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IRES Bicistronic Expression Vectors for Efficient Creation of Stable Mammalian Cell Lines

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Stable transfection of mammalian cells is a widely used technique for the study of gene expression and protein purification. However, selection of cell lines expressing desired genes from a large number of candidate clones is often labor-intensive and time consuming. To improve the efficiency of stable cell line production, we have used a bicistronic mammalian expression vector, pIRES1hyg, which contains the internal ribosome entry site (IRES) of the encephalomyocarditis virus (ECMV). The IRES element permits the translation of two open reading frames from one messenger RNA: one reading frame encoding the recombinant protein of interest and the other an antibiotic resistant marker (e.g. hygromycin). We demonstrate that the use of the bicistronic vector significantly facilitates the creation of stable mammalian cell lines, because all selected antibiotic-resistant colonies express the recombinant gene of interest. Therefore, the use of the pIRES1hyg bicistronic vector for stable transfection eliminates the need to screen large numbers of colonies to find functional clones. We conclude that the IRES bicistronic vector provides a powerful tool for efficient selection of stable transformants in mammalian cells.

Standard methods used to generate stable cell lines require transfecting a host cell line with two expression cassettes, one expressing the protein of interest and the other an antibiotic resistance marker for selection. These cassettes can be introduced into the host cell either by cotransfecting two plasmids each of which contain one of the expression cassettes, or by transfecting one plasmid containing both cassettes. Typically, after transfection and selection, approximately 5-30% of the cells functionally express the recombinant protein of interest (4,5). These relatively low frequencies can be due to many factors including deletion or inactivation of the cassette expressing the gene of interest, or, in the case of cotransfection, the stable integration of only the cassette expressing the selectable marker. Therefore, further screening of selected colonies specific for expression of the gene of interest is often necessary in order to find functional clones. Additionally, the level of gene expression using these standard methods cannot be predicted; expression is generally low and, because the selective pressure is only on the cassette that expresses the antibiotic resistance marker, expression levels can decrease over time in culture.

Unlike most eukaryotic mRNA in which ribosomes scan from the 5′ end until the initiation codon is reached, ribosomes are able to begin translation at internal ribosome entry sites (IRES) in messenger RNA of the picornaviruses (2, 3), such as encephalomyocarditis virus (ECMV). These IRES elements can be removed from their viral setting and linked to unrelated genes to produce polycistronic RNAs. In this report, we demonstrate the utility of the IRES containing bicistronic expression vector, pIRES1hyg, for the rapid and efficient generation of stable mammalian cell lines.

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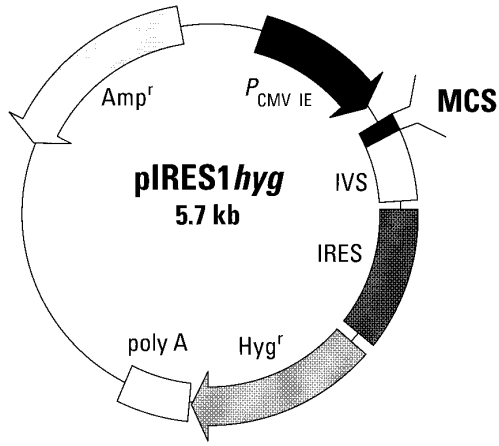

FIG. 1. Map of bicistronic expression vector, pIRES1hyg. The multiple cloning site (MCS) contains BamHI, BstXI, EcoRI and NotI sites. The internal ribosome entry site (IRES) permits the translation of two open reading frames from one messenger RNA (2, 3).

MATERIALS AND METHODS

Cell culture and reagents. CHO-K1 cells (ATCC, Rockville, MD, USA) were cultured in Ham's F-12 medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml of penicillin and 100 μg/ml of streptomycin. Media, sera, and other supplements were purchased from Sigma Chemical Co. (St. Louis, MO). Cultures were maintained at 37°C with 5% CO_2/95% air.

Vector construction. All DNA manipulations were performed using standard methods (5) unless otherwise described. The pIRES1hyg expression vector was constructed by a method described previously (4). The pIRES1hyg-βgal expression vector was constructed and used to evaluate the utility of the bicistronic expression vector for the rapid and efficient production of stable mammalian cells. In the construction, the *E. coli LacZ* gene was excised by enzyme restriction in the *Not* I site of the pCMVβ expression vector (CLONTECH) and inserted into the pIRES1hyg vector through the same restriction site.

