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Retroviral vectors containing putative internal ribosome entry sites: development of a polycistronic gene transfer system and applications to human gene therapy

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ABSTRACT

Recombinant retroviral vectors producing multicistronic mRNAs were constructed. Picornavirus putative internal ribosome entry sites (IRES) were used to confer cap-independent translation of an internal cistron. Internal cistrons were engineered by ligation of various lengths of the IRES of encephalomyocarditis (EMC) virus or polio virus to the E. coli chloramphenicol acetyltransferase (CAT) gene. The IRES/CAT fusions were introduced into retroviral vectors 3′ to the translation stop codon of the neomycin phosphotransferase (NEO) gene, and the molecular constructs transfected into retroviral vector packaging lines. Retroviral vector producer cells efficiently express the internal CAT gene product only when the full length IRES is used. Both the EMC/CAT and polio/CAT retroviral vectors produced high titer vector supernatant capable of productive transduction of target cells. To test the generality of this gene transfer system, a retroviral vector containing an IRES fusion to the human adenosine deaminase (ADA) gene was constructed. Producer cell supernatant was used to transduce NIH/3T3 cells, and transduced cells were shown to express NEO, and ADA. Novel three-gene-containing retroviral vectors were constructed by introducing the EMC/ADA fusion into either an existing internal-promoter-containing vector, or a polio/CAT bicistronic vector. Producer cell clones of the three-gene vectors synthesize all three gene products, were of high titer, and could productively transduce NIH/3T3 cells. By utilizing cap-independent translation units, IRES vectors can produce polycistronic mRNAs which enhance the ability of retroviral-mediated gene transfer to engineer cells to produce multiple foreign proteins.

INTRODUCTION

Retroviral-mediated gene transfer is a highly efficient method of gene transfer that has recently seen its first clinical applications (1). Several types of retroviral vectors have been constructed that use different mechanisms for achieving gene expression. The most common vector designs use the long terminal repeat (LTR) of the retrovirus backbone and, an internal promoter sequence, to promote gene expression (2). Still other manipulations have been used to produce complex splicing vectors which use splice donor/acceptor sequences to generate multiple mRNAs (3,4). One potential problem with retroviral vectors containing multiple transcription units is that if selection is applied for one gene, expression of the other gene can be reduced or lost completely, (this is termed promoter suppression, see references 5,6). To try to avoid promoter suppression and to construct vectors that readily express multiple genes, it may be necessary to exploit alternate expression systems. Gene expression in the picornaviridae family of viruses is unusual in that their 5′ mRNA terminus is pUp... and they possess long untranslated leader sequences (7,8). Analysis of picornavirus gene expression has produced a consistent body of work that suggests that picornaviruses are able to bypass the standard ribosome scanning model of translation and begin translation at internal sites (9–12). Putative internal ribosome entry sites (IRES) have been identified in the long 5′ untranslated regions of picornaviruses (these sequences have also been termed ribosome landing pads, RLP). These IRES elements can be removed from their viral setting, and linked to unrelated genes to produce polycistronic mRNAs (9,11,13). Initial reports describing the application of these elements in retroviral-mediated gene transfer have recently appeared (14,15).

Herein we describe that picornavirus IRES elements can be linked to various genes and that the fusions, when inserted into retroviral vectors, are translated to yield functional gene products. In addition, we extend previous reports (14,15) by producing several three-gene-containing retroviral vectors. These IRES vectors permit multiple proteins to be produced from a single vector without alternative splicing or multiple transcriptions units and avoid the potential of promoter suppression. Furthermore, the coupling of translation of two (or more) different proteins may have significant applications in human gene therapy where the expression, in a given cell, of multiple heterologous proteins or distinct subunits of a multimeric protein is necessary.

