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Construction of a Bifunctional mRNA in the Mouse by Using the Internal Ribosomal Entry Site of the Encephalomyocarditis Virus

D. G. KIM, H. M. KANG, S. K. JANG, AND H.-S. SHIN*

Department of Life Science, POSTECH, Mt 31, Hyoja-Dong, Pohang, 790-330 Republic of Korea, * and Whitehead Institute, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139*

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Picornaviral mRNAs have been shown to possess special structures in their 5′ nontranslated regions (5′NTRs) that provide sites for internal binding of ribosomes and thus direct cap-independent translation. The translational *cis*-acting elements for ribosomal internal entry into the 5′NTR of encephalomyocarditis virus (EMCV), a member of family *Picornaviridae*, have been named the internal ribosomal entry site (IRES). All of the published experiments regarding the IRES function of the picornavirus 5′NTR, however, were performed with cell extracts in vitro or with tissue culture cells in transient assay systems. In this study, we examined the IRES function of the EMCV 5′NTR in chimeric mouse embryos and demonstrated that this element does in fact work stably in mouse embryos as well as in embryonic stem (ES) cells. By using a dicistronic vector, pWH8, consisting of a promoter-driven neomycin resistance gene (*neo*) followed by the EMCV 5′NTR-*lacZ* sequence, we showed that more than half of the ES cells made G418 resistant by the vector stained positive for β-galactosidase (β-gal). On Northern (RNA) blots, all of the clones analyzed revealed a transcript of the expected size containing both the β-gal and the *neo* cistrons. These results indicate that dicistronic mRNAs are produced from the stably integrated vector in those ES clones and that both of the cistrons are translated to produce functional proteins. The chimeric embryos derived from these ES clones also stained positive for β-gal, suggesting that the bifunctional mRNAs are active in the embryos. This dicistronic vector system provides a novel tool by which to obtain temporally and spatially coordinated expression of two different genes driven by a single promoter in a single cell in mice.

The mechanism of initiation of protein synthesis directed by the eukaryotic ribosomes has been best explained by the ribosomal scanning model (12): a ribosomal subunit enters the mRNAs near the capped 5′ terminus, most likely with the help of cap-binding protein complex eIF-4F (29), and then scans the 5′ nontranslated region (5′NTR) of the mRNA until it encounters a proper AUG triplet with a good nucleotide context (RxxAUGR; R = purine, and x = any nucleotide; see reference 13). The first AUG triplet is usually used as the initiator codon in this process in most mRNAs, but occasionally the second or another downstream AUG is used as the initiator codon when the first AUG does not possess a proper nucleotide context (13). This hypothesis gives a reason for the major difference in translation between pro- and eukaryotic mRNAs: the former mRNAs can be polycistronic with multiple internal ribosomal entry sites for initiation of translation, whereas the latter mRNAs are monocistronic with the requirement of the 5′ termini and the cap structure. A few exceptions to the monocistronic rule of eukaryotic mRNA have been reported. For example, small open reading frames preceding the initiating AUG of the GCN4 gene play a key role in controlling the translational efficiency of the main open reading frame (19). The growth-differentiation factor 1 (GDF-1) gene is transcribed as either a monocistronic or a dicistronic mRNA, in which GDF-1 is at the second cistron, depending on the differentiation stages of a mammalian embryo (14). The translational mechanism of the downstream cistron of the GCN4 mRNA was suggested to follow the translational reinitiation model instead of internal ribosomal entry (21), but that of GDF-1 remains obscure.

Several lines of evidence suggest that not all eukaryotic mRNAs comply with the scanning mode of translation. Picornaviral mRNAs have been shown to possess special structures in their 5′NTRs that provide sites for internal binding of ribosomes and thus direct cap-independent translation. The first direct evidence of internal ribosomal entry into picornaviral mRNAs came from translation of artificial di- or tricistronic mRNAs in in vitro and in vivo assay systems. Major progress in illuminating the mechanism of the curious ribosomal internal entry phenomena was achieved by investigating the structure and function of the 5′NTRs of poliovirus and encephalomyocarditis virus (EMCV). When the 5′NTR of poliovirus or EMCV was positioned in intercistronic spaces of multicistronic mRNAs, it directed efficient translation of the immediate downstream gene independently of the translational level of the preceding genes (9, 22). The internal ribosomal entry model was strengthened by the fact that translation of heterologous indicator genes preceded by the EMCV or poliovirus 5′NTR continued even after poliovirus infection of the host cells, in which translation of most of the cap-dependent cellular mRNAs was inhibited by cleavage of an essential component of eIF-4F (8, 22). A known exception to this is BiP mRNA, which has been reported to escape translational inhibition in poliovirus-infected cells and use cap-independent translation mechanisms (15, 26). The translational *cis*-acting elements for ribosomal internal entry into the picornaviral 5′NTRs have been identified, and the sequences responsible for internal ribosomal entry function in the poliovirus and EMCV 5′NTRs have been named the ribosomal landing pad

* Corresponding author.

and the internal ribosomal entry site (IRES), respectively (8, 9, 11, 22, 31). Cellular factors that specifically interact with the IRES have also been identified (11, 16; reviewed in reference 10).

