The Minimal Conserved Transcription Stop-Start Signal Promotes Stable Expression of a Foreign Gene in Vesicular Stomatitis Virus

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A new transcription unit was generated in the 3’ noncoding region of the vesicular stomatitis virus (VSV) glycoprotein gene by introducing the smallest conserved sequence found at each VSV gene junction. This sequence was introduced into a DNA copy of the VSV genome from which infectious VSV can be derived. It contained an 11-nucleotide putative transcription stop/polyadenylation signal for the glycoprotein mRNA, an intergenic dinucleotide, and a 10-nucleotide putative transcription start sequence preceding a downstream foreign gene encoding the bacterial enzyme chloramphenicol acetyltransferase. Infectious recombinant VSV was recovered from this construct and was found to express high levels of functional chloramphenicol acetyltransferase mRNA and protein. The recombinant virus grew to wild-type titers of $5 \times 10^7$/ml, and expression of the foreign gene was completely stable for at least 15 passages involving 10-fold expansion at each passage. These results define functionally the transcription stop/polyadenylation and start sequences for VSV and also illustrate the utility of VSV as a stable vector that should have wide application in cell biology and vaccine development.

Vesicular stomatitis virus (VSV) is the prototype of the rhabdovirus group and has been widely used as a molecular and cell biology tool for the past 30 years. Its popularity results from simplicity of structure and very rapid growth to high titers in most mammalian cells and many other cell types. VSV also provides a paradigm for other RNA viruses having a single, negative-strand RNA genome. These viruses each encode and carry an RNA-dependent RNA polymerase that transcribes the 11,161-nucleotide genomic RNA to generate subgenomic mRNAs (3, 21). This polymerase also replicates the complete genomic RNA by a different mechanism. In the case of VSV, the subgenomic mRNAs encode five structural proteins: the nucleocapsid protein (N) which encases the genome tightly, two polymerase subunits termed L and P, an internal matrix protein (M), and a single transmembrane glycoprotein (G) responsible for both binding virus to cells and membrane fusion (21).

Although the complete sequences of the VSV mRNAs and genome have been known for many years (5, 20, 21, 23), it has not been possible until recently to use recombinant DNA technology to engineer the genomes of infectious viruses. Using a system similar to that first reported for the recovery of rabies virus (22), we and others reported the recovery of VSV from DNA (11, 25). These DNA-based recovery systems for single negative-strand RNA viruses make possible the genetic engineering of infectious viruses.

VSV transcription proceeds sequentially from the 3’ end of the genomic RNA to generate a short leader RNA followed by the five mRNAs (1, 2, 9). The mechanism of sequential transcription involves an obligatory polymerase start at the 3’ end of the genome, which is apparently followed by sequential stops and restarts at each gene junction. We will assume that this stop-start mechanism is in fact used, although the possibility of an RNA cleavage mechanism has not been completely ruled out (21). Nucleotide sequence analysis of the VSV genome showed conserved 23-nucleotide sequences present at each of the gene end and gene start sequences (14, 18). These sequences in the negative sense (genomic) consist of a putative transcription stop/polyadenylation motif 3’-AUACUUUUUUUU that apparently signals repetitive copying of the seven-residue U sequence to generate poly(A). This sequence is followed by an intergenic dinucleotide CA or GA that is not transcribed and a putative transcription start signal, 3’-UG UCNNUAG, complementary to the m7GpppAACAGNNATC sequence present at the 5’ end of all VSV mRNAs. Related conserved sequences are found at the gene junctions in all RNA viruses having single, negative-strand RNA genomes.

In the study described here we show that insertion of this minimal conserved sequence in the 3’ noncoding region of a gene is sufficient to terminate transcription of the gene and promote transcription of a foreign gene (chloramphenicol acetyltransferase [CAT]) introduced downstream. Therefore, no additional unrecognized features of VSV genomic structure are required to promote efficient transcription termination and reinitiation.

