Polymerase Read-Through at the First Transcription Termination Site Contributes to Regulation of Borna Disease Virus Gene Expression

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An unusually long noncoding sequence is located between the N gene of Borna disease virus (BDV) and the genes for regulatory factor X and polymerase cofactor P. Most of these nucleotides are transcribed and seem to control translation of the bicistronic X/P mRNA. We report here that vero cells persistently infected with mutant viruses containing minor alterations in this control region showed almost normal levels of N, X, and P proteins but exhibited greatly reduced levels of mRNAs coding for these viral gene products. Surprisingly, cells infected with these BDV mutants accumulated a viral transcript 1.9 kb in length that represents a capped and polyadenylated mRNA containing the coding regions of the N, X, and P genes. Cells infected with wild-type BDV also contained substantial amounts of this read-through mRNA, which yielded both N and P protein when translated in vitro. Viruses carrying mutations that promoted read-through transcription at the first gene junction failed to replicate in the brain of adult rats. In the brains of newborn rats, these mutant viruses were able to replicate after acquiring second-site mutations in or near the termination signal located downstream of the N gene. We conclude from these observations that BDV uses read-through transcription for fine-tuning the expression of the N, X, and P genes which, in turn, influence viral polymerase activity.

In negative-strand RNA viruses with nonsegmented genomes (Mononegavirales), the polymerase enters the genome at or near the 3’ end and moves toward the 5’ end, sequentially transcribing all genes. Gene start signals direct the polymerase to initiate mRNA synthesis, and transcription termination signals direct the polymerase to release the mRNA (1, 38). Occasionally, viral polymerases ignore termination signals at the gene ends. Such read-through transcription results in the accumulation of capped and polyadenylated mRNAs with two open reading frames, the second of which is not readily accessible to the translation machinery (18). Read-through transcription at gene junctions usually occurs at a low rate and is presumed to play no physiological role in most cases (1, 20, 38). However, in the case of human parainfluenza virus 1 (hPIV-1), termination of transcription after the M gene is surprisingly inefficient because the unusually long noncoding region between the M and F genes contains sequence elements that actively repress termination (3, 4). As a consequence, cells infected with hPIV-1 contain rather low concentrations of the viral F protein and produce relatively few virus particles, a situation that may favor viral persistence. Most other paramyxoviruses also seem to restrict F protein synthesis in infected cells by rendering transcription termination after the M gene rather inefficient (21, 28, 35). Very efficient read-through transcription at the P-M gene junction was observed in the case of a subacute sclerosing panencephalitis (SSPE) patient-derived measles virus (19). Read-through transcription results in the accumulation of capped and polyadenylated mRNAs with two open reading frames, the second of which is not readily accessible to the translation machinery (18). By using either termination sites T3 or T4, the viral polymerase produces either short or long transcripts from the third transcription unit. These transcripts contain two introns which may or may not be removed by splicing. The resulting mature mRNAs are used to synthesize the viral proteins M, G, and L (5, 12, 30, 31) (see also Fig. 1A). BDV-infected cells further contain an abundant transcript of 1.9-kb length that was assumed to represent an incomplete viral replication product (30). This viral transcript is of positive polarity and reportedly lacks a cap structure. It is believed to result from polymerase initiation at the first base of the antigenome and termination at the stop site T2 located at the end of the P gene.

The noncoding region between the N and X genes of BDV has an unusual structure in that the T1 termination site is further modulated by the viral M2-1 protein, which serves as an antiterminator (10, 15, 20). Thus, viruses with nonsegmented negative-strand RNA genomes can, in principal, employ read-through transcription for regulating gene expression.

The RNA genome of Borna disease virus (BDV), a distinct member of the order Mononegavirales, is transcribed and replicated in the nucleus. The viral polymerase uses three transcription start sites and four termination sites to produce several different mRNAs. The product of the first transcription unit is a 1.2-kb mRNA that codes for the N protein. The product of the second transcription unit is a bicistronic 0.8-kb mRNA that codes for X and P. By using either termination sites T3 or T4, the viral polymerase produces either short or long transcripts from the third transcription unit. These transcripts contain two introns which may or may not be removed by splicing. The resulting mature mRNAs are used to synthesize the viral proteins M, G, and L (5, 12, 30, 31) (see also Fig. 1A). BDV-infected cells further contain an abundant transcript of 1.9-kb length that was assumed to represent an incomplete viral replication product (30). This viral transcript is of positive polarity and reportedly lacks a cap structure. It is believed to result from polymerase initiation at the first base of the antigenome and termination at the stop site T2 located at the end of the P gene.

