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RNA Polymerase II-Controlled Expression of Antigenomic RNA Enhances the Rescue Efficacies of Two Different Members of the *Mononegavirales* Independently of the Site of Viral Genome Replication

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De novo generation of negative-strand RNA viruses depends on the efficient expression of antigenomic RNA (cRNA) from cDNA. To improve the rescue system of Borna disease virus (BDV), a member of the *Mononegavirales* with a nuclear replication phase, we evaluated different RNA polymerase (Pol) promoters for viral cRNA expression. Human and mouse Pol I promoters did not increase the recovery rate of infectious BDV from cDNA compared to the originally employed T7 RNA polymerase system. In contrast, expression of viral cRNA under the control of an RNA Pol II promoter increased the rescue efficacy by nearly 20-fold. Similarly, rescue of measles virus (MV), a member of the *Mononegavirales* with a cytoplasmic replication phase, was strongly improved by Pol II-controlled expression of viral cRNA. Analysis of transcription levels derived from different promoters suggested that the rescue-enhancing function of the Pol II promoter was due mainly to enhanced cRNA synthesis from the plasmid. Remarkably, correct 5′-terminal processing of Pol II-transcribed cRNA by a hammerhead ribozyme was not necessary for efficient rescue of BDV or MV. The correct 5′ termini were reconstituted during replication of the artificially prolonged cRNA, indicating that the BDV and MV replicase complexes are able to recognize internal viral replication signals.

The group of negative-strand RNA viruses includes many important human and animal pathogens. Genetic manipulation and molecular analysis of these viruses are complicated by the fact that the naked viral RNA is not infectious. To generate the ribonucleoprotein complex (RNP), the minimal infectious unit of negative-strand RNA viruses, simultaneous expression of viral protein components forming the polymerase (Pol) complex and full-length viral antigenomic RNA (cRNA) from cDNA is required (3, 14). During the past decade, such reverse genetics systems have been established for a variety of negative-strand RNA viruses, relying on different DNA-dependent RNA polymerases for the expression of viral cRNA. Negative-strand RNA viruses with a nuclear replication strategy, such as influenza A virus (15) and Thogoto virus (29), were recovered using cellular DNA-dependent RNA Pol I. Recovery of viruses with a cytoplasmic replication strategy, including most of the nonsegmented negative-strand RNA viruses (*Mononegavirales*) such as measles virus (MV) and rabies virus, relied on the DNA-dependent RNA polymerase from bacteriophage T7 (T7pol) supplied by either stably transfected helper cells or recombinant helper virus (3).

Borna disease virus (BDV) contains a nonsegmented RNA genome of negative polarity. BDV replication is strictly noncytolytic and results in the persistent infection of neurons in the central nervous system (6). The genome codes for six genes and is replicated and transcribed in the nucleus (2, 4). Gene expression involves alternative splicing of two intron sequences (5) and results in a complex pattern of viral transcription and replication products. Based on these unique properties, BDV has been classified in its own family, the *Bornaviridae*. The first reverse genetics systems for BDV using either T7pol (24) or Pol I (16) to express artificial minigenomic RNA molecules from cDNA were established only recently. As in other members of the *Mononegavirales*, the RNP of BDV is composed of viral full-length RNA associated with nucleoprotein (N), phosphoprotein (P), and RNA-dependent RNA polymerase (L). Efficient RNA synthesis requires a distinct ratio of N and P proteins and is inhibited by the presence of the nonstructural viral X protein (22) through its interaction with P (17). Recovery of recombinant BDV was achieved with a plasmid encoding full-length viral cRNA under the control of the T7 promoter in BSR-T7 helper cells (25). To enhance transcription of viral cRNA by T7pol, three G nucleotides were added directly downstream of the T7 promoter, and the generation of correct genome termini was mediated by 5′ hammerhead and 3′ hepatitis delta virus ribozyme sequences, respectively. Although transfection of BSR-T7 cells allowed repeated recovery of wild-type and mutant BDV (25), the rescue efficacy was rather low.

Improvement of virus rescue systems can involve different strategies, including the use of more susceptible cells, improved transfection efficiency, or enhanced expression of viral cRNA. The fact that BDV is the only member of the *Mononegavirales* with a nuclear replication strategy suggests that BDV recovery might benefit from using cellular DNA-dependent Pol I or Pol II, which would express viral cRNA in the nucleus. Pol I is responsible for the synthesis of the majority of cellular RNA molecules. The transcription initiation and termination sites of Pol I are exactly defined by species-specific promoter

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and termination sequences. Thus, Pol I-expressed viral cRNA does not require further processing to generate correct viral genome termini (8). In contrast, the transcription initiation site of Pol II is not precisely defined by the promoter sequence, and transcripts are modified by the addition of a poly(A) tail of variable length to the 3′ end. Thus, neither the 5′ nor the 3′ termini of Pol II-transcribed cRNAs are exactly defined and require posttranscriptional modification for the generation of correct viral genome termini. Furthermore, Pol II-mediated RNA synthesis is closely associated with the nuclear splicing machinery, which mediates efficient export of most cellular mRNAs from the nucleus. Splicing of Pol II-transcribed viral cRNA due to the recognition of natural or cryptic splice acceptor and donor sites by the cellular splicing apparatus could interfere with virus recovery. However, the recent generation of recombinant rabies virus from Pol II-transcribed cRNA (11) demonstrates that the export of unspliced viral cRNA molecules from the nucleus can be efficient enough to support the generation of recombinant viruses.

Here, we demonstrate that RNA polymerase II-mediated expression of viral cRNA greatly improved the recovery of two largely different members of the *Mononegavirales* from cDNA. Our data show that the rescue efficacies of BDV and MV were strongly enhanced by Pol II-controlled expression of viral cRNA, in spite of different replication sites of the two viruses. The enhancing effect of the Pol II promoter was most likely due to increased synthesis of viral cRNA from plasmid. Remarkably, the rescue of BDV and MV from plasmids encoding inactive hammerhead ribozymes was efficient. Subsequent viral replication resulted in the generation of cRNA with correct 5′ termini. These results demonstrate that the polymerases of BDV and MV are able to recognize internal viral replication signals and suggest that rescue systems based on Pol II-directed expression of viral cRNA might generally improve the recovery of negative-strand RNA viruses.