On the basis of the cap-independent translation mechanism of picornaviral IRESs, several mammalian expression vectors have been constructed by using the picornaviral 5'NTRs. Elroy-Stein et al. (5) and Zhou et al. (33) have developed efficient mammalian expression vectors by using the EMCV 5'NTR and heterologous protein-coding sequences under the control of a bacteriophage T7 or T3 promoter and by supplying T7 or T3 RNA polymerase in *trans*. However, all of the experiments published to date were performed with cell extracts in vitro or tissue culture cells in transient assay systems. In this study, we examined the IRES function of the EMCV 5'NTR in chimeric mouse embryos and demonstrated that this element does in fact work stably in embryos derived from embryonic stem (ES) cells transfected with dicistronic vectors. This element allowed construction of a bifunctional mRNA which can be used to obtain coordinated expression of two different genes in a single cell.

MATERIALS AND METHODS

Abbreviations. The following abbreviations are used in this report: β-gal, β-galactosidase; CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's modified Eagle medium; EF, embryonic fibroblast; PGK-1, phosphoglucokinase-1; SDS, sodium dodecyl sulfate; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

Vectors. The promoterless vector pBS-ECAT has been previously described (8). It consists of an EMCV 5'NTR-*cat*-simian virus 40 poly(A) addition signal cloned into the pBS(+) vector. Dicistronic CAT vector pWH5 was constructed as follow. The 2-kb fragment containing the EMCV 5'NTR-*cat* sequence was first isolated from pBS-ECAT after *Hpa*I and partial *Eco*RI digestions, subcloned into the *Eco*RI-*Sma*I site of pKS(+), and then isolated again by cutting the resulting plasmid with *Eco*RV and *Bam*HI. This fragment, after Klenow treatment, was then inserted into the Klenow-treated *Bam*HI site of vector pKJ-1 (1), yielding pWH5 (Fig. 1A). Vector pKJ-1 (gift from Mike McBurney, University of Ottawa, Ottawa, Ontario, Canada) consists of the neomycin resistance (*neo*) gene flanked by the PGK-1 promoter and the poly(A) addition signal sequence of PGK-1 (1). The *Bam*HI site is located immediately downstream of the stop codon of the *neo* gene so that the expected transcript from this vector would contain two cistrons, *neo* and *cat*, intercalated by the 5'NTR sequence.

Dicistronic β-gal vector pWH8 was generated as follows. pE5-LVPO (20) was cut with *Bss*HII and treated with mung bean nuclease to create blunted ends. The 0.6-kb 5'NTR fragment was then isolated by *Eco*RI digestion and inserted into the *Eco*RI-*Sma*I site of pBluescript KS(+). By sequencing the *Bss*HII-*Sma*I junction region, we selected a plasmid that deleted 3 bp from the 5'NTR sequence. Into the *Bam*HI site of this plasmid, the *lacZ* gene, the 3-kb *Bam*HI fragment of pMC1871 (Pharmacia), was inserted. In this configuration, the initiation sequence for translation of *lacZ* is provided by the 5'NTR and yields a fusion β-gal protein including eight amino acids which originate from the EMCV polyprotein (Fig. 1B). The EMCV 5'NTR-*lacZ* unit was isolated from the plasmid by cutting with *Sal*I and *Spe*I, blunt ended by Klenow treatment, and then inserted into the blunt-ended *Bam*HI site of vector pKJ-1. As was the case with pWH5, the expected transcript from this vector would contain two cistrons intercalated by the 5'NTR sequence (Fig. 1A).

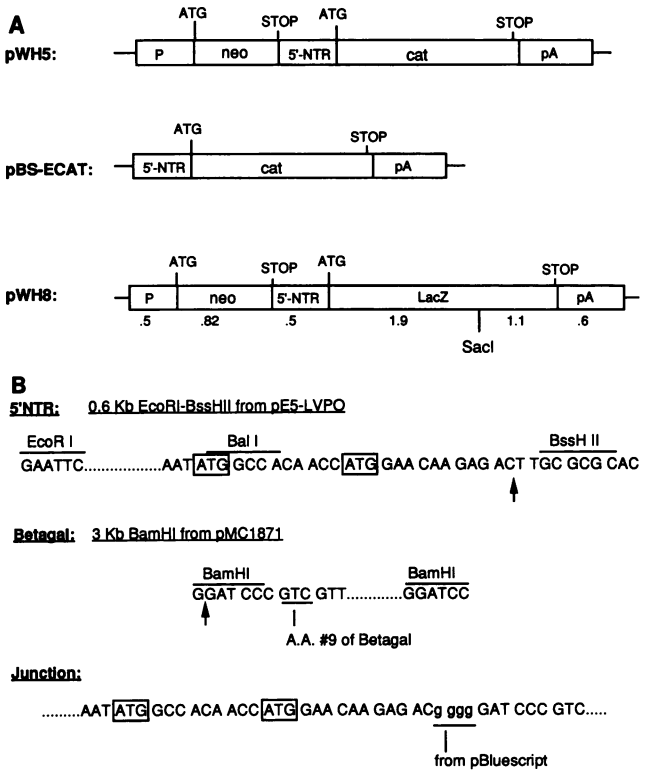


FIG. 1. (A) Schematic diagram of the vectors used for this study. The numbers below the pWH8 diagram indicate the size of each fragment in kilobases. (B) Sequence of the 5'NTR–β-gal fusion region. The arrows indicate the positions of the fusions at the 3' end of the 5'NTR and the 5' end of the β-gal gene in the fusion construct. See Materials and Methods for details. A.A., amino acid.

