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Ambisense Gene Expression from Recombinant Rabies Virus: Random Packaging of Positive- and Negative-Strand Ribonucleoprotein Complexes into Rabies Virions

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We have recovered from cDNA a rabies virus (RV) containing identical, transcriptionally active promoters at its genome (negative-strand) and antigenome RNA and directing efficient expression of a chloramphenicol acetyltransferase (CAT) reporter gene from the antigenome. Transcription of the antigenome CAT gene was terminated by a modified RV gene junction able to mediate transcription stop and polyadenylation but not reinitiation of downstream transcripts. While in standard RV-infected cells genome and antigenome RNAs were present in a 49:1 ratio, the ambisense virus directed synthesis of equal amounts of genome and antigenome RNA (1:1). Total replicative synthesis was reduced by a factor of less than 2, revealing an unexpectedly high level of replication activity of the transcriptionally active promoter in the absence of the parental antigenome promoter. Successful packaging of ambisense ribonucleoprotein complexes (RNPs) into virions demonstrated that the parental 5′ end of the RV genome RNA does not contain putative signals required for incorporation into virions. As determined both for standard RV and ambisense RV, virus particles contained genome and antigenome RNPs in the same ratios as those present in infected cells (49:1 and 1:1, respectively), indicating indiscriminate incorporation of RNPs independent of signals in the RNA. Ambisense expression of multiple foreign genes from RV vectors may circumvent problems with transcriptional attenuation of rhabdovirus housekeeping genes.

Rhabdoviruses and other members of the *Mononegavirales* order, such as paramyxoviruses and filoviruses, contain a single-strand RNA molecule of negative sense (vRNA, genome RNA) which comprises all protein-encoding genes. Both in the virion and in the cytoplasm of infected cells the RNA is present in the form of a ribonucleoprotein complex (RNP), in which it is tightly encapsidated with nucleoprotein (N) and associated with the viral RNA polymerase (L) and a polymerase cofactor (P). Upon infection, *cis*-acting signals of the genome template RNA direct the successive transcription of usually nonoverlapping subgenomic RNAs (1, 2), i.e., a short leader RNA (le), and, in the case of rabies rhabdovirus, five polyadenylated mRNAs in the order 3′-le-N-P-M-G-L-5′. After the accumulation of the viral translation products, the same RNPs are used as templates for replication via the synthesis of RNPs containing full-length antigenome sense RNA (cRNA, antigenome RNA). Antigenome RNPs exclusively serve as templates for replication and produce huge amounts of genome RNPs (for reviews see references 20 and 41).

As indicated earlier by studies of small RNAs of defective interfering (DI) particles (21, 22, 29, 31), and as confirmed more recently by taking advantage of systems that allow recovery of model genomes of rhabdo- and paramyxoviruses from cDNA (for a review see reference 8), the *cis*-acting signals required to direct replication and encapsidation are contained in the 3′-terminal noncoding sequences of the genome and the antigenome RNA (6, 7, 9, 12, 26–28, 35). These RNA ends are regarded as the "genome promoter" and the "antigenome promoter," respectively (6), since the primary sequences that are engaged in common functions during replication, such as polymerase binding, replication initiation, encapsidation, and elongation, are poorly defined. This applies also to the sequences that are specific for the genome promoter and that render it transcription competent. One particular region of the genome promoter, however, which is obviously critical for initiating the transcription mode, i.e., a putative *cis*-acting signal enabling the synthesis of downstream mRNAs, is the junction of the leader template sequence and the first protein-encoding cistron (40). After release of the leader RNA, which probably carries the signal for encapsidation into N protein, the polymerase appears to be fixed in the transcription mode, in which transcription signals present at each gene border and directing stop/polyadenylation and restart are recognized. Indeed, the 3′-terminal part of the Sendai paramyxovirus leader-coding region can be replaced by the corresponding sequence of the antigenome promoter without greatly affecting the transcription of mRNAs (6, 15).

The overwhelming majority of natural DI RNAs of rhabdoviruses and paramyxoviruses lack a transcriptionally active promoter. These so-called "copy-back" DI RNAs have replaced the genome promoter with sequences complementary to the parental 5′ ends, i.e., the antigenome promoter. They are incapable of transcription but interfere greatly with replication of nondefective helper virus and are efficiently assembled into virions (29). In contrast to 5′ copy-back DI RNAs, RNAs with ends derived from the genome promoter (3′ copy-back RNAs) have not been identified. This suggests either a selective replicative disadvantage of the genome promoter or the lack of an essential function, further distinguishing genome from antigenome promoters. For example, putative signals required for assembly of RNPs into virions could be present only in the genome 5′ end, explaining the efficient assembly of 5′ copy-back DI RNAs into virions and the presence of nondefective negative-strand RNAs in standard virions. To address the functions of rabies virus (RV) promoters, we investigated