MATERIALS AND METHODS

Plasmid constructions. Sequences of primers used in PCR assays to amplify promoter and coding sequences are available on request. PCR was performed with proofreading Turbo *Pfu* DNA polymerase (Stratagene) and standard reaction conditions in the GeneAmp PCR Cycler 9600 (Applied Biosystems). The integrity of all PCR-derived DNA fragments was verified by sequencing. All restriction digestions of plasmids and PCR fragments were performed using commercially available enzymes (New England Biolabs and Fermentas) and the supplied buffer systems. Ligation reactions were done using 2.5 Weiss units of bacteriophage T4 DNA ligase (Fermentas) in a total volume of 5 μl. Ligation reaction mixtures were incubated at 16°C for at least 2 h and then used to transform 50 μl of competent Top10 bacteria (Invitrogen).

New promoter sequences were inserted into pBRT7-HrBDVc using an SfiI restriction site upstream of the T7 promoter sequence and a unique SpeI restriction site localized between the T7 promoter and the hammerhead sequence. The human Pol I (hPol I) and murine Pol I (mPol I) promoters were amplified from plasmids pLD91 and pRF42 (8) (kind gifts from R. Flick, Galveston, Tex.) using primer pairs hPol I(+, *SfiI*)/hPol I(−, SpeI) and mPol I(+, *SfiI*)/mPol I(−, SpeI), respectively. The minimal cytomegalovirus-derived Pol II promoter was amplified from vector pCG-MVL (9) (a kind gift of M. Billeter, University of Zurich) using primer pair CMV(+, *SfiI*)/CMV(−, SpeI). To inactivate the autocatalytic activity of the hammerhead sequence, we subcloned the SpeI-XhoI fragment of pBRT7-HrBDVc (500 bp) into the pBluescript-KS(+) vector (Stratagene) and used the plasmid as a template for two rounds of mutagenesis PCR with two different primer pairs, which eliminated five T residues. The mutated hammerhead sequence (Hmut) was excised from pBluescript-KS(+) by digestion with SpeI-XhoI and inserted into pBRT7-HrBDVc and pBRPol II-HrBDVc.

The open reading frames (ORFs) of MV N and P were amplified from pBRT7-MV(+), a derivative of vector pCDV3 (27), using primer pairs MVN(+, NotI)/MVN(−, NheI) and MVP(+, NotI)/MVP(−, *Nhe*), respectively. Amplification products were NotI-NheI digested and ligated into the NotI-NheI-opened pCA-modified vector (24), resulting in pCA-MVN and pCA-MVP. Due to restriction site duplication, construction of pCA-MVL was performed in two steps. First, XhoI-DraIII and DraIII-EcoRI fragments were excised from vector pCG-MVL and ligated into the XhoI-EcoRI-opened pCA-modified vector, resulting in the formation of the incomplete MVL ORF lacking 394 nucleotides at the 5′ end of the ORF. The missing fragment was amplified from pCG-L using primers MVL(+, EcoRI) and MVL(−, *MfeI*), digested with the indicated restriction enzymes, and ligated into the EcoRI-opened and dephosphorylated intermediate pCA construct harboring the incomplete MV L ORF. The strategy took advantage of the fact that EcoRI and MfeI create identical overhangs. The nucleotide exchange introduced by this strategy did not alter the encoded amino acid sequence of the MV L protein.

To insert an MV-specific hammerhead sequence, we first generated an intermediate construct by the digestion of pBRT7-MV(+) with NgoMIV and religation of the backbone fragment. These manipulations resulted in the removal of almost 13,000 nucleotides of the MV antigenomic sequence and created a unique BamHI site 173 nucleotides downstream of the 5′ end of the MV antigenome-encoding sequence. The 5′ end of the MV antigenome was amplified by PCR using primer MVH(+, *AscI*), inserting the hammerhead and a unique AscI restriction site, and primer MV301(−), binding downstream of the BamHI site. The T7 and the Pol II promoters were amplified using primer T7(−, *AscI*) and CMV(−, *AscI*), respectively, together with primer pBR(+, *SfiI*), binding to the plasmid backbone upstream of a unique SfiI site. The promoter fragments were SfiI-AscI digested and ligated together with the AscI-BamHI-digested MV fragment into the SfiI-BamHI-opened intermediate construct. The hammerhead sequence was finally transferred into the full-length vector pBRT7-MV(+) using unique SfiI and SacII sites, resulting in the constructs pBRT7-HrMV and pBRPol II-HrMV. The constructs encoding the inactive hammerhead sequence were constructed in the same way, using primer MVHmut(+, *AscI*) instead of MVH(+, *AscI*).

Cells and transfections. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) for 293T (human embryonic kidney) cells and with 4% FCS for Vero (African green monkey) cells. Cells were kept at 37°C in a 5% CO_2-humidified atmosphere. Transfection of 293T cells in 35-mm (six-well) dishes was performed basically as described previously (24). Briefly, semiconfluent layers of 293T cells were grown for 4 to 6 h in 35-mm (six-well) dishes before transfection with the various plasmids. DNA dilutions were prepared in 100 μl of DMEM and subsequently mixed with 100 μl of DMEM containing 10 μl of Metafectene (Biontex). The transfection solutions were incubated at room temperature for 30 min and then directly applied to the cell supernatants. After overnight incubation, transfection solutions were replaced by fresh DMEM medium.