The noncoding region between the N and X genes of BDV has an unusual structure in that the T1 termination site is located downstream of the S2 start site (Fig. 1A). Furthermore, the noncoding region between the N and X genes is substantially longer than all other intergenic regions of BDV. Most of this region is transcribed and represents the 5’ noncoding region of the bicistronic 0.8-kb mRNA encoding X and P. It contains a short open reading frame that starts at an AUG codon at position −26 and terminates at the first of two con-
secutive stop codons that overlap the translation initiation codon of the X gene (Fig. 1A). This peculiar sequence constellation led to the hypothesis that the intergenic region between the N and X genes may contain unrecognized elements that regulate the synthesis of the viral proteins X and P primarily at the translational level (26). Gene overlaps were reported in other viruses with nonsegmented negative-stranded RNA genomes, including RSV (14), Ebola virus, and Marburg virus (7, 16) and some rhabdoviruses (23, 34, 36), but a possible advantage of this unusual gene arrangement remains obscure.

Here we identified a previously uncharacterized element between the N and X genes of BDV which greatly influences viral fitness by controlling the efficacy by which the viral polymerase terminates transcription at the T1 site. We demonstrate that the resulting 1.9-kb read-through transcript is a functional mRNA of BDV with a cap structure and a poly(A) tail that seems to be used for fine-tuning intracellular levels of the viral factors N, X, and P.

**MATERIALS AND METHODS**

Recombinant viruses. Recombinant BDV was recovered from cDNA as described previously (22, 26, 33). Briefly, semiconfluent 293T cells in 35-mm dishes were transfected with 4 μg of pBRPol H-He/BDV, 0.5 μg of pCA-N, 0.1 μg of pCA-L, and 0.1 μg of pCA-31O. At 72 h posttransfection, the 293T cells were seeded into 94-mm plates together with 10^6 Vero cells. These cells were maintained in coculture for at least 15 days. Indirect immunofluorescence analysis was used to screen for recovery of recombinant BDV.

**Western blot analysis.** Protein extracts derived from 293T cells transfected as described previously (5). Samples were dialyzed for 2 days against phosphate-buffered saline and then titrated on Vero cells.

**Infection of rats.** Four-week-old or newborn Lewis rats (Charles River) were anesthetized by using isoflurane and infected by intracerebral injection of 5,000 or 2,000 focus-forming units of BDV in a 20-μl volume into adult and newborn animals, respectively. Intranasal infections of anesthetized 4-week-old rats were done by applying 50-μl samples of virus-containing solutions into the nose. Animals were examined daily for neurological symptoms.

**Northern blot analysis.** RNA was isolated from infected Vero cells or from infected brain material with peqGOLD TriFast (Peqlab Biotechnologie, Erlangen, Germany) as recommended by the manufacturer. Northern blot analysis using 5-μg samples of total RNA was performed as described previously (32). A DNA probe for detecting viral RNA was amplified by PCR using the primers 1 + (5’-TGGTCGTATCAAGAAGCTATGAC-3’) and 1876 + (5’-GCGCTCGAGTTATG-3’) and radioactively labeled with a Prime-It II random oligonucleotide labeling kit (Stratagene).

**Reverse transcription-PCR (RT-PCR).** Portions (2 μg) of total RNA were reverse transcribed using random hexamer primers and the H-minus first-strand cDNA synthesis kit (Fermentas, St-Leon-Rot, Germany) according to the manufacturer’s protocol. The PCR primers 976 + (5’-TACTGGAGTAAGAAGGAT-3’) and 1749 + (5’-CACCTTCCTCCATGAG-3’) were used to amplify the critical region between the viral N and X genes.

**Western blot analysis.** Protein extracts derived from 293T cells transfected with 1 μg of the indicated pCA expression vectors were lysed in radioimmunoprecipitation assay buffer (33). The protein content was determined by Bradford analysis (Bio-Rad). Proteins were separated by electrophoresis through 15% sodium dodecyl sulfate (SDS) polyacrylamide gels and blotted onto polyvinylidene difluoride membranes (Millipore). Bound proteins were detected by using rabbit antiserum against the BDV proteins X and P. As a loading control, the blot was stained with a mouse monoclonal antibody (Sigma) against β-tubulin.

