The Encephalomyocarditis Virus Internal Ribosome Entry Site Allows Efficient Coexpression of Two Genes from a Recombinant Provirus in Cultured Cells and in Embryos

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Rous sarcoma virus-based retroviral vectors were constructed to compare three different approaches for coexpressing two genes in individual infected cells. All vectors expressed the upstream gene (*lacZ*) from the Rous sarcoma virus long terminal repeat, while the downstream gene (the chloramphenicol acetyltransferase gene [*cat*] or *v-src*) was expressed in one of three ways: from a subgenomic mRNA generated by regulated splicing, from a strong internal promoter, or from the encephalomyocarditis virus internal ribosome entry site (IRES). Both biochemical and immunohistochemical assays of cultured cells showed that the encephalomyocarditis virus IRES provided the most efficient means for coexpressing two genes from a single provirus. Most importantly, most cells infected by a LacZ-IRES-CAT virus expressed both LacZ and CAT, whereas most cells infected by internal promoter or regulated splicing vectors expressed either LacZ or CAT but not both. In addition, viral titers were highest with IRES vectors. Presumably, use of the IRES avoids transcriptional controls and RNA processing steps that differentially affect expression of multiple genes from internal promoter and regulated splicing vectors. Finally, we injected a LacZ-IRES-*v-Src* virus into chicken embryos and then identified the progeny of infected cells with a histochemical stain for LacZ. LacZ-positive cells in both skin and mesenchyme displayed morphological abnormalities attributable to expression of *v-src*. Thus, IRES vectors can be used to coexpress a reporter gene and a bioactive gene in vivo.

When a retrovirus infects a cell, the viral genome is stably integrated into the host chromosome, efficiently expressed, and faithfully passed to the infected cell's progeny. For these reasons, recombinant retroviral vectors have often been used to express exogenous genes in vertebrate cells (reviewed in references 28, 34, and 52). In some of these cases, it is advantageous to express two exogenous genes from a single proviral genome. In strategies being developed for gene therapy, for example, the retrovirus often contains not only the gene of interest but also a selectable marker. The marker is used to facilitate the isolation of infected cells, which are then used as a source of the potentially therapeutic gene product (reviewed in reference 12).

In another set of studies, we and others have used vectors encoding the histochemical marker β -galactosidase, the product of the *Escherichia coli lacZ* gene, as lineage tracers in vivo. A single cell is infected by a retrovirus, the proviral genome is inherited by the cell's progeny, and the clonal relatives are identified with the histochemical stain for LacZ (11, 13, 16, 18, 45; reviewed in reference 17). To extend this work, we wished to construct an efficient double-expression vector to transfer both *lacZ* and a second gene to single cells in vivo. If *lacZ* and a second bioactive gene were reliably coexpressed at high levels, we could use LacZ histochemistry to identify small clones of transgenic cells in a wild-type environment and then seek cell autonomous effects of the second gene by analyzing the number, distribution, and morphology of the labeled cells.

In applications such as these, it is essential that the provirus express both genes within the same individual cells. Retroviruses have been successful in evolving strategies for

coexpressing their own genes. These strategies include synthesis and subsequent processing of fusion proteins, ribosome frameshifting, and regulated splicing to generate subgenomic messages. Unfortunately, achieving balanced expression of multiple exogenous genes from engineered retrovirus vectors has been more problematic. Two approaches have been used previously. The first involves the generation of separate mRNAs by regulated splicing of a single primary transcript expressed from the upstream long terminal repeat (LTR). This strategy mimics that used by retroviruses to generate the *env* gene product (54). With this approach, expression of one gene is always at the expense of the other, and the ratio of spliced to unspliced mRNA is highly dependent on the context (1, 2, 48, 49). The second approach involves expression of the upstream gene from the retrovirus promoter in the LTR and expression of the downstream gene from an internal promoter. This approach has been used most often in vectors designed for gene therapy (reviewed in references 28 and 34) but is compromised by competitive interference between promoters (4, 8, 20, 39). Thus, individual isolates may express one gene or the other, rather than both.

The recent demonstration that the 5' nontranslated region of encephalomyocarditis virus (EMCV) and other picornaviruses allows translation initiation from the internal ribosome entry site (IRES) (22–24) has suggested a third strategy for expressing multiple genes from a single proviral genome: the viral LTR can be used to express a single polycistronic transcript from which several gene products are translated. In this way, transcriptional controls and RNA processing steps that differentially affect expression of multiple genes can be avoided. In this study, we have used three pairs of genes to compare this approach with the double-promoter and regulated-splicing strategies. Biochemical and immuno-

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FIG. 1. Structures of retrovirus vectors used in this study. Notation: straight lines, RSV sequences; wavy lines, bacterial sequences; diagonal lines, truncated U3 region of RSV; squares, CMV immediate-early promoter; vertical lines, sequences of the EMCV 5' nontranslated region; spotted and dark stippled boxes, SV40 promoter and SV40 poly(A) signal, respectively; S.A., splice acceptor site; S.D., splice donor site; B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; N, *Nco*I; Nh, *Nhe*I; S, *Sal*I; X, *Xba*I; Xh, *Xho*I. Brackets indicate sites destroyed by ligation. The diagrams are roughly to scale.

histochemical assays indicate that the EMCV IRES is more effective than either the double-promoter or the regulated-splicing strategy at expressing two exogenous genes from a single proviral genome. Using an EMCV IRES vector that encodes LacZ and v-Src, we then infected chicken embryos in ovo. We show that LacZ-positive cells in the skin and meninges consistently express morphological abnormalities that are attributable to v-src.

