

## Authentic Borna disease virus transcripts are spliced less efficiently than cDNA-derived viral RNAs

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**Borna disease virus (BDV) is a non-segmented, negative-strand RNA virus that replicates and transcribes its genome in the nucleus of infected cells. It uses the cellular splicing machinery to generate a set of alternatively spliced mRNAs from the 2·8 and 7·1 kb primary transcripts, each harbouring two introns. To determine whether splicing of these transcripts is regulated by viral factors, the extent of splicing was studied in infected cells and COS-7 cells transiently transfected with plasmids encoding the 2·8 kb RNA of BDV. Unspliced RNA was found to be the most abundant RNA species in infected cells, whereas viral transcripts lacking both introns were only found in minute amounts. In sharp contrast, plasmid-derived 2·8 kb RNA was predominantly intron 1-spliced and double-spliced. Co-expression of the BDV proteins P, N and X did not influence splicing of plasmid-expressed 2·8 kb RNA. Furthermore, the splicing pattern did not change when the 2·8 kb RNA was expressed in BDV-infected cells. Based on these results we speculate that splicing of authentic BDV transcripts is tightly linked to transcription by the viral polymerase.**

### Introduction

Most pre-mRNAs are spliced and polyadenylated prior to their nuclear export (Nakielnny & Dreyfuss, 1997). Unspliced host pre-mRNAs are normally retained in the nucleus and degraded; however, in many viral systems cytoplasmic accumulation of unspliced or incompletely spliced RNAs is essential for translation of certain viral proteins and particle assembly. Thus, mechanisms have evolved that facilitate nuclear export of intron-containing viral RNAs (Cullen, 1998; Emerman & Malim, 1998; Trono, 1998; Whittaker & Helenius, 1998). In human immunodeficiency virus type 1 (HIV-1) splicing of viral RNAs is circumvented by the activity of the virus-encoded Rev protein (Cullen, 1998; Emerman & Malim, 1998; Trono, 1998). This protein specifically binds to intronic sequences of HIV-1 RNAs, the Rev response element (RRE), and transports the bound RNA into the cytoplasm via the nuclear pore complex (Cullen, 1998; Daly *et al.*, 1989; Emerman & Malim, 1998; Fischer *et al.*, 1994, 1995; Meyer & Malim, 1994; Trono, 1998). The latter activity

requires the leucine-rich region of the nuclear export sequence (NES) of Rev (Fischer *et al.*, 1995; Meyer & Malim, 1994). Mason–Pfizer monkey virus promotes the nuclear export of its unspliced RNAs by a *cis*-acting element (Bray *et al.*, 1994; Ernst *et al.*, 1997; Pasquinelli *et al.*, 1997; Taberero *et al.*, 1997). The function of this constitutive transport element depends entirely on cellular factors (Gruter *et al.*, 1998; Pasquinelli *et al.*, 1997). *Cis*-acting elements that enhance the cytoplasmic accumulation of unspliced or incompletely spliced RNAs are also found in hepatitis B virus (Huang & Liang, 1993; Huang & Yen, 1994) and herpes simplex virus type 1 (Greenspan & Weissman, 1985; Liu & Mertz, 1995). In influenza virus-infected cells nucleocytoplasmic transport of viral and cellular RNAs and pre-mRNA splicing are regulated by the viral NS1 protein (Alonso-Caplen *et al.*, 1992; Chen *et al.*, 1999; Fortes *et al.*, 1994; Lu *et al.*, 1994).

Unlike other non-segmented negative-strand RNA viruses, Borna disease virus (BDV) replicates and transcribes the genome in the nucleus (Briese *et al.*, 1992; Cubitt & de la Torre, 1994). Four major subgenomic RNAs (1·2, 0·8, 2·8 and 7·1 kb RNA) are transcribed from three transcription units of the BDV genome (Briese *et al.*, 1994; Schneemann *et al.*, 1994). Whereas the 1·2 kb RNA, encoding the nucleoprotein (N), is mono-