**Isolation of capped RNA.** A total of 10 μl of the cap-specific monoclonal antibody H-20 (Invitrogen) was diluted with 190 μl of NEN buffer (0.75 mM NaCl, 5 mM EDTA [pH 8], 2.5% NP-40) before it was mixed with 200 μl of presoaked protein G-Sepharose 4 Fast Flow beads (GE Healthcare). After incubation under constant rotation at 4°C for 2 h, the beads were washed three times with NEN buffer. Then, 20 μl of RNA isolated from persistently infected cells was added to the loaded protein G-Sepharose beads, and NEN buffer was added to a final volume of 200 μl. The suspension was incubated at 4°C for 1 h under rotation, and the beads were finally washed three times with 500 μl of chilled NEN buffer. Unbound RNA was treated with phenol-chloroform and precipitated with ethanol. Beads were suspended in 200 μl of RNase-free water and treated with phenol-chloroform before the extracted RNA was precipitated with ethanol.

**Isolation of polyadenylated RNA.** RNA samples (20 μg) in 10 mM Tris [pH 7.5], 1 mM EDTA, and 0.1% SDS were heated to 70°C for 5 min and chilled on ice before 44 μl of 5 M NaCl and 100 μl of presoaked oligo(dT)-cellulose (BioLabs) were added. Unbound RNA was precipitated with ethanol. The cellulose was washed three times with 800 μl of binding buffer (10 mM Tris [pH 7.5], 1 mM EDTA, 0.1% SDS, 0.5 mM NaCl). Polya(+) RNA was eluted from the beads with RNase-free water at 70°C and subsequently precipitated with ethanol.

**RNA self-liquidation and product analysis.** The mRNA cap structure was removed by incubating 2 μg of poly(A)+ RNA at 37°C for 1 h with tobacco acid pyrophosphatase (Epicentre Biotechnologies) at a final concentration of 1 U/μl. Decapped mRNA was self-ligated by incubation at 37°C for 1 h with 20 U of T4 RNA ligase (Fermentas) in a final volume of 20 μl. The primers 1495 + (5’-TATTCAAGAAGCATCTGAC-3’) and 440 + (5’-GAGGATCCGCTTGTC-3’) were used to generate a PCR fragment containing the joining region of the self-ligated 1.9-kb RNA of BDV.

**In vitro translation and immunoprecipitation.** Fragments containing the N gene only, the P gene only, or the complete sequence of the 1.9-kb mRNA were amplified by PCR and cloned between the DraIII and XbaI restriction sites of vector PBS-KS II. Then, 1 μg of plasmid DNA was used for in vitro transcription-translation using the TNT coupled reticulocyte lysate system (Promega) according to the manufacturer’s protocol. Newly synthesized proteins were labeled using [35S]methionine-cysteine. Portions (5 μg) of the TNT reaction products were treated with gel sample buffer and loaded onto 15% SDS polyacrylamide gels. The dried gels were exposed to X-ray film (Kodak). For immunoprecipitation, 15 μl of the TNT reaction products were diluted with 185 μl of radioimmunoprecipitation assay buffer and incubated with antibodies specific for either BDV-N or BDV-P. After 1 h of incubation by rotation at 4°C, protein G-Sepharose 4 Fast Flow beads (GE Healthcare) were added, and the mixture was incubated overnight at 4°C with constant rotation. The beads were washed, and the immunoprecipitated proteins were visualized by SDS-polyacrylamide gel electrophoresis and autoradiography.

**RESULTS**

Mutant viruses with alterations in conserved sequence elements between the viral N and X genes are viable. The 56-nucleotide noncoding region between the N and X genes of BDV includes the transcription termination site T1 and the transcription initiation site S2, which is located upstream of the T1 site (Fig. 1A). Other decisive sequence elements are present, including potential translation initiation signals at antigenic positions 1174 and 1198 (positions −50 and −26 relative to the X gene initiation codon) (Fig. 1A). These AUG codons are in frame with two consecutive translation stop codons that overlap the initiation codon of the X gene. We previously demonstrated that the 5’-noncoding region has a negative effect on the translation of X from the 0.8-kb viral mRNA of BDV (26).