Earlier studies with rabies virus expressing a pseudogene region as a small mRNA (22) and a recombinant VSV in which the G gene was replaced with a G gene from a different serotype (11) suggested the possibility that rhabdovirus recombinants could be made to express foreign genes. The high spontaneous mutation rate in VSV (24) was a major concern in using VSV to express foreign proteins, because expression of the unselected foreign sequence might be lost rapidly through mutation. Surprisingly, we find that the unselected CAT gene is maintained quite stably. Given this stability of expression, the ease with which VSVs expressing foreign proteins can be
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MATERIALS AND METHODS

Plasmid construction. The starting plasmid, designated pSVSVFL-2, was similar to pSVSVFL (+), which has been described previously (11), but contained only a single T7 promoter immediately preceding the sequence encoding the end of the VSV positive strand and was used because it lacked the XhoI site present in pSVSVFL (+). The single Nhel site in the 3′ noncoding region of the G gene was used as the target site for introduction of a linker containing the promoter virus SVS poly(A) signal and stop sequence (Fig. 1A). The linker was prepared from two complementary oligonucleotides, which were annealed and cloned into the unique Nhel site of pVSV. Introduction of this linker in the correct orientation restored the Nhel site immediately next to the XhoI site and eliminated the upstream Nhel site. The resulting plasmid was designated pVSV-XN1, and the presence of the correct sequence was confirmed.

The CAT gene was amplified by PCR with Vent polymerase (Stratagene) from the pSV2-CAT plasmid (6) by using the primers 5′-GCTTCCCCGGGCTCGAGAATAGGAGAAGAAATCACTGGAT-3′ and 5′-GGCGGCGCTTGCAT-3′. The first primer contains an XhoI site (boldface letters) and the first 21 nucleotides of the coding region of the CAT gene (underlined letters); the second primer contains an XhoI site (boldface letters) and the last 21 nucleotides of the CAT gene (underlined letters). The PCR product was digested with XhoI and Xbol and cloned in pVSV-XN1 that was digested with Xhol and Nhel1. The resulting plasmid was called pVSV-CAT.

Transfection and recovery of VSV-CAT. Baby hamster kidney cells (BHK-21; American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum. Cells (∼80% confluent) on 10-cm-diameter dishes were infected with vTF7-3 (4) at a multiplicity of infection (MOI) of 10. After 1 h, the plasmid L encoding the N, P, and L proteins (11) and the pSV-CAT RNA were transfected into the cells using TransfectACE (Bethesda Research Laboratories) (19) as previously described. Plasmids and amounts were as follows: 10 μg of pSV-CAT, 3 μg of pBS-N, 5 μg of pBS-P, and 3 μg of pBS-L. After 48 h of incubation at 37°C in 5% CO2, supernatants from transfected cells were collected and debris was pelleted from the cell lysates by centrifugation at 1,250 × g for 5 min. Lysates were filtered through 0.2-μm pore-size syringe filters (Acrodisc) to remove vaccinia virus, and 5 ml of this filtrate was then added directly to BHK cells on a coverslip in a 35-mm-diameter dish. After 4 h, the cellss were infected with VSV-CAT or wild-type (wt) VSV at an MOI of 0.8; 80% confluent cell monolayers were infected with VSV-CAT at an MOI of 0.1. After 24 h, the cells were washed with Dulbecco's modified Eagle's medium minus serum and incubated at 1 h at 37°C in 1 ml of Dulbecco's modified Eagle's medium lacking unlabelled methionine and containing 80 μCi of [35S]methionine. Cell extracts (2% of the total) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and on a PhosphorImager (Molecular Dynamics). For isolation of VSV-CAT and VSV virus, a monolayer of BHK cells (∼80% confluent) on a 10-cm-diameter dish in 10 ml of Dulbecco's modified Eagle's medium plus 5% fetal bovine serum were infected with virus at an MOI of 0.1. After 24 h, the cells were harvested and centrifuged at 1,250 × g for 5 min and virus was then pelleted from the medium over a 10% sucrose gradient at 80,000 rpm in a Beckman SW41 rotor for 1 h. Virus pellets were resuspended in 0.5 ml of 10 mM Tris-HCL, pH 7.4, and pelleted a second time before separation by SDS-PAGE and staining with Coomassie blue.

