fragments were separated in a 1.5% agarose gel, transferred to a nylon membrane (Hybond, Amersham) and UV crosslinked. The hybridization was performed overnight at 65°C in a buffer containing 1% BSA, 7% SDS, 0.5 M sodium phosphate, pH 7.3 and ^{32}P-labelled SV40 DNA. The filter was washed with 1% SDS, 40 mM sodium phosphate, pH 7.3 at the same temperature, three times for 30 min. X-ray films were exposed for 1–7 days at –70°C.

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 SV40 DNA-specific primers (antisense orientation), 0.5 mM of each dNTP and 200 U MuMLV reverse transcriptase (BRL) in 20 µl enzyme buffer at 37°C 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 1–5 µg isolated genomic DNA, 0.2 mM of each dNTP, 0.5 µg of each of the primers of one pair specific to SV40 DNA and 2.5 U *Taq*I DNA polymerase (Cetus/Perkin Elmer) in 100 µl PCR-buffer. The temperature profile used in 30 cycles of amplification was 1 min at 94°C, 1 min at 52°C and 2 min at 74°C per cycle. The absence of detectable amounts of DNA in the total RNA preparation was always verified by PCRs before cDNA synthesis.

Cloning and sequencing of PCR products

PCR products 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 in the dephosphorylated blunt-ended *Sma*I site of pUC18 (Pharmacia). After ligation overnight at 14°C, 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 analyzed by restriction digests and analytical PCRs. Positive plasmid DNAs, containing the PCR product, were directly sequenced 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 double-stranded DNA for sequencing analysis in the Applied Biosystems Model 373 A.

Table 1. SV40 DNA specific primers used in the PCRs

Index	Nucleotide sequence (5′→3′)	5′-end of primer on SV40	Orientation
a_1	GACAAACTACCTACAGAG	4670	sense
x (Δt)	TCTAAGCGCAATATAAAATTTTTAAGTG	4643	sense
d	GAAATGCCATCTAGTGAT	4496	sense
m	CCTGAACCTGAAACATAA	2708	sense
w	ACTAAACACAGCATGACT	4362	antisense
b	GTTATGATTATAACTGTTATG	4239	antisense
l	CATGGTGACTATTCCAGG	3234	antisense
o	CCAGACATGATAAGATAC	2537	antisense

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

RESULTS

p14T-transformed rat cells synthesize the T1-antigen and T2-antigen

The p14T DNA contains the SV40 *Bst/Bam* DNA fragment as a tail-to-head tandem repeat under the transcriptional control of the early SV40 promoter. As illustrated in Figure 1, the *Bst/Bam* DNA fragment encodes for the second exon of the large T-antigen (aa 83–708) and contains the entire small t-antigen intron. Following microinjection of the p14 T DNA together with the pHVS-106 DNA (which encodes for the HSV *tk* gene) into the nuclei of TK-negative rat 2 cells, five HAT medium-resistant T-antigen positive cell lines were isolated and further analyzed. These cell lines fall into two categories. Cells of the first category (two lines: clones 14T/1 and 14T/2) synthesize one truncated T-antigen, the T2-antigen whereas cells of the second category (three lines; clones 14T/3–14T/5) synthesize two truncated molecules, the T1-antigen and the T2-antigen (Fig. 2, lanes 3–5). As demonstrated elsewhere (25) and confirmed in the current investigation, the T2-antigen contains the wt T-antigen amino acid sequence 109–708; it lacks the first 26 aa of the second exon of the large T-antigen because translation of the T2-mRNA starts with the codon 109 encoding for the first methionine within the second exon (Fig. 1A and C). The T1-antigen has the same sequence as the T2 antigen but it contains an additional 49 aa insert between aa 131 and 132. This insert consists of the T-antigen aa sequence 83–131 (Fig. 1D).

The aim of the first set of experiments was to exclude the possibility that DNA rearrangement accounts for T1-antigen synthesis. As deduced from the amino acid sequence of the T1-antigen (25), generation of the T1-antigen by DNA rearrangement would require the insertion of a 147 bp DNA segment, encoding for the 49 T-antigen amino acids (aa 83–131), between the SV40 wt nt 4425 and 4424. Such DNA modification can be detected by PCR analysis, utilizing the primer pair **d** and **b** for the DNA amplification. As illustrated in Figure 1B, these primers bind upstream and downstream from the insertion site having a distance of 258 bp from each other on the wt SV40 DNA. In the case of DNA rearrangement by a 147 nt insertion the PCR would generate a 405 bp DNA segment. However, with the clone 14T/5 DNA as template, the PCR exclusively generated the 258 bp segment (Fig. 3A, lane 3). DNA sequencing experiments proved that the 258 bp segment corresponded exactly to the wt SV40 DNA sequence 4496–4239. These results demonstrate that the T1-antigen does not have a direct equivalent at the DNA level in these cells. Therefore, RNA splicing has to account for T1-antigen synthesis.

