In vitro synthesized SV40 cRNA is *trans*-spliced after microinjection into the nuclei of mammalian cells

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Received 5 August 1996

Abstract We present for the first time experimental evidence that in vitro synthesized RNA (cRNA) is *trans*-spliced after microinjection into the nuclei of mammalian tissue culture cells. The template used for cRNA synthesis was the early SV40 *Bst*XI/*Bam*HI DNA fragment. This DNA fragment encodes exclusively for the second T-antigen exon and contains the intact small t-antigen intron. To generate the corresponding mRNA (T1-mRNA) by *trans*-splicing, the cells utilize a 5′ cryptic splice site located within the second T-antigen exon of one cRNA molecule which is spliced to the small t-antigen 3′ splice site of another cRNA molecule. Formation of the T1-mRNA by *trans*-splicing was confirmed by RT-PCR analysis and DNA sequencing. Efficient *trans*-splicing required that competitive small t-antigen *cis*-splicing be inhibited by deletion of the small t-antigen 5′ splice site. The T1-mRNA was not generated when the cryptic 5′ splice site was mutated.

Key words: SV40 T1-antigen; Pre-mRNA synthesis; Microinjection; *Trans*-splicing; Translation

1. Introduction

In eukaryotic cells, the majority of the RNA polymerase II transcripts contain introns which are removed from the pre-mRNA molecules by the splicing machinery before nuclear export occurs. Joining different RNA segments together by alternative *cis*-splicing, multiple protein isoforms can be generated from one type of pre-mRNA (for review, see [1]). Splice sites are not only essential to ensure correct intramolecular *cis*-splicing, they are also used to join segments from independent transcripts together by means of *trans*-splicing. *Cis*-splicing and *trans*-splicing have the same consensus sequences, utilize the same splicing machinery and form branched intermediates. Up to now *trans*-splicing has been demonstrated in lower eukaryonts (e.g. trypanosomes, nematodes, trematodes), where an untranslated small leader (SL) RNA is *trans*-spliced, providing the cap structure for each mRNA. Intermolecular RNA splicing has also been observed in plant cell organelles, where new mRNA molecules are formed by self-splicing without the participation of any U snRNPs (for review, see [2–4]). Furthermore, it has been shown in vitro that *Saccharomyces cerevisiae* and HeLa cell extracts catalyze the *trans*-splice reaction [5–8]. Although predicted by computer analysis [9] and suggested in several earlier reports [5,7,10–12], only recently has conclusive experimental evidence been obtained that intact mammalian cells also have the potential to perform RNA *trans*-splicing and that the corresponding mRNA is translated into a functional protein [8]. By deletion of the first SV40 T-antigen exon it was shown that rat cells transformed by an early SV DNA fragment (*Bst*XI/*Bam*HI) generate an SV40-specific mRNA (T1-mRNA) which is 147 nt larger than the primary transcript. Sequencing experiments revealed that the cells create the T1-mRNA by a *trans*-splice reaction between two identical Bst/Bam pre-mRNA molecules. During this *trans*-splicing process a cryptic 5′ splice site, located within the second T-antigen exon of one pre-mRNA molecule (A), is combined with the conventional small t-antigen 3′ splice site of a second pre-mRNA (B), as is illustrated in Fig. 1. Therefore, the T1-mRNA translation product, the T1-antigen, is composed of two distinct SV40 T-antigen segments, a proximal segment with the amino acid sequence 109–131 provided by the pre mRNA molecule (A) and a distal segment with the amino acid sequence 83–708 provided by the pre-mRNA molecule (B). The pre-mRNA molecules that are not engaged in *trans*-splicing are subjected to small t-antigen *cis*-splicing and translated into the T2 antigen. Since translation of the T2-mRNA starts with the first AUG in reading frame, the T2-antigen contains only the T-antigen amino acid sequence 109–708 and lacks the first 29 amino acids of the second T-antigen exon present in the T1-antigen [8,13].