Cells. J-1 ES cells (gift from R. Jaenisch) were cultured on gamma-irradiated (2,000 rads) or mitomycin C (10 μg/ml)-treated primary EF in DMEM supplemented with 15% fetal calf serum (Hyclone), 1× nonessential amino acids (GIBCO), and 0.1 mM 2-mercaptoethanol (Fluka) as previously described (23). NIH 3T3 cells were cultured in DMEM supplemented with 10% newborn calf serum (GIBCO) and 1× antibiotics (Hazleton).

Electroporation. The BTX Transfector (Biotechnologies and Experimental Research Inc.) was used for electroporation. In general, 10^7 cells were electroporated with a vector at 30 μg/ml in 0.6 ml of a buffer consisting of 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.0), 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose, and 0.1 mM 2-mercaptoethanol, at 150 μF and 250 V (30). Sometimes the cell number, DNA concentration, capacitance, and voltage were varied slightly. At 10 min after electroporation, cells were plated at 10^6/100-mm-diameter culture dish containing a monolayer of G418-resistant EF feeder cells. Selection was started 48 h after electroporation with G418 at 200 μg/ml (active dose; GIBCO). At 12 days after selection, independent G418-resistant colonies were picked and cloned.

Staining for β-gal activity. Colonies were stained on a dish 10 days after culture in selective medium as described by Beddington et al. (2). Briefly, the dishes were rinsed with 0.1

M phosphate buffer, pH 7.3, and fixed for 5 min at room temperature with 0.2% glutaraldehyde–5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)–2 mM magnesium chloride in 0.1 M phosphate buffer, pH 7.3. After being washed three times with a solution containing 2 mM magnesium chloride, 0.01% deoxycholate, and 0.02% Nonidet P-40 in 0.1 M phosphate buffer (pH 7.3), the plates were overlaid with a histochemical reaction mixture containing 5 mM potassium ferricyanide (Sigma), 5 mM potassium ferrocyanide (Sigma), and 1 mg of X-Gal (Bachem) per ml in the wash solution. Macroscopic and microscopic examinations were carried out after incubation for 4 h to overnight at 37°C.

Southern and Northern (RNA) analyses. Extraction of genomic DNA from cells, preparation of Southern blots, preparation of a radiolabeled probe, hybridization of blots, and autoradiography were carried out as previously described (28).

From each clone, poly(A)$^+$ mRNAs were isolated by oligo(dT) cellulose column chromatography (27). About 2×10^8 cells were harvested per clone, washed twice with HEPES-buffered DMEM, and homogenized with a Polytron (Brinkmann) in 40 ml of lysis buffer containing 200 μg of proteinase K (Boehringer Mannheim Biochemicals), 0.5% SDS, 0.1 M NaCl, 20 mM Tris-HCl (pH 7.4), and 1 mM EDTA. The homogenate was incubated for 1 h at 37°C. After adjustment of the final NaCl concentration to 0.4 M, the homogenate was mixed with 0.1 g of oligo(dT) cellulose (Collaborative Research) in 1 ml of a high-salt-content buffer containing 0.4 M NaCl, 0.1% SDS, 10 mM Tris-HCl (pH 7.4), and 1 mM EDTA. The mixture was rocked for 1 h at room temperature, washed three times with 40 ml of high-salt-content buffer, and set in a disposable chromatography column (Bio-Rad). Poly(A)$^+$ RNA was eluted four times with 0.5 ml of TES buffer (10 mM Tris-HCl, [pH 7.4], 1 mM EDTA, 0.1% SDS) and precipitated with ethanol. Northern blots were prepared by using 10 μg of poly(A)$^+$ RNA per sample and hybridized.