To test whether the clone 14T/5 cells synthesize two different *Bst/Bam* mRNA species (termed here T1-mRNA and T2-mRNA) the mRNA was isolated from the clone 14T/5 cells, converted into cDNA and amplified by PCR using again the primer pair **d** and **b**. With these primers two SV40-specific DNA segments were generated: the 258 bp DNA segment and the 405 bp segment (Fig. 3A, lane 2; Fig. 3B, lane 4). The 405 bp PCR segment was also obtained with the clone 14T/3 and 14T/4 cell cDNA, although with a lower efficiency (Fig. 3B, lanes 2 and 3) than with the 14T/5 cell cDNA. However, with the clone 14T/2 cDNA as template the PCR generated only the 258 bp DNA fragment (Fig. 3B, lane 1). DNA sequencing of the cloned PCR products revealed that the sequence of the 258 bp DNA segment was identical to the nucleotide stretch 4496–4239 of the wt SV40 DNA; this DNA segment was generated from the T2-mRNA. The 405 bp cDNA segment contained the sequence of the 258 bp cDNA segment, and in addition, a 147 bp insert between the nt 4425 and 4424 (T-antigen aa 131 and 132). This 147 bp corresponded exactly to the wt SV40 nucleotide sequence 4571–4425, which encodes for the wt T-antigen amino acids 83–131 (Fig. 1D). The 405 bp cDNA segment was generated from the T1-mRNA. Further sequencing experiments revealed that this insertion was the only difference between the T1-antigen and the T2-antigen. These results confirm that the clone 14T/5 cells generate the T1-mRNA by splicing. Generation of the T1-mRNA can occur by two different splicing mechanisms namely by alternative-tandem-*cis*-splicing and/or by *trans*-splicing. To obtain the T1-mRNA the cells have to utilize a cryptic splice site within the second T-antigen exon (nucleotides encoding for aa 131/132) and the 147 nt upstream small t-antigen 3′ splice site.

Generation of the T1-mRNA by alternative-tandem-*cis*-splicing

Generation of the T1-mRNA by alternative-tandem-*cis*-splicing requires a transcript that contains the sequence of the two *Bst/Bam* SV40 DNA fragments (2 × 2.2 kb) in the tail-to-head tandem array as shown in Figure 3D (a). In this alternative-tandem-*cis*-splice-process, the cryptic 5′ splice site of the first *Bst/Bam* segment (*Bst/Bam*-A) and the regular small t-antigen 3′ splice site

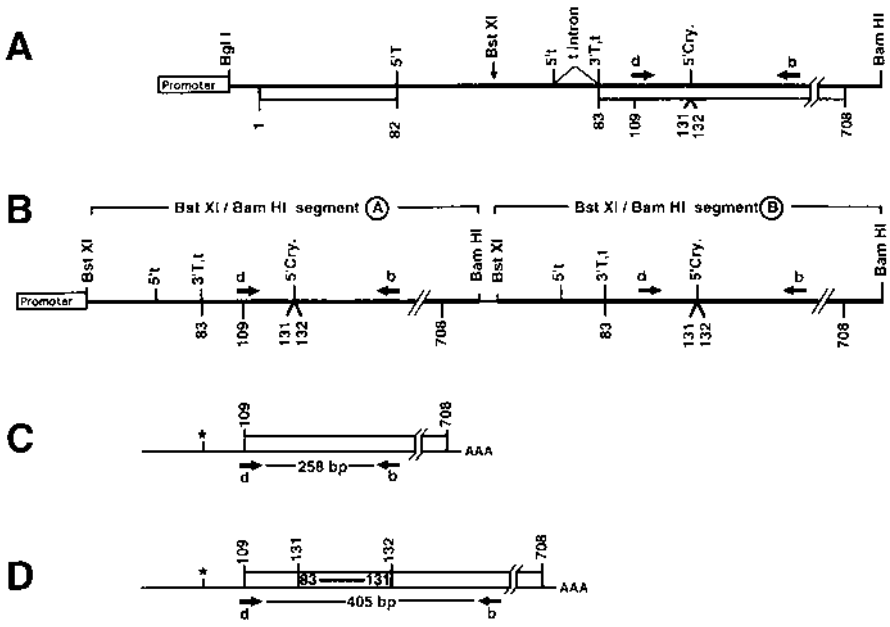

Figure 1. (**A**) Diagram of the early SV40 coding region with the two large T-antigen exons: the first exon contains the aa 1–82 and the second exon the aa 83–708; the aa 109 is the first methionine in the N-terminal part of the second exon. The cryptic 5′ splice site is 147 nt downstream from the small t-antigen and large T-antigen 3′ splice site. The binding position of the primer **d** and **b** at the SV40 DNA is indicated. (**B**) Diagram of the p14T DNA with two *Bst/Bam* SV40 DNA fragments, ligated in a tail-to-head orientation together with the early SV40 promoter (*Hpa*II–*Bgl*I DNA fragment). (**C**) The T2-mRNA and T2-antigen with aa sequence 109–708. The asterisk indicates the small t-antigen intron splice junction. The polyadenylation cleavage site is located 53 nt upstream of the *Bam*HI site. (**D**) The T1-mRNA and T1-antigen with the aa sequence 109–131 followed by the aa sequence 83–708.

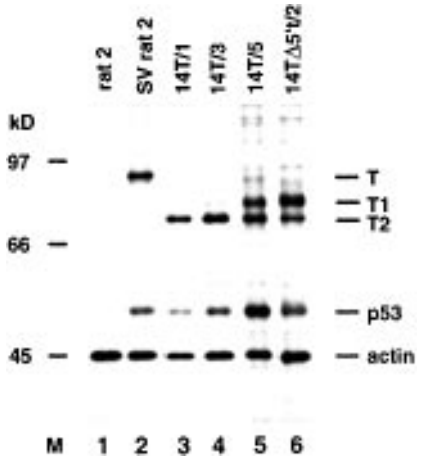

Figure 2. Electrophoretic mobility of large T-, T1- and T2-antigens. Lane 1, control: rat 2 cells; 2, large T-antigen isolated from SV40-transformed rat 2 cells; 3, T2-antigen isolated from the clone 14T/1 cells; 4, T1-antigen and T2-antigen isolated from the clone 14T/3 cells; 5, T1-antigen and T2-antigen isolated from the clone 14T/5 cells; 6, T1-antigen and T2-antigen isolated from clone 14TΔ-5′t/2 cells. The position 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.