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cistronic, all the other RNAs are multicistronic. The 0.8 kb RNA encodes the phosphoprotein (P) and the X-Protein. The 2.8 kb RNA contains the ORFs for the putative matrix protein (M) and the glycoprotein (G). The 7.1 kb RNA encodes the polymerase (L) as well as M and G (Briese *et al.*, 1994). The 2.8 and 7.1 kb RNAs are co-terminal at the 5' end but differ in length due to transcriptional readthrough at a termination signal (Schneemann *et al.*, 1994). Since only about 3% readthrough is observed at this termination signal, the 2.8 kb RNA represents the major transcript of the third transcription unit (Schneemann *et al.*, 1994). Expression of M and G from the 2.8 kb RNA, and L from 7.1 kb RNA, are regulated by splicing (Cubitt *et al.*, 1994b; Schneider *et al.*, 1994). Whereas intron 1 is located within the M-ORF, intron 2 is found within the ORFs for G and L. Therefore, transcripts retaining intron 1 serve primarily as mRNAs for M. Splicing of intron 1 results in the disruption of the M-ORF, leaving a minicistron encoding only eight amino acids, which enhances expression of G (Schneider *et al.*, 1997a). Splicing of intron 2 creates the functional L-ORF in the 7.1 kb RNA (Schneider *et al.*, 1994). Additional splicing of intron 1 is most likely required to facilitate initiation at the AUG of the L-ORF. Thus, differential splicing of both introns is critical for balanced expression of the viral proteins M, G and L.

Little is known about the cytoplasmic levels of the various splice forms of BDV RNAs (Cubitt *et al.*, 1994b; Schneider *et al.*, 1994, 1997b). It is unclear whether virus-specific factors/elements are involved in the regulation of splicing. First indications that such factors might be implicated emerged in a recent study where a *cis*-acting RNA element located within the 3' non-coding region of the 2.8 kb RNA was found to be important for efficient cytoplasmic accumulation of unspliced RNA (Schneider *et al.*, 1997b).

We have examined levels of unspliced and spliced BDV transcripts in infected cells and cells transfected with expression constructs encoding the 2.8 kb RNA. BDV-encoded transcripts were spliced less efficiently than those encoded by expression constructs. The splicing efficiency of transcripts generated from expression constructs was similar in uninfected and infected cells. These findings suggest that splicing of BDV-encoded transcripts may be co-transcriptionally regulated.

## Methods

■ **Cell culture and transient transfection of plasmid DNA.** C6, COS-7, MDCK, Vero and Oligo (OL) cells were cultured in Dulbecco's modified Eagle's medium containing 10% FCS. OL cells were kindly provided by Georg Pauli (Robert Koch Institute, Germany). Transient transfections of COS-7 and Vero cells were carried out with 2 or 10 µg plasmid DNA for six-well or 64 cm<sup>2</sup> plates, respectively, using Lipofectamine (GibcoBRL) according to the manufacturer's recommendations.

■ **Construction of plasmids.** Plasmids pRPAwt and pRPAmt were used for *in vitro* transcription of the wild-type RNase protection assay (RPA) probe (RPAwt) or the mutant RPA probe (RPAmt) and were

generated as follows: To create pRPAwt, the *HindIII*–*EcoRI* fragment of pRPA-1 (Schneider *et al.*, 1997b), encoding part of the BDV M/G-ORFs corresponding to nucleotides 1975–2510 of BDV strain He/80 (Cubitt *et al.*, 1994a), was cloned into pGEM-4 (Promega). Plasmid pRPAmt was derived from pRPAwt by replacing six nucleotides of exon 2 (ccagag to gttaac) of the M/G-ORFs corresponding to nucleotides 2216–2221 of BDV strain He/80 using the Quick-mutagenesis protocol (Roche Biochemicals). To clone the mutated DNA into plasmid pRc2.8 encoding the 2.8 kb RNA of BDV (Schneider *et al.*, 1997b), the *BbsI*–*Bst*1107I fragment of pRPAmt, harbouring the respective region, was first subcloned into plasmid pBS-XbaI containing the *XbaI* fragment of pRc2.8, resulting in plasmid pBS-XbaI/mt. In a final step, the *XbaI* fragment of pRc2.8 was replaced with the corresponding fragment of pBS-XbaI/mt, resulting in plasmid pRc2.8mt. Plasmids pRc2.6 and pRc1.5 were obtained by ligating *NotI* fragments of plasmids p2.6 and p1.5 (kindly provided by Patrick Schneider, UC-Irvine, USA) encoding either the 2.6 kb RNA or 1.5 kb RNA of BDV strain He/80 into the corresponding sites of pRc2.8. Plasmid pG3T.t was used to generate the RPA probe for detection of SV40 t-antigen mRNAs in COS-7 cells. Plasmid pG3T.t was prepared by cloning a PCR product including the sequences from position 4541 to 4978 in SV40 DNA into pGEM-3 (Promega). The primers used for amplification (5' gcgcgcaattcggagaatggagtaaaatgc and 5' gcgcgcggtatcgctccattcatcagttcc) contained additional *EcoRI* and *BamHI* sites. The PCR product was digested with both enzymes and cloned into *EcoRI*- and *BamHI*-digested pGEM-3 DNA. Plasmids encoding the N-ORF (pCMV-N) and X-ORF (pCMV-X) of BDV strain He/80 under control of a CMV promoter were kindly provided by Jürgen Hausmann, University of Freiburg, Germany.