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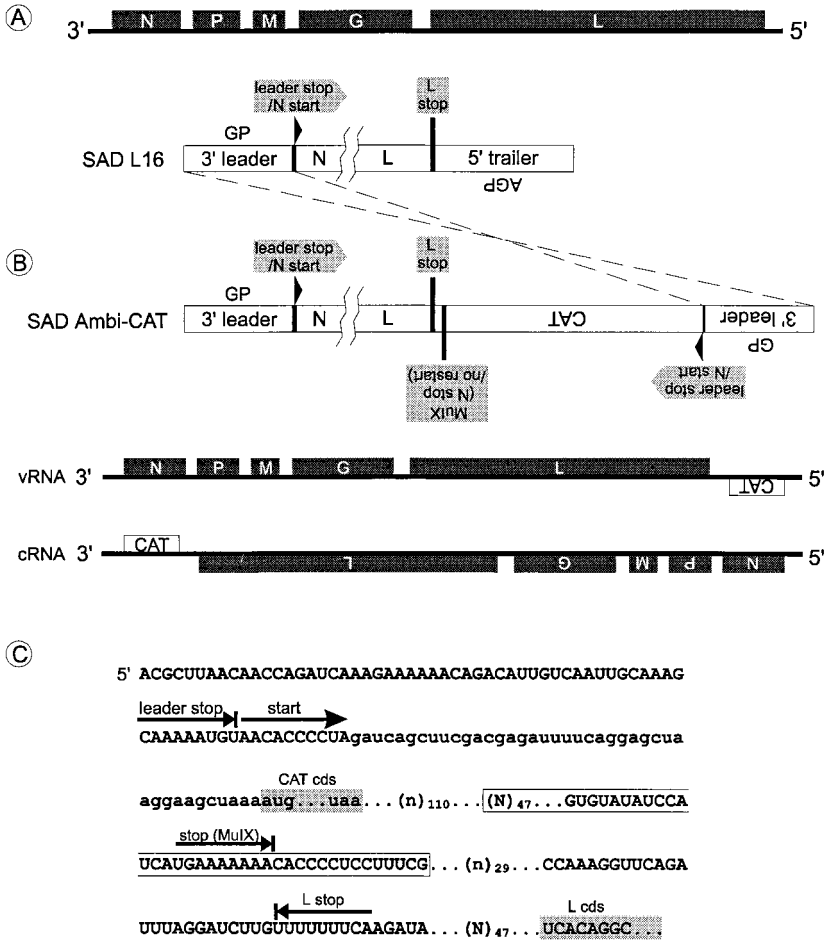

FIG. 1. (A) Organization of the 12-kb RV genome. Shaded boxes represent the open reading frames of the N, P, M, G, and L genes. (B) Organization of recombinant RV SAD Ambi-CAT. The original RV open reading frames are represented by shaded boxes, and the additional CAT gene ORF is indicated by an open box. Transcriptional stop/polyadenylation signals are represented by vertical bars, and restart sequences are represented by arrowheads. In SAD Ambi-CAT the antigenome promoter (AGP) was replaced with the 3′-terminal sequence encoding the entire leader RNA sequence followed by the transcription start signal of the RV N gene. The CAT gene together with a downstream transcriptional stop/polyadenylation signal (MuIX) was inserted downstream of the positive-strand genome promoter (GP) in reverse orientation with respect to the RV open reading frames. The genome organization is shown for the negative-strand genome (template for RV gene expression; vRNA) and the positive-strand antigenome (template for CAT gene expression; cRNA). (C) The nucleotide sequences of the novel RNA end of SAD Ambi-CAT are given in positive orientation. All nonviral sequences that were inserted are in lowercase. Signals involved in transcription are marked by arrows, and protein-coding sequences (cds) are indicated by shaded boxes. The CAT coding sequences (cds) and modified N/P gene border sequence MuIX (indicated by an open box) are separated by 89 nt derived from the 3′-noncoding sequence of the CAT gene and 21 nt of cloning vector sequences, as indicated by $(n)_{110}$. The remainder of the parental trailer sequence (the last 45 nt were deleted) contained the reversed L stop signal and was separated from MuIX sequences by 29 nt derived by cloning vector sequences.

whether it is possible to generate an RV whose replication and transcription is directed exclusively by transcriptionally active genome promoters. In a full-length RV cDNA, the virus 5′-terminal sequences were replaced with sequences specifying the genome promoter and an adjacent cistron encoding chloramphenicol acetyltransferase (CAT). The recovered virus (SAD Ambi-CAT) propagated autonomously to remarkably high titers and expressed CAT from the antigenome RNA efficiently. Replacement of the antigenome promoter not only equalized the relative levels of genome (3′-le-N-P-M-G-L-5′) and antigenome (3′-le-CAT-5′) RNA in infected cells but also resulted in the production of equal amounts of virions containing genome or antigenome RNPs. Since in standard RV the ratio of genome to antigenome RNA in cells also exactly matches that found in virions, we conclude that there is no specific mechanism for incorporation of negative-strand RV RNPs into virions. The possibility of generating viruses with an ambisense gene expression strategy may have practical utility for the development of RV-derived gene delivery vectors and may permit the circumvention of possible problems encountered with transcriptional attenuation of virus gene expression after the introduction of additional cistrons into the genome RNA.

MATERIALS AND METHODS

Construction of cDNA clones. The full-length cDNA clone pSAD L16 (34), which possesses the authentic sequence of attenuated RV strain SAD B19 (11), served as a basis for the assembly of the recombinant RV cDNA clone pSAD Ambi-CAT. The 5′-terminal region of SAD L16 (position 11883 to the end; 45 nucleotides [nt]) was exchanged with the 3′ leader sequence, resulting in a viral cDNA with complementary ends of 68 nt. In addition, the CAT reporter gene and a mutagenized N/P gene border sequence were integrated in reverse orientation relative to the RV genes between the L gene and the novel 5′ end (Fig. 1). For the construction of the recombinant cDNA, the bicistronic model genome plasmid pSDI-CAT/luci(MuIX) (genome organization: 3′-le-CAT-[MuIX]-luciferase-5′), which encoded the CAT and the luciferase gene separated by a modified N/P gene border sequence (MuIX) was used. This model genome plasmid has been constructed for a study of the *cis*-acting transcription signals present in