To evaluate the relevance of putative regulatory elements in the region between the N and X genes, we studied the effects of a series of specific mutations that we introduced into the genome of BDV strain He/80 by reverse genetics technology. In a first set of virus mutants, the AU nucleotides at positions −50 and −26 were changed to GC either individually (rBDV-08gc-50 and rBDV-08gc-26) or in combination (rBDV-08gc-50/26) (Fig. 1B). In another mutant, we removed the two stop codons that overlap the initiating codon of the X gene (rBDV-08gc-50/26). To further study the relevance of the small open reading frame in the intergenic region, we created a mutant with an in-frame stop codon 20 nucleotides upstream of the X gene (rBDV-08u-20) and a mutant with an additional adenine residue immediately downstream of the T1 signal (rBDV-08+ A-31).

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All mutant viruses were successfully rescued from plasmid DNA. The mutants and wild-type virus spread with similar kinetics in Vero cells (Fig. 1B and data not shown). To examine the genetic stability of the newly generated viruses, we isolated RNA from persistently infected Vero cell cultures and analyzed critical parts of the viral genomes by RT-PCR and sequencing. We found that all mutations that we had introduced into the cloned viral DNA were also present in replication-competent viruses. In the sequence of the rBDV-08/H11001A-31 a single second-site mutation had occurred. In rBDV-08/H11001A-31 recovered from Vero cells we found three additional adenosines rather than one at the T1 signal (Fig. 1B), an alteration that restored the short open reading frame. Additional sequencing work showed that the genome of rBDV-08/H11001A-31 was otherwise free of undesired mutations (data not shown).

Mutant viruses with altered RNA expression patterns are attenuated in adult rats. To compare virus spread in brain tissue, we infected 26-day-old rats with 2,000 focus-forming units of either wild-type or mutant viruses. The infected animals were monitored daily for neurological disease. They were sacrificed if moribund or else at day 60 postinfection. To determine the extent of virus infection, brain sections were stained with a monoclonal antibody specific for BDV-N (13, 26).

FIG. 1. Schematic representation and analysis of mutations introduced into the noncoding region between the N and X genes of BDV. (A) Schematic drawing of the BDV antigenome showing the localization of the transcription initiation sites (S1 to S3) and the transcription termination sites (T1 to T4). The blow-up highlights conserved sequence elements upstream of the translational initiation codon of the X gene (position +1). A short open reading frame located in this region is indicated. (B) Nature of point mutations introduced in the N-X intergenic region and growth behavior of resulting recombinant viruses. Successful (+) and unsuccessful (−) propagation of virus mutants in Vero cells, adult rat brains, or newborn rat brains is indicated. Sequence analyses of rescued viruses showed that rBDV-08+A-31 contained two additional adenosine residues at position −31 (indicated in gray letters). Asterisks indicate viruses that acquired compensatory mutations during growth in brains of newborn rats. The detected sequence alterations are shown in Fig. 3. n.d., not done.
of these animals contained viral antigen-positive cells in the brain (Fig. 1B, adult rat brain). One of three rats infected with rBDV-08u-20 and three of four rats infected with rBDV-08gc-2/u+4 developed neurological disease between days 24 and 36 postinfection (data not shown). All diseased rats but none of the healthy rats of this series contained virus antigen-positive cells in the brain (Fig. 1B, adult rat brain). In a second experiment in which a higher virus dose (5,000 focus-forming units) was applied by the intranasal route, all rats infected with either rBDV-08u-20 (n = 4) or rBDV-08-gc-2/u+4 (n = 2) developed neurological disease and contained large numbers of BDV-infected cells in the brain (data not shown). Thus, all virus mutants with grossly disturbed gene transcription patterns showed an attenuated phenotype in adult rats, whereas the other mutants behaved like wild-type virus in this assay system.