■ **RNA preparation.** Isolation of poly(A)<sup>+</sup> RNA from total RNA was carried out using the Oligotex mRNA spin column protocol from Qiagen. RNA from cytoplasmic and nuclear fractions were obtained as described (Nevins, 1980). Briefly, cells were trypsinized 48 h post-transfection, harvested and washed twice with PBS. Cell pellets were lysed in 500 µl Iso-Hi-pH containing 10 mM Tris-HCl (pH 8.4), 140 mM NaCl, 1.5 mM MgCl<sub>2</sub> and 0.5% Nonidet P-40 (0.5%, v/v) for 5 min at 4 °C. Nuclear and cytoplasmic fractions were subsequently separated by centrifugation at 1000 g for 3 min at room temperature. The crude nuclear pellet was immediately washed once in 500 µl Iso-Hi-pH and once in 500 µl Iso-Hi-pH containing 0.33% (w/w) sodium deoxycholate and 0.66% (v/v) Tween 40 to obtain the final nuclear fraction. Total RNA and RNA from both cytoplasmic and nuclear fractions was prepared with peqGOLD TriFast (Peqlab) as recommended by the manufacturer.

■ ***In vitro* transcription and RNase protection assay.** Run-off transcriptions were performed at 37 °C in a total volume of 20 µl containing 0.2 µg linearized DNA, 1 × T7 transcription buffer (Roche Biochemicals), 28 U RNasin (Amersham), 0.5 µM ATP, GTP and CTP, 0.05 µM UTP (Pharmacia), 3 U T7 Polymerase (MBI Fermentas) and 1 µCi <sup>32</sup>P-labelled rUTP (3000 Ci/mmol; Amersham) for 90 min. For subsequent DNase digestion the total volume was raised to a final volume of 30 µl with 1 × transcription buffer including 4 U RNase-free DNase (Ambion) and incubated for 30 min at 37 °C. Purification of the <sup>32</sup>P-labelled RNA and subsequent RPA reactions was done as described in Stalder *et al.* (1998). RPA was performed with approximately 1.4 × 10<sup>5</sup> c.p.m. <sup>32</sup>P-labelled RPA-probe and either 5 µg of total RNA or RNA from the cytoplasmic fraction, 1 µg of RNA from the nuclear fraction or poly(A)<sup>+</sup> RNA, prepared from 50 µg of total RNA. Digestion of unhybridized RNA was performed using 0.02 µg per sample RNase A (Ambion) and 16 U per sample RNase T1 (Roche Biochemicals). The RPA signals were quantified with a phosphorimager (FUJIX BAS1000) using the software package MacBAS version 2.2 (Fuji). Ratios of unspliced and

spliced RNA species of one RPA reaction were calculated based on the signal intensities and were normalized to the number of U residues in the respective protected probe fragments to account for the bias of signal intensities caused by the different lengths of the RNA fragments. RPA size markers were obtained by PCR using pRc2.8 and primers C1 (5' ttgtaggaggacttcacgg), C2 (5' ctgtagctaccaaggatcc), C3 (5' tgaagagcctctgcag) and C4 (5' ctgcatgaaatgacatt), resulting in four DNA fragments of length 535, 484, 434 and 383 bp, respectively. These amplification products (20 ng per fragment) were used in the RPA reaction to generate fragments of the radiolabelled RPA probe corresponding to the length of the expected unspliced and spliced viral RNA species.

**■ Virus stock preparation and titration.** Virus stock from OL cells persistently infected with BDV strain He/80 (Cubitt *et al.*, 1994a) was prepared as described in Briese *et al.* (1992) with slight modifications. Briefly, 25 confluent 90 mm plates cells were washed with 20 mM HEPES (pH 7.4) and incubated with 10 ml 20 mM HEPES (pH 7.4) containing 250 mM MgCl<sub>2</sub> and 1% FCS for 1.5 h at 37 °C. Subsequently, supernatants were harvested and centrifuged twice at 2500 *g* for 5 min to remove cell debris. Virus particles were concentrated by ultracentrifugation for 1 h at 20 °C at 80000 *g* onto a 20% sucrose cushion containing 20 mM HEPES (pH 7.4) and 1% FCS. Virus-containing pellets were resuspended in PBS to approximately 10<sup>8</sup> f.f.u./ml. Persistent BDV infection was established by an initial infection of 10<sup>5</sup> C6, Vero, MDCK or COS-7 cells with 10<sup>4</sup> f.f.u. of virus stock followed by at least 5 weeks of passaging. Complete infection of the cultures was monitored by immunofluorescence assay as described by Hallensleben & Staeheli (1999).

