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Internal Initiation of Translation in Retroviral Vectors Carrying Picornavirus 5′ Nontranslated Regions

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Previous work has shown that picornavirus 5′ nontranslated regions (NTRs) can initiate internal translation of downstream coding regions both in vitro and in transient in vivo assays. We have used 5′ NTR sequences from encephalomyocarditis virus and poliovirus to construct retroviral vectors that are designed to express two proteins from a single mRNA. Inclusion of 5′ NTR sequences did not adversely affect vector titer. Protein expression was studied with stable cell lines generated by vector infection of mouse NIH 3T3 cells and human and canine skin fibroblasts. Expression of a coding region in the downstream position was at levels from 25 to 100% of the same coding region in the upstream position. Expression of downstream coding regions in control vectors that did not contain the 5′ NTR sequences was very low, in agreement with the predictions of the scanning model for eukaryotic translation. These experiments demonstrate coordinate expression of two coding regions from a single mRNA in stable cell lines and provide further support for the model of internal translation initiation by sequences in the 5′ NTRs of picornaviruses.

Translation of typical eukaryotic mRNAs begins with the binding of initiation factors and the 40S small ribosomal subunit to the capped 5′ end of an mRNA, followed by the migration of this complex to the first AUG codon in a context suitable for initiation of translation, where the complete ribosomal initiation complex is formed and protein synthesis begins. Picornavirus infection of cells causes a reduction in the translation of cellular RNA. In the case of poliovirus and several other members of the picornavirus family, the reduction is at the step of cap-dependent binding of ribosomal components to cellular mRNAs and is due to inactivation of cap-binding protein eIF-4F by a viral protease (25, 26). Uncapped viral RNA continues to be translated in a cap-independent manner and is dependent on the presence of unusually long 5′ nontranslated regions (NTRs), ranging from 650 to 1,300 nucleotides, in the viral mRNA. These NTRs contain multiple AUG codons that appear not to initiate translation. In the case of poliovirus, mutation of six of the seven upstream AUG codons had no effect on virus replication in cultured cells, and mutations in the seventh AUG only reduced the replication rate of the virus (20).

Several lines of evidence suggest that picornavirus NTRs provide sites for direct binding of ribosomes and thus allow internal initiation of protein translation from downstream AUG codons. First, although downstream coding regions in multicistronic RNAs are usually expressed poorly in mammalian cells or in in vitro mammalian translation systems, insertion of the 5′ NTR from either poliovirus or encephalomyocarditis virus (EMCV) promotes efficient translation of downstream cistrons in in vitro and transient in vivo assays (4, 5, 22, 28). Second, insertion of a 5′ NTR upstream of a heterologous protein coding region renders translation of that cistron independent of poliovirus infection in cultured cells and in cellular extracts from infected and uninfected cells (4, 21, 22, 28). Last, internal binding of ribosomes to 5′ NTRs has been demonstrated in vitro and involves the binding of additional cellular but not viral proteins (6, 9, 23).

A limitation of the experiments to date is the use of in vitro or transient in vivo assays for measurement of translation initiation. To overcome this, we have examined internal initiation from 5′ NTRs by using bicistronic retroviral vectors for the generation of stable cell lines. We find that insertion of EMCV or poliovirus 5′ NTRs between the two coding regions markedly stimulates translation of the downstream cistron and thus provides further support for the model of internal initiation of translation by picornavirus 5′ NTRs. In addition, because both proteins are coordinately expressed from a single mRNA, this approach to the synthesis of multiple proteins by retroviral vectors has advantages over other methods that employ alternate splicing or internal promoters. Hence, selection for expression of one of the genes should ensure expression of the other, which is not the case when other strategies are used.

MATERIALS AND METHODS

Cell culture. PA317 amphotropic retrovirus packaging cells (11; ATCC CRL 9078), PE501 ecotropic retrovirus packaging cells (12), and NIH 3T3 TK$^-$ cells (11) were grown in Dulbecco modified Eagle medium with high glucose (4.5 g/liter) supplemented with 10% fetal bovine serum (HyClone, Logan, Utah). Human and canine diploid fibroblasts were cultured in Waymouth medium and were isolated from skin biopsies of normal human and canine donors and from adenosine deaminase (ADA)-deficient and purine nucleoside phosphorylase (PNP)-deficient patients.