of the second *Bst/Bam* segment (*Bst/Bam*-B) are used as the splice sites to generate the T1-mRNA. The nucleotide sequence 4424–2533 of the *Bst/Bam*-A segment and the nucleotide sequence 4739–4572 of the *Bst/Bam*-B segment are removed from the 4.4 kb pre-mRNA as a 2.05 kb intron (Figs 1B and 3D). In this way the *Bst/Bam*-A segment provides the amino acid sequence 109–131 and the *Bst/Bam*-B fragment the amino acid sequence 83–708 for the T1-antigen [Fig. 1D (b)]. However, generation of such a 4.4 kb precursor RNA requires that the entire *Bst/Bam* repeat be transcribed without cleavage of the precursor RNA after RNA polymerase II had passed the polyadenylation signal at the *Bst/Bam*-A segment (Fig. 4). The early SV40 polyadenylation signal, with two AAUAAA hexamer sequences at positions 2637 and 2608 and a long poly U/G stretch at positions 2569–2580, is known to be a strong signal that mediates cleavage of the pre-mRNA at nt 2586 and subsequent polyadenylation with a high efficiency (6,29). Since the *Bst/Bam*-A segment contains the intact early SV40 wt polyadenylation signal, synthesis of the 4.4 kb pre-mRNA as a 'polycistronic' transcript had to be confirmed. To demonstrate this, the RNA was isolated from the clone 14T/5 cells and the clone 14T/2 cells, converted into cDNA and subjected to PCR analysis. The primers used for the PCR reaction were the primer **m** for the *Bst/Bam*-A segment and the primer **w** for the *Bst/Bam*-B segment (Fig. 4). With this primer combination the 557 bp PCR product obtained had the size deduced from the tandem 4.4 kb precursor RNA (Fig. 4, lanes 1 and 3). No SV40 specific PCR product was obtained when reverse transcription was omitted, excluding a possible contamination of the RNA preparation with cellular DNA (Fig. 4, lanes 2 and 4). To confirm that the *Bst/Bam* segment A contains the intact poly A signal, the 557 bp fragment was cloned and sequenced. These experiments revealed that the 557 bp fragment corresponded exactly to the wt SV40 DNA sequence including the intact poly(A) signal. We also attempted to demonstrate formation of the *Bst/Bam* tandem-transcript by Northern blot analysis. However, with this method we were only able to detect the ~2.2 kb T2- and T1-mRNA but not the 4.4 kb pre-mRNA (data not shown).

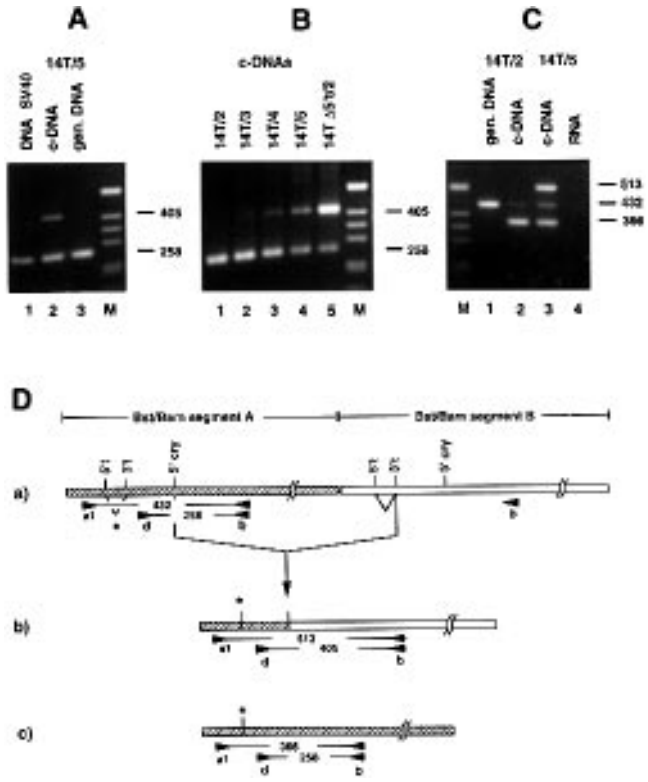

Figure 3. Agarose gel electrophoresis of PCR products generated with the primers **d–b** or **a1–b** and different DNA templates. (**A**) The following templates were used for the PCR utilizing the primer pair **d–b**: lane 1, wt SV40 DNA; 2, clone 14T/5 cDNA; 3, genomic DNA of the clone 14T/5 cells. The 405 bp cDNA PCR fragment was derived from the T1-mRNA and the 258 bp cDNA PCR fragment from the T2-mRNA. The exact size of the PCR DNA fragments was determined by DNA sequencing. M, DNA size marker. (**B**) RT–PCR products generated with the primer pair **d–b**: the RNA was isolated from the following cell lines: lane 1, clone 14T/2 cells; 2, clone 14T/3 cells; 3, 14T/4 cells; 4, clone 14T/5 cells; 5, clone 14TΔ-5′t/2 cells. (**C**) PCR products generated with the primer pair **a1–b**. The following templates were used: lane 1, genomic DNA of clone 14T/2 cells; 2, clone 14T/2 cDNA; 3, clone 14T/5 cDNA; 4, clone 14T/5 RNA; in lane 4 the PCR was performed without prior reverse transcription. M, DNA size marker. (**D**) Schematic representation of the p14T transcript and the corresponding splice products. (**a**) The 4.4 kb pre-mRNA with the two 2.2 kb *Bst/Bam* segments. The position of the small t-antigen intron and the cryptic 5′ splice site are indicated. Excision of the small t-antigen intron by *cis*-splicing is symbolized by *. The position of the primer pairs **a1–b** and **d–b** with the genomic distance of 432 and 258 bp is shown. (**b**) T1-mRNA generated by alternative-tandem-*cis*-splicing. The cells utilize the cryptic 5′ splice site of the *Bst/Bam* segment A (dashed segment) and the small t-antigen 3′ splice site of the *Bst/Bam* segment B (open segment). After excision of the 2.05 kb intron and the proximal small t-antigen intron (66 nt), the RT–PCR generates a 513 bp DNA fragment with the primers **a1–b** and a 405 bp DNA fragment with the primer pair **d–b**. (**c**) T2-mRNA generated from the *Bst/Bam* segment A. After small t-antigen *cis*-splicing, the RT–PCR generates a 366 bp DNA segment with the primer pair **a1–b** and a 258 bp segment with the primer pair **d–b**.