Second-site mutations in newborn infected rats. All BDV mutants that failed to replicate in brains of adult rats were next tested for the ability to grow in the brains of newborn rats, which are intrinsically more susceptible to BDV infection but less susceptible to BDV-induced immunopathology (8, 17, 29). Upon analysis at 28 days postinfection, we observed large numbers of BDV antigen-positive cells in all animals infected with rBDV-08gc-26, rBDV-08gc-50, rBDV-08gc-50/-26, or rBDV-08+A-31 (Fig. 1B, newborn rat brain). To determine whether the introduced mutations were stable in these viruses, RNA from brain homogenates was reverse transcribed, and PCR products containing the critical regions between the N and X genes were sequenced. We found that all nucleotide substitutions that we had introduced in rBDV-08-08gc-26, rBDV-08-gc-50, and rBDV-08-gc-50/-26 were still present, whereas the adenosine insertions in rBDV-08+A-31 had disappeared in all three infected animals (Fig. 3, highlighted in gray). Interestingly, distinct patterns of second-site mutations were found in rBDV-08-gc-26, rBDV-08-gc-50, and rBDV-08-gc-50/-26 (Fig. 3, boxed nucleotides). In each case, the same new mutations were found in three of three virus-infected rat brains, indicating strong selection in favor of these particular virus variants in rats. Variants of rBDV-08gc-26 and rBDV-08-gc-50/-26 capable of replicating in the brain of newborn-infected rats contained one additional adenosine residue in immediate vicinity of the T1 termination site. The signal intensities of the electropherograms resulting from bulk sequencing of RT-PCR products indicated that more than 50% of the viruses in the populations contained the above-mentioned sequence alteration (data not shown). In the case of rBDV-08-gc-50, a U residue in the core of the T1 termination signal was changed to a C residue. Again, bulk sequencing data indicated that viral genomes with the second-site mutation were prominently present (more than 50% of the population) in rat brains (data not shown).

Nature of viral 1.9-kb transcript accumulating in cells infected with mutant viruses. From the Northern blot profiles shown in Fig. 2B and from the second-site mutation analysis shown in Fig. 3, it appeared likely that the novel 1.9-kb RNA of BDV was a viral mRNA generated by read-through transcription at the T1 termination site. If true, the viral 1.9-kb transcript accumulating in cells infected with the mutant viruses should be capped and polyadenylated, and it should extend from S1 to T2.

To evaluate this hypothesis, we first tested whether the viral 1.9-kb transcript carries a cap structure like mRNA typically
does. Northern blot analyses of RNA precipitated with a cap-specific antibody showed that the 1.9-kb transcript from cells infected with rBDV-08gc-26 was enriched as efficiently as the viral 0.8- and 1.2-kb transcripts or the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene transcripts, which represent bona fide mRNAs (Fig. 4A). Interestingly, in cells infected with wild-type BDV the 1.9-kb RNA was also enriched very efficiently by cap-specific antibodies (Fig. 4A). To determine whether the viral 1.9-kb transcripts of rBDV-08gc-26 and wild-type virus are polyadenylated, we subjected RNA from persistently infected Vero cells to oligo(dT)-cellulose chromatography. Northern blot analysis of bound and unbound fractions revealed highly efficient enrichment of 0.8- and 1.2-kb, as well as 1.9-kb transcripts derived from both mutant and wild-type BDV (Fig. 4B). Taken together, these observations indicated that the viral 1.9-kb transcript is capped and polyadenylated and that wild-type and mutant viruses encode this previously uncharacterized viral mRNA.

To characterize the viral 1.9-kb mRNA transcript in more detail, we set out to sequence its 5' and 3' ends. To do this, poly(A)+ RNA from persistently infected Vero cells was decapped and self-ligated. The terminal parts of the viral 1.9-kb RNA were then analyzed by specifically amplifying the joining region of the self-ligated molecule using appropriate primers and RT-PCR technology as described in Materials and Methods. Representative data from these analyses are shown in Fig. 4C. In all cases, the joining region contained at least 50 adenine residues, as predicted from our observation that the viral 1.9-kb transcript is polyadenylated. The 5' end of the viral 1.9-kb RNA mapped to the S1 initiation site at position 44 of the viral antigenome, and the 3' end mapped to the T2 transcription termination site at position 1879 (Fig. 4C). As expected from the Northern blot analyses discussed above, the structures of the 1.9-kb mRNA transcripts from wild-type BDV and mutant rBDV-08gc-26 were identical (Fig. 4C).