CAT assays. NIH 3T3 cells were electroporated with two different vectors, pWH5 and pBS-ECAT (Fig. 1A). About 1.2×10^7 cells were electroporated with 50 μg of a supercoiled vector at 300 μF and 350 V in 0.6 ml of buffer and then plated at 4×10^6/100-mm-diameter dish. As a control, nonelectroporated cells were plated at 2×10^6/100-mm-diameter dish. CAT assays were carried out by published procedures (7). After 24 and 48 h in culture, the cells were harvested by scraping, washed three times with HEPES-buffered DMEM, and then suspended in 1 ml of 0.25 M Tris-HCl (pH 8.0). The cells were homogenized by being frozen and thawed three times. After being heated at 60°C for 10 min to inactivate the endogenous inhibitory factors (17), the homogenates were spun down in a microcentrifuge at 4°C for 10 min. The supernatant was assayed for CAT activity by incubation at 37°C for 20 h with 0.05 μCi of [^{14}C]chloramphenicol (40 mCi/mmol; Amersham) and 4 mM acetyl coenzyme A (Sigma) in 0.25 M Tris-HCl (pH 8.0). After 2 h of the initial incubation, more 4 mM acetyl coenzyme A was added to the reaction. The amount of extract in each assay was normalized to contain 38.8 μg of protein. The protein concentration was determined with a dye reagent (Bio-Rad) by using bovine serum albumin as the standard. Each extract was assayed in duplicate. The reactions were stopped by extraction with ethyl acetate and analyzed by ascending thin-layer chromatography in chloroform-methanol (95:5). Acetylated forms of chloramphenicol were detected by exposure on X-ray film for 1 week.

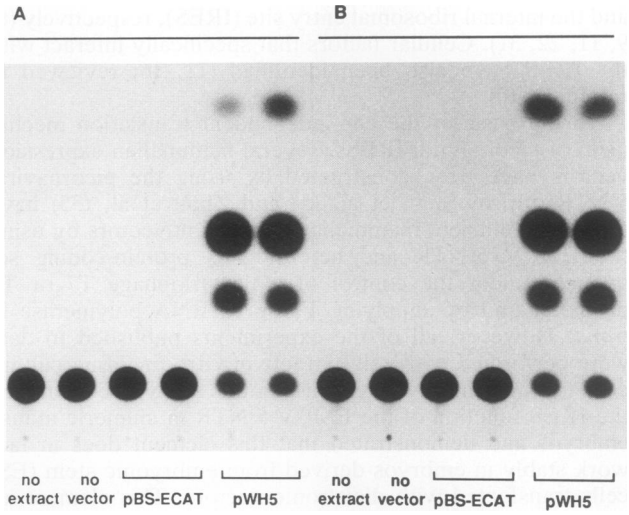


FIG. 2. CAT activity assay. Two vectors, pBS-ECAT and pWH5, described in Fig. 1 were electroporated into mouse 3T3 fibroblast cells to examine the promoter activity of the 5′NTR sequence. After 24 (A) and 48 (B) h of culture, the cells were harvested and CAT activity assays were carried out. pWH5 containing the *cat* gene linked with the 5′NTR under control of the PGK-1 promoter showed strong CAT activity, whereas pBS-ECAT, consisting of only the 5′NTR and *cat*, did not.

Generation and analysis of chimeric embryos. Blastocysts (3.5 days postcoitum) were obtained from 8-week-old, naturally ovulating female mice (C57BL/6J; Jackson Laboratory) mated with male mice of the same strain. The embryos were kept in drops of DMEM supplemented with 10% fetal calf serum covered with silicon oil in a humidified CO_2 incubator (37°C, 5% CO_2). Microinjection of ES cells into blastocysts was performed in a microdrop of HEPES-buffered DMEM–10% fetal calf serum (pH 7.4) as described by Bradley (3). Usually, 10 to 30 ES cells were injected into each blastocoel. Following injection, the blastocysts were cultured for 2 to 3 h and then transferred into the uterine horn of pseudopregnant FVB/N or (B6 × DBA)F1 recipient female mice, 2.5 day postcoitum, which were prepared by mating with vasectomized male mice.

Postimplantation embryos were recovered at appropriate stages, considering the day of plug of the pseudopregnant recipient mother as day 0.5 of development, and then stained for β-gal activity as described above, except for the use of 0.1 M phosphate buffer (pH 7.6) and a longer fixation time of 30 to 60 min, depending on embryo size.

RESULTS

IRES function of the 5′NTR of EMCV in mouse cells in culture. We have extended and confirmed previous results that demonstrated that the IRES element of the 5′NTR of EMCV can function in simian, human (11), or mouse (34) cells. The 5′NTR referred to here is the segment from nucleotides 260 to 833 of the 5′NTR of EMCV RNA which contains all of the essential elements for efficient internal ribosome entry (9, 10) plus 15 nucleotides originating from the EMCV polyprotein (see Materials and Methods). Our approach was to measure the transient CAT activities in mouse 3T3 cells transfected with two different CAT vectors by electroporation (Fig. 2). CAT activity was apparent in cells at 24 and 48 h after electroporation with the pWH5