Transfection and stable selection. Transfection of pIRES1hyg was performed in CHO-K1 cells using CLONfectin transfection reagent (CLONTECH). Briefly, 8×10^5 cells were seeded in 60-mm tissue culture plates one day prior to transfection. The cultures were 60-80% confluent at the time of transfection. Cells were transfected with 6 μg plasmid DNA per plate for 2 hours. After 48 hour incubation in the appropriate growth medium, 200-600 μg/ml of hygromycin was added to the culture and selection was performed for 7-10 days.

In situ β-galactosidase staining of selected culture. Following selection, the remaining cultures were trypsinized and seeded into a new culture dish. β-galactosidase expression was detected by *in situ* staining using the X-gal substrate as described previously (6). Briefly, cells were rinsed in phosphate-buffered saline (PBS), fixed in 2% formaldehyde and 0.2% glutaraldehyde in PBS, rinsed twice with PBS, and stained for 2 hours with 0.1% X-gal in PBS containing 5 mM potassium ferricyanide and 2 mM $MgCl_2$. Cells were photographed on a Leica Leitz light Microscope (Leica Inc., Foster City, CA).

RESULTS AND DISCUSSION

To improve upon the quality and efficiency of producing stable mammalian cell lines, we have tested an IRES containing bicistronic mammalian expression vector, pIRES1hyg (Fig. 1). The pIRES1hyg expression cassette contains the human cytomegalovirus (CMV) major immediate early promoter/enhancer followed by a multiple cloning site (MCS), a synthetic intron known to enhance the stability of the mRNA (1), the EMCV IRES followed by the hygromycin gene, and the polyadenylation signal of the bovine growth hormone. The IRES element permits translation of two open reading frames: one encodes the recombinant protein of interest and the other an antibiotic resistant marker. As shown in Fig. 2, ribosomes can enter the bicistronic mRNA either at the 5′ end to translate the gene of interest or at the ECMV IRES to translate the antibiotic resistance marker.

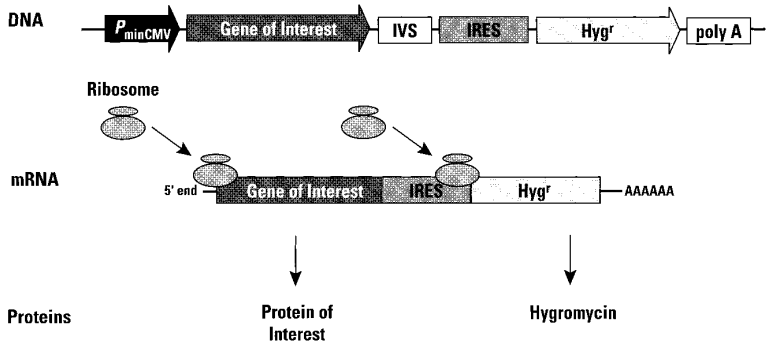

FIG. 2. Schematic diagram of the translation of a bicistronic mRNA. The open reading frames of a protein of interest and an antibiotic selection marker can be translated from the same mRNA by different ribosomes. IVS, intron; hyg, hygromycin.

To demonstrate the utility of the vector for efficient production of stable mammalian cell lines, we cloned the *E. coli LacZ* gene into the pIRES1hyg expression vector, pIRES1hyg-βgal. *In situ* detection of β-galactosidase (β-gal) expression was performed in CHO-K1 cells following transfection and hygromycin selection. We found that the percentage of β-gal positive staining cells depends on the amount of hygromycin added to the culture medium. At low concentrations of hygromycin (200-300 μg/ml), often less than 50% of the selected colonies expressed sufficient levels of β-galactosidase for detection. However, when the concentration of hygromycin was increased to 600 μg/ml, all of the surviving colonies visualized expressed sufficient levels of β-galactosidase for *in situ* detection (Fig. 3). These results demonstrate that using high doses of antibiotics selected only the cells that express sufficient high levels of β-galactosidase for detection.