Viral 1.9-kb mRNA is expressed by different BDV strains in cell culture and in vivo. The transcription analyses described above suggested that gene expression of BDV is more sophisticated than previously assumed. It remained unclear, however, whether this feature was a peculiarity of BDV replicating in Vero cells or even was an artifact of recombinant BDV strain He/80. To clarify the first point, we analyzed the viral gene expression pattern of recombinant BDV in various culture systems. We found large amounts of polyadenylated viral 1.9-kb transcripts in persistently infected monkey Vero, rat C6 glioma, and human oligodendroglial (Oligo) cells, as well as in the brains of infected rats (Fig. 5A). To clarify the second point, we used three different nonrecombinant BDV strains, namely, He/80 (11), No/98 (24), and V (6). We found that Vero cells persistently infected with any of these viruses contained substantial amounts of polyadenylated 1.9-kb transcripts (Fig. 5B). We noted, however, that cells infected with these three different virus strains contained different relative levels of 1.9-kb transcripts.

Coding capacity of the novel viral 1.9-kb mRNA. Since the newly identified viral 1.9-kb mRNA transcript exhibits features of a multicistronic mRNA, it was of interest to experimentally determine its coding capacity. We used a coupled in vitro transcription-translation system for this analysis. At least three prominent radiolabeled products were observed if in vitro-transcribed 1.9-kb mRNA was used as a template for in vitro translation. These products migrated upon SDS-polyacrylamide gel electrophoresis like products translated from mRNAs for BDV-N and BDV-P, respectively (Fig. 6, left panel). To verify the identity of the various bands observed on the gel, the radiolabeled translation products were immuno-
precipitated with BDV-specific antibodies. In reaction programmed with either 1.9-kb viral mRNA or authentic N mRNA, the N-specific antiserum mainly detected a 40-kDa protein and a less prominent 22-kDa polypeptide (labeled with an asterisk in Fig. 6), which presumably represents a degradation product of N (Fig. 6, right panel). The P-specific antiserum specifically recognized a 23-kDa translation product in reactions programmed with either viral 1.9-kb or authentic P mRNA, indicating that the third open reading frame of the 1.9-kb read-through mRNA was indeed used for translation (Fig. 6, middle panel). Since the X protein contains only one cysteine and no methionine residue and since no suitable antibody for specific immunoprecipitation of X is available, this assay system did not allow us to determine whether the 1.9-kb viral mRNA can also direct the synthesis of X. Nevertheless, our results collectively supported the view that the newly identified viral 1.9-kb transcript serves as multicistronic mRNA.

DISCUSSION

The most important conclusion of our present work is that the viral transcriptase frequently ignores the transcription stop...
transcript does not code for viral proteins. At present there is consequently, it had been assumed that this abundant viral described in certain other negative-strand RNA viruses (1). Conclusion product or an extended form of "leader RNA" de-
cluded that the 1.9-kb RNA represents an abortive repli-
the results presented in one of these former studies (30), it was previously been observed in BDV-infected cells (6, 30). From signal is used in BDV.

nucleotides located upstream and downstream of the core sig-
ificant for the proper termination of gene transcription. Other consensus motif found at all termination sites of BDV (31) sequence strongly influence the efficacy by which the T1 element in the immediate vicinity of T1 determines the efficacy of

transcription termination which, in turn, correlated with viral fitness. Our results thus demonstrate that the U(A)₆/₇ consensus motif found at all termination sites of BDV (31) only represents a core signal sequence that is clearly not sufficient for the proper termination of gene transcription. Other nucleotides located upstream and downstream of the core signal sequence strongly influence the efficacy by which the T1 signal is used in BDV.

It should be noted that viral transcripts of 1.9-kb length have previously been observed in BDV-infected cells (6, 30). From the results presented in one of these former studies (30), it was concluded that the 1.9-kb RNA represents an abortive replication product or an extended form of "leader RNA" described in certain other negative-strand RNA viruses (1). Consequently, it had been assumed that this abundant viral transcript does not code for viral proteins. At present there is no good explanation for why the 5' end of the 1.9-kb transcript was previously mapped to antigenome position 1 rather than to the transcription start site S1 at position 44 (30). It is possible that uncapped viral RNA molecules 1.9 kb in length containing the terminal 44 nucleotides in question are indeed present at low levels in infected cells and that these rare molecules were cloned and sequenced in the previous study. In any case, the much more abundant 1.9-kb mRNA described here escaped detection in the previous analysis for unknown reasons. The experiments described in this report unambiguously show that the vast majority of viral 1.9-kb transcripts in BDV-infected cell cultures and rat brains are bona fide mRNAs rather than aberrant replication products.