MATERIAL AND METHODS

Molecular Constructs

EMC/CAT vectors, were constructed from the T7 RNA polymerase expression plasmid pOS6 (also referred to as pT7EMCAT, 16−18). Cla I plus Bsp MII were used to excise the T7-EMC/CAT expression cassette. The resulting fragment was made blunt ended by Klenow fill-in, and ligated into the Hind III cut/Klenow fill-in site of pG1N to produce pG1NECt and pG1NECt-R. EMC deletions mutants were constructed by digesting pOS6 with Bsp MII plus Apa I, Kpn I, or Nco I, followed by cloning into pG1N (as above) to yield pG1NECt-Δ200, pG1ENCt-Δ525, and pG1NCt, respectively. To facilitate further manipulations, the EMC IRES was isolated from pOS6 using polymerase chain reaction (PCR) amplification/restriction enzyme site addition (oligonucleotide primers, 5′-AACGGTTT-CCCTCGAGCGGGATCA-3′ plus 5′-TTTGTTAGCAGCCGG-ATCGT-3′) yielding a fragment with Xho I ends which was cloned into the Xho I site of Bluescript II KS+(Stratagene, La Jolla CA) to produce pEMC-F. PCR was similarly used to produce a fragments containing the ADA gene using the SAX retroviral vector (19) as a template (oligonucleotide primers 5′-TGCGAGACCATGGGACAGACGCCC-3′ plus 5′-CGG-AAGTGTGATCACCTAGGCGAC-3′). The ADA fragment was digested with Nco I, cloned into the Nco I plus Sma I sites in pEMC-F to produce pEMCADA. The EMC/ADA fragment was excised by Sst I digestion/T4 DNA polymerase fill-in plus Xho I digestion and ligated to Apa I cut/T4 DNA polymerase fill-in plus Xho I cut retroviral vector pG1N, to produce pG1NEA. The starting vectors for the triple gene constructs, LSCSN and LNSvCt were produced by inserting the soluble CD4 gene and CAT gene into the Eco RI plus Xho I (for CD4) or Hind III (for CAT) sites of LXSN and LNSX respectively (20). The EMC-ADA fragment was excised from pEMCADA by Xba I digestion/Klenow fill-in plus Xho I digestion and ligated to Bam HI cut/Klenow fill-in plus Xho I LSCSN to produce LSCEASN. To produce LNEASCt, pEMCADA digested with Xho I plus Sst I, filled in with Klenow and ligated to Bam HI cut/Klenow fill-in LNSvCt. The polio IRES element was isolated by PCR amplification/restriction site addition (oligonucleotide primers 5′-CCCAGATCTCCACGTGGCGGC-3′ plus 5′-ACCGGAA-GGCCTATCCAATTC-3′) using pPV16 as a template (21). PCR generated a fragment with Bgl II and Stu I ends which was ligated into Bam HI plus Stu I cut LNSvCt to yield LNPCt. LNEAPCt and LNPCtEA were produced by inserting the EMC/ADA fragment from pEMCADA (Sst I plus Xho I with T4 DNA polymerase fill-in) into the Nru I site or Cla I/Klenow fill-in site of LNPCt respectively. LCtSN was constructed by ligating a Hind HIII cut/Klenow fill-in CAT fragment into the Hpa I site of LXSN (the LXSN vector used in this report has had the normal Moloney U3 promoter region removed and substituted with the U3 region from Harvey murine sarcoma virus) The vectors pG1, pG1N2, and pG1N, are similar to the LN vector (20), but with additional cloning sites (G1 vectors kindly provided by Dr. Paul Tolstoshev, Genetic Therapy Inc. Gaithersburg, MD).

Cell Culture and Vector production

Retroviral vector producer cell lines were generated by the micro-ping-pong procedure (22,23). In brief, 50μg of DNA was used to transfect (via calcium phosphate coprecipitation) a mixture of the ecotropic packaging cell line GP+E-86 (24), and the amphotropic packaging cell line PA317 (25). The packaging cell line mixtures are maintained in culture for at least one week to permit vector amplification. Selection for vector integration is obtained by growth in the presence of the neomycin analog G418 (400 μg/ml active concentration). Transductions of mouse NIH/3T3 cells and Mink lung fibroblasts (ATCC CCL 64) were conducted by incubation of cells with recombinant viral supernatant (MOI = 1)containing 8μg/ml polybrene at 37° for 2hr, followed by removal of virus-containing medium and replacement with fresh culture medium. Transduced cell populations were selected by growth in G418 (400μg/ml) for 10−14 days. Cell clones were obtained using cloning rings following limiting dilution.

Gene Expression Assays

CAT enzyme assays were performed by first lysing cells (at 4°C) in 0.25M Tris-HCl(pH 7.5)/0.1% NP-40, followed by freezing on dry ice, thawing at 37°C (5 min), heating to 60°C (15 min) and removal of cellular debris by centrifugation (top speed, eppendorf microcentrifuge, 4°C, 5 min). After normalization for equal amounts of protein, cell extracts were mixed with acetyl-CoEnzyme A and ^{14}C-chloramphenicol and incubated at 37°C for 1−4 hr. as necessary to stay within the linear range of CAT activity. Chloramphenicol and acetylated products were extracted with ethyl acetate and applied to thin layer chromatography plates. Chromatographs were run in 95% $CHCl_3$, 5% methanol. Imaging was obtained by autoradiography and quantitation by direct beta particle counting of the TLC plates on a Betascope 603 instrument. Southern blot analysis was performed by subjecting restriction enzyme digested DNA samples to agarose gel electrophoresis, transfer to nylon membranes with UV cross-linking, and hybridization with a radiolabeled probe. Northern blot analysis was performed on formaldehyde agarose gels using RNA extracted with RNazol (CINNA Biotecx, Friendswood TX) and selected for poly A containing sequences by oligo-dT linked magnetic beads (Dynal Co. Great Neck, NY). ADA assays were performed on starch gels as described (26) and relative ADA actively was determined by scanning the resultant photographs on a laser densitometer and calculating the ratio of the areas of human ADA to mouse ADA enzymes. Soluble CD4 levels were measured using a CD4/gp120 capture ELISA (American Biotechnologies, Cambridge MA). NEO gene activity was measured using a NPT II ELISA (5 Prime, 3 Prime Inc. West Chester, PA).