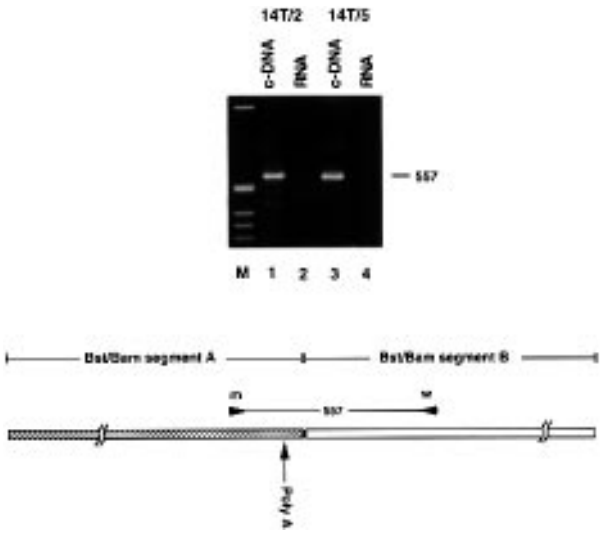

Figure 4. Agarose gel electrophoresis of PCR products generated with the primers **m** and **w**. Lane 1, 557 bp RT–PCR fragment obtained with the total RNA isolated from clone 14T/2 cells; 2, PCR analysis of the clone 14T/2 RNA without prior reverse transcription; 3, 557 bp RT–PCR fragment obtained with the RNA isolated from the clone 14T/5 cells; 4, PCR analysis of the clone 14T/5 RNA without prior reverse transcription. As shown in the diagram below the binding site for the primer **m** is at the proximal *Bst/Bam*-A segment and the binding site for the primer **w** at the distal *Bst/Bam*-B segment of the pre-mRNA.

The second condition for T1-mRNA synthesis is that alternative-tandem-*cis*-splicing precede small t-antigen splicing because deletion of the 3′ splice site by small t-antigen *cis*-splicing on the *Bst/Bam*-B segment would exclude subsequent alternative-tandem-*cis*-splicing [Fig. 3D (a)]. Our hypothesis is that those cells which synthesize only the T2-antigen (e.g. clone 14T/2) excise the small t-antigen intron with a greater efficiency than the clone 14T/5 cells. To demonstrate this, the clone 14T/2 and clone 14T/5 cDNAs were PCR-amplified with primer pair **a1** and **b**. As shown in Figure 3D (a) the primer **a1** binds upstream and the primer **b** downstream from the small t-antigen splice site, having a distance of 432 bp at the SV40 DNA. With the clone 14T/2 cDNA as template the PCR mainly generated the 366 bp DNA fragment, but the 432 bp fragment being generated only to a very low extent. The 366 bp DNA fragment was generated from the small t-antigen intron minus T2-mRNA and the 432 bp DNA segment from unspliced pre-mRNA (Fig. 3C, lane 2). With the clone 14T/5 cDNA as the template however, the PCR generated the 432 bp DNA fragment with a significantly higher efficiency (Fig. 3C, lane 3), confirming that excision of the small t-antigen intron occurs less efficient in the clone 14T/5 cells than in the clone 14T/2 cells. Formation of the 432 bp DNA fragment was not due to contamination of the RNA preparation with cellular DNA because no PCR product was obtained when the PCR was performed without prior reverse transcription (Fig. 3C, lane 4). The origin of the 513 bp DNA fragment was the T1 mRNA. To demonstrate that small t-antigen splicing is indeed in competition with T1-mRNA synthesis, the small t-antigen 5′ splice site of the *Bst/Bam*-B fragment at the p14T DNA was destroyed by site-directed mutagenesis, as described in Materials and Methods. The corresponding construct (p14TΔ-5′t) was co-transfected with the pHSV-106 DNA into TK-minus rat 2 cells and HAT medium-resistant cell lines were selected. From three arbitrarily chosen T1-antigen positive cell lines (e.g. 14TΔ-5′t/2) the RNA was extracted, converted into cDNA and subjected to PCR analysis. With the primer pair **d** and **b** the amount of the 405 bp DNA fragment generated was in all three cases 2–4-fold higher than that obtained with the clone 14T/5 cDNA (Fig. 3B, lane 5). A 2–4-fold higher rate of T1-antigen synthesis was also demonstrable by SDS gel electrophoresis, as is shown for the 14TΔ-5′t/2 cells (Fig. 2, lane 6).