## Results

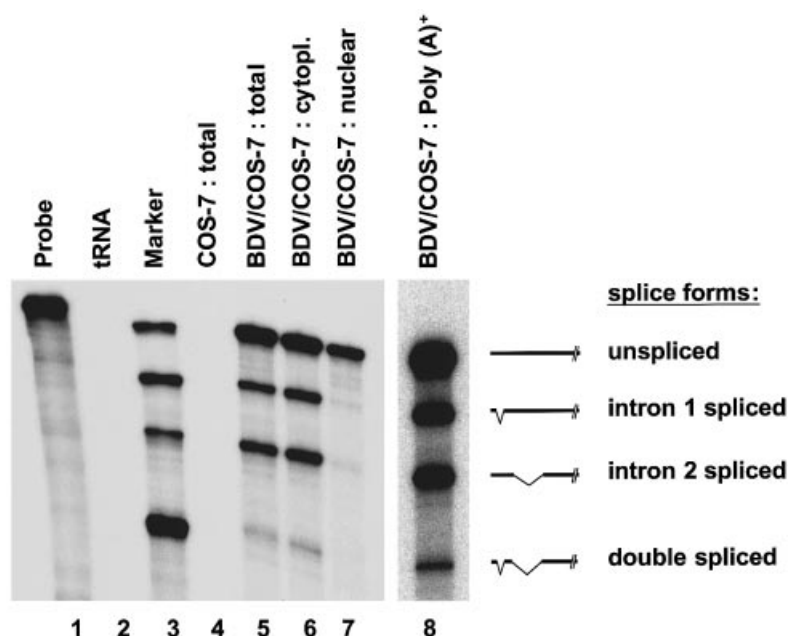
### Ratios of spliced and unspliced viral mRNA species in BDV-infected cells

Unspliced and spliced viral transcripts in the cytoplasm of BDV-infected COS-7 cells were detected by RPA using an

**Table 1.** Quantification of RPA signals of cytoplasmic unspliced and spliced RNA species from various BDV-infected cell lines

Cell line...	Splice form (%)				
	C6	Vero	COS-7	MDCK	OL
Unspliced	47	72	48	31	65
Intron 1-spliced	33	16	19	30	18
Intron 2-spliced	12	8	26	22	13
Double-spliced	8	4	7	16	4

RNA probe (RPAwt) complementary to a part of intron 1, exon 2 and intron 2 of the M/G ORFs (nt-1975–2510). Unspliced RNA was the most abundant RNA species in both total RNA and the cytoplasmic fraction of BDV-infected COS-7 cells followed by lower levels of intron 1- or intron 2-spliced RNAs and minute amounts of RNAs lacking both introns (Fig. 1, lanes 5 and 6). Nearly all RNA detected in the nuclear fraction was unspliced (Fig. 1, lane 7). Quantification of the cytoplasmic RPA signals revealed that 48% of the RNA species were unspliced, 19% intron 1-spliced, 26% intron 2-spliced and 7% double-spliced (Table 1). As a control to assess whether the unspliced RNA observed in the cytoplasmic fraction was viral mRNA and not antigenomic viral RNA that leaked from the nucleus during the fractionation procedure, poly(A)<sup>+</sup> RNA from BDV-infected COS-7 cells was used for RPA. The ratios of unspliced and spliced transcripts were similar in experiments with poly(A)<sup>+</sup> RNA and RNA obtained



**Fig. 1.** Levels of spliced and unspliced viral RNA species in COS-7 cells persistently infected with BDV. RPAs were performed using a 535 nt long RNA probe complementary to the M/G ORF of strain BDV He/80 (lane 1). RNA samples (5 µg) were prepared from total cell lysates (lane 5) or from cytoplasmic (lane 6) and nuclear (lane 7) fractions of BDV-infected COS-7 cells. In lane 8, poly(A)<sup>+</sup> RNA prepared from 50 µg of total RNA of BDV-infected COS-7 cells was used in the RPA. Controls for RNase digestion efficiency were 10 µg of tRNA (lane 2) and 5 µg of total RNA from uninfected COS-7 cells (lane 4). Lane 3 contains markers corresponding to the expected unspliced and spliced transcripts protected by the RNA probe. The potential repertoire of RNA species is indicated (right-hand side).