RESULTS

Construction of CAT IRES vectors

To determine if the picornavirus IRES elements could function in a retroviral vector we constructed a series of CAT reporter gene vectors using the IRES elements from both the EMC and polio viruses. As starting material we used the EMC/CAT gene fusion from the plasmid pOS6 (16). The full length EMC/CAT fusion was excised and transferred in both orientations into the retroviral vector G1N to generating G1NECt (sense orientation) and G1NECt-R (reverse orientation). The distance between the NEO stop codon and the CAT start codon in the G1NECt construct is approximately 700 bp, and contains 9 intervening start codons plus 19 interrupting stop codons in all three reading frames. Next we made three deletions of the EMC IRES sequence and transferred these into the same retroviral backbone. In G1NECt-Δ200, G1NECt-Δ525, and G1NCt we deleted respectively; 200 bp, 525 bp, or all of the sequences between

the 5′ end of the EMC IRES leader sequence and the start of the CAT gene. These constructs along with a control CAT vector were then introduced into retroviral vector packaging cell lines and the coculture expanded for two weeks to allow vector spread. Southern blot analysis indicated that equivalent amounts of each vector were present in these lines (data not shown). Cell lysates were prepared and equal amounts of protein used to assay for CAT activities as described (see methods).

Figure 1A, shows significant CAT activity from the G1NECt IRES vector in comparison to the activity driven by the strong chimeric LTR present in LCtSN. Quantitation of CAT activity, determined in the linear range of the assay, indicated that G1NECt containing cells produce 72% of the LCtSN activity. To rule out the possibility that the EMC IRES was serving as a promoter element in the context of a retroviral vector, the construct G1NECt-R, with the EMC/CAT fusion in the reverse orientation, was produced and tested. No CAT activity was observed from the reverse orientation EMC/CAT vector. Analysis of the EMC deletion mutants indicate that efficient expression of the internal CAT gene is dependent on the presence of a full length IRES element. Deletion of 200 bp from the EMC IRES decreases activity of G1NECt-Δ200 to 4% of the full length EMC construct. The amount of CAT activity gradually increases as the EMC CAT start codon is brought closer to the NEO stop codon, with CAT activity increasing to 6% of control for G1NECt-Δ525, and 18% of control for G1NCt.

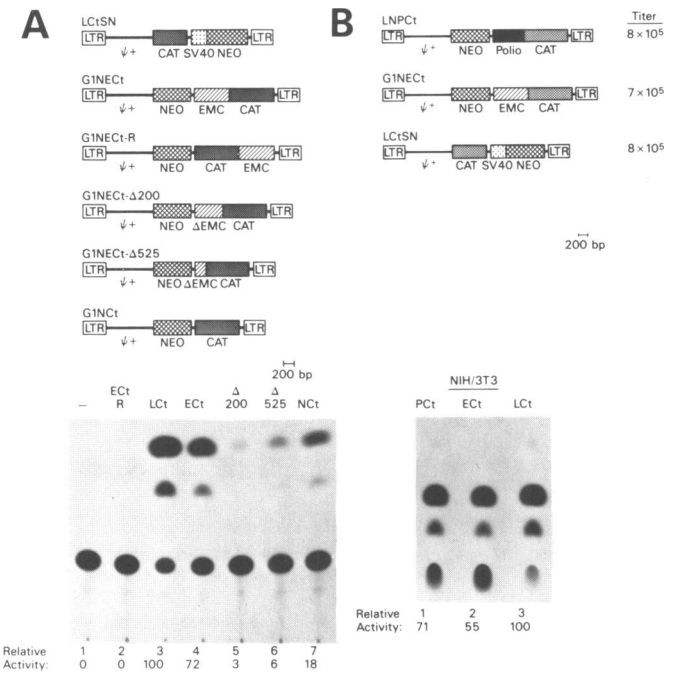

Figure 1. IRES CAT Vectors. Shown on the top of the figure are diagrams of the indicated IRES CAT vectors. Below is shown the autoradiograms from CAT enzyme analysis (1hr incubation). Panel A, lane 1, producer cells transfected with pOS6; lane 2, G1NECt-R; lane 3, LCtSN; lane 4, G1NECt; lane 5, G1NECt-Δ200; lane 6, G1NECt-Δ525; lane 7, G1NCt. In panel B, LNPCt, G1NECt, and LCtSN retroviral vector-containing supernatant (titer of producer cells populations are indicated to the right of each vector diagram) was used to transduce NIH/3T3 cells. After selection in G418 containing medium cell lysates were prepared for CAT assays; lane 1, LNPCt transduced NIH/3T3 cells; lane 2, G1NECt; lane 3, LCtSN. Relative activity was calculated from the mean of at least three independent assays (all samples within linear range) where the percent conversion of the LCtSN control was set to 100%.