the N/P gene border. Mutation IX directed effective transcriptional stop and polyadenylation but no detectable restart of transcription (unpublished results). After *Nhe*I-*Eco*RV restriction of pSDI-CAT/luci(MuIX) and generation of blunt ends by a fill-in reaction with Klenow enzyme, an *Sma*I-*Eco*47III fragment of pT7T-L, encoding the C-terminal part of the L gene and the L transcription stop signal, was joined in reverse orientation downstream of MuIX. In a second step, the 5′-terminal part of the RV full-length cDNA clone pSAD L16 (position 8226, *Mlu*I site to the end) was replaced by the *Mlu*I-*Not*I fragment {*Not*I-3′-le-CAT-[MuIX]-[Lstop]-L(partial)-*Mlu*I} of the subclone described above. The resulting recombinant RV cDNA was named pSAD Ambi-CAT (Fig. 1).

Recovery of recombinant virus. Recombinant RV SAD Ambi-CAT was recovered as described previously (34) after coexpression of the full-length cDNA and plasmids encoding the RV proteins N, P, and L (9) in BSR cells expressing T7 RNA polymerase from recombinant vTF7-3 (14). Two days after DNA transfection, cell culture supernatants were harvested, partially cleared of vaccinia virus by centrifugation, and transferred to fresh BSR cells. Residual vaccinia virus was removed by filtration (Millipore; 0.1-μm-pore-size filter) of the supernatant harvested after the third virus passage. DNA transfections were performed after $CaPO_4$ precipitation (Stratagene) in 8-cm^2 culture dishes containing $\sim 10^6$ cells.

RNA analysis and RT-PCR. Total RNA from cells was isolated after 1 to 3 days of infection and analyzed as described previously, including denaturation of RNA with glyoxal and electrophoresis in denaturing gels containing 5% formalin (10). Virion RNA was isolated from virion pellets (Beckmann TLA45 rotor; 45,000 rpm, 1 h, 4°C) obtained after purification of virus particles on a 60 to 20% sucrose gradient (Beckmann TST41 rotor; 35,000 rpm, 1.5 h, 4°C). RVN and CAT DNA fragments were labeled with [α-^{32}P]dCTP (3,000 Ci/mmol; ICN) by nick translation (nick translation kit; Amersham). Alternatively, [α-^{32}P]UTP (>600 mCi/mmol; ICN)-labeled strand-specific riboprobes were generated by in vitro runoff transcription from the T3 or T7 RNA polymerase promoter of pT7T-N or pSK-CAT after linearization with *Xho*I or *Bam*HI, respectively. pSK-CAT was constructed for probe synthesis by insertion of a 417-bp *Eco*RI-*Sca*I fragment of pCM7 (Pharmacia) into *Eco*RI-*Sma*I-restricted pBluescriptSKII− (Stratagene). After hybridization, RNA amounts were measured in a phosphorimager (Fuji). For quantitation of genome and antigenome RNAs, dilutions of in vitro runoff transcript (pT7T-N) were used as a standard after removal of nonincorporated nucleoside triphosphates (QIAquick nucleotide removal kit; Qiagen).

Reverse transcription-PCR (RT-PCR) was performed on viral RNA isolated from purified virions. RT by avian myeloblastosis virus reverse transcriptase was primed with the RV L-gene-specific oligonucleotide L2P (5′-ATCCTGAG GCACTTCAAC-3′; SAD B19 nt 11408 to 11425). DNA amplification was done as described previously (34) by using primers L2P and T7G (5′-GAATTCCTGC AGTAATACGACTCACTATAGACGCTTAACAACCAG-3′), which is complementary to the first 15 nt of the SAD B19 leader RNA sequence (underlined). PCR products resulting from 45 cycles (30 s at 94°C; annealing for 60 s at 37°C [first 10 cycles] and 43°C [further 35 cycles]; elongation for 120 s at 72°C) were analyzed on 1% agarose gels and were subcloned in pBluescriptSKII− (Stratagene) for sequence analysis.

CAT assay. Cell extracts were analyzed by thin-layer chromatography with [^{14}C]chloramphenicol (53 mCi/mmol; Amersham) as described previously (9). Alternatively, CAT activity was determined after "CAT phase exchange" in a liquid scintillation counter: 10 μl of cell extract was incubated for 1 h at 37°C with 0.25 μCi of [^{3}H]acetyl-coenzyme A (CoA) (200 mCi/mmol; ICN)–0.2 mM chloramphenicol–0.2 mM acetyl-CoA–50 mM Tris-HCl (pH 7.5) in a total volume of 50 μl. After the addition of 250 μl of stop solution (0.1 M H_3BO_3, 5 M NaCl [pH 9]) the solution was mixed with 1.5 ml of organic scintillation fluid (Betamax, ICN). Quantitation of CAT protein was performed by using the CAT-ELISA kit (Boehringer Mannheim) according to the supplier's instructions.