Since it is possible to analyze mRNA maturation and translation efficiently in microinjected mammalian tissue culture cells [14,15] we combined in the present investigation in vitro pre-mRNA synthesis and in vivo mRNA processing to entirely exclude DNA rearrangement as a possible alternative mechanism to *trans*-splicing. The Bst/Bam pre-mRNA (cRNA) was synthesized in vitro and microinjected into the nuclei of TC7 and rat 2 cells. Immunofluorescence staining, RT-PCR and DNA sequencing experiments revealed that the injected cRNA was *trans*-spliced after nuclear injection and translated into the T1-antigen.

2. Materials and methods

2.1. DNA constructs

The p14-T7 DNA was obtained by inserting the early SV40 *Bst*XI/*Bam*HI (Bst/Bam) 2.2 kb DNA segment into the multiple cloning site (*Acc*I/*Bam*HI) of pSPT19 DNA (Pharmacia), oriented in the same sense as the T7 promoter within the plasmid. The p14Δ5′t-T7 DNA was constructed by eliminating the consensus sequence (G/GTA changed to G/CGC) of the small t-antigen 5′ splice site of the p14-T7 DNA by site-directed mutagenesis as described elsewhere [8].

2.2. cRNA synthesis

For runoff cRNA synthesis [16], the p14-T7 DNA and the p14Δ5′t-T7-DNA were linearized by *Sma*I which cuts 6 bp downstream from the *Bam*HI site; completeness of the cleavage was confirmed by Southern blot analysis. In vitro transcription including 1 μg of linearised template DNA, the cap nucleotide m7G(5′)ppp(5′)G (Ambion) and T7 polymerase (T7 Cap-Scribe; Boehringer Mannheim) was performed according to the supplier's protocol. The quality and correct size of the cRNA preparations were confirmed by Northern blot anal-

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PII S0014-5793(96)00957-X

ysis. The absence of any template DNA in the cRNA preparation after RQ-I-DNase (Promega) digestion was confirmed by PCR analysis with 1 ng cRNA, the SV40 specific primers **d** (5'GAAATGCCATCTAGTGAT3') and **b** (5'GTTATGATTATAACTGTTATG3') and 3 U Taq polymerase (BRL) after 35 PCR cycles (30 s at 94°C, 30 s at 53°C, 1 min at 74°C each cycle). Control PCRs included 1 ng reverse-transcribed (RT) cRNA. The Southern blots were hybridized with ^{32}P-labelled SV40 DNA [18].

2.3. cRNA injection and immunofluorescence staining

Cells grown on glass slides [15] were microinjected with capped or non-capped cRNA molecules (concentration 1 μg/μl, injection volume 10^{-8} μl/cell) and stained for T-antigen as described previously [16]. The cRNA was subjected to agarose gel electrophoresis under denaturing conditions (formamide/formaldehyde buffer) and processed for Northern blotting as described elsewhere [17,18].