In the next series of experiments, we isolated the IRES from poliovirus and used it to construct a retroviral vector. PCR was used to generate a fragment containing the IRES element from the 5′ untranslated region of poliovirus (Mahoney strain). The polio IRES was inserted 3′to the NEO stop codon and upstream of a CAT reporter gene to generate LNPCt. This vector along with the EMC IRES construct (G1NECt), and the positive control vector (LCtSN) were transfected into packaging cell cocultures. The cultures were grown for one week in standard medium and selected for vector containing cells by growth for two weeks in G418-containing culture medium. Completely selected cultures were used to harvest retroviral-vector-containing supernatant for NEO^R titer determination. The titer for all three vectors was similar when assayed on NIH/3T3 cells (approximately 7.5×10^5 $G418^R$ cfu/ml, fig 1B). Retroviral vector supernatant from the G1NECt LNPCt, and LCtSN producer cells was used to transduce NIH/3T3 cells. Following transduction, the cells were cultured for 10 days in the presence of G418. After selection, lysates were prepared for CAT assays. The representative CAT activity for each transduction is shown in fig 1B. The data indicate that both the G1NECt and LNPCt IRES vectors can produce functional retroviral vector particles that productively transfer and express an IRES/reporter gene in target cells (LNPCt 70%, and G1NECt 55% of LCtSN).

Construction of an EMC human ADA vector

To evaluate the use of IRES elements in the construction of retroviral vectors for potential human gene therapy applications, a fusion between the EMC IRES and the human adenosine deaminase (ADA) gene was assembled and introduced into a

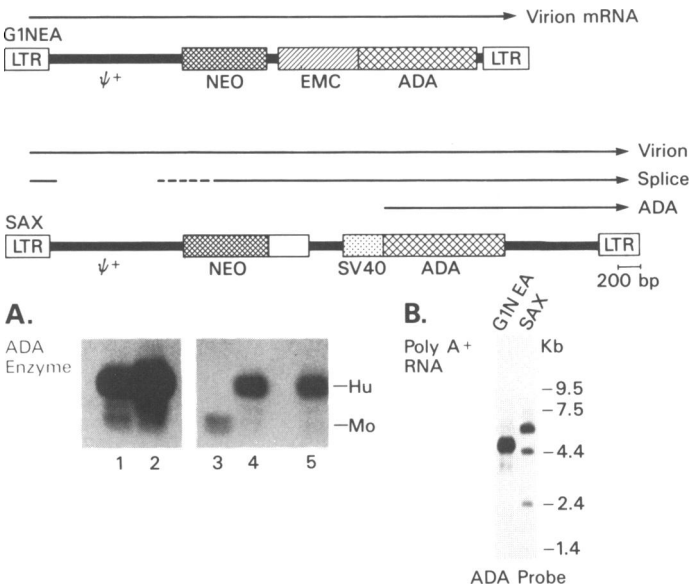

Figure 2. ADA IRES Vector. Shown on the top of the figure is a diagram of the EMC/ADA vector G1NEA, and the control ADA vector, SAX. Arrows indicate location of mRNA species, the exact location of the SAX splice acceptor site has not been mapped (dashed line). Panel A, starch gel analysis for ADA enzyme activity, equal amounts of total cell lysates were used for each sample, the location of the human (Hu) and mouse (Mo) ADA enzymes are indicated. Lane 1, SAX producer cells; lane 2, G1NEA producer cells; lane 3, NIH/3T3 cells; lane 4, SAX transduced 3T3 cells; and lane 5, G1NEA transduced 3T3 cells. Panel B, Northern blot analysis using 5μg of poly A+ mRNA hybridized with a human ADA probe. The origin of each sample is indicated on the top of the lanes.

retroviral vector. To do this, a DNA fragment containing the human ADA gene was synthesized, using PCR, and cloned into the pEMC-F plasmid to generate pEMCADA. The EMC/ADA fusion was excised from pEMCADA and inserted into the retroviral vector G1N yielding G1NEA (Fig. 2, top).

DNA for the G1NEA vector and the control ADA vector SAX were transfected into packaging cell line cocultures. The cocultures were grown for 1 week in standard culture medium, then selected for stable vector integration by culture for 2 weeks in the presence of the neomycin analog G418. The G418 selected producer cell populations were used to generate vector containing supernatant for titer determinations, and were subjected to gene expression analysis.

Figure 2, panel A shows the results of ADA starch gel analysis on the G1NEA producer cells (lane 2) and SAX control producer cells (lane 1). Both producer cell populations made large amounts of human ADA. Retroviral-vector-containing supernatant from the producer cell populations were then used to transduce NIH/3T3 cells and to determine the vector titer on 3T3 cells. Both producer cell populations yielded high titer vector supernatants with SAX being 1.9×10^6 $G418^R$cfu/ml and G1NEA being 1.2×10^6 $G418^R$cfu/ml. The $G418^R$ 3T3 cells were next assayed for ADA activity. ADA starch gel analysis demonstrated functional transfer of the human ADA gene into the 3T3 cells by the G1NEA IRES vector (Fig. 2, panel A, lane 5). Northern blot analysis (Fig. 2, panel B), was used to visualize the RNA transcripts from the two vectors in transduced 3T3 cells. For SAX, as previously reported (19), a full length LTR transcript as well as the internal SV40 transcript and a spliced subgenomic transcript were detected with the ADA probe. In the case of G1NEA, only one full length transcript is identified by Northern blot analysis with the ADA probe (a very small amount of what could be a spliced transcript may also be seen in RNA from the G1NEA cells).