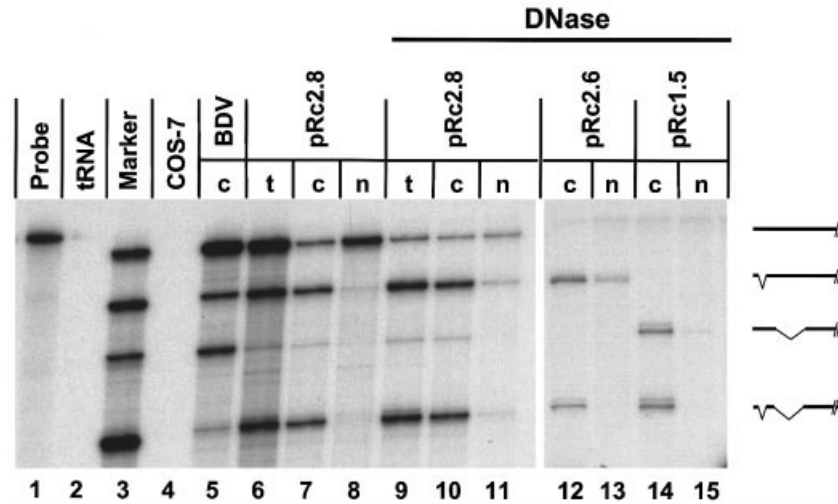


Fig. 2. Plasmid-encoded 2·8 kb RNA is preferentially intron 1-spliced and double-spliced. Levels of unspliced and partially spliced BDV RNA species in infected COS-7-cells (lane 5) and uninfected COS-7-cells transiently transfected with expression plasmids encoding either the 2·8 kb RNA (pRc2·8, lanes 6–11) or mutant 2·8 kb RNAs lacking intron 1 (pRc2·6, lanes 12 and 13) or intron 2 (pRc1·5, lanes 14 and 15) were analysed by RPA (see Fig. 1). RNA samples (5 µg) used in lanes 9–15 were pretreated with DNase I. The source of RNA samples is indicated: t, total cell lysate; c, cytoplasmic fraction; n, nuclear fraction. Controls for RNase digestion efficiency were 10 µg of tRNA (lane 2) and 5 µg of total RNA from uninfected COS-7 cells (lane 4). Lane 3 contains markers corresponding to the expected unspliced and spliced transcripts protected by the RNA probe. The potential repertoire of RNA species is indicated (right-hand side).

from the cytoplasmic fraction (Fig. 1, lanes 6 and 8). Quantification by RPA confirmed this similarity (data not shown).

To address the question whether the ratios of the different splice forms of BDV transcripts may vary with host cell type, RPAs were performed with cytoplasmic RNA extracted from infected renal (Vero and MDCK) and neural (C6 and OL) cell lines. With the exception of BDV-infected COS-7 cells, where levels of the intron 2-spliced RNAs were slightly higher than intron 1-spliced RNAs, decreasing concentrations of unspliced, intron 1-spliced, intron 2-spliced and double-spliced RNAs were observed in BDV-infected C6, Vero, MDCK and OL cells (Table 1). In summary, these data indicate that a characteristic splicing pattern is maintained in all tested BDV-infected cells.

#### Splicing of plasmid-encoded 2·8 kb RNA differs from that of virus-encoded transcripts

To test the hypothesis that the ratio of spliced and unspliced RNAs in BDV-infected cells is controlled by intrinsic features of the viral 2·8 kb or 7·1 kb RNA we transfected plasmid pRc2·8, which expresses only the 2·8 kb RNA, into COS-7 cells (Fig. 2). Two days after transfection we prepared RNA from total cell lysates or from cytoplasmic and nuclear extracts, and analysed the RNA samples for the presence of the various splice forms using the RPA probe described above. As residual plasmid DNA in the RNA preparations would provide signals indistinguishable from unspliced 2·8 kb RNA, parallel RNA samples were treated with RNase-free DNase prior to the RPA. The importance of this control is illustrated by the presence of a strong signal at the position of unspliced

#### Table 2. Quantification of RPA signals of unspliced and spliced RNA species

COS-7 cells were infected with BDV or transfected with plasmids expressing the viral 2·8 kb RNA (pRc2·8) shown in Fig. 2 and the 2·6 kb RNA (pRc2·6) or the 1·5 kb RNA (pRc1·5). Values are means of three independent experiments.