Retroviral vector construction. Scale drawings of the retroviral vectors are shown in Fig. 1. Vector descriptions are based on the order of sequence elements within the vector, including promoters, coding regions, and viral translation initiation sites (see legend to Fig. 1). Plasmid forms of retroviral vectors are preceded by "p," while the viral forms have no prefixes. Construction of pLPNSN-2 has been described previously (15). Plasmid pLNPN was made from pLNSPN (15) by deletion of the simian virus 40 (SV40)

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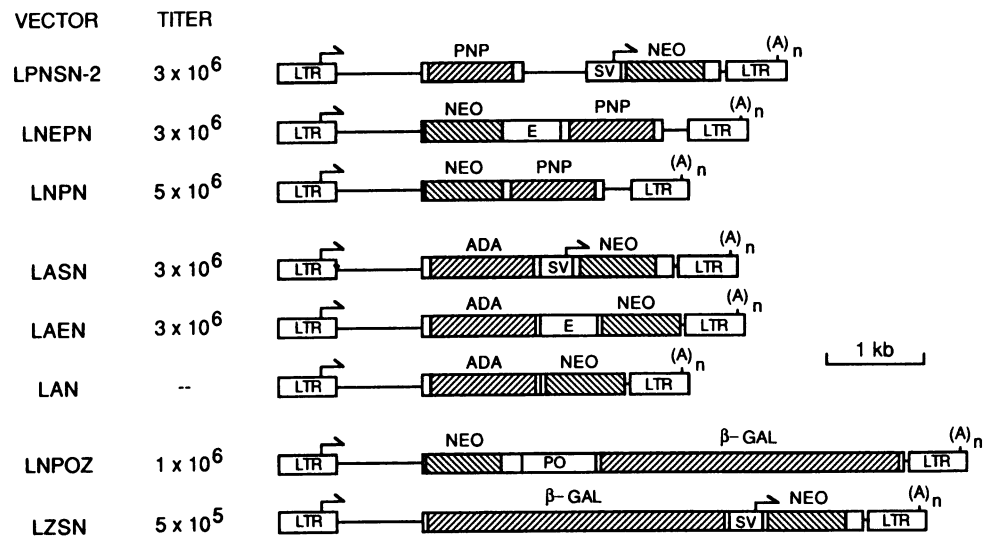

FIG. 1. Retroviral vectors. Abbreviations and symbols are as follows: LTR, retroviral long terminal repeat; SV, SV40 early promoter and enhancers; E, EMCV 5′ nontranslated region; PO, poliovirus 5′ nontranslated region; PNP, human PNP cDNA; NEO, bacterial NPT cDNA; ADA, human ADA cDNA; β-GAL, β-gal cDNA with synthetic mammalian translation start codon; $(A)_n$, polyadenylation signal. Hatched areas indicate protein coding regions; lines indicate viral sequences other than the LTR sequences, including the extended virus packaging signal (1) that follows the retroviral LTR. Vector names are based on the order of genetic elements within the vector: L, LTR; PN, PNP; S, SV40 promoter; N, NEO; A, ADA; E, EMCV 5′ sequences; PO, poliovirus 5′ sequences; Z, *lacZ* gene or β-gal.

promoter. Plasmid pLNEPN was made from pLNSPN (15) by replacement of the SV40 promoter with the EMCV 5′ nontranslated region from bases 260 to 837 obtained as an *Eco*RI-to-*Bal*I fragment from pBS-ECAT (4). This segment contains the internal ribosomal entry site and sequences that augment efficient initiation of translation (6). Plasmid pLASN has been described previously (3). Plasmid pLAN was made by insertion of the ADA coding region from pLASN into pLXSN (12) in place of the SV40 promoter. Plasmid pLAEN was made by insertion of the EMCV fragment described above between the *neo* and ADA genes of pLAN. Plasmid pLNPOZ was made by replacing the SV40 sequences in pLNSX (12) with the 5′ nontranslated sequences from bases 1 to 732 of poliovirus type 2 (Lansing) obtained as a *Hin*dIII-to-*Eco*RV fragment from pP2-5′ (21) followed by β-galactosidase (β-gal) sequences isolated from pLacD (kind gift from Jacques Peschon and Richard Palmiter, University of Washington, Seattle). pLacD contains a synthetic translation initiation codon and proper surrounding sequences (ACCATGG; 7) for efficient translation of the bacterial β-gal gene in mammalian cells. pLZSN (2) was made by inserting a *Bgl*II fragment containing the β-gal coding region from pLacD into the *Bam*HI site of pLXSN (12).