Construction of triple gene vectors

To test the versatility of IRES elements in the construction of complex retroviral vectors, we inserted the EMC/ADA fusion gene into two different double-gene vectors to generate three-gene-containing vectors. The first recipient vector, LSCSN, uses the LTR to promote the expression of the anti-HIV agent soluble CD4 (sCD4) and an internal SV40 early region promoter to drive the NEO selectable marker gene (27). The EMC/ADA fragment was introduced after the sCD4 translation stop codon and 5′ to the start of the SV40 promoter to generate LSCEASN (Fig. 3). LSCEASN DNA was transfected into a packaging cell line coculture that was grown for one week before being passaged, at limiting dilution, into G418-containing medium. Twelve $G418^R$ producer cell clones were isolated and expanded for analysis. All 12 $G418^R$ producer cell clones synthesize both the human ADA enzyme and the sCD4 protein (fig. 3).

The second two-gene retroviral vector used as recipient for the EMC/ADA fragment was LNSCt, a vector that uses the LTR to drive NEO expression and has an internal SV40 promoter directing CAT expression. EMC/ADA was inserted 3′ to the NEO gene stop codon and upstream of the SV40 promoter to generate LNEASCt (Fig. 4). LNEASCt DNA was transfected into packaging cells, cultured for one week, and $G418^R$ producer cell clones were isolated by limiting dilution. Twelve producer cell clones were expanded and used to isolate vector containing supernatant to determine $G418^R$ titer, and analyzed for both CAT and ADA gene expression. Figure 4 shows that all twelve producer cell clones had both CAT (panel A) and human ADA (panel B) enzyme activity. The titer from the twelve clones showed a typical distribution, ranging from 4×10^4 $G418^R$cfu/ml for clone 10 to 4×10^6 $G418^R$cfu/ml for clone 4.

Retroviral vector-containing supernatant from LNEASCt producer cell clone 12 was used to transduce NIH/3T3 cells. Six $G418^R$ 3T3 cells clones were isolated by limiting dilution, and expanded for analysis. To verify the integrity of the integrated provirus, genomic DNA was isolated from each clone digested with restriction enzyme Sst I, and the digested DNA subjected to Southern blot analysis using a NEO probe (Fig 5, Southern). All six clones produced the predicted 5593 bp band with no apparent rearrangements or deletions (note, the images seen below the proviral band were the result of contamination of the intensifying screen with previous CAT assay samples). Next, RNA was isolated and subjected to formaldehyde gel

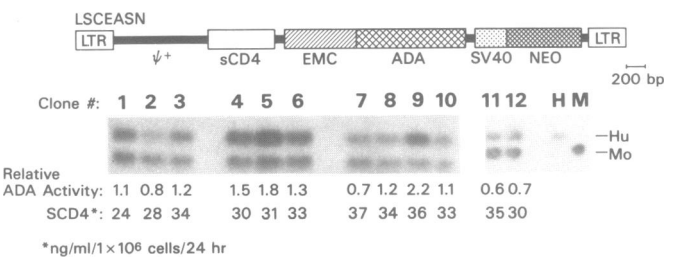

Figure 3. sCD4-ADA-NEO Triple-Gene Vector. Shown on the top of the figure is a diagram of the LSCEASN (sCD4, ADA, and NEO) triple-gene vector. Below is shown the results of ADA starch gel analysis and sCD4 ELISA from 12 $G418^R$ producer cell clones (numbers 1−12, H=human control, M=mouse control). Relative ADA activity is determined as the ratio of the intensity of the human to mouse ADA enzyme bands while the amounts of sCD4 produced in the culture medium was determined using standards supplied by the manufacturer.