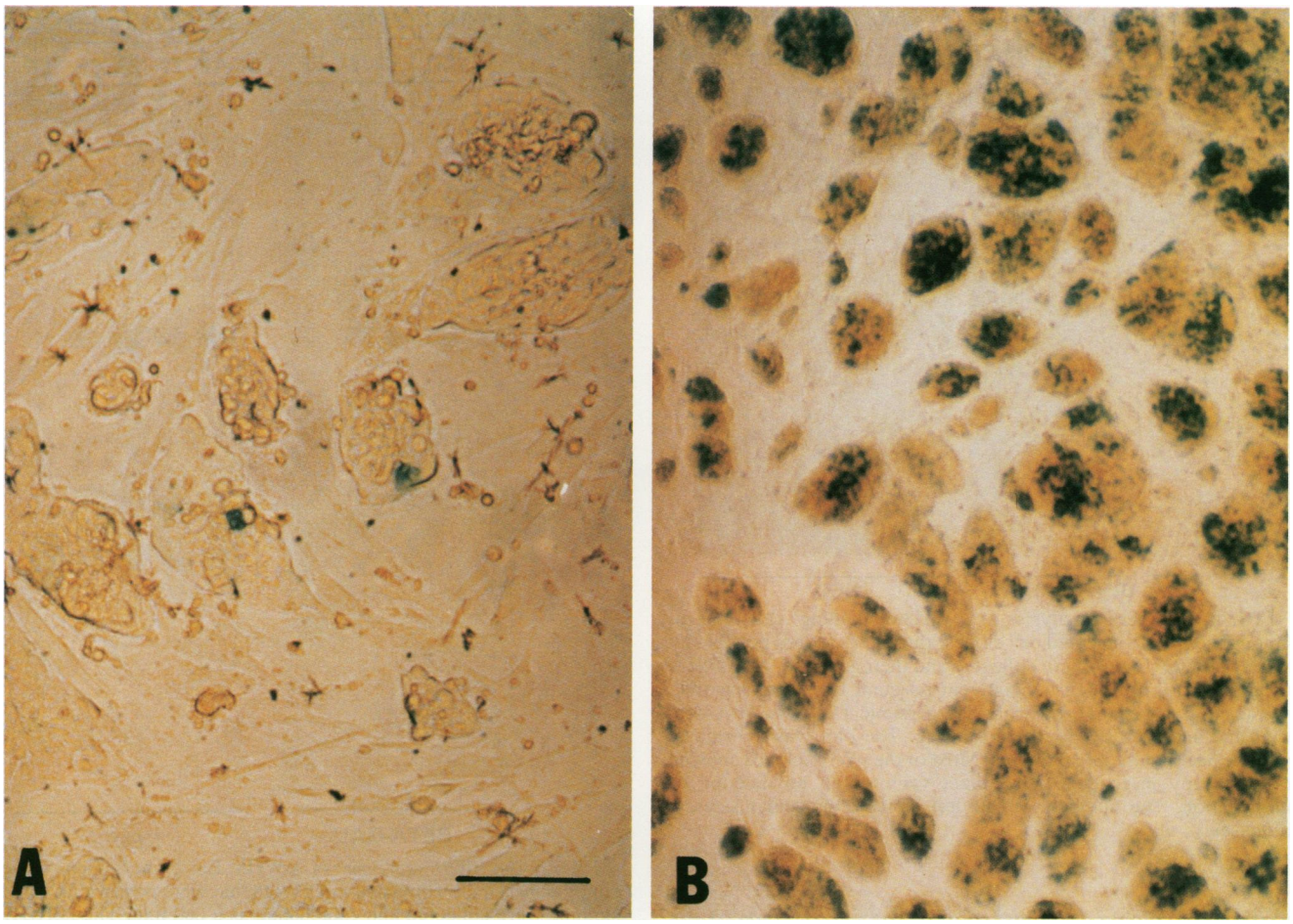


FIG. 3. X-Gal staining of ES clones obtained after electroporation with vector pWH8 and selection. Plasmid pWH8, containing the *lacZ* gene as the second cistron preceded by the EMCV 5'NTR, was electroporated into ES cells, selected with G418, and then stained with X-Gal. (A) ES cells during selection (~10 days). (B) Cloned G418-resistant ES cells.

vector, which contains the 5'NTR-*cat* sequence as the second cistron downstream of the eukaryotic promoter-driven *neo* gene. No CAT activity was detectable in the extract of control cells that were electroporated with pBS-ECAT, which contains only the 5'NTR-*cat* sequence without a promoter. This indicated that the CAT activity detected by transfection of pWH5 was not attributed to translation of a monocistronic mRNA which might have been produced from a cryptic promoter in the EMCV 5'NTR.

Having confirmed the IRES function of the EMCV 5'NTR in mouse cells in transient assays, we carried out the following experiments to examine whether the EMCV 5'NTR functions stably after being integrated into the mouse genome.

Expression of β-gal from a dicistronic vector in cells in culture. To enable histochemical analyses of dicistronic expression in vivo, we constructed a dicistronic vector, pWH8, consisting of a promoter-driven *neo* gene followed by the 5'NTR-*lacZ* sequence (Fig. 1A). Pluripotent ES cells were transfected with supercoiled pWH8 by electroporation, and G418-resistant colonies were obtained as described in Materials and Methods. G418-resistant colonies were obtained at a frequency of 5.8×10^{-5} per cell electroporated. Upon staining, about 55% of the G418-resistant colonies were positive for β-gal. The β-gal-negative clones among the G418-resistant cells may have resulted from inactivation of *lacZ* in the process of random integration of the circular plasmid into the chromosomal DNA. We suspect that the rate of β-gal-positive clones among G418-resistant clones could be increased by using linear DNA in the process of DNA transfection into the ES cells. Some of the β-gal-negative clones contained a vector that had actually lost the *lacZ* gene sequence, as shown below. The staining pattern among the cells in a given colony was not uniform (Fig. 3A). Colonies from remaining dishes were cloned, and duplicate cultures were stained for β-gal. The staining was more uniform once the cells were cloned and plated again (Fig. 3B).