3. Results and discussion

3.1. In vitro cRNA synthesis

In previous studies we have demonstrated that microinjection is a powerful tool for analyzing the function of biologically relevant macromolecules directly in intact mammalian tissue culture cells [14,15]. One of the results we obtained was that in-vitro-synthesized early SV40 RNA (cRNA) is *cis*-spliced and translated into wt T-antigen after intranuclear injection [16,17,19–21]. Subsequently, *Xenopus* oocytes were also used as recipients to study RNA processing and nuclear RNA export [22–26]. To investigate whether the in-vitro-synthesized pre-mRNA (cRNA) molecules are *trans*-spliced in mammalian cells, the early SV40 Bst/Bam DNA fragment was inserted into the multiple cloning site of the pSPT19 DNA in the sense orientation with regard to the T7 promoter (p14-T7). As shown in Fig. 1B, the Bst/Bam DNA encodes only for the second T-antigen exon and contains the intact small t-antigen intron (66 nt). Before in vitro transcription by the T7 RNA polymerase (T7 Cap-Scribe, Boehringer), the p14-T7 template DNA was linearized by *Sma*I endonuclease treatment which cuts 6 bp downstream from the *Bam*HI site. Synthesis of the cRNA started with the formation of the cap structure and the runoff transcript (cap-Bst/Bam-cRNA) was liberated from the template DNA by two consecutive DNase treatments [16]. The size of the cRNA was determined by Northern blot analysis. As shown in Fig. 2A (lane 1), a single transcript of about 2.2 kb was obtained, which was the size predicted from the Bst/Bam template DNA. To verify the absence of the template DNA, the cRNA was subjected to PCR analysis using the SV40 specific primer pair **d** and **b** for DNA amplification (Fig. 2A). These primers bind within the coding region of the second exon of the large T-antigen and are at a distance of 258 bp from each other on the SV40 DNA (Fig. 1B). As shown in Fig. 2, no SV40-specific PCR product was obtained when the reverse transcription was omitted, excluding the contamination of the cRNA preparation with trace amounts of the template DNA (Fig. 2B, lane 1). As expected, the 258 bp DNA fragment was generated when the cRNA was subjected to RT-PCR analysis (Fig. 2B, lane 2).

3.2. The cRNA is trans-spliced and translated into the T1-antigen after intranuclear microinjection

In a first set of experiments, synthesis of SV40-specific proteins was analyzed by immunofluorescence staining after intranuclear injection of the cap-Bst/Bam-cRNA into TC7 and rat 2 cells. To demonstrate that the cRNA was processed into mRNA and translated in vivo, the cells were fixed and stained at different points in time after the cRNA injection. The antibodies used were a hamster polyclonal antibody (HPA), which recognizes both the T1-antigen and the T2-antigen, and a monoclonal antibody (Ab-2; Oncogene Science) that specifically recognizes an epitope within the T-antigen amino acid sequence 83–108; this epitope is present in the T1-antigen but not in the T2-antigen (Fig. 1C,E). In both cell types, T-antigen synthesis was already demonstrable 30 min after injection, with the maximum number of T-antigen positive cells being obtained 5–6 h after cRNA transfer. At this time, more than half of the injected cells exhibited strong intranuclear T-antigen fluorescence after staining with the HPA-antibody. However, only 3–5% of these cells were T1-antigen-positive when stained with the Ab-2 antibody (Table 1). This demonstrates that the injected cells preferentially synthesized the T2-antigen. With prolonged cultivation time of the microinjected cells, the number of positive cells and the intensity of the T-antigen-specific fluorescence decreased, and about 36 h after injection the cells were once again T-antigen negative.

In an earlier investigation [8,13] we demonstrated that the efficiency of T1-antigen synthesis increased significantly in stable transformed rat cells that carry the p14-Δ5't DNA as a *trans*-gene. The p14-Δ5't construct contains the early SV40 Bst/Bam DNA fragment but the cells cannot excise the small t-antigen intron, since the small t-antigen 5' splice site was deleted from the Bst/Bam DNA fragment by site-directed mutagenesis before microinjection. Small t-antigen *cis*-splicing and T1-mRNA formation by *trans*-splicing are in competition, because both splice reactions utilize the same 3' splice acceptor site.