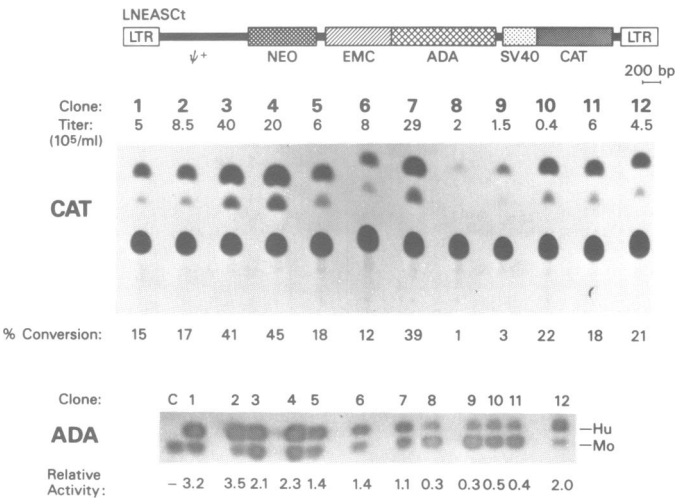

Figure 4. NEO-ADA-CAT Triple-Gene Vector. Shown on the top of the figure is a diagram of the LNEASCt (NEO, ADA, and CAT) triple-gene vector. Shown first below, autoradiogram of resultant CAT activity (1hr incubation, % conversion as indicated) from 12 $G418^R$ producer cell clones (numbers 1−12). The titer, measured on NIH/3T3 cells, of $G418^R$cfu/ml is indicated below the producer cell clone number. Lower panel, ADA starch gel analysis from the 12 producer cell clones (numbers 1−12), C = NIH/3T3 cells, human (Hu) and mouse Mo) ADA bands are indicated. Relative ADA activity is determined as the ratio of the intensity of the human to mouse ADA enzyme bands.

electrophoresis/Northern blot analysis with a ADA probe (fig 5, Northern). All six clones produced a single RNA transcript of the proper length using an ADA probe for detection. Enzyme assays were then performed to measure CAT and ADA with NEO being measured by an NPT II ELISA. ADA and CAT activity was documented in 6 of 6 3T3 clones and further, NPT II protein was also detected in all clones (Fig. 5, ADA, CAT, NPT II).

For the last series of vectors, we inserted the EMC/ADA gene fragment into the bicistronic LNPCt vector. The EMC/ADA fragment was inserted 5′ to the polio IRES to produce LNEAPCt or 3′ to the CAT gene to produce LNPCtEA (Fig. 6). Both DNA's were transfected into producer cell cocultures along with the control bicistronic vectors LNPCt and G1NEA. The cultures were grown for one week, then selected for vector-containing cells by growth in G418 containing medium for an additional 7 days. Retroviral-vector-containing supernatant from the $G418^R$ producer cells was collected and cells harvested for DNA and RNA isolation. The supernatant was used for titer determination on NIH/3T3 cells and to transduce a mink lung cell line.

The stability of the LNEAPCt and LNPCtEA vectors was analyzed by subjecting producer cell DNA to digestion with the Sst I restriction enzyme (Sst I cuts once in each LTR), followed by Southern blot analysis (Fig. 6, Southern). Each vector produced a band of the expected size of 5860bp, with no sign of rearranged species. Northern blot analysis, additionally revealed no significant species of RNA's other than those predicted to originate from a single LTR promoted transcript (Fig. 6, Northern). Analysis of vector-containing-supernatant on NIH/3T3 cells yielded titers of 1×10^5 $G418^R$ cfu/ml for LNPCtEA, 3×10^5 $G418^R$ cfu/ml for LNEAPCt, 6×10^5 $G418^R$ cfu/ml for LNPCt, and 8×10^5 $G418^R$ cfu/ml for G1NEA. The same supernatants were used to transduce mink lung cells that were then selected for vector-containing cells by growth in G418 containing medium. Mink cells were then harvested for ADA and CAT enzyme analysis (Fig. 6, ADA and CAT). The results of these assays indicate that each of the tricistronic vectors was fully functional, they each produced all three gene products, ADA, CAT, and were $G418^R$ (ie. NEO^R producing). For each enzyme tested, the bicistronic vector yielded more activity than