In vitro transcription studies using rabbit reticulocyte lysates suggested that the newly discovered 1.9-kb mRNA of BDV not only serves as a template for the synthesis of N but also might be used for the translation of the viral P protein (Fig. 6) and possibly X. At present, it is unclear whether the results of these in vitro studies can be taken as evidence for a similar multiple use of the 1.9-kb mRNA in infected cells. It is expected that the 1.9-kb mRNA is a much better template for N than for X and P since secondary structure prediction analyses yielded no evidence for an internal ribosomal entry site upstream of the X or P open reading frames in the 1.9-kb mRNA. It should be noted, however, that the presence of a functional internal ribosomal entry site has not been tested rigorously by appropriate experiments. Thus, read-through of the viral transcriptase at T1 may deprive infected cells from templates for X and P. This deprivation is not critical for virus growth in highly susceptible Vero cells but, apparently, it abolishes virus replication in the brain of adult rats. It is well documented that the concentrations of X and P play a critical role in BDV-infected cells and that the activity of the viral polymerase is greatly influenced by the abundance of these proteins (25–27, 32). Our results thus indicate that BDV must actively regulate its polymerase activity to overcome growth restrictions by the host and that such regulation is hampered if the element which regulates read-through transcription at T1 is defective. Surprisingly, we observed that the steady-state levels of N, X, and P were almost normal in Vero cells infected with rBDV-08gc-26 and rBDV-08gc-50, in which termination at T1 is permanently repressed. To account for this finding, we speculate that viral proteins exhibit greatly enhanced metabolic stability if they can form complexes with other viral components. If this hypothesis is correct, we might not observe strong effects on the steady-state levels of viral proteins in persistently infected Vero cells although de novo synthesis of X and P is substantially reduced.

The first and the second transcription units of the BDV genome are overlapping by 17 nucleotides (Fig. 1A). We propose that this structural feature may relate to the particular use of the transcription start and stop signals in this region. If transcription of the BDV genome obeys the general rules of negative-strand RNA viruses which have been worked out mainly with vesicular stomatitis virus (VSV) as a model (1, 2), we would assume that after having synthesized the poly(A) tract of the 1.2-kb mRNA, the viral polymerase has to move back on the template in order to reinitiate transcription at start site S2 and to synthesize the 0.8-kb mRNA. From our data we speculate that the BDV polymerase can perform this difficult task only if the template has assumed a particular secondary structure which may not be formed if the highly conserved AU dinucleotides at antigenome positions 1174 or 1198 (positions −50 and −26 relative to the X open reading frame) are mutated to GC as in mutants rBDV-08gc-26, rBDV-08gc-50, and rBDV-08gc-50/-26. These viruses accumulated compensatory second-site mutations rather than simple reversions during replication in brains of newborn rats. These compensatory mutations all occurred in the immediate vicinity of the core element in the T1 signal sequence, changing the U(A)₆ signal to either U(A)₆ in two virus mutants or to C(A)₆ in a third virus. We assume that these mutations increased the efficiency by which the termination signal is recognized by the viral transcriptase. For RSV and VSV, it appears that the rate of transcriptional read-through at gene junctions is mainly determined by the length and not so much by the structure of the intergenic region (2, 37). However, based on the analysis of SSPE-derived mutant viruses that lost the ability to make mRNAs for M, Cattaneo et al. (9) proposed a model for

![FIG. 6. The 1.9-kb mRNA serves as a template for N and P protein synthesis. Expression constructs for viral N, P, or 1.9-kb mRNAs were used for in vitro translation in the presence of [³⁵S]methionine-cysteine. Portions (5 μl) of the in vitro translation products were directly loaded onto a 15% SDS-polyacrylamide gel (left panel). Portions (15 μl) of the translation products were treated with a rabbit antiserum directed against BDV-P (anti-P) or BDV-N (anti-N) and precipitated with protein G-Sepharose. Bound fractions were loaded onto 15% SDS-polyacrylamide gel (middle and right panels). The asterisk marks an N-specific fragment of unknown identity.](http://jvi.asm.org/content/82/20/9543/suppl/DC1/F6.jpg)
transcription termination at the P-M gene junction of measles virus that strikingly resembles the model we propose for transcription termination at the N-X/P gene junction of BDV. As in the case of BDV, it was observed that a few point mutations upstream and possibly also downstream of the core polyadenylation signal of measles virus determine whether or not the signal is used efficiently.