Virus production and assay. Virus was generated from the vector plasmids as previously described (12, 13). Briefly, plasmids were introduced into PE501 cells by calcium phosphate-mediated transfection, and virus was harvested 2 days later and used to infect PA317 cells. Clonal PA317 cell lines were isolated following selection of the infected cells in 0.75 mg of G418 per ml (actual concentration). About 10 infected PA317 clones for each vector were screened for high-titer virus production by assay of secreted virus on NIH 3T3 TK^- cells. Clonal PA317 cell lines were isolated which produced from 5×10^5 to 5×10^6 G418-resistant CFU/ml of medium harvested from the cells for all of the vectors except LAN (see Results).

Enzyme assays. Activities of ADA and PNP were determined by spectrophotometric assays by using adenosine and inosine, respectively, as substrates (14, 16). Relative amounts of human, mouse, and dog ADA or PNP in cell extracts were determined by starch gel electrophoresis (16), which separates enzymes from different species. Neomycin phosphotransferase (NPT) activity was determined as described previously (24) by measuring [^{32}P]ATP phosphate transfer to neomycin by cell extracts. Nonspecifically labeled protein was removed by phenol-chloroform extraction of the reaction mixture prior to product adsorption to DEAE anion-exchange paper and quantitation by scintillation counting.

RESULTS

Retroviral vector construction and virus production. The retroviral vectors used here are all derivatives of the Moloney murine leukemia virus-based vector LNL6 (1). The viral sequences surrounding the inserted genes are identical and include the extended packaging signal required for high-titer virus production (1). This signal includes a portion of the *gag* gene of Moloney murine leukemia virus, but the start codon has been changed to TAG to prevent *gag* translation and to provide efficient translation of downstream sequences (12).

Included in the vectors are cDNAs encoding human ADA, human PNP, and bacterial NPT, all of which can be quantitated by enzymatic assays. EMCV and poliovirus 5′ NTRs have been inserted between the cDNAs in LNEPN, LAEN, and LNPOZ in order to study their effects on downstream translation. The sequences separating the coding regions in these vectors are from 677 to 1,001 bp and contain from 11 to 13 ATGs. The vectors LNPN and LAN serve as controls for expression of downstream coding regions in bicistronic mRNAs. Approximately 100 bp separate the coding regions in these vectors, and there are no intervening ATGs in LNPN and only two ATGs in nonoptimal contexts for

TABLE 1. Enzyme levels in vector-infected PA317 cells

Virus	Level of enzyme[a]		
	ADA	PNP	NPT
None	1.8	2.1	<2
LPNSN-2	1.8	6.8	48
LNEPN	1.9	6.7	320
LNPN	1.2	1.6	170
LASN	16.2	2.0	60
LAEN	19.4	2.1	120
LNPOZ	1.8	1.4	330

[a] ADA and PNP activities are expressed as micromoles per hour per milligram of protein. The mean coefficient of variation of these assays was 5%. NPT activity is expressed as picomoles per hour per milligram of protein, and the results from duplicate determinations varied by no more than 10%.

TABLE 2. Enzyme levels in vector-infected PNP$^-$ human diploid fibroblasts

Virus	Level of enzyme[a]	
	PNP	NPT
None	<0.01	<2
LPNSN-2	5.49	120
LNEPN	4.50	260
LNPN	0.10	180
Normal[b]	0.74	ND

[a] Units are the same as in Table 1. ND, not done.
[b] Uninfected normal human fibroblasts.

initiation of translation (7) in LAN. Additional controls involve the insertion of an SV40 early promoter and enhancers between the coding regions to promote translation of the second coding region from an SV40-initiated subgenomic mRNA.