For analysis of VSV-CAT virus after 15 passages, virus isolated from 12 single plaques (∼10⁶ PFU) was used to infect twelve 3.5-cm-diameter dishes of BHK cells. After 24 h, cells were harvested and analyzed for CAT activity. Supernatants were clarified by centrifugation at 12,000 × g for 5 min, and virus was pelleted from the medium at 15,000 rpm in a TOMY MTX-150 centrifuge for 1 h. Virus pellets were resuspended in 0.2 ml of 0.5% SDS-0.2 M sodium acetate, pH 8.0, followed by extraction with phenol-chloroform. RNA was precipitated with 95% ethanol and 5 μg of carrier RNA, pelleted by centrifugation at 12,000 × g for 15 min, and resuspended in water with 1 U of RNasin (Promega). For reverse transcriptase PCR, the first-strand DNA synthesis reaction was carried out in 50 μl of PCR buffer (Promega) containing 5 mM MgCl₂, 1 mM each (each) deoxynucleoside triphosphate, 1 U of RNasin (Promega), 1 U of Taq DNA polymerase, and a 32P-labeled pSVSPF-2 or pSV2-CAT as probes.

CAT enzyme assay. For analysis of CAT activity, VSV-CAT- or wt VSV-infected cells were washed twice with phosphate-buffered saline and cell extracts were prepared by scraping the cells from the dish following three rounds of freeze-thawing (∼70 and 37°C) in 500 μl of Tris-HCl (pH 7.5). Cell extracts (10%) were analyzed in CAT assays by standard procedures adapted from those of Gorman et al. (6).

RESULTS

To construct a DNA clone allowing insertion and expression of a foreign gene in the genome of VSV, we generated a synthetic DNA linker containing the sequence illustrated in Fig. 1A. This DNA encodes the minimal sequence elements that are conserved at all four VSV gene junctions: a transcription stop/polyadenylation signal, an intergenic dinucleotide, and the terminal transcription start sequence, all of which are shown in the positive-strand (mRNA) sense. These sequences were followed by unique Xhol and Nhel sites to allow insertion of a foreign gene. The linker was inserted at the unique Nhel site that we had previously engineered in the 3′ noncoding region of the VSV G mRNA (11).

We next inserted the complete sequence encoding the bacterial CAT protein (6) between the Xhol and Nhel sites. The complete material encoding the VSV-CAT positive-strand RNA, along with the T7 RNA polymerase promoter, ribosome, and T7 terminator, is diagrammed in Fig. 1B. If the stop-start signals preceding the CAT gene junction sequence were functional in recovered virus, we anticipated that G mRNA would terminate at the new poly(A) site and that CAT mRNA would initiate at the new start site and terminate at the original G poly(A) site. This particular site downstream of G was chosen for insertion of the new transcription unit because it was likely to have minimal effects on viral replication, perhaps only reducing L expression slightly because of a polar effect on downstream transcription.