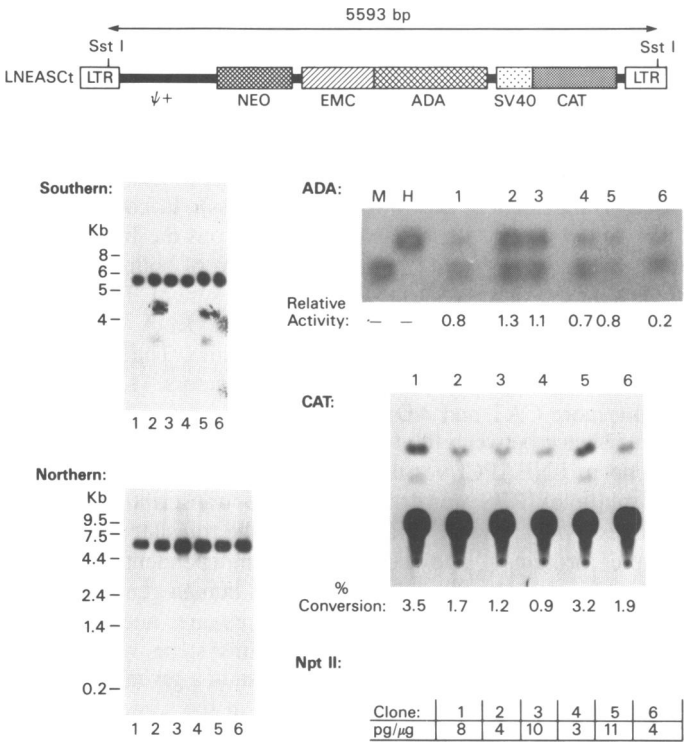

Figure 5. Analysis Triple-Gene Transduced Cells. Shown on the top of the figure is a diagram of the LNEASCt (NEO, ADA, and CAT) triple-gene vector. Southern, 20μg of genomic DNA from 6 independent NIH/3T3 cell clones was digested with Sst I and subjected to Southern blot analysis using a human ADA probe (note, the signals below the proviral band were the result of contamination of the intensifying screen with ^{14}C from a previous CAT assay). Northern, 20μg of total cell RNA from the 6 3T3 clones was subjected to Northern blot analysis using a human ADA probe. ADA, starch gel analysis of 6 3T3 cell clones, relative ADA activity is determined as the ratio of the intensity of the human to mouse ADA enzyme bands. CAT, CAT enzyme analysis (4hr incubation, % conversion as indicated) from 6 3T3 cell clones. Npt II, data from NPT II ELISA using cell lysates from 6 3T3 cell clones (expressed as pg NPT II/μg total protein). The numbering of each lane corresponds to the particular NIH/3T3 cell clone used.

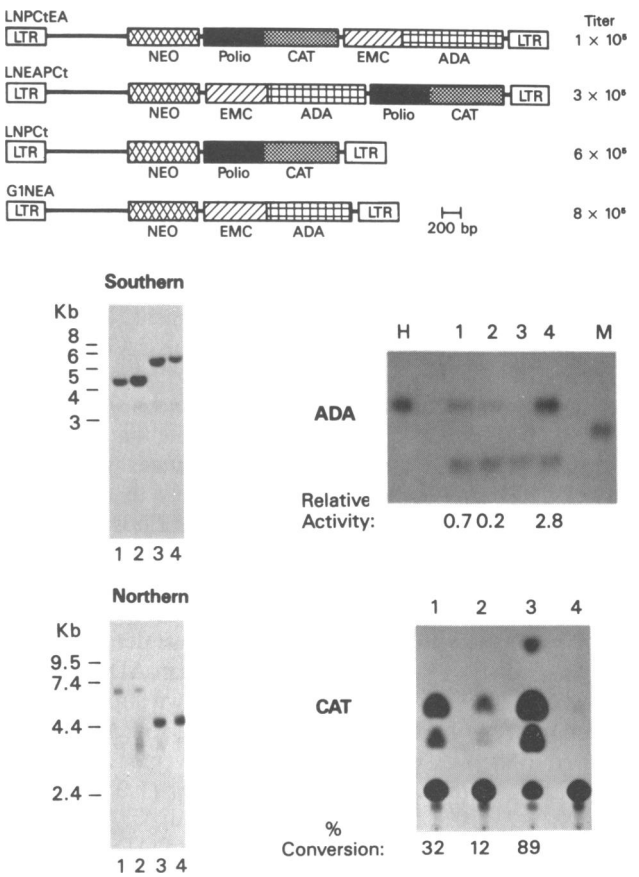

Figure 6. Analysis of Tricistronic Vectors. Shown on the top of the figure are diagrams of each of the individual vectors used along with the resultant titer of the producer cells, $G418^R$ cfu/ml measured on NIH/3T3 cells. Southern, 20μg of genomic DNA from producer cells LNPCt (lane 1), G1NEA, (lane 2), LNEAPCt (lane 3), and LNPCtEA (lane 4) was digested with Sst I and subject to Southern blot analysis using an NEO probe for detection. Northern, 20μg of total RNA from producer cells LNEAPCt (lane 1), LNPCtEA (lane 2), LNPCt (lane 3), and G1NEA (lane 4) was subjected to Northern blot analysis using a human ADA probe for transcript visualization. ADA, starch gel analysis of mink lung cells transduced with the following vectors; LNEAPCt (lane 1), LNPCtEA (lane 2), LNPCt (lane 3), and G1NEA (lane 4). Lane H is a human ADA control, lane M is a mouse cell control (the mink ADA enzyme migrates below the mouse control). Relative ADA activity is determined as the ratio of the intensity of the human to mink ADA enzyme bands. CAT, CAT enzyme assays (% conversion as indicated) from mink lung cells transduced with the following vectors; LNEAPCt (lane 1), LNPCtEA (lane 2), LNPCt (lane 3), and G1NEA (lane 4).

the corresponding tricistronic vector, with LNEAPCt yielding both greater CAT and ADA activity than LNPCtEA (see discussion).

DISCUSSION

We describe retroviral vectors in which the expression of an internal protein coding sequence is facilitated by the use of picornavirus-derived putative internal ribosome entry sites (IRES vectors). As test constructs, we assembled retroviral vectors in which the polio virus or EMC virus IRES was linked to the prokaryotic reporter gene CAT (fig. 1). Both of these constructs yielded functional retroviral vectors that were able to transfer productively the reporter genes to NIH/3T3 cells. The relative amount of reporter gene enzyme activity was approximately 50%−75% of that generated by direct transcription/translation using the strong promoter element in LCtSN (the retroviral LTR in LCtSN is derived from Harvey murine sarcoma virus and is 2−3×more active than the standard Moloney virus LTR used in the IRES vectors, unpublished observations).