Amphotropic helper-free virus was made from the plasmid forms of the vectors by using PA317 retrovirus packaging cells (10, 11), as previously described (see Materials and Methods). G418-resistant PA317 clones that contained single unrearranged proviruses were chosen for study. Virus with titers from 5×10^5 to 5×10^6 CFU/ml were obtained from all of the constructs except pLAN (Fig. 1), for which six of six G418-resistant clones examined by Southern analysis contained rearranged proviruses. The rearrangements involved partial or complete deletion of ADA sequences, and we conclude that *neo* expression from the downstream coding region in this bicistronic vector was not sufficient to confer G418 resistance to the PA317 cells without the deletion of ADA sequences.

Compared with results for the parent vectors, the inclusion of 5′ NTR sequences from EMCV or poliovirus did not significantly alter the vector titer. For example, the three vectors LPNSN-2, LNEPN, and LNPN all contain *neo* and PNP coding regions and either an SV40 promoter, 5′ NTR, or no additional sequence, and all had titers from 3×10^6 to 5×10^6 CFU/ml (Fig. 1). Similarly, LASN and LAEN, which both contain *neo* and ADA coding regions, had the same titers. LNPOZ and LZSN, which both contain *neo* and β-gal coding regions, had similar titers, which are somewhat lower than those for the other vectors, probably because of the presence of the β-gal coding region.

Analysis of enzyme activities in vector-infected PA317 cells. Vector-producing cells infected with the PNP vectors LPNSN-2, LNEPN, and LNPN were assayed for ADA, PNP, and NPT activities (Table 1). Insertion of the EMCV 5′ NTR between *neo* and the PNP coding region in LNEPN resulted in a threefold increase in PNP activity in PA317 cells infected with this vector compared with that in uninfected cells. A similar increase in PNP activity was seen in cells infected with the LPNSN-2 vector, where PNP is the first coding region after the viral long terminal repeat (LTR). Starch gel analysis showed that the PNP activity in uninfected PA317 mouse cells was due to mouse PNP, while the observed increases in the vector-infected cells were due to human PNP (data not shown). Positioning PNP downstream of *neo* in LNPN resulted in PNP activity equivalent to levels found in cells not infected with a PNP vector. ADA activities in the PNP vector-infected PA317 cells were similar to those in uninfected control cells. Thus, positioning the EMCV 5′ NTR between two coding regions in a single transcriptional unit elevates expression of the downstream coding region to levels obtained when the coding region is in the upstream position.

ADA, PNP, and NPT activities in PA317 cells infected with the vectors carrying *neo* were also measured. Positioning *neo* as the first coding region following the LTR in LNEPN, LNPN, and LNPOZ resulted in an average of 270 U of NPT activity. When *neo* was positioned downstream of the ADA coding region in the LAN vector, G418-resistant PA317 clones containing an unrearranged provirus were not obtained, suggesting poor *neo* expression by the LAN vector. In contrast, when the EMCV 5′ NTR was inserted between ADA and *neo* in LAEN, PA317 cells infected with this vector expressed 120 U of NPT activity, or about half of the activity seen when *neo* was positioned near the LTR. PA317 cells infected with LAEN made about 10-fold-higher levels of ADA than cells not infected with an ADA vector, and the increase in ADA was shown by starch gel analysis to be due to human enzyme (data not shown); thus, the ADA coding region in LAEN was functional. Expression of *neo* from an internal SV40 promoter in LASN or LPNSN-2 resulted in 60 U of NPT activity. This is in agreement with our previous results showing the viral LTR to be a stronger promoter than SV40 (3, 15, 17). These data confirm that inserting the EMCV 5′ NTR between two coding regions in a single transcriptional unit results in translation of the downstream coding region at a rate approaching that of the upstream coding region.

Analysis of vector expression in PNP$^-$ human fibroblasts. Human skin fibroblasts from a patient with PNP deficiency were infected with the three PNP vectors and selected in G418, and enzyme activities were determined (Table 2). Uninfected cells had undetectable PNP activity. PNP$^-$ fibroblasts infected with LPNSN-2 or LNEPN vectors expressed PNP at about eight times the level found in uninfected normal human fibroblasts. PNP activity could be detected in LNPN-infected cells but was only about 15% of that found in normal human skin fibroblasts. NPT activities in the vector-infected cells were similar to those found in PA317 cells infected with the same vectors (Table 1). Analysis of RNA from the human PNP$^-$ fibroblasts infected with LNEPN or LNPN vectors by using either a *neo* or PNP probe confirmed the existence of single genome-length mRNAs of the expected sizes transcribed from each vector and ruled out the possibility that spliced or internally promoted mRNAs were responsible for expression of the downstream PNP cDNA in these vectors (Fig. 2). Thus, although there was some expression of PNP as the second gene in a bicistronic mRNA lacking any special sequences between the coding regions, expression of PNP was increased about