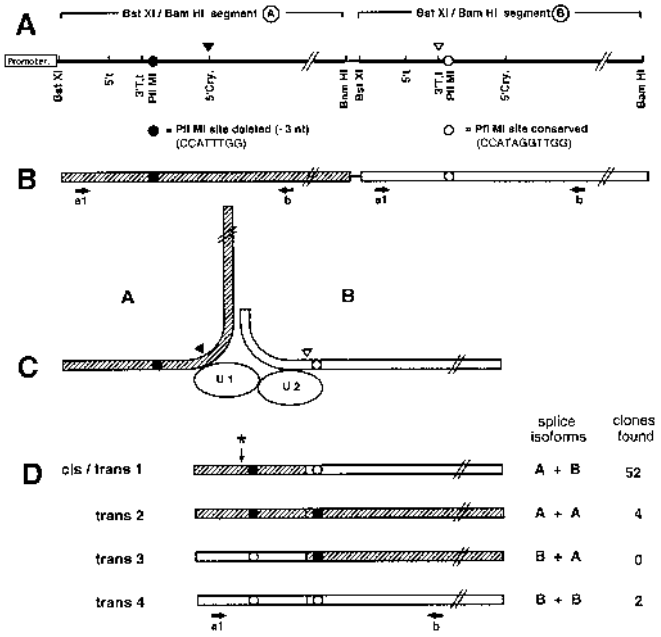

Figure 5. *Trans*-splice model and molecular interactions between two pre-mRNA molecules in the *trans*-splicing pre-spliceosome complex. (**A**) represents the p14TΔ3 DNA with the 3 bp deletion at the proximal *Bst/Bam* segment A (CCATTTGG), destroying the *Pfl*MI restriction site and the distal *Bst/Bam* segment B (CCATAGGTTGG) with the conserved *Pfl*MI site. The small t-antigen intron and the cryptic 5′ splice site are shown. (**B**) represents the 4.4 kb 14TΔ3 pre-mRNA. (**C**) Illustrates the interaction of the two pre-mRNA molecules and the formation of the (pre)spliceosome complex. The U1snRNP binds via its snRNA to the cryptic 5′ splice site on the pre-mRNA (A) and U2 snRNP to the branch site on the pre-mRNA molecule (B). The two pre-mRNA molecules can then be brought together via a U1/U2 snRNP association. (**D**) The four possible T1-mRNA isoforms. Among the 58 clones analyzed, 52 clones contained the combination A+B, four clones the combination A+A and two clones the combination B+B.

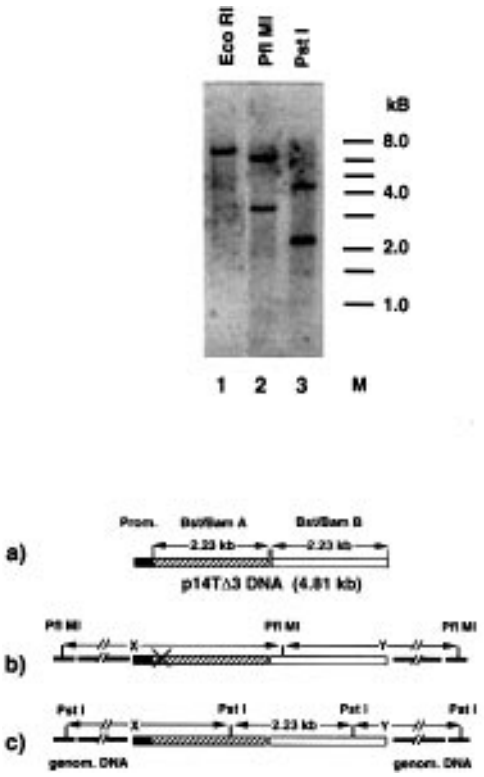

Figure 6. Southern blot analysis of 14TΔ3/6 cells (soft-agar cloned). The cellular DNA was isolated and restricted with: lane 1, *Eco*RI endonuclease; 2, *Pfl*MI endonuclease; 3, *Pst*I endonuclease. The blot was hybridized with ^{32}P-labelled SV40 DNA. The drawings below indicate (**a**) the p14TΔ3 *trans*-gene in the clone 14TΔ3/6 cells; (**b**) the cleavage sites of the *Pfl*MI enzyme and (**c**) the cleavage sites of the *Pst*I enzyme within the p14TΔ3 and the genomic DNA. As shown the *Eco*RI enzyme (does not cut the 14TΔ3 DNA) generates a single SV40-specific DNA segment when only one copy of the 14TΔ3 DNA is integrated into the host genome, and the *Pfl*MI enzyme generates two SV40 specific DNA fragments. After restriction of the cellular DNA with the *Pst*I enzyme a 2.23 kb fragment has to be generated from the p14T DNA (lane 3).

The T1-mRNA molecules are also generated by *trans*-splicing

To analyse whether the T1-mRNA is also generated by *trans*-splicing, a 3 bp deletion was introduced as a marker into the *Bst/Bam*-A segment of the p14T DNA by site-directed mutagenesis to generate the p14TΔ-3 DNA. This deletion (SV40 nt: 4563–4561: CCT) destroyed the *Pfl*MI recognition site (CCAACCTATGG) of the *Bst/Bam*-A segment and deletes the large T-antigen aa 85 while leaving the *Pfl*MI site within the *Bst/Bam*-B segment intact (Fig. 5A). This 3 bp deletion, which was confirmed by DNA sequencing, allowed the differentiation between the *Bst/Bam*-A and the *Bst/Bam*-B transcripts. To generate the T1-mRNA by *trans*-splicing the cells have to use two distinct p14TΔ-3 transcripts as pre-mRNA molecules to obtain one T1-mRNA molecule. Therefore, four distinct T1-mRNA isoforms can be predicted, as illustrated in Figure 5D. Utilizing two *Bst/Bam*-A RNA molecules as precursors, the resulting *trans*-splice product will contain the 3 nt *Pfl*MI deletion twice, once in the proximal segment, the second time in the distal RNA segment (splice isoform A+A); when two *Bst/Bam*-B RNA molecules are used, the T1-mRNA contains the intact *Pfl*MI site twice (splice isoform B+B) and so on.