To confirm that the *trans*-splice efficiency also increases when the in-vitro-synthesized cRNA molecules carry the above deletion, the early SV40 Bst/BamΔ5't DNA fragment was inserted into the multiple cloning site of the pSPT19 DNA (p14-Δ5't-T7) for in vitro cRNA synthesis (cap-Bst/BamΔ5't-cRNA). The purified cRNA was then microinjected and the recipient cells were stained with the T-antigen antibodies. As summarized in Table 1, this deletion significantly increased the efficiency of T1-antigen synthesis in terms of both the number of T1-antigen-positive cells and the brightness of the immunofluorescence staining. 5 h after injection about 20–30% of the injected cells exhibited strong intranuclear T1-antigen-specific fluorescence after staining with the Ab-2 antibody (Table 1). We also stained the injected cells with the HPA-antibody and observed that the deletion of the small t-antigen 5' splice site did not significantly affect the efficiency of T2-antigen synthesis (Table 1). This observation demonstrates that excision of the small t-antigen intron is not essential for the nuclear export of the T2-mRNA. Our RT-PCR and DNA sequencing experiments confirmed that the T2-mRNA still contained the 66 nt small t-antigen intron (see below). The high rate of T2-antigen synthesis observed after intranuclear microinjection of the cap-Bst/BamΔ5't-cRNA further implies that the small t-antigen intron does not affect the translation efficiency of the T2-mRNA. Our protein sequencing experiments confirmed that translation of both the T2-mRNA and T1-mRNA starts with the large T-antigen codon 109 [8]. This AUG is the first potential translation initiation signal in the reading frame at these mRNA molecules, with a distance of 269 nt to the cap site. There are five additional AUGs proximal to the T-antigen codon