Sixteen clones that were strongly positive for β-gal and appeared to be undifferentiated were expanded for further analyses. Genomic DNAs were extracted from each of the clones for Southern analysis. DNAs from β-gal-negative clone 5 and from the EF feeder cells were included as control samples. The DNA samples were digested with *Sac*I, and Southern blots were prepared. *Sac*I cuts once in the vector, splitting the *lacZ* gene into two pieces (Fig. 1). Therefore, the β-gal probe is expected to reveal two bands in a clone

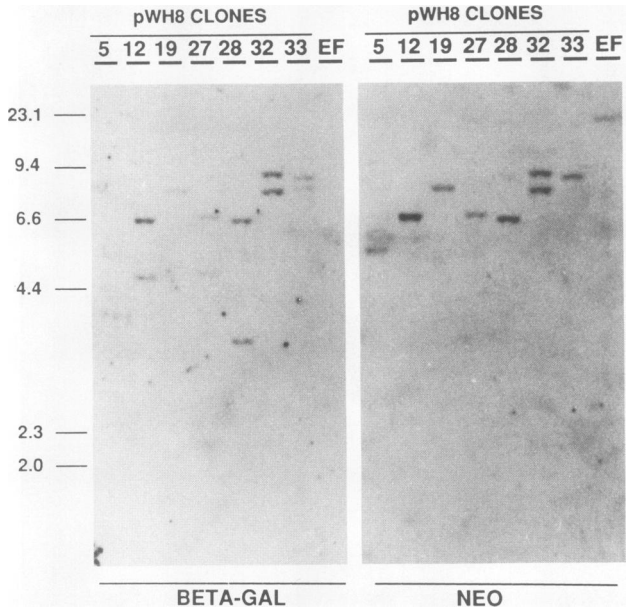


FIG. 4. Southern analysis of pWH8-containing cell clones. Among the pWH8-selected cell clones, those that scored positive by X-Gal staining (Fig. 3) were collected and their genomic DNAs were isolated. Clone 5, which is neomycin resistant but β-gal negative, was used as a negative control. The genomic DNA was cut with *Sac*I and analyzed by Southern blotting. The blot was hybridized with the *neo* and the β-gal probes. Most of the pWH8 clones showed a single-copy insertion of the intact vector with no deletion or modification. The numbers on the left indicate molecular sizes in kilobases.

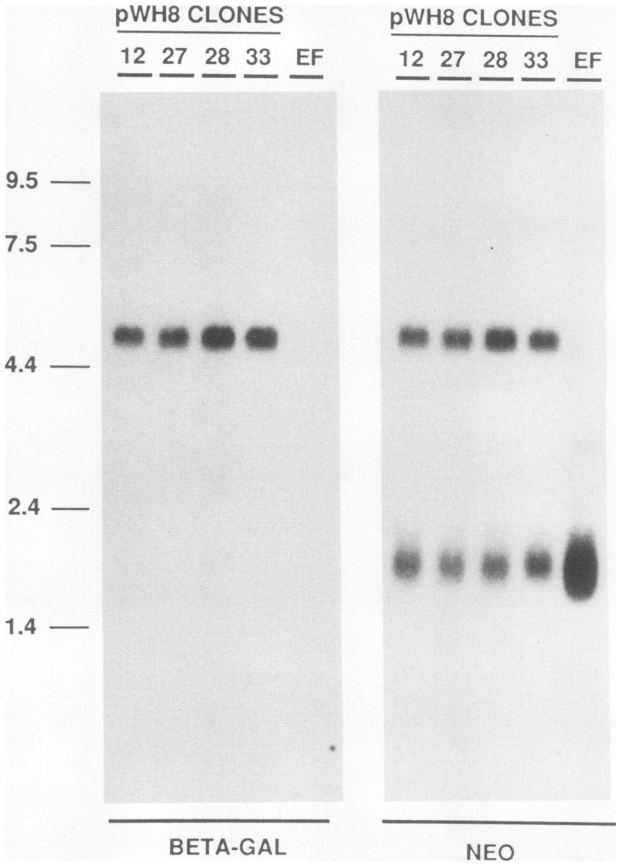


FIG. 5. Northern analysis of pWH8 ES clones. Cell clones containing a single-copy insert of pWH8 that were positive for X-Gal staining were collected and established as a cell line. The poly(A)$^+$ RNAs from the ES clones were purified and separated in a 1% agarose gel containing formaldehyde. After transfer of RNA to nylon membranes, the blot was hybridized with the *neo* and β-gal probes. Each pWH8 clone shows that both the *neo* and β-gal genes are contained in a single transcript. The numbers on the left indicate molecular sizes in kilobases.