Recovery of VSV-CAT from DNA. The recovery of VSV from DNA involves simultaneous transfection of the plasmid encoding the full-length antigenomic RNA along with three plasmids encoding the VSV N, P, and L proteins into cells infected with the vaccinia virus vector vTF7-3 (11). VSV nucleocapsids are assembled in these cells, but because the assembly of the VSV nucleocapsid is a relatively inefficient process, recoveries are not obtained in all transfections (11, 25). We therefore set up 10 transfections to recover VSV-CAT virus. We found in other experiments that recoveries of VSV from DNA were obtained just as frequently by transfection with a cationic lipidosome reagent (19) 1 U of RNApol₂; therefore, the simpler lipidosome transfection method was adopted. After 2 days, supernatants from the initial transfections were filtered to remove vaccinia virus and added to BHK cells. Infection of BHK cells with supernatants from this first passage showed typical VSV cyto-
pathic effects for 9 out of the 10 transfections. Further passage of these supernatants and analysis of infected cells by indirect immunofluorescence revealed surface expression of VSV G protein and a diffuse cytoplasmic fluorescence of CAT protein. Examples of the immunofluorescence results for a low-multiplicity VSV-CAT infection are shown so that some uninfected cells are visible (Fig. 2A and B).

**Proteins expressed by VSV-CAT.** Immunofluorescence showed that CAT protein sequences were expressed in cells infected with VSV-CAT, and the results in Fig. 3A show that the CAT protein expressed was functional. To determine if a full-length CAT protein was being produced and to quantitate expression, we infected BHK cells for 4 h with two independently derived VSV-CAT viruses or with wt VSV. Infected cells were then labeled with \[^{35}S\]methionine, and crude lysates were fractionated by SDS-PAGE. Because VSV infection shuts off host mRNA translation, the viral proteins can be visualized without immunoprecipitation. The results in Fig. 3B show that the VSV L, G, N, P, and M proteins as well as an additional protein of the size expected for CAT were synthesized in VSV-CAT-infected cells. The CAT band is not seen in the VSV-infected cells. There are also two proteins of unknown identity flanking

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FIG. 1. Construction of VSV-CAT. (A) VSV genome diagram and sequence of the synthetic linker encoding minimal, consensus transcription start-stop sequences. The letters in boldface type represent the nucleotides within the consensus sequence that are conserved at all four gene junctions. Intergenic dinucleotides are italicized. The putative stop/poly(A) sequences as well as the transcription start sequences are labeled. The linker was cloned at the unique NheI site introduced in the 3' noncoding region of the VSV gene and eliminates the upstream NheI site. (B) The diagram shows the pVSV-CAT plasmid with the positions of the T7 RNA polymerase promoter (T7P) and terminator (T7 term) and hepatitis virus delta ribozyme (RBZ) indicated. The NheI site was eliminated when joined to the XbaI site in the CAT PCR product.
CAT that are seen in the wt infection also. Quantitation of CAT protein expression on the PhosphorImager (correcting for the number of methionine residues) showed that CAT was expressed at a level of 58% of that of VSVG protein.

**VSV-CAT expresses CAT protein from a sixth mRNA.** To determine if CAT protein was being expressed from an mRNA of the expected size [approximately 800 nucleotides without poly(A), including the untranslated portion from G], we electrophoresed RNA from cells infected with VSV-CAT or VSV and carried out the Northern blot shown in Fig. 4. Using a probe generated from the complete pVSVFL-2 plasmid, we detected similar mRNA patterns from both VSV- and VSV-CAT-infected cells. The specific probe for CAT sequences showed an mRNA comigrating with the VSV M and P mRNAs, which are both approximately the same size as CAT. The faint RNA bands migrating above the 9.5-kb marker are probably the full-length genomic and antigenic RNAs.

**VSV-CAT grows to the same titer and produces the same number of particles as VSV.** Two different VSV-CAT isolates were plaque purified and then grown on BHK cells. Final titers on BHK cells were $2 \times 10^9$ or $5 \times 10^9$ PFU/ml, equivalent to those obtained with wt VSV. To determine if there were any differences in the protein compositions or amounts of total protein produced by VSV or VSV-CAT, identical numbers of BHK cells ($5 \times 10^6$) were infected with virus from single plaques of either VSV or VSV-CAT. After 24 h when the infection was complete, the virus was pelleted from the supernatant and 5% of the total from each pellet was fractionated by SDS-PAGE and subjected to Coomassie staining. The photograph of the gel (Fig. C) shows identical protein compositions and amounts for both viruses, with no CAT protein present in the particles. We conclude that the presence of the CAT gene does not have a detectable effect on the growth of VSV, although VSV-CAT presumably replicates marginally more slowly than wt VSV because of its longer genome.