MATERIALS AND METHODS

Plasmid constructions. DNA manipulations were done by standard methods (44). The main features of the plasmids described below are illustrated in Fig. 1.

All vector plasmids are derivatives of pLZ20, which is similar to the previously described pLZ10 (13) except for the addition within the plasmid of a Tn5 neomycin resistance gene (*neo*) flanked by a simian virus 40 (SV40) promoter and

poly(A) signal. Both pLZ10 and pLZ20 are derived from the SRA-2 molecular clone of the Rous sarcoma virus (RSV) genome (50) and produce LacZ as a freely soluble Gag-LacZ fusion protein. For each new vector, the *Xba*I-*Sal*I fragment of pLZ20 was replaced by a different fragment of interest. Plasmid pLZΔIC contains a truncated EMCV IRES extending from the *Eco*RI site at nucleotide (nt) 2334 to the *Hind*III site at nt 2566 of pE5LVP0 (40); nt 2334 of the plasmid is equivalent to nt 260 of the EMCV 5' nontranslated region. This fragment was linked to a 773-bp *Hind*III-*Bam*HI fragment of pSV2CAT which contains the bacterial chloramphenicol acetyltransferase (*cat*) gene (15). The IRES fragment in pLZIC1 extends from nt 2334 to the EMCV initiation codon at nt 2915, which was converted to a *Hind*III site (conversion from AAUAUGGCC to AAGCUUGCC extending from nt 2912 to 2920 of pE5LVP0) by site-directed mutagenesis (36). The oligonucleotide ACACGAATGATA AGCTTGCCACAACCA used for mutagenesis was obtained from the protein chemistry laboratory at Washington University. In pLZIC2, pLZIC3, pLZIS, and pLZIN, the EMCV sequence extends to an *Nco*I site 9 bp downstream of the EMCV initiation codon, which was altered in the cases of pLZIC2, pLZIS, and pLZIN. The *cat* gene in pLZIC2 and pLZIC3 has a 29-bp sequence of the 5' nontranslated *cat* region deleted and the initiation codon converted to an *Nco*I site. The *Xba*I-*Bgl*II fragment in pLZUC and pLZUS includes a truncated RSV U3 region, extending from nt -220 (an artificial *Bam*HI site) to -10 (a *Taq*I site), joined to a short linker sequence ATACCGTCCCAGATCT. To generate pLZUC, this fragment was linked to the *Hind*III end of the *cat* fragment by using a 56-bp *Sfi*I-*Hind*III filler fragment derived from the SV40 T-antigen leader sequence that was modified at the *Sfi*I end by addition of a *Bgl*II linker. pLZCC was derived from pLZIC1 by substitution of the *Xba*I-*Hind*III EMCV fragment with a 780-bp *Xba*I-*Hind*III fragment that harbors the enhancer/promoter region of a cytomegalovirus (CMV) immediate-early gene (3). The *v-src* splice acceptor site used in pLZSAC is derived from the region upstream of SRA *v-src* and was transferred as a *Bam*HI-*Nco*I fragment from a previously described construction (21). The *v-src* gene in pLZIS, pLZUS, and pLZSAS was derived from this same construction and was transferred as a *Bam*HI-*Eco*RI fragment into pLZUC and pLZSAC to make pLZUS and pLZSAS and as an *Nco*I-*Eco*RI fragment to make pLZIS. Finally, the *Nco*I-*Nhe*I fragment of pLZIN, which includes the *neo* gene, was generated by the polymerase chain reaction, using primers which converted the *neo* AUG to an *Nco*I site and introduced an *Nhe*I site immediately downstream of the translation termination site. Joining to the 3' LTR was effected by introduction of an *Nhe*I linker at the *Bam*HI site that separates the CAT sequences in pLZIC1 from the downstream viral sequences. The helper plasmid employed in these experiments used the CMV promoter fragment described above to promote expression of SRA viral sequences that extended from a *Sac*I site 120 bp 5' to the *gag* AUG (6) to an *Rsa*I site 6 bp 3' to the end of *env* (5). A polyadenylation signal was provided from the herpes simplex virus *tk* gene (32).

Transfections and virus recovery. These experiments used QT6 cells, a chemically transformed quail fibroblast cell line (37). Cells were grown at 37°C and 5% CO₂ in Earle's 199 medium supplemented with 5% tryptose phosphate buffer, 5% fetal bovine serum, 1% dimethyl sulfoxide, penicillin, and streptomycin. Cells were plated at 2 × 10⁶ cells per 60-mm dish 24 h prior to transfection and were fed fresh

medium 1 h before transfection. The cells were transfected by the calcium phosphate procedure (53) with one of the recombinant plasmids and the helper plasmid, which provided the viral genes necessary to produce viral particles. The calcium phosphate coprecipitates were formed by using 6 μg each of the recombinant and helper plasmids. Seven hours after the precipitate had been added, the cells were treated with dimethyl sulfoxide as described by Lopata et al. (31). The cells were then incubated for 40 h before the virus-containing media were harvested and cell extracts were made.