BDV rescue. Semiconfluent 293T cells in 35-mm dishes were transfected with 4 μg of plasmids encoding the full-length BDV antigenome in combination with 0.5 μg of pCA-N, 0.05 μg of pCA-P, and 0.1 μg of pCA-L (24). To express viral cRNA from pBRT7-HrBDVc in 293T cells, 1 μg of pCA-T7 (a kind gift of R. Flick, Galveston, Tex.) encoding T7pol was added to the transfection mixture. In T7pol-independent rescue experiments, the amount of transfected DNA was kept constant by the addition of 1 μg of empty pCA vector. Three days posttransfection (p.t.), the cells were trypsinized and seeded onto 94-mm dishes together with 10^6 Vero cells. The cocultures were kept in DMEM containing 4% FCS and were split twice a week at a ratio of 1:3. Rescue efficacy was evaluated by immunofluorescence analysis of approximately 10^5 cells, and the cells in which BDV N accumulated in nuclear dot-like structures, a reliable visual sign for ongoing BDV replication, were counted.

Fluorescence microscopy. Approximately 10^5 cocultured 293T and Vero cells were seeded into 12-mm (24-well) dishes. The next day, the cells were fixed for 10 min in 3% paraformaldehyde and permeabilized by incubation for 5 min in phosphate-buffered saline (PBS) containing 0.5% Triton X-100. BDV antigen was detected, as described previously (24), using a rabbit polyclonal antiserum directed against the BDV N protein.

MV rescue. Semiconfluent 293T cells in 35-mm dishes were transfected with 4 μg of plasmids encoding the full-length MV antigenome in combination with 0.5 μg of pCA-MVN, 0.1 μg of pCA-MVP, and 0.5 μg of pCA-MVL. To express viral cRNA from pBRT7-HrMV in 293T cells, 1 μg of pCA-T7 encoding T7pol was added to the transfection mixture. In T7pol-independent rescue experiments, the amount of transfected DNA was kept constant by the addition of 1 μg of empty pCA vector. Three days p.t., the cells were trypsinized and seeded onto 94-mm dishes together with 10^6 Vero cells. The cocultures were kept in DMEM containing 4% FCS for 7 days until virus harvest.

Plaque assay. Cocultured cells were scraped into 2 ml of ice-cold PBS, and cell-associated MV was harvested by three freeze-thaw cycles, followed by centrifugation at 4°C for 2 min at 1,500 × *g* (Heraeus Biofuge) to remove cell debris. Ten-fold serial dilutions of isolated MV were prepared in DMEM plus 2% FCS containing 20 mM HEPES (pH 7.3). Dilutions were added onto 70% confluent Vero cells in 35-mm dishes and incubated at 37°C for 2 h. The cells were washed with 1 ml of PBS and overlaid with 4 ml of DMEM plus 2% FCS containing 20 mM HEPES (pH 7.3), 0.4% Noble agar, and 0.002% dextran. Three days after infection, the agar overlay was removed, and cells were stained using 1% crystal violet dissolved in 20% ethanol.

RNA preparation and Northern blot analysis. Total RNA for Northern blotting was prepared from 35-mm dishes of 293T cells with peqGOLD TriFast reagent (PeqLab) as recommended by the manufacturer. The isolated RNA was treated with 2 units of Turbo DNase (Ambion) at 37°C for 1 h as recommended by the manufacturer. To ensure complete digestion of transfected plasmid DNA, an additional 2 units of Turbo DNase were added after 30 min of incubation. Northern blot analysis using 10 μg of total RNA was performed as previously described (24). The DNA probe for detection of primary full-length cRNA transcripts was prepared by PCR using primer pair 4014(+)/4729(−), which amplifies part of the BDV L gene. The numbering refers to the position and orientation of the primers on the BDV antigenome. The PCR product was radioactively labeled using a Prime it II random primer labeling kit (Stratagene).

In vitro transcription. Full-length plasmids pBRT7-HrBDVc and pBRT7-HmutrBDVc were linearized with the single-cutter XhoI, cleaving the plasmids 445 nucleotides downstream of the BDV antigenomic 5′ end. Full-length plasmids pBRT7-HrMV and pBRT7-HmutrMV were digested with BamHI, creating a 13-kb fragment containing the T7 promoter and the hammerhead sequence followed by 174 nucleotides of the 5′ end of the MV antigenome. DNA fragments were isolated by electrophoresis on a 1% agarose gel. In vitro RNA transcription was performed at 37°C for 1 h in a total volume of 50 μl. The transcription mixture contained 1 μg DNA, 40 units recombinant T7 RNA polymerase (Fermentas), 40 units RNase inhibitor (Fermentas), 1 mM of each rNTP, and 2 μCi of [α-^{32}P]CTP (Amersham). The transcription products were incubated with 40 units DNase I (Ambion) at 37°C for 30 min and then incubated at 65°C for 10 min. Ten microliters of the transcription sample was analyzed for autocatalytic cleavage on a 5% polyacrylamide gel containing 7 M urea.

Determination of 5′-terminal sequences of BDV and MV cRNA. The BDV sequence was determined as previously described (25). The 5′-terminal sequence of MV cRNA was determined as follows. RNA was recovered from purified MV particles (23) with peqGOLD TriFast reagent (PeqLab) according to the manufacturer's protocol. Isolated MV RNA was first treated with 1 unit of calf intestine alkaline phosphatase (Fermentas) for 1 h at 37°C in the supplied buffer (0.1 M Tris-HCl [pH 7.5], 0.1 M $MgCl_2$) to remove triphosphate groups from the 5′ termini of the RNA. After phenol-chloroform extraction, the RNA was incubated with 10 units of T4 polynucleotide kinase (Fermentas) in the presence of 5 μM ATP and the supplied buffer A (500 mM Tris-HCl [pH 7.6], 100 mM $MgCl_2$, 50 mM dithiothreitol, 1 mM spermidine, 1 mM EDTA), which favors the addition of monophosphate groups to the 5′ end of the RNA. After phenol-chloroform extraction, the monophosphorylated RNA was ligated to a synthetic RNA oligonucleotide (5′-CGACTGGAGCACGAGGACACTGACATGGACT GAAGGAGTAGAAA-3′) using the GeneRacer kit (Invitrogen). Modified RNA was reverse transcribed using random hexamer primers, and the 5′ end of MV cRNA was amplified by PCR (30 cycles) using primer MV 396(−) and GeneRacer 5′ primer (Invitrogen). Nested PCR (30 cycles) was performed using 2 μl of the PCR samples and primer MV 301(−) and GeneRacer 5′ nested primer (Invitrogen).