Figure 4 shows a representative culture selected using 600 μg/ml of hygromycin. After 10

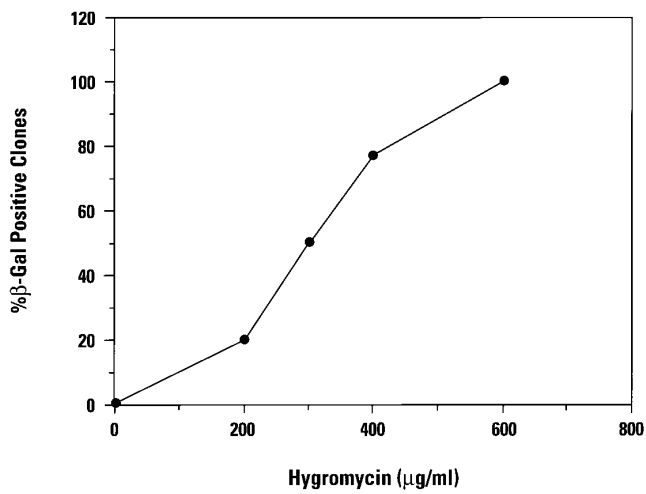

FIG. 3. Hygromycin dose-dependent selection of β-gal expressing clones. CHO-K1 cells were transfected with the pIRES1hyg-βgal expression vector as described in the Methods. Various concentrations of hygromycin were added to cultures 48 hours post-transfection and selection was performed for 7-10 days. *In situ* β-gal staining was performed as described in Methods. Generally, 4-6 representative fields for each selection were scored for β-gal expression under a Leica light microscope (Leica Inc., Foster City, CA).

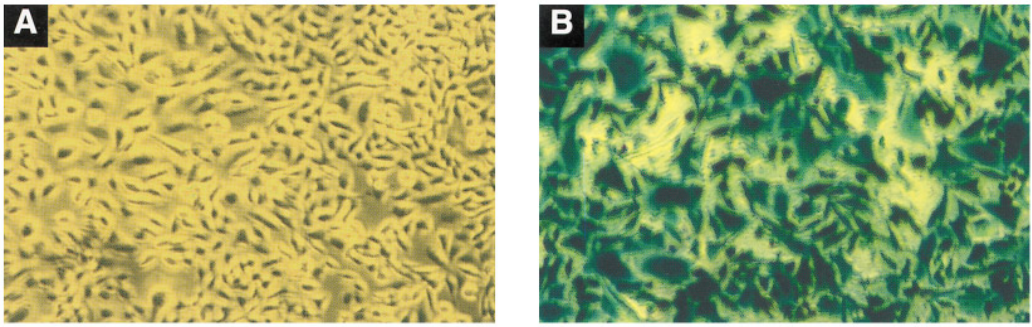

FIG. 4. Visualization of CHO-K1 cells stably transfected with the pIRES1hyg-βgal bicistronic vector. CHO-K1 cells were transfected with pIRES1hyg-βgal using CLONfectin transfection reagent as described in Methods. Antibiotic selection and *in situ* β-galactosidase staining were performed as described in Methods. β-gal staining with the selected culture shows that essentially all of the cells which survived selection express β-galactosidase (Panel B). Untransfected cultures do not stain for β-gal (Panel A).

days of selection with hygromycin, cells were trypsinized and seeded into a new culture dish. All of the surviving cells expressed β-galactosidase as shown by *in situ* β-gal staining. These results further demonstrate the effectiveness of the IRES bicistronic expression vector in the creation of functional stable cell lines. Thus, the IRES bicistronic mammalian expression vector provides an excellent system for rapidly producing stable cell lines. In addition, the IRES bicistronic expression vector has the potential to produce stable cell lines with high-level expression of the gene of interest. Since the selective pressure provided by the antibiotic is on the entire expression cassette, a high dose of antibiotic will select only for those cells expressing a high level of the gene of interest (4).

Unless a pure population of cells is required for expression experiments, the pIRES1hyg bicistronic expression vector allows researchers to use the pool of cells surviving selection, typically following 7-10 days in culture. This saves considerable time and manpower during the generation of a stable cell line, and eliminates the need for screening a large number of colonies to identify positive clones. Taken together, these studies indicate that the IRES bicistronic expression vector provides a powerful tool for the rapid and efficient production of stable mammalian cell lines.

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