The harvested media were passed through a 0.45-μm-pore-size filter to remove intact cells, and Polybrene was added to a final concentration of 8 μg/ml to enhance the efficiency of infection. The virus-containing media were then added to fresh QT6 cells, which were grown for at least 48 h before expression of virus-encoded genes was assessed as described below.

Enzyme assays. Extracts of transfected cells were prepared by three cycles of freezing and thawing in 100 μl of 0.25 M Tris-HCl (pH 8.0), followed by centrifugation at 4°C for 5 min to remove cell debris. LacZ activity was measured by the method of Miller (35), with the amounts of extract and time of incubation adjusted so that the A₄₂₀ was between 0.2 and 1. CAT activity was measured by the method of Gorman et al. (15). Briefly, extract of transfected cells was added to a reaction mixture containing 37.5 μl of Tris-HCl (pH 7.5), 1 μl of 50-mg/ml chloramphenicol, and 0.1 μCi of [¹⁴C]chloramphenicol (60 mCi/mmol; New England Nuclear Corp.). The mixture was adjusted to a final volume of 135 μl and preincubated for 5 min at 37°C. The reaction was then started by adding 15 μl of 50 mM acetyl coenzyme A. The amount of extract was adjusted so that the percent conversion of the nonacetylated form to the acetylated form of chloramphenicol was between 10 and 50%. For CAT assays on infected cells, unlabeled chloramphenicol was omitted.

Histochemistry. For histochemical staining of cells expressing the LacZ gene product, cells were fixed in 2% formaldehyde in phosphate-buffered saline (PBS) for 10 min. The cells were then stained for LacZ at room temperature, overnight, in a mixture containing 1 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) per ml, 4 mM potassium ferricyanide, 4 mM potassium ferrocyanide, and 2 mM MgCl₂ in PBS (45). For immunohistochemical detection of LacZ and CAT, infected cells were seeded on coverslips 24 h prior to staining. The cells were fixed in 1% formaldehyde in PBS for 30 min and then incubated with antibodies. Primary antibodies were mouse monoclonal anti-β-galactosidase, prepared in our laboratories, rabbit anti-CAT (5 Prime → 3 Prime Inc., West Chester, Pa.), and rabbit antiserum to bacterially produced v-Src, generously provided by P. Maness, University of North Carolina (33). Secondary antibodies were (i) fluorescein-conjugated goat anti-mouse immunoglobulin G (IgG) plus IgM and (ii) rhodamine-conjugated goat anti-rabbit IgG (Boehringer Mannheim, Indianapolis, Ind.). For LacZ and CAT, each incubation was for 30 min at room temperature in PBS containing 5% goat serum and 0.01% Triton X-100. The *v-src* gene product was detected by incubating the cells overnight at 4°C with primary antibody, followed by a 30-min incubation with biotin-conjugated anti-rabbit IgG (Sigma, St. Louis, Mo.) and a 30-min incubation with Texas red-conjugated streptavidin (Bethesda Research Laboratories). Finally, coverslips were washed with PBS and mounted in 80% glycerol-*p*-phenylenediamine.

Infection of embryos. Methods for inoculation of embryos

and processing of tissue have been described previously (13, 16, 29). Briefly, fertilized White Leghorn eggs were obtained from SPAFAS (Roanoke, Ill.) and incubated at 37°C. Embryos were exposed through a window in the shell and staged by the criteria of Hamburger and Hamilton (19). Virus was concentrated from the medium of virus-producing QT6 cells by centrifugation, mixed with fast green and Polybrene, and drawn into a 10- to 30- μ m-tip glass capillary microelectrode. A total of 0.5 to 3 μ l of this mixture was pressure injected over the skin, subdermally, and into the lumen of the neural tube. No attempt was made to limit the injections to particular regions of the embryo. The shell was then closed with tape, and the egg was returned to the incubator.

At appropriate times thereafter, embryos were recovered, eviscerated, fixed for 1 h with 2% formaldehyde plus 0.4% glutaraldehyde in PBS, washed thoroughly in PBS, incubated overnight in X-Gal solution (see above), rinsed in PBS, and refixed in 2% formaldehyde–2% glutaraldehyde. The embryos were then examined under a dissecting microscope at $\times 20$ to $\times 40$. Small pieces of tissue bearing LacZ-positive cells were cut out and either mounted whole in 90% glycerol–10% PBS or dehydrated in ethanol, embedded in Araldite, and sectioned at 5 μ m.

RESULTS

In this study, we compared three different strategies for coexpressing *lacZ* and a downstream gene from RSV-based retrovirus vectors. The upstream gene was always fused in frame with the *gag* polypeptide and expressed from the LTR of the SRA-2 clone of RSV (50). The downstream gene was expressed in one of three ways: from a spliced subgenomic message, from an internal promoter, or from the EMCV IRES. To generate spliced mRNA from primary transcripts, we used the splice acceptor site of the RSV *v-src* gene (51). In these vectors, the RSV LTR promotes expression of primary transcripts, which are either used intact for packaging and for translation of the upstream gene or spliced to form a subgenomic RNA from which the downstream gene is translated. In cells infected with the wild-type RSV, about half of the primary transcripts are spliced to generate *env* and *v-src* subgenomic mRNAs, although the precise ratio of spliced to unspliced RNA varies among strains (49). For an internal promoter, we used a fragment of the U3 region of the RSV LTR (50). This fragment includes promoter and enhancer sequences but excludes both the inverted repeat sequence at the 5' end, which is required for integration, and the polyadenylation signal at the 3' end. In one vector, we also used the immediate-early promoter of CMV as an internal promoter. Finally, a fragment of EMCV that contains an IRES was used to promote internal ribosome binding. *lacZ* was paired with three genes for these tests: *cat*, because a simple and sensitive test is available for its products; *v-src*, because we expected it to alter cellular morphology in ways that would be detectable *in vivo*; and *neo*, because it is a selectable marker that can be used in studies of transcriptional activation.