RESULTS

Variable rescue efficiencies of BDV using different promoters for the expression of viral cRNA from cloned cDNA. To determine the rescue support activity of different cellular RNA polymerase promoters, we replaced the T7 RNA polymerase promoter in pBRT7-HrBDVc (25) with either a minimal cytomegalovirus-derived RNA Pol II promoter or an RNA Pol I promoter of human (hPol I) or murine (mPol I) origin (Fig. 1A). All constructs contained identical hammerhead (H) and hepatitis delta ribozyme (δ) sequences for correct processing of the 5′ and 3′ termini of the artificial BDV cRNA and a diagnostic restriction site to distinguish recombinant from authentic BDV (25).

The available BDV rescue system (25) was modified in several aspects. Highly transfectable 293T cells were used instead of BSR-T7 (baby hamster kidney) cells. We transfected standard amounts of expression vectors pCA-N (0.5 μg), pCA-P (0.05 μg), and pCA-L (0.1 μg), encoding the protein components of the BDV polymerase complex (24), but used 4 μg instead of 1.5 μg of the various full-length plasmids (Fig. 1A). To supply T7pol, expression vector pCA-T7 (1 μg), encoding the T7 RNA polymerase, was cotransfected with pBRT7-HrBDVc. Three days after transfection, the cells were seeded onto 94-mm dishes and mixed with Vero cells. The extent of viral replication was determined at 15, 22, and 29 days p.t. Since BDV is a very poor producer of infectious particles, isolation and titration of rescued viruses at these early time points were not suitable. Instead, we determined the number of rescue events by immunofluorescence analysis as previously described (25). We counted the cells in which the BDV nucleoprotein accumulated in nuclear dots, which is an easily detectable and reliable visual sign for ongoing BDV replication. Three independent experiments were performed. The use of 293T cells and plasmid pCA-T7 instead of helper BSR-T7 cells improved the efficiency and robustness of virus recovery from pBRT7-HrBDVc, resulting in earlier detection of virus replication in all rescue attempts (data not shown). The full-length vectors under the control of the hPol I or mPol I promoter supported BDV recovery with an efficiency nearly identical to that of pBRT7-HrBDVc. Only a few cells with BDV N accumulating in nuclear dots were detected at 15 days p.t., and the number of positive cells increased to approximately 200 cells at 29 days p.t. (Fig. 1B). In contrast, Pol II-controlled expression of BDV cRNA from the full-length vector allowed the detection of viral replication as early as 7 days p.t. (data not shown), and the number of BDV-positive cells increased from 113 cells at day 15 p.t. to almost 4,000 cells at 29 days p.t. (Fig. 1B), demonstrating a nearly 20-fold improvement of the rescue efficiency compared to the T7 and the Pol I promoter systems.

To determine the transcription levels of viral cRNA mediated by the different promoters, we transfected 293T cells as described above but omitted pCA-L to prevent viral RNA synthesis. To determine the background signal derived from plasmid DNA, we transfected 293T cells with pBRT7-HrBDVc, pCA-N, and pCA-P but replaced pCA-T7 by an empty pCA vector to prevent cRNA synthesis (T7-ctr.). Northern blot analysis of total RNA showed that both Pol I promoters were only minimally active, whereas cRNA expression was efficient from the T7pol promoter and very strong from the Pol II promoter (Fig. 1C). These data suggest that the rescue-promoting function of the Pol II promoter is due mainly to enhanced transcription of cRNA from plasmids.

Improved MV recovery by Pol II-controlled expression of MV cRNA from cDNA. We determined whether the rescue of a member of the *Mononegavirales* with a cytoplasmic replication strategy would similarly benefit from the use of a Pol II promoter for the expression of viral cRNA. We subcloned the genes encoding the essential protein components of the MV polymerase complex, the MV L, N, and P proteins, into the pCA expression vector. In vector pBRT7-HrMV, we further inserted a hammerhead for correct processing of the 5′ termi-