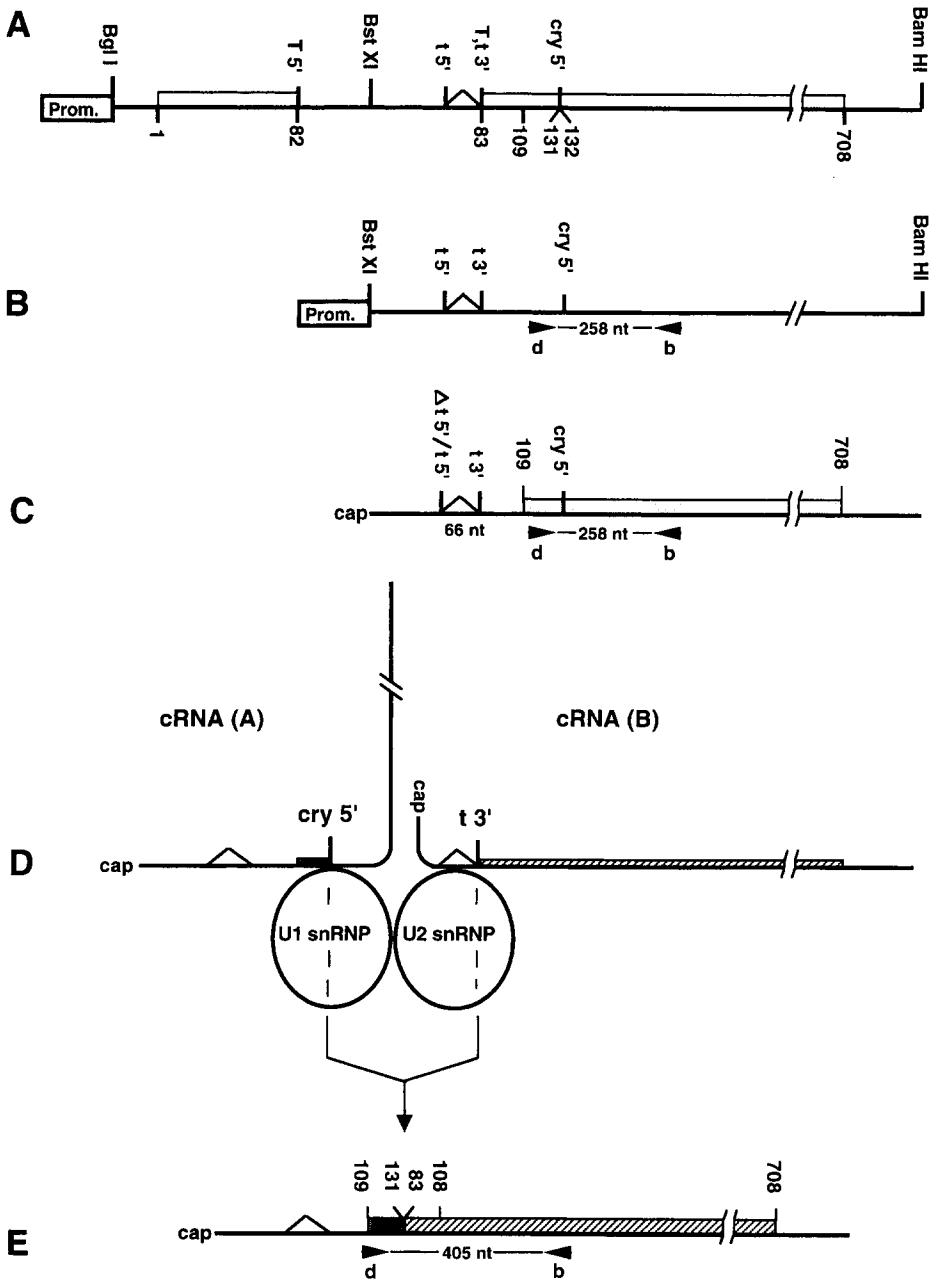

Fig. 1. (A) Diagram of the early SV40 DNA region from the *Bgl*I to the *Bam*HI site, encoding for the large T-antigen (amino acid sequence 1–708) and small t-antigen (not shown). The amino acid 109 is the first methionine within the second T-antigen exon (amino acid sequence 83–708). The small t-antigen 5′ and 3′ splice sites and the position of the cryptic 5′ splice site (amino acids 131–132) are shown. (B) Diagram of the pT7-Bst/Bam and pT7-Bst/BamΔ5′t-DNA used for in vitro cRNA synthesis. The binding sites of the primer pair **d** and **b** with a genomic distance of 258 bp are shown. (C) Schematic illustration of the in-vitro transcripts (Cap-Bst/Bam-cRNA and cap-Bst/BamΔ5′t-cRNA), with the small t-antigen intron (66 nt). The shaded box represents the T2-antigen with the amino acid sequence 109–708. (D) Model for the interaction of two cRNA molecules during *trans*-splicing. (E) Schematic structure of the T1-mRNA and T1-antigen, composed of two segments (amino acids 109–131 and 83–708). The t-antigen intron of the cRNA (A) segment is removed after the *trans*-splicing. With the primer pair **d** and **b** and the T1-cDNA as template, the PCR generates a 405 bp DNA fragment.

109 which are immediately followed by termination signals. This implies that in monkey and rat cells, the ribosomes scan and translate these mRNA molecules with a high efficiency despite the long untranslated leader sequences. This is not unusual, however, since long untranslated leader sequences (5′ UTR) are found in about 5–10% of eukaryotic mRNAs, for example, in the retinoic-acid-receptor-beta 2 mRNA, which has a 461-nucleotide-long 5′UTR segment [27,28].

Synthesis of the T2-antigen also occurred when the cap-Bst/BamΔ5′t-cRNA was microinjected into the cytoplasm of the TC7 and rat 2 cells, although at a lower efficiency than after nuclear injection (Table 1). However, T1-antigen synthesis was not demonstrable in this case. This was not unexpected, since splicing is known to be a nuclear process and cRNA molecules are inefficiently transported from the cytoplasm into the nuclei of the injected cells, as shown by in-situ hy-