Structure of the EMCV IRES fragment. Translation of EMCV mRNA occurs in a cap-independent fashion: ribosomes bind internally at the initiating AUG without scanning the 5' nontranslated region of the transcript. Sequences between nt 403 and 811 of the EMCV 5' nontranslated region are required for efficient translation. They include a stem-loop structure which interacts with a 57-kDa cellular protein and a stretch of pyrimidine-rich sequences near the initiation codon, both of which are essential for IRES function (24).

Synthesis of the EMCV polypeptide initiates at the 11th AUG codon, which is located in a perfect Kozak consensus sequence (27).

In earlier studies which used the IRES, the *cat* gene was expressed either from the EMCV initiation codon as a fusion protein (22) or from an engineered *Nco*I site placed at the EMCV initiation codon (7). We wanted to construct a vector in which foreign genes could be expressed either from their own initiation codons or from one in the vector. To do so, we took note that the 12th EMCV AUG, located 9 bp downstream of the EMCV initiation codon (the 11th AUG), is part of an *Nco*I site and is surrounded by a Kozak consensus sequence. To assess whether we could use that site as an initiation codon and to provide a site for insertion of genes with their own initiation codons, we converted the natural translation start site of the EMCV to a *Hind*III site. Thus, the sequence 5'-AAU AUG GCC ACA ACC AUG GAA-3' was mutated to 5'-AAG CUU GCC ACA ACC AUG GAA-3'. The efficiency of translation from the 12th AUG was tested by placing the *lacZ* gene at that site in the wild-type context and in the altered context and then measuring the levels of LacZ in cells transfected with the two plasmids. Translation is presumed to start at the 11th AUG in the wild-type context but must start at the 12th AUG in the altered vector. Nonetheless, levels of LacZ were roughly the same in both cases (data not shown).

LacZ-CAT vectors. Unselected coexpression of the bacterial *lacZ* and *cat* genes was tested by expressing the upstream *lacZ* gene from the RSV LTR as a Gag-LacZ fusion protein and expressing the downstream *cat* gene in each of several ways: from a spliced subgenomic message (pLZSAC), from an internal RSV promoter (pLZUC), from an internal CMV promoter (pLZCC), or from EMCV IRES fragments (Fig. 1). Because our primary aim was to test the efficacy of the EMCV IRES strategy, and because the distance between the pyrimidine stretch in the IRES and the initiation codon has been reported to be critical (24), we constructed four different IRES-containing vectors (pLZIC1, pLZIC2, pLZIC3, and pLZΔIC). In the case of pLZIC1, the *cat* gene from pSV2CAT (15) was inserted at the new *Hind*III site of the EMCV IRES fragment (see above). In this vector, a 29-bp sequence of the CAT 5' nontranslated region separates the CAT initiation codon from the 3' end of the EMCV IRES. To assess the effect of this separation on CAT expression, we also converted the CAT initiation codon to an *Nco*I site and joined it to the *Nco*I site of either the altered EMCV IRES (pLZIC2) or the wild-type EMCV IRES (pLZIC3). Finally, plasmid pLZΔIC was constructed as a control for internal initiation, with the *cat* gene placed at the upstream *Hind*III site of the EMCV fragment. The EMCV fragment was only 232 bp long in this case and did not include the entire IRES sequence.

QT6 cells were transfected with each plasmid to confirm the integrity of the two genes. Cell extracts were prepared 40 h after transfection, and CAT activity relative to LacZ activity was measured (Table 1). All of the vectors expressed LacZ, and all except pLZΔIC expressed CAT, but the ratios of CAT to LacZ varied among them. The relative levels of CAT expression were roughly twofold higher when the *cat* gene was placed directly at the 3' end of the EMCV IRES (pLZIC2 and -3) than when it was separated by a 29-bp untranslated fragment (pLZIC1) or expressed from the RSV promoter (pLZUC). The highest levels of CAT expression were obtained from the CMV promoter, which was previously shown to be more powerful than the RSV promoter in mammalian expression vectors (10). CAT expression from a

TABLE 1. Gene expression LacZ-CAT vectors

Vector	Relative CAT activity (%) ^a	
	In transfected cells	In infected cells
pLZΔIC	0.6	0.75
pLZIC1	49, 48	46, 62
pLZIC2	89, 84	75, 101
pLZUC	26, 58	68
pLZCC	274, 468	56, 74
pLZSAC	5, 6	4, 4

^a For each vector, the amount of CAT activity was determined per unit of LacZ activity in the same extract. These values were then expressed as percentages of the values from pLZIC3-transfected or -infected cells. Numbers from two independent experiments are represented. CAT activities in pLZΔIC-infected or -transfected cells were similar to those of mock-transfected or -infected cells.

spliced message was only 5 to 10% that of the IRES vectors. There was no CAT expression from the 232-bp EMCV fragment of pLZΔIC, which confirms the requirement of an IRES for internal initiation. Thus, the ratio of CAT to LacZ activities depended on the mechanism used to drive expression of CAT.