COS-7 ...	Splice form (%)			
	BDV	pRc2·8	pRc2·6	pRc1·5
Unspliced	47	9	–	–
Intron 1-spliced	20	39	52	–
Intron 2-spliced	28	4	–	33
Double-spliced	5	49	48	67

transcripts shown in Fig. 2, particularly in the total and the nuclear RNA fraction (lanes 6 and 8); this signal was indeed substantially reduced in DNase-treated samples (lanes 9 and 11). All four splice forms were detected in total RNA and the cytoplasmic fraction (Fig. 2, lanes 6, 7, 9 and 10). Transcripts originating from 2·8 kb RNA expression plasmids were preferentially intron 1-spliced and double-spliced (Fig. 2, lane 10). In contrast, higher levels of unspliced transcripts were observed in BDV-infected COS-7 cells (Fig. 2, lane 5). Cytoplasmic concentrations of unspliced and intron 2-spliced transcripts were 9 and 4%, respectively, as compared to 47 and 28% in BDV-infected COS-7 cells (Table 2).

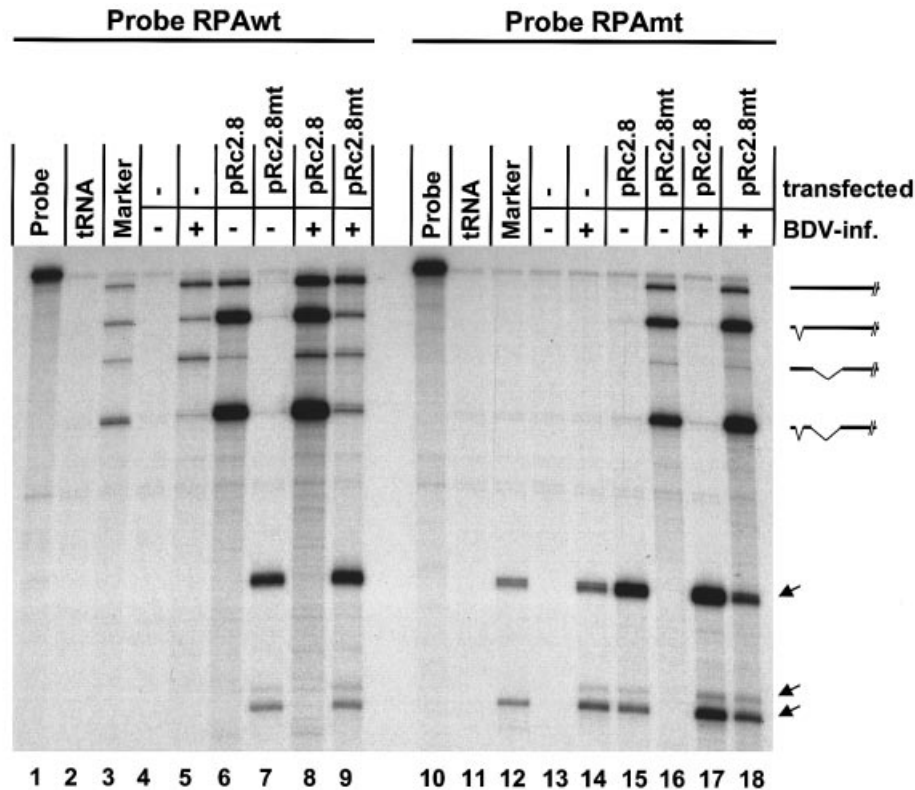


Fig. 3. The splicing of a plasmid-encoded 2.8 kb RNA variant is similar in uninfected and BDV-infected COS-7 cells. Uninfected or BDV-infected COS-7 cells were transiently transfected with plasmids expressing either the wild-type form of the 2.8 kb RNA (lanes 6, 8, 15 and 17) or mutant 2.8 kb RNA (lanes 7, 9, 16 and 18). The mutant RNA is identical to wild-type 2.8 kb RNA with the exception of six nucleotide exchanges within exon 2. RPAs were performed using probes complementary to either wild-type 2.8 kb RNA (RPAwt, lanes 1–9) or mutant 2.8 kb RNA (RPAmt, lanes 10–18). Lane 3 contains markers corresponding to the expected unspliced and spliced transcripts protected by the RNA probe. Lane 12 represents the RPA reaction product with the marker material used in lane 3. Arrows indicate signals from specifically degraded RPA probes due to the six nucleotide mismatch between wild-type and mutant RNAs. Controls for RNase digestion efficiency were 10 µg of tRNA (lanes 2 and 11) and 5 µg of total RNA from uninfected COS-7 cells (lanes 4 and 13). The potential repertoire of RNA species is indicated (right-hand side).