RESULTS

Recovery of recombinant RV SAD Ambi-CAT. Replication and transcription of RV are coordinated by the *cis*-acting functions of the 3′-terminal genome and antigenome promoters. As demonstrated by the successful recovery of defective RV RNAs from cDNA, the first 68 nt of the genome including the leader RNA and the N-gene start signal are sufficient for directing both replication and transcription. The sequence specified by the last 169 nt including the 5′-nontranslated region, i.e., the antigenome promoter, only directs replication (9). Similarities of the 3′ ends of genome and antigenome RNAs are observed only in the terminal regions. The ends consist of short stretches of identical nucleotides (11 nt in RV SAD B19) and are followed by a U-rich (negative-strand) region. Sequences resembling the junction of leader RNA and the N gene are not present in the antigenome RNA. To analyze whether the transcriptionally active genome promoter can substitute for the antigenome promoter in an RV full-length clone, we exchanged the 5′-terminal 45 nt with the complement of the 3′ end encoding the entire leader RNA sequence and the transcription start signal of the N gene. To facilitate analysis of the transcription activity of the novel promoter at the antigenome RNA 3′ end, a complete CAT reporter gene was inserted immediately downstream of the promoter in reverse orientation with regard to the virus genes (Fig. 1). To provide a signal directing the termination of transcription of the CAT gene and to prevent probably deleterious synthesis of RNAs complementary to RV protein mRNAs, a mutagenized RV gene border (MuIX) was introduced downstream of the CAT-encoding sequences. MuIX was derived from the SAD B19 N/P gene border by deletion of 4 nt (negative-strand sequence: ACUUUUUUUGAUUGUGGGGA; nucleotides deleted in MuIX are underlined), including the complete intergenic region (GA). MuIX caused effective transcriptional stop and polyadenylation but no detectable transcriptional restart in a bicistronic RV model genome (unpublished results).

The final pSAD Ambi-CAT was used in virus recovery experiments as previously described (34). After infection of BSR cells with recombinant vaccinia virus vTF7-3 (14) providing T7 RNA polymerase and transfection of T7 promoter-controlled plasmids encoding RV proteins N, P, and L and of pSAD Ambi-CAT, RV was recovered. The rescue efficiency was comparable to that observed previously for standard RV cDNA clones, yielding recombinant virus in 5 of 20 experiments.

Although lacking sequences derived from the parental 5′ end, SAD Ambi-CAT viruses were able to propagate autonomously. Vaccinia virus was removed by filtration of supernatants after three virus passages, and the recombinant RV was further grown for two passages to obtain virus stocks. The identity of RV SAD Ambi-CAT was examined by RT-PCR with primers L2P and T7G as detailed in Materials and Methods. As predicted, a 1.5-kb cDNA fragment was amplified and was cut correctly into fragments of 0.5 kb and 1 kb by *Pst*I (data not shown). After the 1-kb cDNA fragment was subcloned, the presence of the cDNA-derived nucleotide sequence of the new genome 5′ end was verified.

As determined by end point dilution of supernatants from infected cell cultures over a period of 15 virus passages, the infectious titers of SAD Ambi-CAT were constantly 10- to 15-fold lower than that of standard SAD L16. Three days after infection at a multiplicity of infection (MOI) of 0.01, virus titers were in the range of 5×10^6 to 1×10^7 focus forming units (FFU)/ml for SAD Ambi-CAT and 10^8 FFU/ml for SAD L16.

CAT reporter gene expression. The recovery of an efficiently propagating virus demonstrated that the identical genome promoters of the two RNA strands support the functions necessary for RNP replication, incorporation of RNPs into virions, and expression of the proteins encoded on the genome RNA. As shown previously by expression of CAT and other reporter genes from monocistronic model genomes, the 3′-terminal 68 nt of the RV genome RNA are sufficient to direct transcription of downstream reporter genes when present on the genome RNA strand (9). To determine whether the CAT gene located on the antigenome RNA of SAD Ambi-CAT is expressed, CAT assays with cell extracts were carried out. In parallel experiments cells were infected with recombinant RVs SAD V-CAT and SAD X-CAT, which express CAT from the genome RNA. Both viruses contain the CAT gene inserted between the RV G and L genes and express different amounts of CAT due to the presence or absence of a nontranslated pseudogene region in the CAT cistrons (23).

After infection of cells at an MOI of 1 and incubation for

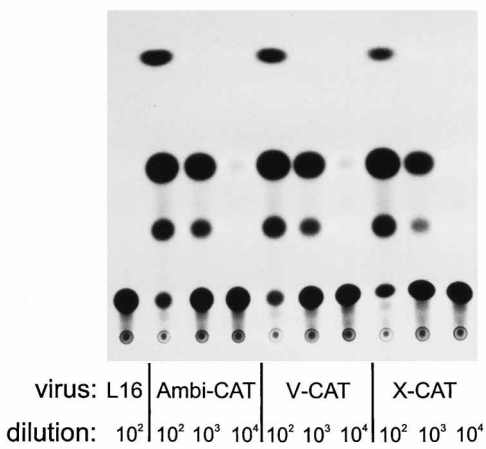

FIG. 2. CAT activity in extracts from cells infected with SAD Ambi-CAT, SAD V-CAT, and SAD X-CAT. Cells were infected at an MOI of 1, and cell extracts were harvested 20 h postinfection. For a comparison of CAT expression levels, the enzyme activities were determined for a dilution series of cell extracts. RV SAD L16 was used as a negative control.