To test for virus particle production and for expression of the *lacZ* and *cat* genes from a proviral genome, virus particles were generated by cotransfection of QT6 cells with a helper plasmid and one of the retroviral plasmids. Media were collected 40 h after transfection and used to infect fresh QT6 cells. After another 48 h, the infected cells were assayed in three ways. First, we estimated the number of LacZ infectious units per microgram of transfected DNA (LacZ titers) by staining with X-Gal and counting the resulting blue cells. LacZ titers were consistently higher from the IRES vectors than from the other proviruses. For example, in five experiments in which pLZIC1 and the U3 internal promoter vector were directly compared, the titer of the double-promoter vector was $23\% \pm 4\%$ (mean $\pm$ standard error of the mean) that of the IRES vector. Thus, even if LacZ titers underestimate true titers of the double-promoter vector by twofold (see below), the IRES vectors still produce higher levels of viral particles. Similarly, in a single experiment, the LacZ titers of pLZSAC and pLZCC were found to be 35 and 40% that of pLZIC1, respectively. These results are consistent with the possibilities that negative interactions between promoters partially inhibit the production of genomic RNA from the LTR (8, 9) and that expression of spliced subgenomic mRNA is at the expense of full-length genomic RNA (see below).

Second, we estimated the amount of CAT expression relative to LacZ expression by preparing cell extracts from the infected cells and assaying them for the two enzymes. As for the transfectants, more CAT than LacZ was expressed from vLZIC2 and vLZIC3 (v denotes the proviral state) than from vLXIC1, vLZUC, or vLZSAC (Table 1). Surprisingly, the ratio of CAT to LacZ was severalfold lower for vLZCC than for pLZCC. This difference may reflect an effect of the integrative state of the provirus, although this was not observed in mammalian cells (10). For the other six vectors, however, the relative expression of the two genes was not greatly affected by the state of integration.

The biochemical assays of infected cells showed that the levels of CAT relative to LacZ from the double-promoter and IRES vectors varied less than twofold. They did not, however, address the issue of whether both genes are

reliably coexpressed within individual cells. To test for coexpression, we performed a third set of assays in which we doubly stained infected cultures with antibodies to LacZ and CAT. Cells from infected cultures were seeded on coverslips 40 h after infection and grown for another 24 h. The cells were then fixed and stained with rhodamine-tagged anti-CAT and fluorescein-tagged anti-LacZ. As shown in Fig. 2 and Table 2, the extent of coexpression varied greatly among vectors. More than 90% of the vLZIC1-, -2-, and -3-infected cells that were LacZ positive were also CAT positive. In contrast, in vLZUC-infected cultures, only 6% of infected cells were stained for both CAT and LacZ; two-thirds of the infected cells made detectable levels of only the upstream *lacZ* gene, and one-quarter expressed only the downstream *cat* gene. This result suggests that the two promoters in the vector interfered with each other. To determine whether the interfering effect of the two promoters occurred only when the two promoters in the vector were identical, we substituted the internal U3 fragment with a fragment harboring an immediate-early CMV promoter. In this case, expression of LacZ and CAT was also noncoordinate in most (60%) of the infected cells; the other 40% stained positively for both LacZ and CAT. There was little CAT staining detectable above background in the vLZSAC-infected cells and none in the vLZΔIC-infected cells. The low levels of CAT activity measured by enzymatic assays in cells infected with vLZSAC presumably limited detection by immunostaining. Thus, IRES vectors direct more reliable coexpression of LacZ and CAT than do double-promoter or regulated-splicing vectors.

LacZ-v-Src vectors. To test the general utility of the IRES approach for expressing two genes from a proviral genome, we constructed a second set of vectors in which the viral oncogene *v-src* was placed downstream of the *lacZ* gene (Fig. 1). The downstream *v-src* gene was expressed from a spliced subgenomic mRNA (pLZSAS), from an internal RSV promoter (pLZUS), or from the EMCV IRES fragment (pLZIS). In all vectors, LacZ was expressed as a Gag-LacZ fusion protein from the 5' LTR, and gene expression was estimated by visual inspection of immunohistochemically stained infected cells.

Virus particles were generated by cotransfection of QT6 cells with a helper plasmid and a LacZ-v-Src vector. Media were harvested 40 h after transfection and used to infect fresh QT6 cells. The vector pLZ20, which expresses LacZ alone, was used as a control. The infected cells were stained with X-Gal 48 h after infection to estimate LacZ titers. LacZ titers from four independent experiments indicated that the LacZ titers of pLZUS and pLZSAS were $8\% \pm 3\%$ and $16\% \pm 10\%$ that of pLZIS. This order is the same as that observed with the LacZ-CAT vectors and supports the idea that production of subgenomic RNA occurs at the expense of genomic RNA. The titers of vLZIS were 78, 125, and 169% those of vLZ20 in three experiments, indicating that insertion of the IRES does not interfere with virus production.