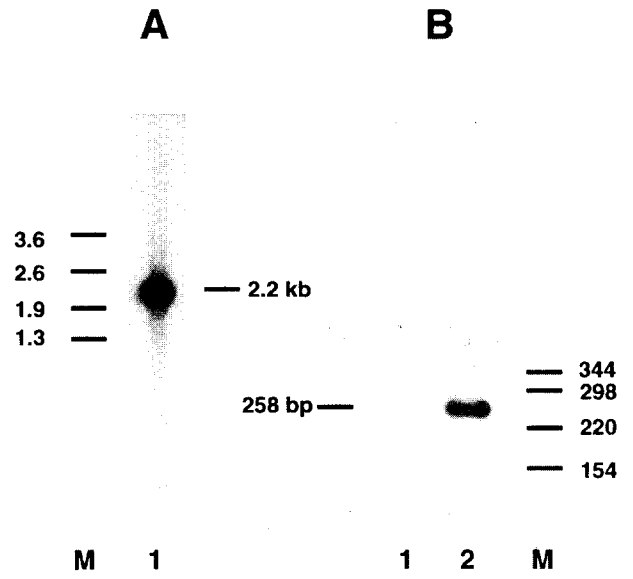

Fig. 2. (A) Northern blot analysis: (lane 1) the in vitro synthesized 2.2 kb cap-Bst/BamΔ5′t-cRNA; (M) RNA size marker (Promega). (B) PCR analysis of the cap-Bst/BamΔ5′t-cRNA: (lane 1) PCR without reverse transcription (RT) to exclude template DNA contamination; (lane 2) PCR with prior RT of the cRNA (RT-PCR); (M) DNA size marker (BRL).

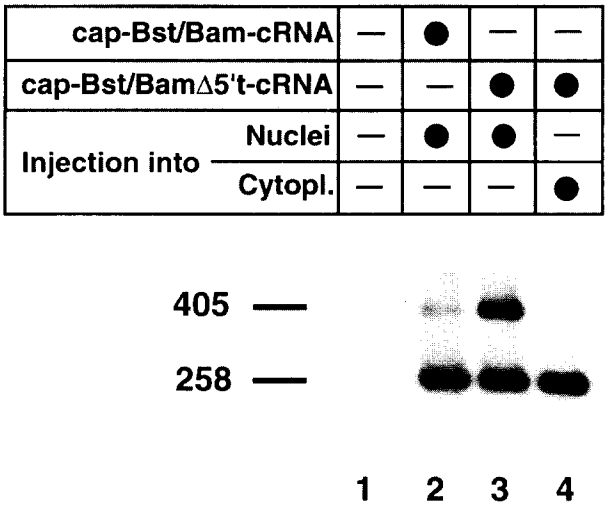

Fig. 3. RT-PCR analysis: (lane 1) RNA extracted from mock injected TC7 cells RNA; (lane 2) RNA extracted from TC7 cells 3 h after intranuclear injection of the cap-Bst/Bam-cRNA; (lane 3) RNA extracted from TC7 cells 3 h after intranuclear injection of the cap-Bst/BamΔ5′t-cRNA; (lane 4) RNA extracted from TC7 cells 3 h after cytoplasmic injection of the cap-Bst/BamΔ5′t-cRNA. The RT-PCR products were separated by agarose gel electrophoresis and subjected to Southern blot analysis. The blot was hybridized with the early SV40 Bgl/Bam DNA fragment. The 405 bp PCR product was obtained from the T1-mRNA, the 258 bp segment was generated from the T2-mRNA or respectively from the unprocessed cRNA.

bridization experiments (M. Graessmann, personal observation).

The aim of the second set of microinjection experiments was to demonstrate *trans*-splicing directly on the level of T1-mRNA synthesis by means of RT-PCR analysis. For this, 100 TC7 cells were microinjected with the cap-Bst/BamΔ5′t-cRNA and 3 h later the RNA was extracted, converted into cDNA and PCR-amplified with the SV40-specific primer pair **d** and **b**. These primers bind upstream and downstream from the 5′ cryptic splice site (Fig. 1), respectively. With this primer pair it is possible to differentiate between the unprocessed cRNA (or T2-mRNA) and the 147 nt larger *trans*-spliced T1-mRNA. With the unprocessed cRNA as template, the RT-PCR generates a 258 bp DNA segment (Fig. 1C); with the T1-mRNA as template it generates a 405 bp DNA segment (Fig. 1E). As shown in Fig. 3, lane 3, with the RNA re-extracted from the microinjected TC7 cells the RT-PCR generated both the 258 bp and the 405 bp DNA segments (the exact sizes of the PCR products were determined by DNA sequencing as shown below).