To test this, the p14TΔ-3 DNA was transfected with the pHSV-106 DNA into rat 2 cells and HAT medium-resistant cell clones were isolated. From 10 randomly chosen HAT medium-resistant cell clones, six clones exhibited a strong intranuclear T-antigen fluorescence after staining with anti T-serum. From these six cell lines, cells of four lines synthesized both the T1-antigen and the T2-antigen (e.g. 14TΔ3/6), whereas two lines contained only the T2-antigen. For further analyses, the clone 14TΔ3/6 was selected because cells of this line synthesize the T1-antigen with the greatest efficiency (data not shown). To determine the copy number of the p14TΔ-3 DNA, the 14TΔ3/6 cells were recloned in soft agar, the cellular DNA was extracted from one soft agar clone and subjected to Southern blot analysis. As shown in Figure 6, only a single SV40-specific band was obtained when the cellular DNA was treated with the *Eco*RI enzyme. This restriction enzyme does not cleave the *Bst/Bam* tandem repeat. After treatment of the cellular DNA with the *Pfl*MI endonuclease, which cleaves the p14TΔ3 DNA only in the distal *Bst/Bam* segment (Fig. 5A), two SV40 specific fragments were generated. These results demonstrate that the 14TΔ3/6 cells contain only a single copy of the p14TΔ3 DNA.

To further characterize the T1-mRNA, PCR experiments were performed with the clone 16TΔ3/6 cDNA again using the primer pair **a1** and **b** for DNA amplification. These primer bind upstream

and downstream from the *Pfl*MI restriction site (Fig. 5B). With the 14TΔ3/6 cDNA as template the PCR generated the 363 and 510 bp DNA fragments (Fig. 7, lane 1). The two PCR DNA fragments were restricted with the *Pfl*MI enzyme and subjected to agarose gel electrophoresis. As shown in Figure 7 (lane 2) the 363 bp DNA fragment could not be cleaved by the PflMI restriction enzyme. In contrast, when the clone 14T/5 cDNA which contains two intact *Pfl*MI sites was used as the template both PCR fragments were cleaved by the *Pfl*MI enzyme (Fig. 7, lane 4). We further cloned and sequenced the 14TΔ3/6 cell 363 bp cDNA PCR fragment. These experiments confirmed that this fragment was generated by T2-mRNA; it lacked the small t-antigen intron and contained the 3 bp deletion, indicating that the *Bst/Bam*-A DNA segment was the origin of this T2-mRNA. Treatment of the 14TΔ3/6 cell 510 bp cDNA PCR fragment with the *Pfl*MI enzyme generated DNA fragments of 187 and 323 bp. These results indicated that the first but not the second segment of the T1-mRNA harboured the 3 nt *Pfl*MI deletion (Fig. 7B) which was confirmed by DNA sequencing (see below). This T1-mRNA could be generated either by alternative-tandem-*cis*-splicing or by *trans*-splicing (Fig. 5D; splice isoform A+B).

Importantly, ~10–15% of the 510 bp PCR DNA segments were resistant to *Pfl*MI enzyme treatment (Fig. 7A, lane 2). This indicated that both *Pfl*MI sites were deleted from the corresponding T1-mRNA. Such mRNA molecules can only be generated by *trans*-splicing and not by alternative-tandem-*cis*-splicing. Generation of T1-mRNA molecules with two *Pfl*MI deletions, one in the first and the other in the second part of the molecule, requires that two *Bst/Bam*-A transcripts be used as precursors as shown in Figure 5.

To confirm this by DNA sequencing the 510 bp T1-mRNA RT–PCR product was inserted into the multiple cloning site of pUC18 and propagated into *E.coli*. One hundred independent colonies were isolated and further analysed. From these hundred colonies, 58 isolates contained the pUC18 DNA with the 510 bp insert (pUC18-510). These 58 pUC18 510 bp DNA segments were reisolated, restricted with the PflMI endonuclease and subjected to agarose gel electrophoresis. These experiments revealed that 52 isolates contained the 3 bp deletion only within the *Bst/Bam*-A segment; PflMI digestion generated the 323 and 187 bp DNA fragments (Fig. 7A, lane 6). Four of the 510 bp DNA segments, however, were entirely resistant to PflMI restriction enzyme treatment (Fig. 7A, lane 8). DNA sequencing experiments confirmed that all four DNA segments contained the 3 bp deletion twice: one deletion was in the proximal (*Bst/Bam*-A) the other was in the distal segment (*Bst/Bam*-B); the size of these four DNA segments was only 507 bp (Fig. 7B). Therefore, generation of the corresponding T1-mRNA molecules was only possible when two *Bst/Bam*-A pre-mRNA segments were used as the precursor for the *trans*-splicing reaction (splice isoform A+A). Moreover, two of the 510 bp DNA segments contained the intact PflMI restriction site two times (Fig. 7A, lane 10) and PflMI treatment generated three DNA fragments (43, 147 and 323 bp). The size of these two DNA segments was 513 bp and the nucleotide sequence was identical to the sequence of the clone 14T/5-specific 513 bp PCR DNA fragment, having the intact PflMI sequence in both the proximal and the distal segments (Fig. 7A, lane 4). This means that the corresponding T1-mRNA molecules were derived from two *Bst/Bam*-B segments by *trans*-splicing (splice isoform B+B). Taken together these results prove that the 14TΔ3/6 cells generate