Coexpression in cells infected by LacZ-v-Src vectors was assessed by immunofluorescence using fluorescein-tagged anti-LacZ and Texas red-tagged anti-v-Src. Background staining by anti-v-Src was relatively high in all cultures, possibly because of cross-reaction of the antibody with endogenous c-Src. Nonetheless, specific staining attributable to v-Src was detectable, and it was evident that proviruses expressing v-Src from the EMCV IRES showed higher rates of coexpression than did the other two LacZ-v-Src proviruses (Fig. 3). Coexpression of LacZ and v-Src was

FIG. 2. Coexpression of LacZ and CAT from proviral genomes. Cultures of QT6 cells that had been infected with LacZ-CAT vectors were doubly stained with antibodies to LacZ and to CAT as described in Materials and Methods. Fields were then photographed with fluorescein optics to show LacZ (a, c, e, g, and i) and with rhodamine optics to show CAT (b, d, f, h, and j). Vectors were the IRES vector, pLZIC1 (a and b); the internal U3 promoter vector, pLZUC (c and d); the internal CMV promoter, pLZCC (e and f); the regulated splicing vector, pLZSAC (g and h); and the truncated IRES vector, pLZ Δ IC (i and j). LacZ and CAT are reliably coexpressed only in pLZIC1-infected cells. Arrows mark corresponding points on the two micrographs of each pair. In all cultures, <5% of the cells were antibody stained; unstained cells are only barely visible in these micrographs. The bar represents 50 μ m.

TABLE 2. Coexpression of LacZ and CAT in QT6 cells infected with LacZ-CAT vectors^a

Vector	% of infected cells expressing:		
	CAT only	LacZ only	LacZ and CAT
pLZΔIC	0	100	0
pLZIC1	3	0	97
pLZIC2	7	3	90
pLZIC3	2	2	96
pLZUC	25	69	6
pLZCC	34	26	40
pLZSAC	3	90	7

^a Cells were doubly stained for CAT and LacZ as described in Materials and Methods. In each case, 200 stained cells were counted from randomly chosen fields.

detected in more than 90% of the vLZIS-infected cells but in less than 10% of the vLZUS- and vLZSAS-infected cells. Most of the vLZUS-infected cells that expressed LacZ stained weakly if at all above the background level with the v-Src antibody, and most of the v-Src-positive cells were negative for LacZ expression. In the case of vLZSAS-infected cells, there were roughly threefold more LacZ-positive than v-Src-positive cells, although again weak expression of v-Src might have been obscured by the endogenous immunoreactive material. Thus, LacZ and v-Src were more reliably coexpressed from the IRES vector than from the double-promoter or regulated-splicing vectors.

In examining the immunostained cultures, a second indication of v-Src expression became apparent: most v-Src-positive cells were rounded and refractile, as has been noted previously with fibroblasts infected or transfected with v-Src expression vectors (30). In vLZIS-infected cultures, most of the LacZ-positive cells detected immunohistochemically dis-

FIG. 3. Coexpression of LacZ and v-Src from proviral genomes. Cultures of QT6 cells that had been infected with LacZ-v-Src vectors were doubly stained with antibodies to LacZ and to v-Src as described in Materials and Methods. Fields were then photographed with fluorescein optics to show LacZ (a, c, e, and g) and with rhodamine to show v-Src (b, d, f, and h). Vectors were the IRES vector, pLZIS (a and b); the internal U3 promoter vector, pLZUS (c and d); the regulated-splicing vector, pLZSAS (e and f); and the LacZ-only vector, pLZ20 (g and h). LacZ and v-Src are reliably coexpressed only from pLZIS-infected cells. Arrows mark corresponding points on the two micrographs of each pair. The bar represents 50 μ m.

played this altered phenotype, whereas most LacZ-positive cells were morphologically normal in vLZUS- and vLZSAS-infected cultures. This result supports the conclusion that vLZIS is the best of the three vectors tested at coexpressing LacZ and v-Src.

LacZ-Neo^r vector. As a third test of the IRES strategy, we constructed two vectors in which the *lacZ* and *neo* genes were separated by the EMCV IRES. In the first, the *lacZ* gene was expressed from the 5' LTR as a Gag-LacZ fusion protein and the *neo* gene was placed downstream of the IRES sequence at the *NcoI* site (Fig. 1). In the second, the *neo* gene was expressed from the LTR as a Gag-Neo^r fusion protein and the *lacZ* gene was placed downstream of the IRES at the *NcoI* site. Virus was produced from both vectors and used to infect QT6 cells. Neo^r cells were selected by growth in G418 and stained for LacZ. More than 90% of the Neo^r cells were LacZ positive after infection with either vector. Thus, the IRES can be used to express LacZ as a downstream gene and to reliably coexpress a reporter gene and a selectable marker.

LacZ-v-Src expression in ovo. Because LacZ and v-Src were efficiently coexpressed from pLZIS in vitro, this vector was selected for tests in ovo. Virus concentrate, derived either from pLZIS or from the LacZ control vector, pLZ10, was injected into embryos at 55 to 70 h of incubation (Hamburger and Hamilton stages 13 to 17). The embryos were recovered, fixed, and stained for LacZ 3 to 6 days after injection (stages 30 to 34 are equivalent to embryonic days 6 to 8 [E6 to E8]).