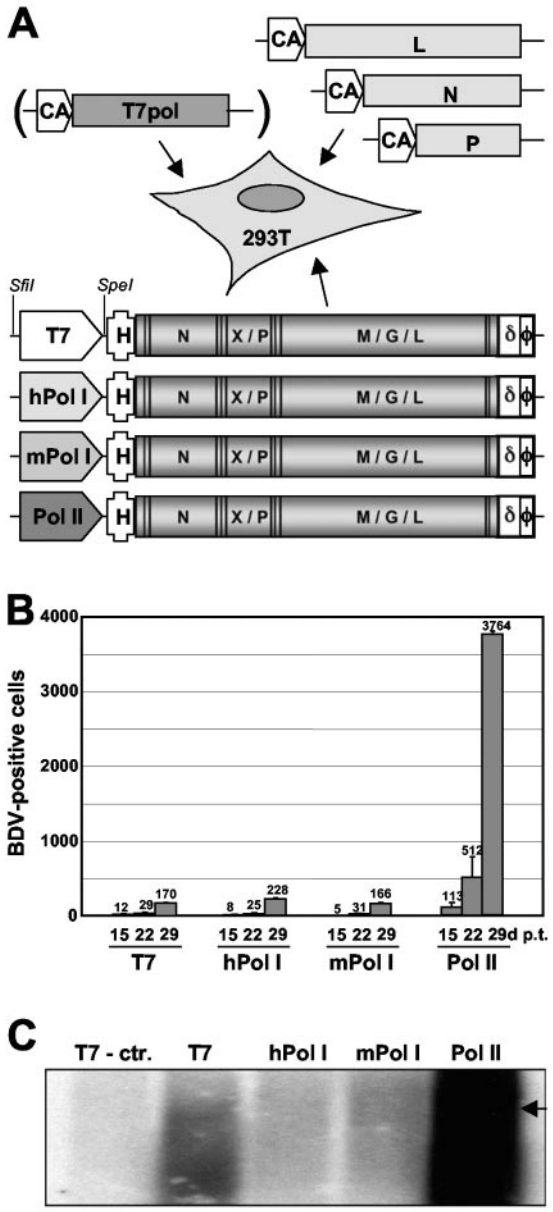

FIG. 1. Rescue of recombinant BDV using different RNA polymerases for the expression of full-length antigenomic RNA from cDNA. (A) Schematic representation of the modified rescue system. Plasmids encoding the viral proteins L, N, and P under the control of the chicken β-actin promoter (CA) were transfected with either of the plasmids encoding the full-length BDV antigenome into 293T (human embryonic kidney) cells. All full-length plasmids were identical, except for the SfiI-SpeI fragment containing the promoter sequences used to control RNA expression. The promoters tested were the T7 RNA polymerase promoter (T7), the human (hPol I) or murine (mPol I) RNA polymerase I promoter, and a cytomegalovirus-derived RNA polymerase II promoter (Pol II). A BDV-specific hammerhead sequence (H) and the hepatitis delta virus ribozyme sequence (δ) were used to generate the correct 5′ and 3′ ends of the antigenomic RNA, respectively. To test the T7 RNA polymerase promoter in 293T cells, the T7 RNA polymerase (T7pol) was supplied by transfection of plasmid pCA-T7. The total amount of transfected DNA was kept constant by transfection of the empty pCA vector instead of pCA-T7 in all setups not requiring the T7 RNA polymerase. (B) Evaluation of rescue support functions of different RNA polymerases. Subconfluent 293T cells in six-well plates were transfected with 0.1 μg pCA-L, 0.5 μg pCA-N, 0.05 μg pCA-P, 1 μg pCA-T7, or pCA (empty) and 4 μg of the full-length plasmid containing the indicated promoter sequence. Three days p.t., the 293T cells were mixed with 10^6 Vero cells and seeded onto a 94-mm dish. At 15, 22, and 29 days p.t., approximately 10^5 cells were fixed, and rescue efficacy was evaluated by immunofluorescence analysis. The bars represent the average numbers of BDV-positive cells in three independent experiments. The standard deviations and the numbers of BDV-positive cells are indicated. (C) Northern blot analysis of cRNA synthesis from plasmids. 293T cells were transfected as described above, except that plasmid pCA-L was omitted to prevent viral RNA synthesis. To distinguish primary transcription from background signals, we transfected cells with pBRT7-HrBDVc but without pCA-T7 to prevent the synthesis of T7pol. Three days p.t., total RNA was isolated and intensely treated with Turbo DNase (Ambion) to remove transfected plasmid DNA. Ten micrograms of DNase-treated total RNA was analyzed by Northern blot using a radiolabeled DNA probe corresponding to nucleotides 4014 to 4729 of the BDV genome, representing part of the L gene. The arrow indicates the size of the BDV genome, which was determined by loading total RNA from BDV-infected cells into a slot on the same gel (not shown).

TABLE 1. Titers of recombinant MV recovered from Pol II- and T7-transcribed cRNA

Rescue[a]	Titer (PFU/ml)		
	pBRPol II-HrMV	pBRT7-HrMV	pBRPol II-HmutrMV
1	3.1×10^5	3.2×10^3	3×10^4
2	1×10^5	1×10^3	4×10^4
3	1×10^4	+[b]	4×10^3
4	1×10^4	+[b]	5×10^3

[a] Four independent rescue experiments were performed. 293T cells (50% confluent in six-well plates) were transfected as described in Materials and Methods. Three days p.t., 293T cells were mixed with 10^6 Vero cells and seeded onto 94-mm dishes. Seven days p.t., cells were scraped into 2 ml fresh medium, and cell-associated MV was recovered by three freeze-thaw cycles. Titers were determined in plaque assays and are given as pfu/milliliter.

[b] Rescued viruses were observed by syncytium formation, but titers were too low to be determined.

nus of the MV cRNA. The T7 promoter was replaced by the Pol II promoter using unique SfiI and AscI restriction sites located upstream of the T7 promoter sequence and in between the T7 promoter and the hammerhead sequences, respectively. The MV rescue system was basically identical to the BDV system represented in Fig. 1A. 293T cells were transfected with expression vectors pCA-MVN (0.5 μg), pCA-MVP (0.1 μg), and pCA-MVL (0.5 μg) in combination with 4 μg of either pBRT7-HrMV or pBRPol II-HrMV. T7pol was supplied by cotransfection of plasmid pCA-T7 (1 μg) with pBRT7-HrMV. Three days p.t., the 293T cells were seeded onto a 94-mm dish together with 10^6 Vero cells. Transfection of pBRT7-HrMV resulted in the appearance of only a few syncytia between 4 and 7 days p.t., whereas transfection of pBRPol II-HrMV induced the formation of multiple syncytia at day 4 p.t., suggesting many independent rescue events per transfection. To quantify rescue efficiency, we harvested cell-associated MV at day 7 p.t. by repeated freeze-thaw cycles and determined viral titers by plaque assay (Table 1). Although the titers obtained from different rescue experiments varied strongly, the data clearly indicate a highly improved rescue efficacy upon Pol II-controlled expression of MV cRNA, resulting in approximately 100-fold-increased MV titers (Table 1).