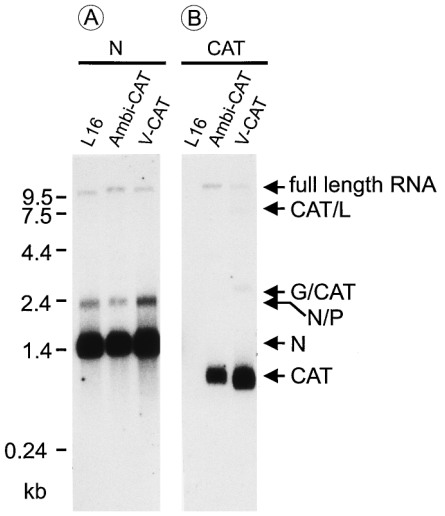

FIG. 3. RNA synthesis of SAD Ambi-CAT. Total RNA from cells infected with SAD L16 (L16), SAD Ambi-CAT (Ambi-CAT), and SAD V-CAT (V-CAT) was isolated 2 days after infection at an MOI of 1. (A) Hybridization with a RV N-gene-specific DNA probe. As for the controls, the majority of N transcripts of SAD Ambi-CAT are monocistronic mRNAs. In addition, minor amounts of bicistronic read-through RNA (N/P) are present. Due to the 1-kb insertion (CAT gene plus MuIX) the mobility of the viral full-length RNA is slightly decreased compared with that of SAD L16 RNA. (B) Transcription of the positive-strand-encoded CAT gene was detected by hybridization with a DNA probe specific for CAT gene sequences. In SAD Ambi-CAT-infected cells, abundant amounts of CAT mRNA but no read-through transcripts were detectable. In contrast, in SAD V-CAT-infected cells, bicistronic G/CAT and CAT/L transcripts could be visualized.

20 h, SAD Ambi-CAT expressed significant amounts of CAT. Moreover, the expression level appeared similar to that observed for SAD X-CAT or SAD V-CAT (Fig. 2). For a precise quantitation of CAT protein synthesis, cell extracts were harvested 48 h after infection and analyzed in a CAT enzyme-linked immunosorbent assay. In SAD Ambi-CAT-infected cells, 200 ng of CAT protein per 10^6 cells was found. The amount of CAT protein synthesis was comparable to the amount of expression determined for SAD X-CAT (210 ng), whereas SAD V-CAT expressed a fourfold-higher amount (800 ng). In SAD L16-infected cells no CAT activity was detectable. As determined previously for SAD X-CAT and SAD V-CAT, expression of the foreign genes introduced into the genome RNA of RV vectors is highly stable (23). To examine whether this also applies to the positive-strand-encoded CAT gene, SAD Ambi-CAT was passaged in cell culture successively 15 times by infection of BSR cells at an MOI of 0.01 and harvesting of viruses 3 or 4 days after infection. At every fifth passage, single FFU were isolated by end point dilution and tested for their abilities to express CAT after infection of fresh cells. After five virus passages, all of the isolated 29 clones exhibited significant CAT expression. In the 10th passage, 5 of 23 analyzed virus clones were CAT negative. However, the number of CAT-negative virus clones did not further increase from the 10th to the 15th passage.

RNA synthesis of SAD Ambi-CAT. Although supporting the pivotal functions in replication and transcription, the presence of identical, transcriptionally active promoter sequences at the 3′ ends of both genome and antigenome RNA was expected to severely affect the regulation of RV RNA synthesis. To investigate these effects, RNA produced in SAD Ambi-CAT-infected cells (MOI, 0.1) was characterized in Northern hybridizations after isolation of total cellular RNA at 48 h postinfection. For comparison, cells infected with SAD L16 or SAD V-CAT were included. Hybridization with a DNA probe specific for RV N sequences confirmed that the N gene was transcribed correctly from SAD Ambi-CAT (Fig. 3A). Consistent with the insertion of nearly 1 kb of foreign RNA, the genome size of SAD Ambi-CAT was increased compared to that of SAD L16. Although the CAT gene in SAD Ambi-CAT is located on the antigenome RNA strand (Fig. 1), large amounts of CAT mRNA of the predicted size were transcribed (Fig. 3B). In contrast to cells infected with SAD V-CAT, in which bicistronic RNAs containing the CAT sequence (G/CAT and CAT/L RNAs) were prominent, no CAT-encoding read-through transcripts were detectable (Fig. 3B). Thus, termination of CAT gene transcription from SAD Ambi-CAT occurred correctly. The responsible N/P gene border mutant MuIX mimics the L-gene end in functioning solely as a signal for polymerase stop and polyadenylation but not for transcription restart.

As estimated from hybridizations with the DNA probes, the abundance of CAT mRNA in SAD Ambi-CAT-infected cells was apparently only slightly lower than that of N mRNA, which is transcribed from genome RNPs (Fig. 3). Since both genes were under the control of promoters with identical sequences, this finding indicated that the respective templates for transcription might also be present in similar amounts. To allow a quantitative comparison of genome and antigenome RNAs, hybridizations with riboprobes specific for CAT sense or antisense sequences were carried out (Fig. 4). The positive-strand CAT probe recognizes negative-strand full-length RNA of SAD V-CAT and positive-strand full-length RNA of SAD Ambi-CAT. With this probe, similar signals were obtained for both viruses. With a negative-strand CAT probe, which should recognize negative-strand SAD Ambi-CAT RNA and positive-strand SAD V-CAT RNA, a signal was only obtained for SAD Ambi-CAT-infected cells. This finding showed that the promoter exchange provoked severe effects on the regulation of replication, resulting in an obvious increase of antigenome RNA.