We noted with interest that the compensatory mutation that appeared in rBDV-08-gc-26 and rBDV-08-gc-50/26 during newborn rat passage was identical to the mutation we intended to introduce in rBDV-08-A-31 but failed to detect in the rescued virus (Fig. 1B). The virus we were able to rescue from a plasmid construct carrying an extra A residue at position −31 acquired two additional A residues that changed the termination signal motif from U(A)n to U(A)n. At present, we do not know how these additional residues might affect the termination efficacy. However, the fact that some additional A residues are tolerated in the context of the wild-type sequence while others are tolerated only in the context of a mutated sequence strongly suggests that there is a delicate balance between the activity of the core signal sequence and the adjacent control element. Obviously, sophisticated new in vivo analysis systems are required for future delineation of the proposed regulatory element that modulates the termination and reinitiation efficacy of the BDV polymerase at this intergenic region.

A closer inspection of the Northern blots in Fig. 2 and 5 suggests that BDV transcription may not obey the general rules of negative-strand RNA viruses that have been worked out mainly with VSV as a model (1, 2). If the viral transcriptase entered the linear genome of BDV exclusively at or near the 3′ end as in VSV, the first gene should be transcribed with equal or higher efficacy than the second transcription unit. We find, however, that in cells infected with wild-type virus the steady-state levels of the 1.2-kb transcript are substantially lower than those of the 0.8-kb transcript. More work is required to determine whether the metabolic stability of the 1.2-kb mRNA is reduced or whether the second transcription unit is indeed expressed at higher rate than the first.

Due to the unusual position of the S2 start signal, the 0.8-kb mRNA of BDV carries a rather long 5′ nontranslated region (Fig. 1A). From the results of a study with plasmid-based expression constructs carrying or lacking this sequence we previously showed that the 5′ noncoding region regulates the efficacy by which the X and P proteins are translated from the bicistronic 0.8-kb mRNA (26). The 5′ noncoding region of the 0.8-kb mRNA contains a short open reading frame (Fig. 1A) which could modulate the use of the initiation codon of the X and P open reading frames. In rBDV-08u-20 and rBDV-08-gc-2/(u+4) the short open reading frame upstream of the X and P coding regions was either shortened or extended. Surprisingly, these mutant viruses grew well in Vero cells and rat brains (Fig. 1B), indicating that regulation of viral gene expression at the translation level plays a minor role in BDV-infected cells. To evaluate the possibility that the peptide encoded by the open reading frame near the 5′ end of the 0.8-kb mRNA might regulate the activity of the BDV polymerase, we used the BDV minireplicon system. We found that an expression construct designed to express the peptide in question did not alter the activity of the viral polymerase in this system (data not shown). This negative result does not exclude the possibility that the role of the putative small peptide can only be measured in the context of virus infections of neurons. To evaluate this possibility, new recombinant viruses will need to be constructed.

In summary, our results demonstrate that transcription termination at intergenic signal sequences of negative-strand RNA viruses is not necessarily set to a default value. In the case of the termination signal after the N gene of BDV, the flanking sequences seem to constitute a regulatory element that promotes termination if present and suppresses termination if altered by subtle mutations. By modulating read-through transcription at this intergenic region, BDV may efficiently regulate the levels of X and P, which in turn regulate polymerase activity. A conceptually similar role was previously assigned to sequences located between the M and F genes of hPIV-1 (3). However, in hPIV-1 the regulatory element suppresses rather than stimulates transcription termination. Further, it is much larger than the element we identified in BDV, and it is not transcribed into mRNA. Since part of the newly identified regulatory element in BDV is transcribed, it is tempting to speculate that it acts cotranscriptionally. One might envision a scenario in which the critical element may only fold into an active conformation on newly synthesized mRNA or, alternatively, on the template when the ribonucleoprotein complex is locally destabilized as the polymerase is about to transcribe the critical region of the viral genome.

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