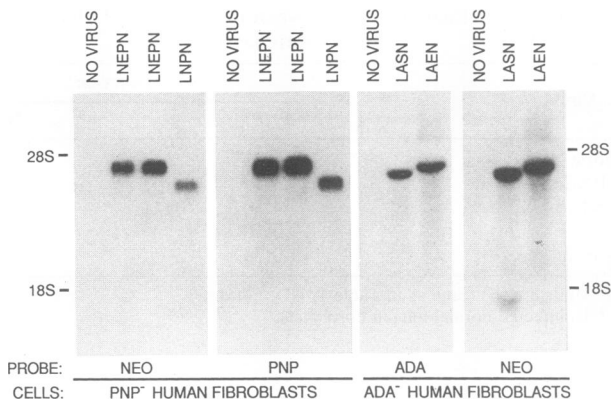

FIG. 2. Analysis of RNA from infected human fibroblasts. Total RNA was prepared from cells infected with the indicated vectors and selected in G418 or from cells that were not infected or selected. RNA was subjected to electrophoresis in denaturing formaldehyde gels, transferred to nylon membranes, and hybridized with the indicated radiolabeled probes. 28S (4.5 kb) and 18S (1.8 kb) ribosomal markers are indicated. The sizes of the expected mRNAs from the different vectors, assuming 150-bp poly(A) at the 3′ ends, are as follows: LNEPN, 4.4 kb; LNPN, 3.8 kb; LASN, 4.3 and 1.7 kb; and LAEN, 4.4 kb.

50-fold by inclusion of the EMCV 5′ NTR between the coding regions.

Analysis of vector expression in ADA⁻ human fibroblasts. Human skin fibroblasts from a patient with ADA deficiency were infected with the LASN and LAEN vectors and selected in G418, and enzyme activities were determined (Table 3). Both of the vectors expressed ADA at very high levels, up to 190-fold higher than normal human skin fibroblasts. Cells infected with LAEN expressed 60 U of NPT activity, which is 60% of the NPT level in cells infected with LASN, in which *neo* is driven by an internal SV40 promoter, or about 25% of the NPT levels seen in the PNP⁻ human fibroblasts infected with LNEPN or LNPN, in which *neo* is driven by the viral LTR (Table 2). Analysis of RNA from the human ADA⁻ fibroblasts infected with LASN or LAEN vectors by using either a *neo* or ADA probe confirmed the existence of single genome-length mRNA of the expected sizes transcribed from each vector and a smaller mRNA in LASN-infected cells that hybridized only to the *neo* probe and that had the size expected of an SV40-promoted subgenomic mRNA (Fig. 2). Thus, the insertion of the EMCV 5′ NTR between ADA and *neo* coding regions in a bicistronic mRNA promotes expression of the downstream *neo* coding region at a level approaching that directed by an internal SV40 promoter and at fourfold-lower levels than that directed by the viral LTR.

TABLE 3. Enzyme levels in vector-infected ADA⁻ human diploid fibroblasts

Virus	Level of enzyme[a]	
	ADA	NPT
None	<0.01	<2
LASN	69	100
LAEN	169	60
Normal[b]	0.9	ND

[a] Units are the same as in Table 1. ND, not done.
[b] Uninfected normal human fibroblasts.

TABLE 4. Enzyme levels in vector-infected dog skin fibroblasts

Virus	Level of enzyme[a]		
	ADA	PNP	NPT
None	0.9	0.94	<2
LASN	26	0.69	57
LAEN	44	0.61	140

[a] Units are the same as in Table 1.