To quantitate genome and antigenome RNAs in cells infected with standard RV SAD L16 and SAD Ambi-CAT by phosphorimaging, three independent infection experiments were carried out. Total RNA was isolated and hybridized to

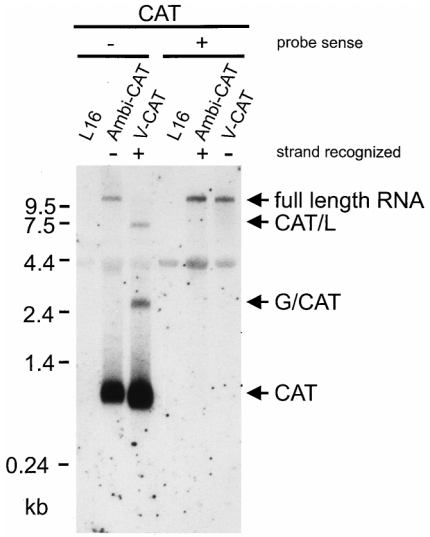

FIG. 4. Antigenome synthesis of SAD Ambi-CAT is increased. Total RNA was isolated from virus-infected cells as described in the legend for Fig. 3 and hybridized with riboprobes specific for sense or antisense CAT sequences. Given the reverse orientation of the CAT gene in SAD Ambi-CAT and SAD V-CAT (Fig. 1), and defining the strand encoding the RV protein genes as the genome (negative-strand) RNAs, the CAT positive-sense (+) probe recognizes the antigenome (+) RNA of SAD Ambi-CAT and the genome (−) RNA of SAD V-CAT. The CAT negative-sense (−) probe recognizes genome (−) RNA of SAD Ambi-CAT as well as the CAT mRNA of SAD Ambi-CAT. The CAT negative-sense probe should recognize the antigenome (+) RNA of SAD V-CAT and also the CAT mRNA of SAD V-CAT. In contrast to what was found for SAD Ambi-CAT, for which both genome and antigenome RNAs were readily detected, antigenome (+) RNA could not be demonstrated in SAD V-CAT-infected cells under these conditions.

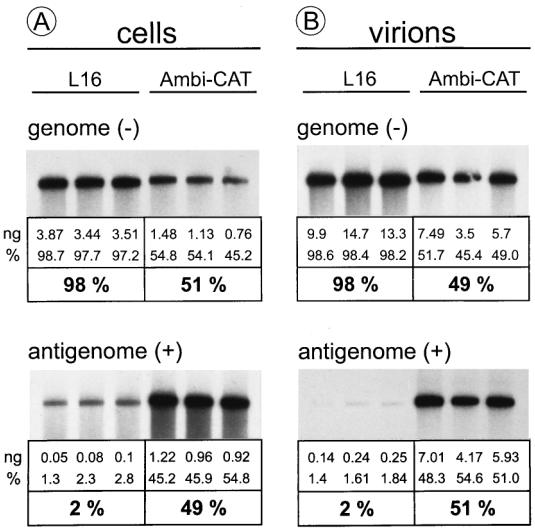

FIG. 5. Quantitation of genome and antigenome RNAs in virus-infected cells (A) and in virions (B). BSR cells were infected with SAD L16 or SAD Ambi-CAT at an MOI of 1. After 48 h total RNA was isolated from cells and from virions purified by sucrose gradient centrifugation of cell culture supernatant. Three micrograms of total cellular RNA or one-sixth of the RNA isolated from virions was electrophoresed in denaturing agarose gels. After Northern blotting the RNA was hybridized with strand-specific riboprobes recognizing RV N-gene sequences. For quantitation of virus RNAs in a phosphorimager, in vitro-transcribed RNA standards were used (data not shown). Each quantitation was performed with RNA isolated from three independent infections. Below the images the total masses (in nanograms) and the proportions (in percent) with respect to total full-length virus RNA are given. On the bottom line the average percentages from three experiments are shown. The total amount of viral full-length RNA in SAD Ambi-CAT-infected cells (2.2 ng/3 μg of total cellular RNA) was decreased by a factor of 1.7 compared with SAD L16 (3.7 ng/3 μg of total cellular RNA). The ratios of genome to antigenome RNAs (SAD L16, 49:1; SAD Ambi-CAT, 1:1) were identical for cells and virions.

strand-specific riboprobes derived from N-gene sequences by in vitro transcription (Fig. 5). Hybridization signals were compared to in vitro-transcribed cRNA standards. Accordingly, in SAD L16-infected cells an average of 2% of the full-length RNA was of positive polarity. In contrast, about half of the full-length RNA produced in SAD Ambi-CAT-infected cells was antigenome RNA (49%), confirming that the balance between RNA strands is determined by their terminal promoters (Fig. 5A). With regard to the assumed replicative disadvantage of the genome promoter, it was interesting to notice that total RNP synthesis in SAD Ambi-CAT occurred very efficiently (Fig. 3). As determined in phosphorimaging experiments, the total amount of full-length RNA in SAD Ambi-CAT-infected cells was 60% of that found in SAD L16-infected cells (2.2 and 3.7 ng/3 μg of total cellular RNA, respectively; Fig. 5A). Remarkably, the amount of genome promoter-derived full-length antigenome RNA was nearly 10-fold higher in SAD Ambi-CAT-infected cells, although the amount of genome RNA, which is needed as a template for antigenome RNA synthesis, was decreased 3-fold compared with that in SAD L16-infected cells (1.1 and 3.6 ng/3 μg of total cellular RNA, respectively).