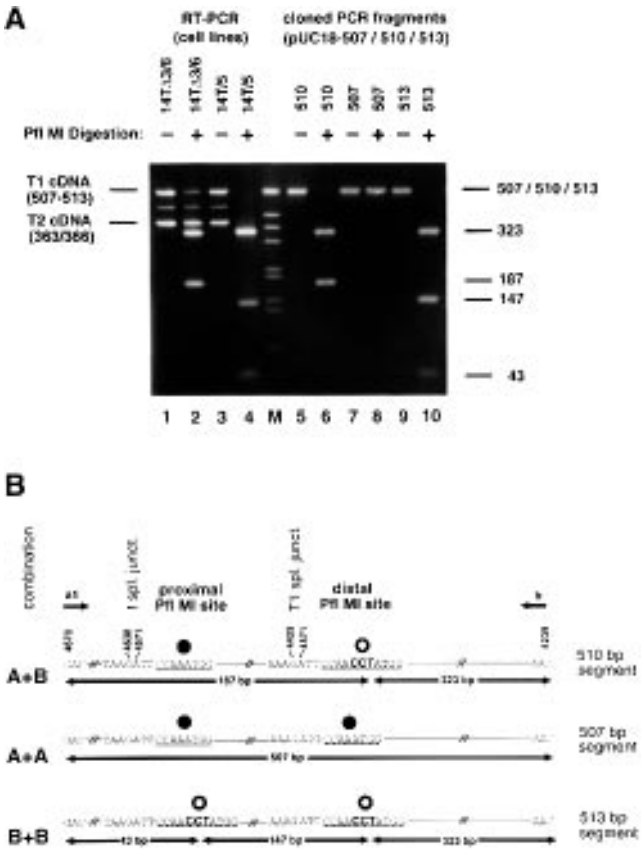

Figure 7. Analysis of RT–PCR products and of cloned PCR DNA fragments. (**A**) Agarose gel electrophoresis of PCR products generated with the primers **a1** and **b**: lane 1, contains the RT–PCR fragments obtained with the RNA isolated from the clone 14TΔ3/6 cells; 2, the same RT–PCR DNA fragments as in lane 1 but restricted with the *Pfl*MI enzyme; 3, contains the RT–PCR fragments obtained with the RNA isolated from the clone 14T/5 cells; 4, the same RT-DNA fragments as in lane 3 but restricted with the *Pfl*MI enzyme; 5–10, contain the inserts isolated from three pUC18-510 clones without (–) and with (+) *Pfl*MI restriction enzyme treatment. The nucleotide sequence and the exact size of the three pUC18-510 inserts (507/510/513 bp) were determined by DNA sequencing. (**B**) The sequence of the 510/507/513 bp DNA fragments. Only the relevant nucleotides are shown (e.g. small t-antigen splice-junction; T1-antigen splice-junction; PflMI sequence on the proximal and on the distal segment). The size of the DNA fragments after *Pfl*MI digestion is indicated. The 510 bp segment is obtained from splice isoform A+B (Fig. 5D), the 507 bp segment is obtained from splice isoform A+A, the 513 bp segment from splice isoform B+B.

the T1-mRNA by two different RNA processing mechanisms: alternative-tandem-*cis* splicing and by *trans*-splicing.

DISCUSSION

Trans-splicing is an RNA processing mechanism frequently used by lower eukaryotic cells such as trypanosomes, nematodes and Euglena to generate functional mRNA molecules (16–20). *Trans*-splicing has further been observed in plant organelles (21–24) but had not been conclusively demonstrated to occur *in vivo* in mammalian cells until recently (25). In the current investigation we have demonstrated that rat cells containing the *Bst/Bam* DNA fragment as a tail-to-head tandem repeat generate the T1-mRNA by two distinct mechanisms, namely alternative-tandem-*cis*-splicing

and *trans*-splicing. Both splice processes are mechanistically related and identical donor and acceptor splice sites are used for the *cis*-splice and the *trans*-splice reaction. In the case of alternative-tandem-*cis*-splicing (Fig. 3D) the two splice sites are on one pre-mRNA molecule whereas in the case of *trans*-splicing these sites are located on two distinct precursor molecules (Fig. 5).

Alternative-tandem-*cis*-splicing

In order to generate the T1-mRNA by alternative-tandem-*cis*-splicing it is essential that the cells synthesize the 4.4 kb pre-mRNA containing the entire *Bst/Bam* sequence in the tail-to-head duplication. Synthesis of such a 'polycistronic' pre-mRNA was not predictable, however, because the early SV40 polyadenylation signal would have to efficiently mediate the cleavage of the pre-mRNA after the RNA polymerase II had passed the wt nt 2587 of the proximal *Bst/Bam* DNA segment (Fig. 4). Mutation of the poly(A) signal at the *Bst/Bam*-A segment as a possible reason for this read-through transcription in the 14T/5 cells was excluded by DNA sequencing. Nevertheless, it has been demonstrated that splicing signals directly upstream and downstream from a poly(A) site can affect the polyadenylation efficiency. Valid 3′ splice sites of upstream introns are required for poly- adenylation (8) and introduction of a 5′ splice site upstream from the poly(A) site may reduce the polyadenylation efficiency, as has been shown elsewhere (8,30,31). Furthermore, it has been demonstrated that poly(A) signals can be ignored when they are followed by a downstream 3′ splice site (7,30). In the case of the *Bst/Bam* tail-to-head tandem repeat the poly(A) signal of the *Bst/Bam*-A segment, located 53 nt proximal of the *Bam*HI site (Fig. 1; 4), is bordered by the upstream cryptic 5′ splice site and by the downstream 3′ splice site of the *Bst/Bam*-B segment, which may reduce the strength of this signal. However, our experiments imply that the 4.4 kb pre-mRNA is very unstable and/or immediately further processed, since the existence of the 4.4 kb transcript was only demonstrable by RT–PCR but not by Northern blot analysis. As in nematodes, where *trans*-splicing and *cis*-splicing occur in parallel, polycistronic pre-mRNA molecules are very unstable and in most cases it was not possible to obtain cDNA clones from the polycistronic transcripts (19).