In initial experiments, we analyzed LacZ-positive cells in the skin because it is simple, thin, and easily examined in whole mount. Clusters of closely spaced LacZ-positive cells were assumed to be clones on the basis of statistical and double-labeled studies that have been detailed elsewhere (13, 45). In vLZ10 (LacZ only)-injected embryos, typical clones were composed of flat, polygonal cells with large central nuclei and relatively scant cytoplasm (Fig. 4a and b); the average number of cells per clone was about 40 at stages 30 to 34. Plastic sections through such clones revealed that the marked cells were well integrated into the epidermis and identified them as typical cells of the basal and peridermal layers (Fig. 4c and d; see Fig. 7 in reference 45 for similar studies of mouse skin). In contrast, most cells in vLZIS (LacZ-v-Src)-marked clones were markedly abnormal: they were large and irregularly shaped and contained multiple LacZ-negative inclusions that might have been vacuoles or supernumerary nuclei generated by disordered cytokinesis (Fig. 4e and f). Sections through these clones showed that the LacZ-positive cells were poorly integrated into the epidermis (Fig. 4g) and frequently seemed to be delaminating from its apical surface (Fig. 4h). In addition the number of cells per clone was greatly reduced when v-Src was present; the average number of cells per vLZIS-marked clone, counting each potential syncytial aggregate as a single cell, was around five at the stages examined. Finally, it is important to note that the incidence of such abnormalities was high: in one set of vLZIS-injected embryos analyzed at stage 30, 14 of 15 clones of skin cells were markedly aberrant. In embryos analyzed at stage 34, about 25% of the clones contained groups of normal-appearing cells, either in isolation or intermingled with abnormal cells, suggesting that expression of v-Src may be reduced or rendered less toxic at later developmental stages (data not shown). Nonetheless, more than 90% of the clones analyzed at this stage contained one or more clearly aberrant cells. Thus, at least some progeny of most LacZ-positive vLZIS-marked epidermal

cells infected in ovo expressed sufficient levels of v-Src to perturb normal developmental processes.

In addition to skin, we dissected and examined the central nervous system of vLZIS-injected embryos. Only a few clones of LacZ-positive cells were found within the brain or spinal cord, and these will be described elsewhere. In roughly 50% of the embryos, however, we found aggregates of LacZ-positive cells in association with the meninges that ensheath the brain and spinal cord and with the loose mesenchyme that overlies the meninges. Examples from the lumbar segments of the spinal cord and from the optic tectum of the mesencephalon are shown in Fig. 5. In both areas, the LacZ-positive cells were heterogeneous in size and shape but were not grossly abnormal in morphology. Most striking, however, was the large size of the clones: each cluster contained hundreds to thousands of LacZ-positive cells. No comparably large clones of meningeal or mesenchymal cells were seen in vLZ10-injected embryos (data not shown), nor have we seen such clones in previous studies of cell lineage in optic tectum (13, 16, 18) or spinal cord (29). From these results, we conclude that vLZIS promotes coexpression of LacZ and v-Src in mesenchymal tissue as well as in skin.

DISCUSSION

The aim of this work was to construct a recombinant retroviral vector which would allow efficient coexpression of two genes in single infected cells. We have shown that use of the EMCV IRES to produce a bicistronic message provides a more efficient means of expressing two genes within individual cells than does use of two promoters or a regulated-splicing mechanism.

Retroviral vectors containing internal promoters are often compromised by the problem of promoter interference, which is dependent on the strength of the promoter and the context in which it is placed. Cullen et al. (4) showed that expression of the preproinsulin gene from the 3' LTR of the avian leukosis virus occurred only when the upstream LTR was inactivated. This phenomenon was called transcriptional interference and is reminiscent of a more natural case observed in avian leukosis virus-induced bursal tumors (38, 41). Such tumors have been associated with insertion of the virus upstream of *c-myc*. In most of these tumors, the provirus was found to bear mutations near its 5' end that were correlated with expression of *c-myc* from the 3' LTR (14). Independently, Emmerman and Temin (8, 9) showed that in a double-promoter spleen necrosis virus vector, one gene is usually suppressed when there is selection for the other, regardless of the position of the genes relative to each other. This suppression was found to be epigenetic, reversible, and *cis* acting. It was attributed to a change in chromatin conformation near the active promoter, which in turn inhibited transcription from nearby promoters. More recently, Hoebe et al. (20) have suggested that suppression is associated with methylation and is dependent on the site of integration of the virus. This, however, was not observed by Emmerman and Temin (8).

Different problems occur when splice acceptor sites are used to construct double-expression vectors. If the aim were to produce maximal levels of both gene products, optimal expression would be reached when half of the RNA is spliced. Thus, each gene would be expressed at only 50% of the level expected if no splicing occurred. In addition, the amount of genomic RNA available for packaging, and potentially the viral titer, would be reduced by half. Finally,

FIG. 4. Clones of LacZ-positive cells in the skin of chicken embryos that had been injected with vLZ10 (LacZ only; a to d) or vLZIS (LacZ-IRES-v-Src; e to h). Embryos were injected on E3 and examined on E6 to E8. (a, b, e, and f) Whole mounts, photographed with bright field optics; (c, d, g, and h) plastic sections photographed with Nomarski optics. Aberrant morphology of LacZ-positive, vLZIS-marked cells demonstrates that these cells express v-Src. The bar represents 50 μ m.

regulation of alternative splicing is not well understood in any system, and it is already clear that splicing efficiency in RSV is context dependent and that discrete intronic sequences are responsible for the regulation of splicing (1, 2, 25, 48, 49). Thus, it is not currently possible to engineer vectors that produce spliced and unspliced RNAs in predict-

able ratios. In addition, for RSV the translational start site for *gag* is retained in the spliced message and could interfere with translation from the AUG of the downstream gene.