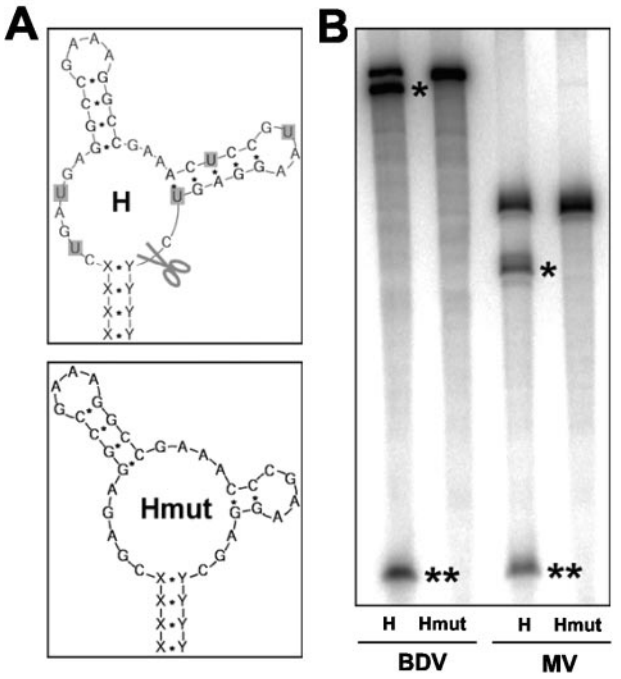

FIG. 2. Autocatalytic activity of intact and mutated hammerhead sequences. (A) Schematic representation of intact (H) (top panel) and mutated (Hmut) (lower panel) hammerhead sequences. The secondary structures of the RNA sequences were predicted using the Mfold 3.2 program (13, 30). The autocatalytic cleavage site within H is indicated, and the five U residues deleted in Hmut are shown in gray boxes. Nucleotides belonging to the viral antigenome are represented by Y, and the complementary nucleotides required for stem-loop formation are indicated by X. (B) Analysis of RNA cleavage by intact and mutated hammerhead sequences. Plasmids pBRT7-H/HmutrBDV and pBRT7-H/HmutrMV were cut with restriction enzymes XhoI and BamHI, respectively. One microgram of linearized plasmids was isolated and in vitro transcribed using recombinant T7 polymerase (Fermentas) and 2 μCi of [α-^{32}P]CTP (Amersham) for radiolabeling. Transcription products were separated on a 5% polyacrylamide gel containing 7 M urea. Runoff transcription of linearized plasmids pBRT7-H/HmutrBDV and pBRT7-H/HmutrMV resulted in uncleaved transcripts of 504 nucleotides (HrBDV) or 499 nucleotides (HmutrBDV) and 233 nucleotides (HrMV) or 228 nucleotides (HmutrMV), respectively. RNA species resulting from autocatalytic cleavage of the hammerhead sequence are indicated by single asterisks for the processed antigenomic RNA of BDV (445 nucleotides) and MV (174 nucleotides) with correct 5′ ends and double asterisks for the removed hammerhead (59 nucleotides).

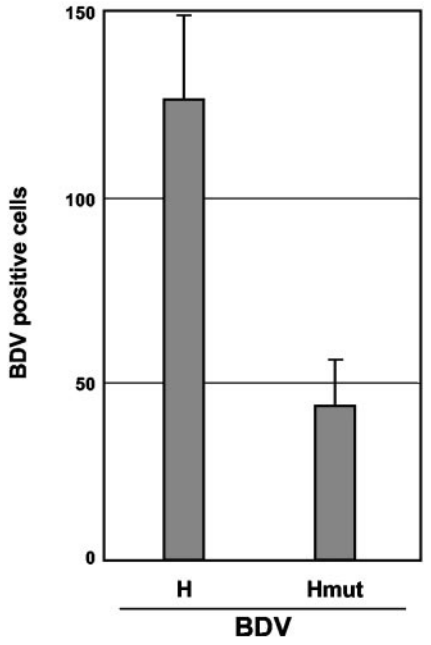

FIG. 3. A functional hammerhead sequence is not required to recover BDV from cDNA. Data from an evaluation of rescue efficacy from plasmids pBRPol II-HrBDV and pBRPol II-HmutrBDV are shown. Subconfluent 293T cells were transfected as described in the legend to Fig. 1. At 15 days p.t., approximately 10^5 cells were fixed, and rescue efficacy was determined by immunofluorescence analysis. The bars represent the average number of BDV-positive cells in three independent experiments. The standard deviations are indicated.

Functional hammerhead sequences are not required for the recovery of BDV and MV. For Sendai virus, another member of the order *Mononegavirales*, internal initiation of the viral polymerase complex during replication of an artificial minigenome has been demonstrated (28). These data suggested that viral polymerases are able to recognize internally located genome termini, thereby eliminating additional sequences located upstream of the viral termini during the process of genome replication. To test this hypothesis for BDV and MV in the context of replicating viruses, we inactivated the functional hammerhead ribozyme (H) (Fig. 2A, top panel) by the deletion of five U residues in the autocatalytic hammerhead domain of the BDV and the MV hammerhead (Hmut) (Fig. 2A, bottom panel), respectively. As shown in Fig. 2B, the deletions completely abrogated autocatalytic cleavage of in vitro-transcribed, radiolabeled RNA molecules. The rescue support function of the inactivated hammerhead sequences was tested in the context of the Pol II promoter-controlled full-length vectors (Table 1 and Fig. 3). Remarkably, pBRPol II-HmutrBDVc supported efficient BDV recovery from cDNA. The number of BDV-positive cells at day 15 p.t. was only threefold lower than that of cells transfected with pBRPol II-HrBDVc (Fig. 3) containing the functional hammerhead but was still 4- to 10-fold higher than in rescue experiments using pBRT7-HrBDVc, pBRhPol I-HrBDVc, and pBRmPol I-HrBDVc (compare Fig. 1B and 3). Similarly, recovery of MV from pBRPol II-HmutrMV was efficient and resulted in only a 2- to 10-fold reduction of titers of cell-associated MV at 7 days p.t. compared to titers obtained after transfection of pBRPol II-HrMV (Table 1). These data indicate that processing of the 5′ terminus of viral cRNA is not essential for the recovery of BDV and MV from cDNA.