**CAT protein is expressed at high levels.** To determine the amount and the time course of CAT protein expression by VSV-CAT, cells were infected with VSV-CAT at an MOI of $\sim 20$ and after 4, 6, 8, and 24 h cell lysates were prepared from cell pellets. The amount of CAT protein as a percentage of cell protein was determined by an ELISA with a CAT protein standard. The results showed that CAT protein was already 1% of total cellular protein at 4 h and was 1.7, 1.6, and 1.3% of total cellular protein after 6, 8, and 24 h, respectively.

**Stability of the CAT gene and protein expression over 15 passages.** Because RNA viruses including VSV are known to have high spontaneous mutation rates (24), we were concerned that expression of the CAT protein might be lost rapidly during passaging of VSV-CAT. To determine genomic stability, we passaged a VSV-CAT stock obtained from a single recovery. Virus ($10^6$ PFU) was then added to $10^7$ BHK cells and grown for 24 h, yielding $10^{10}$ infectious VSV particles. Approximately $10^5$ particles from this stock were then added to fresh cells, and the entire process was repeated for a total of 15 low-multiplicity passages. We then infected BHK cells with the passaged virus at an MOI of 1 and carried out double-label immunofluorescence microscopy for CAT protein and VSV G protein as...
shown in Fig. 2. Scanning of over 10,000 infected cells expressing G protein revealed none that had lost CAT expression.

We next carried out a plaque assay on the passaged virus and picked 12 individual plaques. Virus from these plaques was used to infect separate dishes of BHK cells, and CAT assays were performed on the cell lysates from each dish (Fig. 5). This experiment showed that all cells expressed functional CAT protein and suggested that no major changes such as frame-shift mutations or deletions of the CAT sequence had occurred, but the results did not rule out minor base changes that might affect CAT sequence without eliminating enzyme activity. To look for such changes, we used reverse transcriptase PCR with Vent polymerase to amplify the CAT sequences from six of the individual plaques and carried out direct sequencing of the PCR products. At least 400 bases could be read reliably from each PCR product. Four of the sequences agreed completely with the sequence of the starting pVSV-CAT plasmid. In two sequences we found different single-base changes that would change single amino acids in CAT at positions 79 and 88, but these mutations apparently did not eliminate CAT activity. Thus, even after 15 passages involving 10³-fold expansion at each passage, functional CAT expression was not lost in a significant proportion of the virus population and nucleotide sequence changes were still relatively rare.

DISCUSSION

The homologies among the sequences at the junctions of the VSV genes have been known for many years (14, 18), but until very recently it has not been possible to test the function of these conserved sequences as terminators and promoters for expression of foreign genes. Here we have described a modified VSV containing the minimal 23-nucleotide conserved sequence, and we report that it is sufficient to direct expression of a foreign gene from a new mRNA species in VSV. Therefore, no other special features of VSV genome structure, such as transcription termination, polyadenylation, or reinitiation signals, are required.

We chose the bacterial CAT gene for these studies because it is small (~700 nucleotides) and might fit within any existing packaging constraint. In other recent studies, we have found that VSV can accommodate and express extra genes of at least 2.5 kb and still produce normal yields of virus particles (our unpublished results). VSV virions contain a helical nucleocapsid with approximately 35 turns that is tightly packed within a membrane-enveloped, bullet-shaped particle (21). Defective VSVs have been known for many years, and these contain shorter nucleocapsids packed in shorter, bullet-shaped particles. Thus, it is not unreasonable that a longer RNA might simply be accommodated in a longer nucleocapsid. In fact, other rhabdoviruses do contain an extra gene between G and L (10). On the basis of our results to date, we believe that there is no strict packaging limit in VSV, although the packaging of very large nucleocapsids will likely become inefficient at some point.