Analysis of vector expression in canine skin fibroblasts. Normal dog skin fibroblasts were infected with the LASN and LAEN vectors, selected in G418, and analyzed for enzyme activities (Table 4). In both LASN- and LAEN-infected cells, ADA activities of 30- to 40-fold greater than in uninfected cells were observed. The PNP activity levels were similar in infected and uninfected cells. The NPT activity of LAEN infectants was about 2.5 times higher than that of cells infected with the LASN vector. This is similar to the results seen in PA317 cells, for which LAEN-infected cells produced twofold-higher NPT activity than LASN-infected cells (Table 1). In contrast, the SV40 promoter provided about twofold-higher levels of NPT activity than the EMCV 5′ NTR in human skin fibroblasts (Table 3). Thus, on average the EMCV 5′ NTR allowed about the same levels of *neo* expression in a downstream position as the SV40 promoter.

Use of the poliovirus 5′ NTR. We have also constructed a vector, LNPOZ (Fig. 1), with an internal poliovirus 5′ NTR and upstream *neo* and downstream β-gal cDNAs. While we have not characterized LNPOZ as completely as the vectors containing the EMCV 5′ NTR, LNPOZ provides similar levels of β-gal in infected cells, as judged by blue staining after incubation with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), as the LZSN vector, in which β-gal is driven directly by the viral LTR (data not shown).

DISCUSSION

We have demonstrated stable expression of both genes in bicistronic mRNAs transcribed from retroviral vectors in which EMCV or poliovirus 5′ NTR sequences have been inserted between the two coding regions. Analysis of RNA from infected cells reveals only full-length genomic messages, and no subgenomic spliced or internally initiated mRNAs that could account for expression of the downstream coding region. The level of PNP expression in such bicistronic mRNAs was similar when PNP was in the upstream or downstream position. Expression of *neo* in the downstream position was 25 to 50% of that observed when *neo* was in the upstream position. These results provide strong evidence for internal initiation of translation in mammalian cells and document a mode of translation in stable cell lines that is distinct from the scanning model for translation (7).

In the vectors described here, the poliovirus 5′ NTR sequences were truncated before the natural poliovirus start codon, while the EMCV 5′ NTR sequences still contain the natural EMCV start codon. This start codon is out of frame with the downstream coding regions in the LNEPN and LAEN vectors. In LNEPN, the EMCV start codon is followed two codons downstream by a stop codon, and we

presume that such a short open reading frame does not interfere with translation initiation at the downstream start codon of PNP. In the LAEN vector, the EMCV start codon is followed 21 codons downstream by a stop codon which overlaps the start codon of *neo*. That we find relatively efficient translation of the downstream *neo* coding region in the LAEN vector is consistent with previous results showing that a coding region that lies downstream of an open reading frame can be efficiently translated when the start codon of the downstream coding region is close to the stop codon of the upstream open reading frame (8, 18, 19, 27). Presumably, under these conditions the ribosomal complex reinitiates translation at the nearby start codon before the components have had time to dissociate. It is still possible, however, that elimination of the EMCV start codon or an in-frame fusion of the downstream coding region with this start codon might increase expression of the downstream coding regions in these vectors.

Insertion of the 5′ NTRs from EMCV or poliovirus into several retroviral vectors did not interfere with the production of high-titer virus from the vectors. It is tempting to speculate that retroviral vectors containing a string of coding regions separated by 5′ NTRs could be designed to allow coordinate expression of multiple cDNAs. Such constructions would be limited by size constraints on retroviral vectors, around 7 to 10 kb, and the potential for recombination between identical 5′ NTRs, resulting in cDNA deletion.

Cells infected with vectors containing ADA linked to the viral LTR, LASN and LAEN, produced from 4- to 30-fold-higher ADA activities than the PNP activities produced by cells infected with the corresponding PNP vector LPNSN-2. This difference is not due to increased protein synthesis from the ADA vectors but is due to the 10-fold-higher specific activity of the ADA protein compared with that of PNP (14, 15).

Although expression of the two coding regions in vectors containing 5′ NTRs is linked at the transcriptional level by their presence on the same RNA, there are conditions that can alter the ratio of translation by cap-directed or internally initiated mechanisms. Poliovirus infection of cells drastically reduces the rate of cap-dependent translation, while internally initiated translation is greatly increased because of facilitated access to the translational machinery (26). It will be interesting to see whether there are conditions that influence the ratio of translation by these two mechanisms in normal cells. The use of retroviral vectors for the promotion of unrearranged, single-copy insertions of these bicistronic genes will facilitate these studies.

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