Artificially prolonged 5′ termini of the BDV and MV cRNAs are repaired during viral replication. To demonstrate that unspliced hammerhead sequences were removed during viral replication, we determined the 5′-terminal cRNA sequences of rescued BDV and MV. RNA was isolated from purified viral particles, and 5′ rapid amplification of cDNA ends experiments were performed by ligation of an RNA oligonucleotide and subsequent reverse transcription, followed by nested PCR. The PCR products were directly sequenced. The 5′-terminal sequences of the BDV cRNA were identical for viruses res-

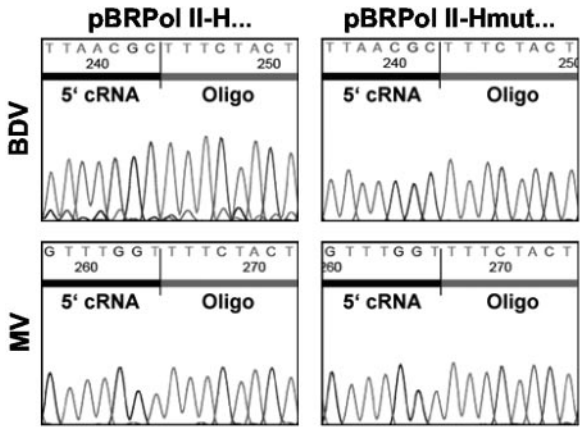

FIG. 4. Uncleaved hammerhead sequences are eliminated from the 5′ ends of the BDV and MV cRNA during viral replication. Viruses rescued from plasmids pBRPol II-H/HmutrBDVc and pBRPol II-H/HmutrMV, respectively, were propagated in Vero cells, and BDV and MV particles were isolated and purified as described previously (1, 23). Sequences were determined by ligation of a synthetic RNA oligonucleotide (Oligo) to the 5′ termini of isolated RNA molecules and subsequent reverse transcription-PCR amplification. Shown are sequences from near the 5′ terminus of the cRNA (black line) including part of the synthetic RNA oligonucleotide (gray line). Numbering indicates the nucleotide position in the electropherogram. Note that the electropherogram shows the complementary strand.

cued from plasmids pBRPol II-HrBDVc (Fig. 4, upper left panel) and pBRPol II-HmutrBDVc (Fig. 4, upper right panel). Similarly, analysis of cRNAs derived from rescued MV showed that viruses rescued from plasmids pBRPol II-HrMV (Fig. 4, lower left panel) and pBRPol II-HmutrMV (Fig. 4, lower right panel) have identical 5′-terminal sequences.

DISCUSSION

The efficiency of a reverse genetics system is of critical importance for the recovery of mutant recombinant viruses with reduced replication competence. Efficiency considerations are an important topic, especially for BDV, which multiplies slowly. We show here that the rescue efficacies of BDV and MV, two largely different members of the *Mononegavirales*, can be drastically increased. In a first step, we modified the available rescue system of BDV (25) by replacing the BSR-T7 helper cell line with 293T cells and by using expression vector pCA-T7 encoding T7pol under the control of the chicken β-actin promoter. The modified system resulted in the improved efficiency and robustness of the BDV rescue system. For MV, two systems for the recovery of recombinant virus from cDNA relying either on a helper cell line (20) or on helper virus (21) for the expression of T7pol have previously been established. Modification of these rescue systems in the same way as described above for BDV, including the insertion of a hammerhead ribozyme, expression of viral proteins from pCA vectors, the use of 293T cells, and expression of T7pol from pCA-T7 vector, similarly resulted in efficient and reliable recovery of MV. To the best of our knowledge, these are the first rescue systems that no longer rely on either helper cells or helper viruses for the expression of T7pol. These systems are thus able to support virus recovery in any permissive cell line without helper virus contamination.

Based on these modified systems, we tested different RNA polymerase promoters for the expression of viral cRNA in order to increase rescue efficiencies (Fig. 1B). Despite the nuclear replication of BDV, rescue efficacy was similar for the T7, human Pol I, or murine Pol I promoter system. In contrast, when we used a minimal Pol II promoter derived from cytomegalovirus to control viral cRNA expression, virus-positive cells were observed much earlier after transfection and at increased numbers (Fig. 1B). Northern blot analysis of cRNA transcription from the various plasmids in human 293T cells (Fig. 1C) showed that the T7 promoter supported efficient cRNA expression, which was further enhanced by the use of the Pol II promoter. In contrast, the human and murine Pol I promoters supported only minimal cRNA expression from plasmid DNA, suggesting low activity of these promoters. These results suggest that the improved rescue efficacy from pBRPol II-HrBDVc is due mainly to enhanced transcription of viral cRNA from plasmids. However, the observation that the minimal cRNA expression from the Pol I promoters and the much stronger cRNA synthesis from the T7 promoter resulted in nearly identical numbers of rescue events indicates that BDV recovery also benefits from nuclear expression of cRNA. In fact, considering the low transcriptional activity of both Pol I promoters, the number of rescue events seems remarkably high. This suggests that Pol I-transcribed cRNA is efficiently packaged into RNP structures, which might be explained by colocalization of BDV replication and Pol I transcription in the nucleolus (18, 26). However, it should be noted that immunofluorescence analysis detected the BDV N, P, and L proteins (24) exclusively in the nucleoplasm and not in the nucleolus of infected cells, which calls the proposed nucleolar replication of BDV into question (18). We cannot explain the low transcriptional activity of the hPol I promoter in human 293T cells. Sequence analysis confirmed the integrity of the hPol I promoter and demonstrated that our hPol I promoter is identical to the promoter sequence present in plasmid pHH21, which was used for the rescue of influenza A virus and Thogoto virus (15, 29). Evaluation of the human and murine Pol I promoters in the BDV minreplicon system further showed that in human 293T cells, the hPol I promoter induced approximately 20-fold-higher reporter gene expression than the mPol I promoter (our unpublished data). This observation suggests that the increased length of the full-length cRNA affected the transcriptional activity of the human Pol I promoter more strongly than that of the murine Pol I promoter. Our data thus imply that BDV recovery from Pol I-transcribed cRNA would, in principle, be highly efficient but is hampered by the intrinsic weak activity of this frequently used human Pol I promoter fragment.