There is a polarity in VSV transcription that follows the gene order $N > P > M > G > L$. As polymerase proceeds along the genome during transcription, it apparently terminates after polyadenylating each mRNA. This is followed by reinitiation of about 70 to 80% of the polymerases on the next gene (9). The level of expression of the CAT protein was less than that of G, consistent with such a polarity. We might also expect that inclusion of the CAT gene between G and L would reduce levels of L expression relative to those of the other genes. Although a 20 to 25% reduction in levels of L expression relative to those of $N$ or $P$ was noted for the recombinants compared with those of wt VSV in the experiment shown (Fig. 3B), this difference does not appreciably affect the growth of the recombinant virus.

VSV offers a number of advantages over other live virus-based expression systems. First, the virus life cycle is so rapid that recombinants expressing the protein of interest can be generated in 1 to 2 days after the gene to be expressed is cloned into the pVSV-XN1 vector. Virus plaque assays require only 12 to 18 h, and stocks with titers of $5 \times 10^9$/ml can be prepared overnight from single plaques. The very useful live-virus expression systems based on vaccinia virus recombinants require significantly more time and effort, because recombination in vivo is used and then recombinants must be screened or selected (15). Second, the gene to be included in the VSV vector needs only an effective translation initiation site and a termination codon because it is expressed from a new mRNA species. This contrasts with expression in positive-strand RNA viruses such as poliovirus, which cleave all proteins from a larger polyprotein. Proteolytic cleavage sites must therefore be introduced on either side of protein to be expressed, and the polyprotein structure is also important (7). Third, the expression level in VSV is quite high. CAT protein was 1.7% of total cell protein after 6 h, and it is expressed with only the five other VSV proteins because host protein synthesis is shut off. In contrast, vaccinia virus expresses a very large number of additional polypeptides from its 190-kb genome (15). For the generation of specific immune responses in vaccine applications it will likely prove advantageous to have only a limited number of proteins expressed.

There are also other very useful expression systems based on defective alphaviruses derived from Sindbis and Semliki Forest viruses. Because of RNA packaging limits in these viruses, mostly defective derivatives expressing the gene of interest replacing the viral structural genes are normally employed. These defective viruses are generally packaged by employing a helper virus and are limited to a single cycle of replication (13, 26). Recently, the use of a nondefective Sindbis virus as a vaccine vector was described. However, in this system expression of the foreign gene is lost after one to five passages and the recombinant viruses grow to lower titers than the wt virus (17).

Polymerase errors during replication were a major concern in using an RNA virus such VSV to express a foreign gene. Neutral mutation frequencies in VSV have been estimated at 1 in $10^3$ to $10^4$ for specific nucleotides in the genome (8, 24). However, there are examples of remarkable VSV genome sequence stability based on analysis of VSV genome sequence
evolution in nature. For example, a region of the P gene that is highly variable in sequence among VSV strains isolated from diverse geographical areas was extremely stable over 7 years of replication in sandflies and pigs in one enzootic focus (16).

Our results show that an additional unselected gene is very stable in VSV. Even after 15 low-multiplicity passages, all viruses examined expressed functional CAT protein. We did, however, find single-base changes in the CAT gene in two out of six viruses after 15 low-multiplicity passages. Extensive analysis of the number of sequence changes in this CAT gene occurring over a very large number of passages could be useful for obtaining an accurate measure of the neutral mutation rate in VSV.

Most importantly, the mutation rate in VSV is so low that it does not present a problem when the virus is used to express foreign genes, even during extensive passaging. In a single does not present a problem when the virus is used to express foreign genes, even during extensive passaging. In a single

dition would produce 200 g of virus. However, one could imagine circumstances in which the expression of the foreign gene could be inhibitory to VSV replication. One might then expect an accumulation of mutations eliminating expression.

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