The question of whether mechanisms exist that allow selective incorporation of negative-strand RV RNPs into mature virions was of particular interest. We therefore analyzed the particles released into the supernatant from cells infected with SAD L16 or SAD Ambi-CAT. Virions were purified by sucrose gradient centrifugation, and the polarities of their RNAs were determined by Northern hybridization with strand-specific probes (Fig. 5B). As determined by phosphorimaging, the ratios between genome and antigenome RNA were found to be highly similar in virions and in virus-infected cells (Fig. 5A and B). For SAD Ambi-CAT 51% of virion RNA was of positive sense, whereas 2% of the SAD L16 virion RNA exhibited positive polarity. Incorporation of antigenome RNPs into SAD L16 virions was apparently limited only by their low level of availability in SAD L16-infected cells. Thus, it can be assumed that the 5′ end of the RV genome does not contain signals discriminating genome and antigenome RNA for incorporation of RNPs into virus envelopes. The efficient incorporation of both genome and antigenome SAD Ambi-CAT RNPs further indicates a nonselective incorporation of RNPs into rabies virions.

DISCUSSION

In this report we describe the successful generation of a nondefective 3′ copy-back RV that expresses genes both from its genome and its antigenome RNP. Sequences that were usually present only in the 68 3′-terminal nt of the genome RNA, and that in the novel virus replaced the parental 45 3′-terminal nt of the antigenome RNA, rendered the antigenome RNP transcriptionally active. All *cis*-acting functions attributed to the antigenome promoter or its complement were fulfilled by the genome promoter-derived sequences. Moreover, the promoter exchange resulted in a virus with an unexpectedly high level replicative activity, while maintaining transcription levels similar to that of wild-type virus.

One key feature for the successful recovery of viruses transcribing nucleotide sequences from genome and antigenome RNA may be the prevention of antigenome-derived antisense RNA synthesis, which may inhibit viral protein expression.

This hypothesis is supported by the recent finding of significant inhibition of vesicular stomatitis virus (VSV) propagation by antisense L-gene sequences (38). Therefore, the presence of the mutated N/P gene border directing efficient transcriptional stop but no detectable restart might be essential for efficient propagation of SAD Ambi-CAT. MuIX represents a gene border in which the stop/polyadenylation signal ACUUUUUUUU overlaps by 2 nt (underlined) the RV consensus transcription start sequence UUGURRNGA. While transcription stop and polyadenylation are carried out, reinitiation is not observed, indicating an important role for the nontranscribed intergenic region in the regulation of viral transcription. A similar result for VSV was recently described. After deletion of the intergenic dinucleotide, transcription of a downstream gene from a bicistronic model genome was completely abolished (4).

A typical feature of rhabdo- and paramyxovirus infections is the presence of overwhelming amounts of genome sense RNA versus antigenome sense RNA in the cytoplasm of infected cells. The finding of equal amounts of genome and antigenome RNAs in SAD Ambi-CAT-infected cells confirms the assumption that solely the terminal promoters of the viral RNAs are responsible for keeping the natural balance of genome and replicative intermediate RNA. At least in the case of RV, putative *cis*-acting factors located in the internal parts of the RNAs and affecting, for example, the stability of RNAs or RNPs, the rate of polymerization, or the rate of premature, abortive termination obviously do not contribute.

Two different factors may account for the observed 50-fold preponderance of genome over antigenome RNA in wild-type RV: (i) an intrinsic advantage of antigenome promoter sequences in replication, i.e., in binding the viral polymerase (replicase) and/or in initiation of replication and elongation, and (ii) the dual function of replication and transcription of the genome promoter. In all stages of the viral life cycle transcription is by far the dominant type of RNA synthesis. If transcription and replication compete for the same polymerase, only a few molecules entering at the genome promoter would be able to perform replication, while most would be engaged in transcription.

The observation that 5′ copy-back DI particles interfere greatly with the propagation of wild-type helper virus has been used as an argument to support the hypothesis of an intrinsic replicative advantage of the antigenome promoter over the genome promoter, but the same effect can be expected in the case of competition between transcription and replication. The hypothesis that the antigenome promoter sequence indeed has an intrinsic advantage in replication has only recently been given more substance. Calain and Roux (6) have rescued transcriptionally active Sendai virus DI RNA possessing a chimeric genome promoter, in which the terminal 42 nt had the sequence of the antigenome promoter. Although it was transcriptionally active, the DI particle replicated as efficiently as nontranscribing copy-back DI particles, indicating that transcription does not necessarily interfere with replication. Upon introduction into full-length Sendai virus cDNA, the resulting nondeficient virus was found to interfere with replication of wild-type Sendai virus (15). Findings obtained with a bicistronic model genome of human respiratory syncytial virus are also in favor of a lack of competition between transcription and replication: upon deletion of the transcriptional start signal of the first gene, transcription was abolished while replicative antigenome synthesis was not found to be augmented (19).

Considering, therefore, the RV genome promoter to be a "weak" replication promoter, we expected to recover a very inefficiently replicating virus from pSAD Ambi-CAT. However, the two weak virus promoters directed the synthesis of RNP amounts reduced by less than a factor of 2 compared to that synthesized by wild-type virus (2.2 and 3.7 ng/3 μg of total cellular RNA, respectively). When the ratios of template and replicative products (1:1 and 49:1, respectively) are taken into account, the replication rate of the genome RNA, whose 3′ promoter was left unchanged, was raised by a factor of approximately 30 by the alteration of its 5′ end. This shows that the genome promoter sequence is only weak in the presence of the antigenome promoter and may indicate that a factor limiting replication of RNPs is the amount of replicase for which both promoters compete.