It has previously been shown that in uninfected cells, nearly 100% of plasmid-derived BDV mRNA molecules carrying the authentic BDV intron sequences become spliced and that the splicing efficiency in this artificial system is not affected by the presence of the viral proteins N, P, and X (12). Since splicing of the Pol II-transcribed full-length cRNA molecules would interfere with the recovery of BDV, it is conceivable that splicing is less efficient in our system. To a certain extent, the reduced splicing efficiency might be explained by the fact that

we used a minimal Pol II promoter lacking intron sequences that usually help to direct mRNAs to the splicing machinery before mRNA export from the nucleus. The presence of a hammerhead ribozyme that removes the 5′-capping group from the majority of the Pol II-transcribed cRNAs (Fig. 2B) might inhibit splicing. However, the fact that a functional hammerhead is not required for BDV rescue from Pol II-transcribed cRNA (Fig. 3) suggests that the removal of the 5′-capping group is not the main cause for inefficient cRNA splicing. The best way to protect the Pol II-transcribed BDV cRNA from splicing would be efficient formation of the RNP. If this is the case, packaging of the cRNA molecule has to occur early after synthesis, which would imply that free proteins of the BDV polymerase complex and possibly BDV replication itself are closely associated with the site of Pol II transcription in the nucleus. In case of the influenza A virus, an association of the viral polymerase with cellular RNA Pol II has been demonstrated (7). This observation supports the view that enhanced cRNA synthesis from plasmid DNA is the main, but probably not the only, reason for the rescue-promoting function of the Pol II promoter. It should be noted that the polymerase complexes of viruses of the order *Mononegavirales* are able to produce 5′-capped mRNA in the transcription modus and that the cellular mRNA cap guanylyltransferase has recently been identified as a submolar component of the vesicular stomatitis virus transcriptase complex (19). Although viral and cellular cap synthesis are mechanistically distinct processes, this finding indicates that viral and cellular Pol II-mediated RNA synthesis have similar requirements for cellular cofactors.

The observation that the recovery of MV, a member of the *Mononegavirales* with a cytoplasmic replication strategy, is also improved by the usage of the Pol II promoter (Table 1) suggests that Pol II-transcribed MV cRNA is efficiently exported from the nucleus without abundant splicing of cryptic splice sites. The mechanisms by which the MV cRNA is exported and protected from the cellular splicing machinery are unknown, but it has been shown that plasmid-expressed MV nucleoprotein migrates predominantly to the nucleus in the absence of MV P (10). It is well possible that even in the presence of MV P, minor fractions of the MV nucleoprotein migrate to the nucleus and associate with Pol II-transcribed viral cRNA. Such an association might protect the cRNA from splicing and might further enhance the transport of the long RNA molecule from the nucleus to the cytoplasm. Our data imply that the rescue of probably most of the members of the *Mononegavirales*, if not of most negative-strand RNA viruses, would benefit from Pol II-driven expression of full-length cRNA.

Genome and antigenome synthesis of negative-strand RNA viruses is initiated at promoters located in the noncoding regions at the 5′ and 3′ termini of the viral genome. The terminal nucleotides forming inverted terminal repeats at both sides of the genome are of critical importance for viral replication. RNA synthesis of the viral polymerase initiates at the precise 3′ end of genome RNA, and therefore, it was assumed that plasmid-expressed viral cRNA requires autocatalytic processing to generate free genome termini at both ends of the RNA molecule. This view has been questioned by the observation that replication of a Sendai virus minireplicon with a duplication of the 5′ noncoding region results in the elimination of the additional noncoding region by replication initiation at the internally localized viral promoter (28). Many rescue systems, including our novel MV and BDV rescue systems, employ an autocatalytic hammerhead sequence to process the 5′ terminus of the full-length cRNA and the hepatitis delta virus ribozyme to process the 3′ end. In contrast to the hepatitis delta virus ribozyme, which cleaves independently of the viral genome sequences, the hammerhead is virus specific, since the cleavage site is determined by a stem-loop that involves up to 20 nucleotides from the 5′ terminus of the viral cRNA (Fig. 2A). Thus, for each new virus and for each virus containing mutations in the nucleotides involved in stem-loop formation, a new hammerhead has to be generated, which is labor intensive and cumbersome, since not every hammerhead is functional. To test whether 5′ processing of Pol II-transcribed cRNA is actually required for virus rescue, we abolished the autocatalytic activity of both MV and BDV hammerheads by the deletion of five U nucleotides (Fig. 2A). The inactivation of the hammerhead results in the exclusive expression of full-length cRNA molecules from a plasmid, which contain 47 additional 5′-terminal nucleotides upstream of the correct viral genome termini. Interestingly, BDV and MV were recovered from plasmids encoding inactivated hammerheads (Table 1 and Fig. 3), and the correct 5′ termini were restored during viral replication (Fig. 4). This provides the first evidence for the recognition of internal replication signals at the level of full-length genome replication. The fact that two largely different viruses like BDV and MV showed similar capacities to recover from artificially prolonged cRNA molecules indicates that polymerases of negative-strand RNA viruses might generally possess the ability to initiate or terminate RNA synthesis at internal viral replication signals. This finding will simplify the application of the Pol II expression system in other viral systems.

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