The second condition for T1-mRNA synthesis is that the small t-antigen intron at the second *Bst/Bam* segment not be removed by conventional *cis*-splicing since the small t-antigen 3′ splice site is also the acceptor site for alternative-tandem-*cis*-splicing. We obtained experimental evidence that those cells that synthesize only the T2-antigen perform small t-antigen splicing more efficiently than those cells that contain both the T2-antigen and the T1-antigen. Consequently, deletion of the small t-antigen 5′ donor splice site at the *Bst/Bam*-B segment increased the efficiency of T1-antigen synthesis ~2–4-fold, verifying the competition between these two splicing processes. It has been demonstrated elsewhere that the efficiency of small t-antigen splicing is cell type dependent and requires certain cellular factors such as ASF/SF2 (32). Therefore we would suggest that a variation in the expression rate of these splicing factors also occurs within cells of established cell lines, a subject not further addressed in the current investigation.

Trans-splicing

By destroying the recognition site for the *Pfl*MI restriction enzyme within the *Bst/Bam*-A segment of the p14T DNA (p14TΔ3), we have further demonstrated that rat cells, in addition to generating the T1-mRNA by alternative-tandem-*cis*-splicing, also employ *trans*-splicing. This 3 bp deletion allowed us to test which of the *Bst/Bam* transcripts are used to generate the T1-mRNA. In contrast to alternative-tandem-*cis*-splicing, when the T1-mRNA molecules are generated by *trans*-splicing, utilizing two unlike pre-mRNA molecules, four particular T1-mRNA isoforms can be predicted. Indeed, our *Pfl*MI restriction enzyme analysis and DNA sequencing experiments of the cloned T1-mRNA-specific RT–PCR products revealed that the 14TΔ3/6 cells generate at least two out of the three possible T1-mRNA isoforms that can be generated only by *trans*-splicing (Fig. 5). We found that four out of the 58 clones analyzed carried the 3 bp deletion in both the proximal and the distal segments (Figs 5 and 7; 507 bp PCR fragment). To obtain T1-mRNA molecules that contain the 3 bp deletion twice, the cells have to utilize two *Bst/Bam*-A transcripts to generate one of these mRNA-isoforms. Furthermore, two isolates did not carry any deletion (513 bp PCR fragment). The precursors of this T1-mRNA must have been two *Bst/Bam*-B RNA segments. Since the clone 14TΔ3/6 cells contain only one copy of the p14TΔ-3 DNA (Fig. 6) and rearrangement of the 14TΔ3 transgene was excluded by DNA sequencing, these T1-mRNA isoforms can only be generated by *trans*-splicing but not by alternative-tandem-*cis*-splicing. We conclude that in the 14TΔ3/6 cells ~10% of the T1-mRNA molecules are generated by *trans*-splicing and 90% by alternative-tandem-*cis*-splicing. This observation was unexpected, since for kinetic reasons, intramolecular tandem *cis*-splicing should exclude intermolecular *trans*-splicing. These findings extend our previous observation that *trans*-splicing occurs not only in cells, which contain the transgene as a monomer, but also in those cells which contain the *Bst/Bam* segment in a tail-to-head tandem repeat.

Figure 5 illustrates our suggestions as to how that *trans*-splicing occurs. The precursors for the *trans*-splicing reaction could either be two large 4.4 kb tandem transcripts or two 2.2 kb *Bst/Bam* pre-mRNA molecules, a topic not further addressed in the present investigation. Our model suggests further that the *trans*-splicing mechanism is similar to that of the *cis*-splicing reaction, requiring the formation of a similar *trans*-splicing pre-spliceosomal complex. Therefore, in analogy to *cis*-splicing the U1-snRNP binds via its snRNA to the cryptic 5′ splice site on the pre-mRNA molecule A and the U2-snRNP binds to the branch site on the pre-mRNA molecule B. The donor 5′ splice site and the branch site of the two RNA molecules may be brought together by U1-/U2-snRNP interaction/ association as has been suggested for *cis*-splicing (33–37).

Activation of cryptic splice sites

For generation of the T1-mRNA it seems to be important that the pre-mRNA does not contain the coding sequence of the first T-antigen exon. The lack of the large T-antigen 5′ splice site mediates the activation of the cryptic 5′ splice site within the second T-antigen exon. Since the 5′ cryptic splice at the *Bst/Bam* transcript is not followed by a functional 3′ splice site, the cells have to utilize the 3′ splice-site of a second *Bst/Bam* transcript. The site used is the conventional small t-antigen 3′ *cis*-splice site, the only functional acceptor site within the early SV40 region.

Activation of cryptic splice sites, as a consequence of splice-site deletion or inactivation, is a well-documented phenomenon in mammalian cells (17,36). Mutation of conventional splice-sites may convert *cis*-spliced genes into *trans*-spliced genes (38,39). This implies further that *trans*-splicing may be a more general feature in mammalian cells. Candidates are mainly those genes containing

potential cryptic splice-sites that are not followed by valid downstream acceptor or upstream donor splice-sites.

Biological significance of T1-mRNA synthesis

Through these intra- and inter-molecular-splicing processes, the T1-mRNA gains a 147 nt segment, encoding for the 49 large T-antigen aa 83–131. Its translation product, the T1-antigen, therefore contains the entire amino acid sequence of the second large T-antigen exon and in addition the aa sequence 109–131 as a duplication (Fig. 1D). In contrast, the T2-antigen lacks the first 29 aa (83–108) because translation of both the T1-mRNA and the T2-mRNAs starts with the T-antigen codon 109 which is the first potential translation initiation signal within the second T-antigen exon (Fig. 1C) as has been confirmed by protein microsequencing (25). As a consequence of T1-antigen synthesis cells are fully malignant transformed while those cells that synthesize only the T2-antigen do not grow in soft agar and do not form tumours after inoculation into nude mice (Graessmann *et al.*, manuscript in preparation). This implies that the T-antigen aa sequence 83–108, which bears the binding site for the RB tumour suppressor protein family (40,41) is crucial for cell transformation

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