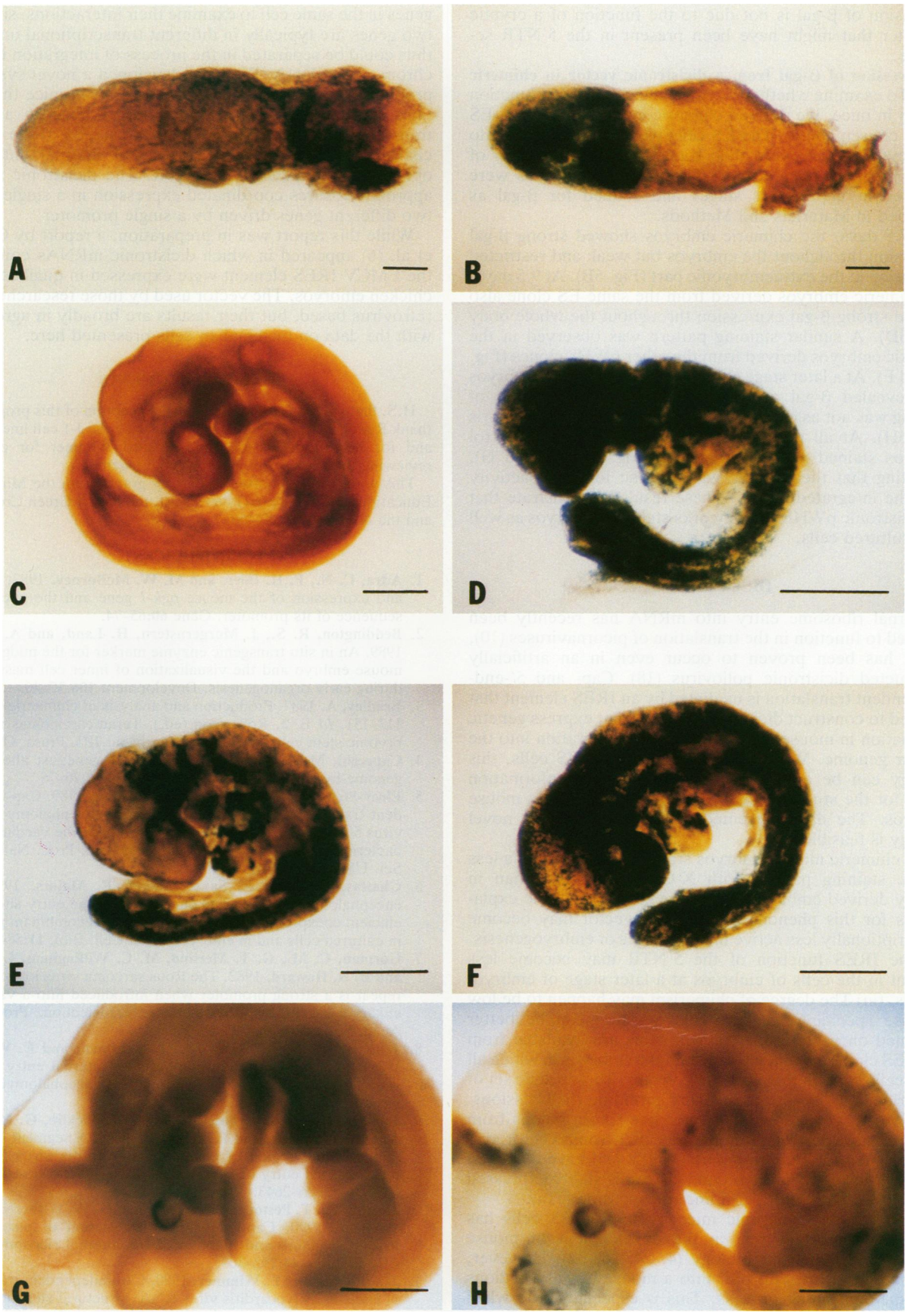
containing a single-copy insertion of the vector. One of the two bands would also be hybridized with the *neo* probe. An autoradiogram of a sample Southern blot is shown in Fig. 4, and it revealed patterns of hybridization expected for single-copy insertion of the vector. The EF and the β-gal-negative clones showed only the *neo* band. We conclude that the vector sequence, containing the *neo* and the β-gal genes in a single transcription unit, was stably integrated into different locations in the cellular genome.

Clones 12, 27, 28, and 33 were chosen for further analyses. They all contained a single-copy integration, revealed the expected pattern on Southern analyses, and stained strongly positive for β-gal.

Bifunctional mRNA active in ES cells. To examine the nature of the transcript derived from the integrated vector, poly(A)$^+$ RNAs were isolated from ES clones 12, 27, 28, and 33, as well as from the EF cells. A Northern blot containing these RNAs was hybridized with the β-gal probe, stripped, and then hybridized with the *neo* probe. If a dicistronic mRNA were transcribed from the pWH8 vector under control of the PGK-1 promoter, one would expect to see a transcript that is somewhat bigger than 4.4 kb (Fig. 1A) and hybridizes with both the β-gal and *neo* probes. As shown in Fig. 5, the four ES clones revealed a transcript of about 5 kb which hybridized with both the β-gal and *neo* probes. The ES lanes also showed a small *neo*-containing transcript which was also detected in the EF cells. These small *neo* transcripts in the ES lanes must, therefore, be derived from the EF cells, since significant numbers of EF cells were present in the ES cell preparations from which the RNAs were extracted.