We repeated the microinjection with the cap-Bst/Bam cRNA, which still contained the intact small t-antigen intron. As shown in Fig. 3, lane 2, the amount of the 405 bp RT-PCR

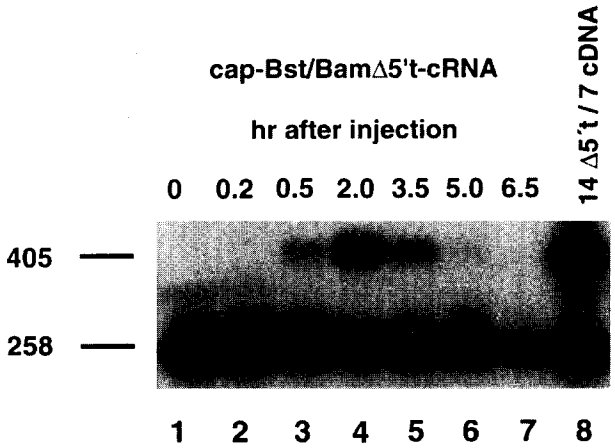

Fig. 4. RT-PCR analysis: (lanes 1–7) RNA re-extracted from TC7 cells at different points in time (0–6.5 h) after nuclear injection of the cap-Bst/BamΔ5′t-cRNA; (lane 8) RNA isolated from the 14Δ5′t/7 cell. Each test point is based on 100 microinjected TC7 cells. The RT-PCR products were separated an agarose gel and subjected to Southern blot analysis as described in Fig. 3.

Table 1
Percentage of T-antigen positive cells

Cells injected with	Injected into	Percentage of T-antigen positive cells	
		HPA-stained	Ab-2-stained
cap-Bst/Bam-cRNA	nuclei	50–60	3–5
cap-Bst/BamΔ5′t-cRNA	nuclei	50–60	20–30
cap-Bst/BamΔ5′t-cRNA	cytoplasm	20–30	0

TC7 cells microinjected with cRNA molecules were fixed and stained for SV40 T-antigen 6 h later. The hamster polyclonal antiserum (HPA) allows detection of both the T2- and T1-antigens, whereas the mouse monoclonal antibody Ab-2 exclusively monitors T1-antigen. The wt SV40 T-antigen is recognized by both types of antisera.