To investigate splicing efficiency of each of the two introns, COS-7 cells were transiently transfected with either plasmid pRc2.6, which expresses a 2.6 kb RNA that lacks intron 1, or plasmid pRc1.5, which expresses a 1.5 kb RNA that lacks intron 2. Two days post-transfection, RNA samples from the cytoplasmic fraction were subjected to RPA. As shown in Fig. 2, the 2.6 kb RNA (lane 12) and the 1.5 kb RNA (lane 14) were both partially spliced. Quantification of the corresponding RPA signals from three independent experiments revealed that 67% of 1.5 kb and 48% of 2.6 kb RNAs lack their corresponding introns (Table 2). These data suggested that intron 1 was more efficiently spliced than intron 2.

#### Splicing of cDNA-derived 2.8 kb RNA remains unchanged in the presence of viral proteins

The differences in the splice ratios of cDNA-derived 2.8 kb RNA and virus-encoded RNAs could be explained by viral factors. Pilot experiments indicated that co-transfection of

plasmids encoding the viral proteins N, P or X either alone or in combination into Vero cells expressing the viral 2.8 kb RNA, did not influence splicing of intron 1 and intron 2 (data not shown). To address the possibility that other transacting viral factors might be missing, we studied splicing of the 2.8 kb RNA in BDV-infected COS-7 cells. To facilitate discrimination between virus and plasmid-encoded RNAs we used a plasmid (pRc2.8mt) that coded for a mutant form of the 2.8 kb RNA carrying six nucleotide exchanges in exon 2. RNA samples were analysed 48 h post-transfection by RPA using RPAmt as a probe, which is complementary to mutated 2.8 kb RNA. Since virus-encoded RNAs do not perfectly match RPAmt, the RNases cleave the probe into two fragments of smaller size. As shown in Fig. 3, expression of the mutated 2.8 kb RNA revealed a splice pattern in BDV-infected COS-7 cells (lane 18) similar to that observed in uninfected COS-7 cells (lane 16). This pattern was identical to that seen in COS-7 cells transfected with pRc2.8, which encodes the wild-type form of the 2.8 kb RNA (lane 6). Comparable splice ratios were

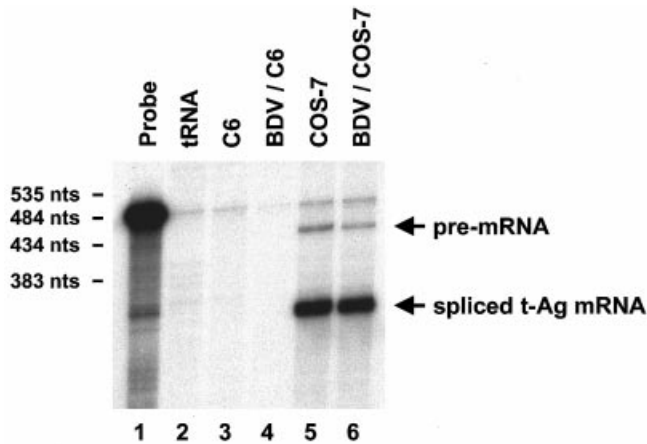


Fig. 4. Levels of unspliced and spliced t-Ag mRNA remain unchanged in BDV-infected and uninfected COS-7 cells. RPAs were performed using a 437 nt long RNA probe (lane 1) complementary to the SV40 early transcripts including the t-Ag mRNA (340 nt). RNA samples (5 µg) were prepared from total cell lysates of uninfected and BDV-infected COS-7 cells (lanes 5 and 6). Controls for RNase digestion efficiency were 5 µg of tRNA (lane 2), 5 µg of total RNA from uninfected and BDV-infected C6 cells lacking the endogenous SV40 transcription unit (lanes 3 and 4). The positions of unspliced pre-mRNA and spliced t-Ag mRNA are indicated by arrows.

observed in uninfected and BDV-infected COS-7 cells using different amounts of pRc2.8mt per transfection (ranging from 0.1 to 1 µg; data not shown). Thus, BDV infection did not seem to influence splicing of cDNA-derived 2.8 kb RNA. However, expression of the mutant form of the 2.8 kb RNA resulted in a subtle change in the splice pattern of virus-encoded RNAs (compare Fig. 3, lane 5 and 9): the amounts of intron 1-spliced and double-spliced transcripts seemed to increase under these conditions.