These results indicate that a dicistronic mRNA is produced from the stably integrated vector in those ES clones and that both of the cistrons are translated to produce functional proteins. Furthermore, they also confirm that

FIG. 6. Chimeric embryos stained with X-Gal. Cells containing a single-copy insert of pWH8 that were positive for β-gal were injected into blastocysts, and then chimeric embryos were obtained by uterine transfer. The chimeric embryos were stained with X-Gal to examine in vivo expression of β-gal from a dicistronic message. Panels A and B show 7.5-day embryos. Bars, 100 μm. β-gal was highly expressed in the embryonic ectoderm, indicating that the injected cells contributed mainly to the embryonic ectoderm. Panels C, D, E, and F show 9.5-day embryos. Bars, 500 μm. Panels G and H show 11.5-day embryos. Bars, 1 mm. The chimeric embryos in panels B, D, and H were derived from ES clone 12, and those in panels E and F were derived from clones 27 and 33, respectively. All of the chimeric embryos (B, D, E, F, and H) expressed β-gal, whereas the control embryos (A, C, and G) did not.


A
B
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expression of β-gal is not due to the function of a cryptic promoter that might have been present in the 5'NTR sequence.

Expression of β-gal from a dicistronic vector in chimeric mice. To examine whether dicistronic messages can function in vivo in mice, we derived chimeric embryos from the ES clones chosen above. The ES cells were microinjected into blastocysts, which were then transferred into the uteri of pseudopregnant female mice. The chimeric embryos were collected at appropriate stages and stained for β-gal as described in Materials and Methods.

At 7.5 days, the chimeric embryos showed strong β-gal expression throughout the embryos but weak and restricted expression in the extraembryonic part (Fig. 6B). At 9.5 days, the chimeric embryos derived from the same ES clone also showed strong β-gal expression throughout the whole body (Fig. 6D). A similar staining pattern was observed in the chimeric embryos derived from the other pWH8 clones (Fig. 6E and F). At a later stage (11.5 days), the chimeric embryos also revealed β-gal activity although the distribution of staining was not as extensive as in the earlier-stage embryos (Fig. 6H). At all of the stages examined, normal control embryos stained negative for β-gal (Fig. 6A, C, and G), indicating that the staining was specific for β-gal activity from the integrated vector. These results demonstrate that the dicistronic pWH8 vector works stably in embryos as well as in cultured cells.

DISCUSSIONS

Internal ribosome entry into mRNA has recently been reported to function in the translation of picornaviruses (10), and it has been proven to occur even in an artificially constructed dicistronic poliovirus (18). Cap- and 5'-end-independent translation is mediated by an IRES element that we used to construct dicistronic vectors that express genetic information in mouse cells after random integration into the cellular genome. When expressed in mouse ES cells, this strategy can be applied to introduce genetic information useful for the study of embryonic development into mouse embryos. The data presented here show that this novel strategy is feasible.

The chimeric mouse embryos studied here revealed a less intense staining pattern with X-Gal in 11.5-day than in 9.5-day derived embryos. There are several possible explanations for this phenomenon. (i) The vector may become transcriptionally less active in later stages of embryogenesis. (ii) The IRES function of the 5'NTR may become less efficient in the cells of embryos at a later stage of embryogenesis. (iii) The degree of chimerism may happen to be low in those specific chimeras. This question can be better examined once germ line transmissions are available from those ES clones so that we can stain the embryos in which all of the cells contain the dicistronic vector. This specific set of chimeric mice did not produce any germ line transmissions. We have, however, obtained germ line transmissions from chimeric mice derived from another dicistronic vector system designed for gene trap mutagenesis and have observed that the β-gal gene in the second cistron is stably expressed in embryos, even after germ line transmission (11a).

Production of transgenic mice by use of ES cells has played an important role in the development of mouse genetics and molecular biology (4, 24, 25, 32). However, introduction of a useful trait into a mouse is still a tedious and time-consuming process. This is especially true when one wants to obtain coordinated expression of two different genes in the same cell to examine their interactions, since the two genes are typically in different transcriptional units and thus could be separated in the process of integration into the chromosomal DNA. We have developed a novel system to produce a bifunctional dicistronic mRNA in mice that contains a selection marker and a target gene in a single transcription unit. In this system, translation of the second cistron is directed by the ribosomal internal entry site obtained from the EMCV 5'NTR. The dicistronic mRNA approach ensures coordinated expression in a single cell of two different genes driven by a single promoter.

While this report was in preparation, a report by Ghattas et al. (6) appeared in which dicistronic mRNAs containing the EMCV IRES element were expressed in quail cells and chicken embryos. The vector used by those researchers was retrovirus based, but their results are broadly in agreement with the data on mammalian cells presented here.

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