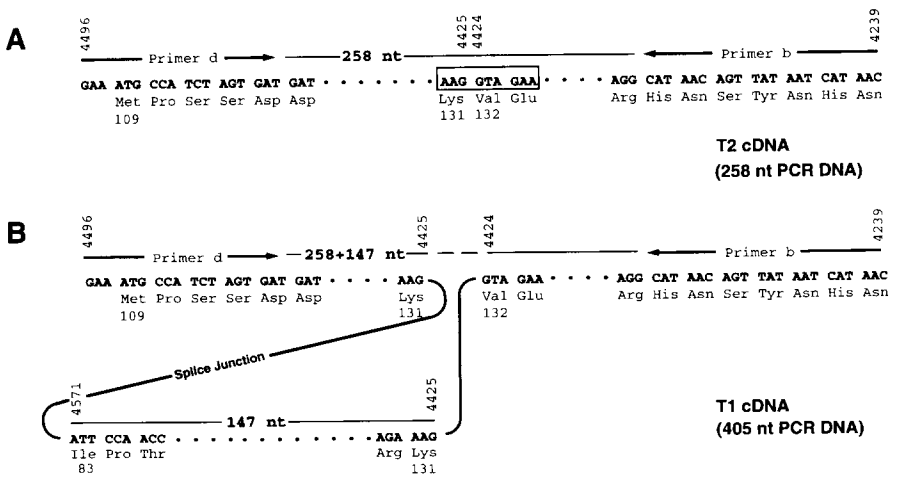

Fig. 5. (A) Nucleotide (nt) sequence of the 258 bp RT-PCR product obtained from the T2-mRNA. The nucleotide sequence is exactly the wt SV40 DNA nucleotide sequence 4496–4239. The positions of the primers **d** and **b** are indicated. The frame shows the cryptic 5′ splice site and the corresponding amino acid sequence. (B) Nucleotide sequence of the 405 bp RT-PCR product obtained from the T1-mRNA. This fragment contains the same sequence as the 258 bp DNA fragment and a 147 nt 'insertion' between nt 4425 and 4424 (amino acids 131/132). This 147 nt segment corresponds to the wt SV40 nt sequence 4571–4425 encoding for the large T-antigen amino acid sequence 83–131.

DNA segment synthesized was significantly lower than that obtained after microinjection of the cap-Bst/BamΔ5′t-cRNA, confirming that small t-antigen *cis*-splicing and T1-mRNA *trans*-splicing are in competition with each other. The RT-PCR analysis further confirmed the above observation that T1-mRNA synthesis requires that the cRNA molecules be microinjected into the nuclei of the recipient cells. Following cytoplasmic microinjection, the RT-PCR generated only the 258 bp DNA fragment (Fig. 3, lane 4).

To determine more precisely when *trans*-splicing occurs after microinjection of the cap-Bst/BamΔ5′t-cRNA, the TC7 cells were lysed at different points in time and the cRNA was re-extracted and subjected to RT-PCR analysis. As shown in Fig. 4, *trans*-splicing was already demonstrable 0.5 h after cRNA injection and the maximal *trans*-splice reaction occurred 1–2 h later. At this time about 10–20% of the injected cRNA molecules were converted into T1-mRNA by *trans*-splicing (Fig. 4).

Finally, we cloned and sequenced the SV40-specific PCR products. These experiments revealed that the 258 bp segment corresponded exactly to the wt SV40 DNA sequence 4496–4239 (Fig. 5A). The 405 bp segment contained the same sequence and an additional 147 bp segment (4571–4425) between nt positions 4425 and 4424 (cryptic 5′ splice site; T-antigen amino acids 131/132). These 147 bp encode for the wt large T-antigen amino acid sequence 83–131, as indicated in Fig. 5B. We also repeated the RT-PCR analysis with primers which bind downstream and upstream from the small t-antigen intron, as described elsewhere [8]. This and subsequent DNA sequencing experiments confirmed that the T1- and T2-mRNA still contained the small t-antigen intron with the 5′ donor splice site mutated (data not shown).

3.3. The cryptic 5′ splice site is essential for trans-splicing

In the model shown in Fig. 1D we propose that *trans*-splice and *cis*-splice mechanisms are closely related and that mammalian cells utilize the same splicing machinery for both pathways. With two cap-Bst/BamΔ5′t-cRNA molecules as precursors, *trans*-splicing is favored because the cryptic 5′ splice site of the donor cRNA (A) is not followed by a functional 3′ splice site, and the 3′ splice site at the acceptor cRNA molecule (B) is not removable by small t-antigen *cis*-splicing. Consequently, *trans*-splicing should not occur when the cryptic 5′ splice site is not accessible. To demonstrate this, the G/GTA splice consensus sequence [29] of the cryptic 5′ splice at the p14 DNA (it contains the SV40 Bst/Bam fragment and the early SV40 promoter [8]) was converted into G/CTT by site-directed mutagenesis, generating the p14Δcry5′ DNA. The p14Δcry5′ DNA was then transfected into rat 2 cells and different independent T-antigen positive cells lines were isolated and further analyzed. As shown by RT-PCR analysis and by immunofluorescence staining, cells of all lines synthesized the T2-mRNA and the T2-antigen exclusively. DNA sequencing confirmed that the T-antigen amino acid 132 of this T2-antigen was converted from Val to Leu. Generation of the T1-mRNA was not demonstrable in any of the analyzed cells.

Acknowledgements: We thank E. Guhl and J. Fuhrhop for excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (GR. 384/13-2).

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