An intriguing observation has been made recently in a study utilizing a reconstituted system for replication of artificial nontranscribing VSV DI particles. An increased replication efficiency was observed for RNAs possessing extended complementary ends derived from either terminus of the genome (42). The augmentation of the genome promoter-directed replication rate in SAD Ambi-CAT-infected cells may be consistent with those findings, while an extended terminal complementarity was not found to influence the replication efficiency of Sendai virus DI constructs (39). Extending the terminal complementarity in the VSV DI particles also resulted in a decrease of transcription, while transcription of SAD Ambi-CAT was apparently unchanged. The efficient transcription of N mRNAs of SAD Ambi-CAT in addition to the synthesis of nearly equal amounts of CAT gene transcripts suggests that replication is independent of transcription in RV. However, we can so far not definitely rule out the possibility that the observed replicative ~30-fold enhancement of the SAD Ambi-CAT genome promoter is accompanied by some reduction in transcription. In view of the overwhelming excess of N mRNA molecules over full-length antigenome RNA molecules, a putative shift toward replication may not be detectable. While for paramyxoviruses it appears clear that the ability to transcribe does not reduce the replication efficiency of the genome promoter (6, 19), a similar conclusion for RV has to await additional data.

Viruses have acquired elaborate mechanisms for ensuring efficient propagation. One key event is packaging of the appropriate nucleic acids into virus particles. In many RNA viruses this is achieved by the presence of specific packaging signals present in the genomes and distinguishing them from replicative intermediates, subgenomic mRNAs, or cellular RNAs (see, for example, references 13, 25, and 43). Most probably, nonsegmented negative-strand RNA virus particles containing positive-strand RNPs cannot initiate replication autonomously, since for replication the synthesis of nucleoprotein N is required. Positive-strand RNPs may therefore only be replicated when cells are coinfected with a negative-strand "helper" RNP. This raises the question of whether mechanisms exist that exclude the incorporation of positive-strand RNPs into virions. A stringent selection of genome RNPs was assumed for VSV, because no antigenome RNPs could be found in virions (3, 36). In contrast, the assembly of antigenome RNPs into virions has been reported for Newcastle disease virus (30) and Sendai virus (18). The ratios of Sendai virus genome to antigenome RNPs in virus-infected cells and in virions were nearly identical (17, 24). In contrast to what is found for rhabdoviruses, however, polyploidy is common in the pleomorphic Sendai virus, and a less stringent incorporation of positive-strand RNPs might not have deleterious effects on the formation of infectious virions.

The finding of identical ratios of genome to antigenome RNAs in standard SAD L16 rabies virions and in SAD L16-infected cells strongly suggests that genome RNPs do not have a selective advantage in RV assembly. As demonstrated by the

successful recovery and propagation of SAD Ambi-CAT, the parental RV 5′ genome end does not encode a signal required for assembly of RNPs into virus envelopes. Moreover, the presence of putative strand-specific signals within internal sequences of the genome RNA is ruled out, since both strands of SAD Ambi-CAT, containing identical termini, were incorporated equally. The only factor determining the ratio of genome to antigenome virions therefore appears to be the replicative competition between genome and antigenome promoters. These observations also suggest that the lack of natural 3′ copy-back DI particles has to be attributed exclusively to a replicative disadvantage of such RNAs compared to standard helper virus.

The strategy of keeping levels of genome RNA above those of antigenome RNA in order to guarantee preferential production of infectious virions over positive-strand virus particles, which cannot autonomously initiate a replication cycle, has been lost in ambisense virus SAD Ambi-CAT. In addition, the lack of the antigenome promoter resulted in a decrease of total RNP synthesis. Together, these effects may contribute considerably to the observed 10- to 15-fold decrease of infectious (negative-strand) virus titers compared to standard RV titers, indicating a 5- to 7.5-fold decrease of total (positive- and negative-strand) virion synthesis. Negative-strand RNAs, which should directly determine the yield of infectious virions both by representing the genome RNA component and by being responsible for production of the necessary virus proteins, were reduced by a factor of 3 (1.1 versus 3.6 ng) in SAD Ambi-CAT-infected cells. Moreover, in contrast to what is found for standard RV, a considerable proportion of RNPs not contributing to productivity (50%) have to be provided with proteins for replication and virus assembly. The availability of virus proteins may therefore be another major factor limiting production of infectious SAD Ambi-CAT virions to the observed values. However, SAD Ambi-CAT expressed an additional gene at levels similar to those of standard "monosense" RV vectors. Additional genes have so far been expressed from recombinant rhabdo- and paramyxoviruses by insertion of a cistron between virus protein-encoding cistrons (5, 23, 33). At each gene border the expression of downstream genes is attenuated by partial dissociation of the polymerase. In the case of VSV, a decrease in transcription of 29 to 33% was determined for the N/P, P/M, and M/G gene borders (16). Expression of the 5′-terminally located L polymerase will therefore probably become a limiting factor after integration of multiple cistrons into the viral genome RNA. Whereas the introduction of a single CAT-encoding cistron upstream of the L gene had no obvious effect on RV and VSV propagation (23, 33), a 20-fold decrease of virus titers was observed after introduction of a CAT cistron between the G and F genes of human respiratory syncytial virus (5).

The ambisense gene expression strategy of SAD Ambi-CAT circumvents the problem of transcriptional attenuation of virus "housekeeping" genes, while providing additional coding capacities by exploitation of the complementary antigenome RNA as a template for transcription. In particular, the introduction of multiple genes may not result in further attenuation of essential virus genes. These considerations and the observed significant CAT protein expression in SAD Ambi-CAT-infected cells make ambisense RVs promising candidates for the development of new expression vectors and multivalent vaccines, mimicking a gene expression strategy naturally observed for segmented arenaviruses (family *Arenaviridae*) and some members of the *Bunyaviridae* family (for reviews see references 32 and 37).

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