To obviate the problems encountered with the double-expression vectors that use internal promoters or regulated splicing, we tested the EMCV IRES. In the vectors that we

FIG. 5. Clones of LacZ-positive cells in the spinal (a to c) and tectal (d to f) meninges of chicken embryos that were injected with vLZIS on E3 and examined on E8. (a) Large clone of cells surrounding and extending laterally from the spinal cord. (b) Photomontage of a section through vertebral column shows that LacZ-positive cells ensheath but do not penetrate the spinal cord (SC) and extend into mesenchyme surrounding vertebral bodies (V) and dorsal root ganglia (D). Groups of LacZ-positive mesenchymal cells more distant from the cord were also present in this clone but are not visible in this field. (c) At higher power and with Nomarski optics, some of the perispinal cells are seen to be associated with the meninges (M) external to a fiber tract in the spinal cord. (d) Clone of LacZ-positive cells on the surface of the optic tectum. (e) At higher power in whole mount, the cells seem to be disordered, heterogeneous in shape, and numerous. (f) Section photographed with Nomarski optics showing LacZ-positive meningeal cells external to the apical surface (arrowheads) of the tectum. The bar represents 1 mm in panel a, 125 μ m in panel b, and 50 μ m in panel c, e, and f, and 0.5 mm in panel d.

constructed, both genes were expressed from the 5' LTR of RSV and translation of the downstream gene in the dicistronic message occurred by ribosomes binding internally to the IRES. The efficiency of translation of the second cistron

in such dicistronic mRNAs has been shown to be similar to that of the corresponding gene in a monocistronic message, indicating that interference does not occur at the translational level (23). Several lines of evidence presented here

indicate that the IRES provides the best available method for coexpressing two genes from a retroviral vector. (i) In a population of infected cells grown under no selection, more than 90% of cells infected with an IRES vector coexpressed LacZ and CAT, whereas most cells infected with an internal promoter vector expressed either one gene or the other. (ii) Similarly, coexpression of LacZ and v-Src in infected QT6 cells was also substantially more efficient from the IRES vector than from either an internal promoter or a regulated splicing vector. (iii) The IRES vector permits reliable coexpression of Neo^r and LacZ (>90%) in either order (Neo^r-IRES-LacZ or LacZ-IRES-Neo^r). (iv) The levels of expression of LacZ, CAT, and v-Src determined either by enzymatic assays or by visual inspection of immunostained cells were high. (v) For both LacZ-CAT and LacZ-v-Src vectors, LacZ titers were consistently higher with the IRES vectors. Together, these results provide strong evidence that the EMCV IRES is more efficient than either of the previously used approaches at coexpressing two genes from a recombinant provirus.

We chose the LacZ-IRES-v-Src vector (pLZIS) for injection into embryos because previous studies *in vivo* and *in vitro* (26, 30, 42, 46, 47), as well as our observations on cultured cells (Fig. 3), suggested that expression of v-Src would be likely to affect cellular morphology. Indeed, in both skin and meninges, vLZIS-marked cells were consistently different from vLZ10-marked cells in number or size, indicating that most LacZ-positive cells also expressed v-Src. In addition, although our aim was not to study the biology of v-Src, these experiments provide new data on the effects of v-Src on cells in complex tissue. First, expression of v-Src appears to block the normal proliferation of epidermal cells. This inhibition might result either from a general toxic effect of the oncogene or from a more specific interference with endogenous mechanisms of growth control; we cannot distinguish between these alternatives. Our results are somewhat different from those of Stoker et al. (46), who saw disturbances of growth control in only one of several clones of epidermal cells marked by a LacZ-v-Src vector. One possible explanation for the difference is that Stoker et al. used a regulated splicing vector in which many LacZ-positive cells (about 40%) did not express detectable levels of v-Src, and other cells may have expressed lower levels than occur in vLZIS-infected cells. In addition, whereas we analyzed clones at E6 to E8, Stoker et al. did not examine tissue until E12 to E14, by which time v-src-poisoned cells might have died and only unaffected cells survived. In contrast, expression of v-Src appears to cause loss of proliferative control in the mesenchyme and meninges. This result is consistent with the known ability of v-src to transform mesenchymal cells (e.g., fibroblasts) *in vitro* and to induce fibrosarcomas *in vivo* (42). The large clones of mesenchymal and meningeal cells that we observed might have eventually formed tumors, as has been observed previously following infection with replication-defective v-src-containing virus (47); however, we confined our observations to short times (3 to 6 days) after infection.

IRES-based retroviral vectors of the sort described in this report may be useful for a variety of purposes. (i) Vectors encoding the LacZ marker and a second bioactive gene can be used to generate small clones of transgenic cells in a wild-type environment. As shown here for vLZIS, the transgenic cells can be identified histochemically, and the effects of the second gene can be assessed by analyzing the phenotype of the labeled cells. (ii) The LacZ-Neo^r vector can be used in transcription activation studies: mutations are

introduced in a promoter, Neo^r clones are isolated, and the effect of the mutation on promoter activity is measured by LacZ assays (13a). (iii) Similarly, if vectors for gene therapy were constructed by linking *neo* and the gene of interest by an IRES, selection for G418 resistance should ensure expression of the second gene. Finally, it is important to note that use of the IRES strategy described here is not limited to retroviral vectors. For example, transgenic mice expressing a marker gene (e.g., *lacZ*) and a bioactive gene from a single promoter would allow direct correlation of patterns of transgenic expression with patterns of transgenic effect.

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