We further analysed whether virus replication may influence splicing of host cell-derived transcripts by comparison of the spliced and unspliced levels of t-Ag pre-mRNA in COS-7 cells carrying the SV40 early region in the genome. Similar concentrations of t-Ag mRNA and unspliced transcripts were observed in the absence (Fig. 4, lane 5) or presence of BDV (Fig. 4, lane 6), suggesting that splicing of cellular RNAs is not affected in general.

## Discussion

We have shown that splicing of BDV transcripts in infected cells is quantitatively different from splicing in cells transfected with a plasmid encoding the 2.8 kb BDV RNA. Whereas transcripts generated by the viral polymerase were predominantly unspliced, the majority of plasmid-encoded 2.8 kb RNAs were either intron 1-spliced or double-spliced. Based on these differences and the observations that splicing of plasmid-derived 2.8 kb RNA is unchanged in virus-infected cells, we propose that BDV cotranscriptionally regulates the splicing of its own primary transcripts.

Attenuation of transcription at the gene borders is a feature of nonsegmented negative-strand RNA viruses that results in a concentration gradient of mRNAs encoding sequential ORFs (Conzelmann, 1998). The pattern of gene expression is more complex in BDV because M and G are derived from the multicistronic 2.8 and 7.1 kb RNAs. The unspliced and intron 2-spliced mRNAs that encode M are more abundant in the cytoplasm of BDV-infected cells than the intron 1-spliced mRNAs that encode G. Thus, BDV establishes a concentration gradient similar to that observed in other *Mononegavirales* at least in part by regulating the splicing of intron 1 and intron 2. A comparison with plasmid-encoded 2.8 kb RNA indicates that splicing in BDV-infected cells is not solely due to intrinsic features of the viral RNA. We therefore speculate that BDV-encoded factors may interfere with splicing of both introns. This could be achieved by promoting nuclear export of unspliced and partially spliced mRNAs. Intrinsically inefficient splicing of intron 2 might cause the preferential cytoplasmic accumulation of intron 1-spliced RNA. This view is supported by our observation that plasmid-encoded splice intermediates lacking intron 1 were not spliced as efficiently as those intermediates lacking intron 2. However, recent observations indicate that transcription and processing of the cellular polymerase II transcripts is tightly linked, most likely due to the spatial relationship of the different enzymes involved (McCracken *et al.*, 1997). Therefore, an alternative explanation for the preferential splicing of the plasmid-derived transcripts might be that these transcripts are generated in an environment more active in RNA processing than the compartment in which BDV transcription occurs.

In this study we were unable to confirm the results of our earlier report wherein the unspliced plasmid-encoded 2.8 kb RNA predominantly accumulated in the cytoplasm due to an RNA element located in the 3' non-coding region (Schneider *et al.*, 1997b). Since we used the same detection technique (RPA), plasmid (pRc2.8) and cell type (COS-7 cells), we propose that the differences may relate to the fact that the RNA samples in this study were subjected to DNase treatment before analysis by RPA. Furthermore, expression of 2.8 kb RNA lacking the putative export sequence did not result in an enhanced cytoplasmic accumulation of spliced RNA (C. Jehle, N. Horscroft, W. I. Lipkin & M. Schwemmler, unpublished results), indicating that regulation of viral RNA splicing in infected cells most likely occurs via other mechanisms and not as proposed through the 3' non-coding region (Schneider *et al.*, 1997b). One alternative is that nuclear export of unspliced BDV transcripts may be coupled to transcription by the viral polymerase. Support for this hypothesis was found in the observation that the cytoplasmic levels of unspliced 2.8 kb RNA originating from transfected plasmid DNA were low in both infected and uninfected COS-7 cells (Fig. 3, lanes 16 and 18). Intriguingly, we detected subtle changes in virus-encoded RNA species when large amounts of 2.8 kb RNA were expressed from transfected plasmids (Fig. 3, lane 9), suggesting

interference with viral factors required for regulation of splicing.

BDV infection does not appear to interfere with the processing of cellular intron-containing RNA polymerase II transcripts including t-antigen pre-mRNAs in COS-7 cells (Fig. 4) or trKc pre-mRNAs (Valenzuela *et al.*, 1993) in brains of newborn rats (C. Sauder, personal communication). Regulation of differential splicing at the co-transcriptional level provides a mechanism for control of BDV gene expression that does not compromise the viability of the host cell or the potential for establishment of persistent infection.

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