To verify that the expression of the internal CAT gene was the result of putative cap-independent translation mediated by the IRES element, we constructed a series of control vectors (fig. 1). First, the opposite orientation vector, G1NECt-R, displays no CAT activity suggesting that EMC sequences are not functioning as general promoters. Second, deletion mutants indicate that removing 200bp from the 5′ end of the EMC IRES element results in near abolishment of CAT activity. Third, in the G1NCt vector, deletion of all of the IRES element, (leaving no interrupting start or stop codons), results in the CAT start codon being brought to within 20 bp of the NEO stop codon. This vector produces 18% of control CAT activity versus 72% for the complete EMC IRES. These data demonstrate that insertion of the full length IRES element greatly facilitates second gene translation. The mechanism for this is likely to be the same employed by picornaviruses, that is, internal ribosome binding and subsequent cap-independent translation.

The next construct produced was devised to mimic retroviral vector designs that are currently being used in a human gene therapy protocol to treat adenosine deaminase deficiency (28). Here, the EMC IRES was joined to the human ADA gene, and the fusion inserted into a retroviral vector down stream from the stop codon of the NEO gene (fig. 2). The G1NEA vector was compared to the well described SAX ADA vector and found to behave similarly to SAX with respect to titer (1.9×10^6 cfu/ml for SAX, 1.2×10^6 cfu/ml for G1NEA) and ADA expression (see fig. 2, panels A and B). The results of ADA starch gel analysis indicate that EMC mediated expression of the internal ADA gene is approximately equivalent to that generated in the SAX vector. Northern blot analysis failed to demonstrate significant amounts of subgenomic ADA transcripts suggesting that the IRES is facilitating internal translation initiation.

In the IRES vectors G1NECt, LNPCt, and G1NEA a putative bicistronic mRNA is produced by transcription initiating in the 5′ LTR promoter. The retroviral LTR is a strong promoter and has been shown to be more active than internal promoters in vitro (4,29) and simple LTR vectors have been successfully utilized in vivo (30−32). While retroviral splicing vectors can also achieve the translation of two gene products using the LTR promoter element, it has been difficult to predict the efficiency of splicing in these vectors and activation of cryptic splice donor sequences can result in deletion of vector sequences (4,33).

Many retroviral vectors have been constructed that express two independent genes using an internal promoter element to drive the expression of the second gene (2). IRES vectors may prove to be an improvement over standard two-promoter vectors. Because only one promoter is used in IRES vectors, these vectors should avoid the phenomenon of promoter suppression (5,6). Furthermore, reporter gene expression from an internal promoter can be unstable (in long term culture), with or without selection for the LTR driven gene (34). To date, the LTR promoted bicistronic IRES vectors have been stable in culture (eight week culture period, data not shown).

We extended the use of IRES elements in retroviral vector design to assemble three-gene retroviral vectors (figs. 3, 4, and 6). Triple-gene IRES vectors avoid the use of multiple promoter elements (again avoiding possible promoter suppression) and are compatible with the generation of high titer functional retroviral vectors. The construction of triple-promoter-containing retroviral vectors has been previously reported (35). In this report, significant differences in the levels of RNA transcripts were observed. The LTR, SV40, and HSV tk promoters used in this previous report, yielded relative RNA levels of 42:6:1 respectively. This observed disparity in RNA levels could be avoided in IRES vectors.

We assembled two types of three-gene-containing vectors by inserting the EMC/ADA fusion into either existing internal promoter vectors (Fig. 3 and 4), or into the bicistronic LNPCt vector (Fig. 6). All arrangements of genes and promoters in the various three-gene vectors were capable of generating high titer stable producer cells which can productively transduce target cells. Further analysis of the various three-gene-containing vectors will be required for quantitative comparisons to assess which vector arrangement is best (work in progress). In comparison of the reporter gene expression produced from the bicistronic versus tricistronic vectors, greater expression of both the CAT or ADA gene is observed using the bicistronic system. The nature of this observation (either differences in RNA stability, translatability or both) is not know at this time. The observed difference between the two tricistronic vectors (LNEAPCt yielding more CAT and ADA than LNPCtEA) may be related to the distance between the two IRES elements (approximately 1300 bp in LNEAPCt versus 800 bp in LNPCtEA).

In multiple IRES constructs, a single transcription event generates a polycistronic mRNA that can be translated to yield multiple proteins. The coupling of independent protein translations can have several advantages in human gene therapy situations where multiple protein subunits are needed (eg. immunoglobulins, or T-cell receptors) or in systems where two heterologous proteins may be more efficient at a given task (eg. by combining multiple anti-HIV proteins in the same vector). It may further be advantageous to have the production of a potentially physiologically dangerous protein be coupled to that of a conditional cell lethal protein (eg. tumor necrosis factor with herpes virus thymidine kinase). It is these types of applications where IRES vectors may find their greatest usefulness.

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