pIRES-CD4t, a Dicistronic Expression Vector for MACS- or FACS-Based Selection of Transfected Cells

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ABSTRACT

To facilitate the selection of cell lines expressing transfected genes of interest, a plasmid vector has been constructed that directs the co-expression of heterologous cDNAs and a 3'-positioned cassette encoding a truncated CD4 marker. An encephalomyocarditis virus internal ribosomal entry site (IRES) mediates translational initiation from this 3' cassette, and a cytomegalovirus promoter drives dicistronic transcript expression. To test the utility of this vector, a luciferase reporter gene was inserted, and this construct (pIRES-CD4t-luc) was electroporated into myeloid FDCW2 cells. As monitored by flow cytometry and luciferase assays, three rounds of magnetic cells sorting (MACS) yielded ~90% CD4t-positive cells with an average density of 17 000 CD4t molecules per cell. In ten clonal sublines analyzed, luciferase expression was uniformly high and stable over a test period of three months. Finally, a comparison of MACS- vs. FACS-based isolation of transfected cells showed two to three rapid rounds of MACS to be somewhat more effective. Thus, pIRES-CD4t should prove useful in the direct and rapid selection of relevant stably or transiently transfected cells.

INTRODUCTION

Dicistronic vectors recently have been developed that increase the frequency of heterologous gene expression in transfected cells (7,10,11,13, 14). In these vectors, a single promoter drives the transcription of tandem cDNAs, and translation of the 3'-positioned cDNA (typically a selectable marker) is directed by an internal ribosomal entry site (IRES) from encephalomyocarditis virus (EMCV) (5). EMCV possesses an uncapped RNA

strand including a 600-nucleotide (nt) IRES with a 5' untranslated region (UTR) that initiates translation by means of a cap-independent mechanism (5). When positioned between heterologous cDNAs, this element can mediate the translation of 3'-positioned constructs (7,9), thereby coupling the expression to 5'-inserted cDNAs (7,11,14). This provides an advantage over co-transfected or dual-promoter vectors in which expression is often uncoupled (7,8). Among IRES-containing dicistronic vectors, several distinct 3' marker cassettes exist, including: pCIN (14) and pMK (11) vectors include neomycin phosphotransferase, the tetracycline inducible vector pTETiβgeo (10) encodes a lacZ-neomycin phosphotransferase fusion gene and the retroviral vector pED4 (7) contains a 3'-positioned dihydrofolate reductase (DHFR) cassette. Tet-inducible vectors such as pTETiβgeo, however, require extended induction and incubations with fluorescein di-β-D-galactopyranoside, while pED4 requires the use of DHFR^{-/-} target cells. Dicistronic vectors encoding green fluorescent protein (GFP) (e.g., pTR-DC/GFP; Reference 13) allow selection by fluorescence-activated cell sorting (FACS), but in at least certain systems, clones stably expressing S65T GFP can be inactivated by rearrangement or mutation (3).

For the selection of transfected cells, immunomagnetic methods (i.e., magnetic cell sorting [MACS]) using ferritin particle-coupled antibodies against cell-surface markers provide a direct approach (12,17). For MACS, pSRαMSV-TKCD8 and pMT-IRES-CD4 vectors have been previously developed. However, the pSRαMSV-TKCD8 vector is not dicistronic (6), and in studies of stably transfected cells, pMT-IRES-CD4 was co-transfected with a neomycin-encoding plasmid; it is unclear whether this vector alone can be used to efficiently select stably transfected lines (18). In addition, the use of a full-length CD4 cDNA in this vector is potentially complicating. To provide for the selection of stably transfected cells co-expressing marker and heterologous cDNAs at high frequencies, a vector presently has been developed in which a cytomegalovirus (CMV) promoter drives ex-

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pression of dicistronic transcripts that contain a centrally positioned EMCV IRES and a 3' cassette encoding a truncated form of human CD4 gene (1). To test the utility of this vector, a luciferase reporter gene was inserted, and this construct was electrotransfected into myeloid FDCW2 cells (16). Cells stably expressing CD4t were isolated by either MACS or FACS. Analyses of marker and reporter expression show that this pIRES-CD4t vector provides for the direct and efficient isolation of transfected cells and stably drives the coupled expression of inserted heterologous cDNAs.

MATERIALS AND METHODS

Dicistronic Expression Vectors

pIRES-CD4t was constructed by replacing the neomycin phosphotransferase gene in pIRES_{1neo} (CLONTECH Laboratories, Palo Alto, CA, USA) with a cDNA encoding the extracellular and transmembrane domains of human CD4 (1). This cDNA was excised from pMACS 4 (Milenyi Biotec, Bergisch Gladbach, Germany) using *EcoRI* (site located 81 nt upstream from the AUG start codon) and *PstI* (site located 12 nt 3' to a stop codon positioned five amino acids after the transmembrane domain) and was cloned (1363-bp fragment) into pBlue-script[®] KS(+) (Stratagene, Palo Alto, CA, USA). pBS-CD4t then was digested with *EcoRI*, blunt-ended using the Klenow fragment of DNA polymerase I (New England Biolabs, Beverly, MA, USA) and digested with *XbaI* to yield a 5'-blunted, 3' *XbaI* fragment. pIRES_{1neo} was digested with *SmaI* and *XbaI* to excise the neomycin phosphotransferase gene, and the above CD4t cDNA fragment was ligated into these sites to yield pIRES-CD4t. pIRES-CD4t-*luc* was constructed as follows. A luciferase cDNA was isolated from pSP-*luc+* (Promega, Madison, WI, USA) by digestion with *HindIII*, blunt-ending with the Klenow fragment of DNA polymerase I and digestion with *EcoRI*. This 1669-bp fragment then was cloned into pIRES-CD4t at *EcoRV* and *EcoRI* sites to yield pIRES-CD4t-*luc*.

Table 1. Sustained Expression of Luciferase Activity in Polyclonal MACS 4 FDCW2-pIRES-CD4t-*luc* Cells and Ten Clonal Sublines

	6 weeks	8 weeks	12 weeks
MACS 4	11.48 ± 5.82	16.69 ± 2.55	10.95 ± 1.92
NonSelected	0.25 ± 0.04	0.15 ± 0.02	0.02 ± 0.01
Clone A12	19.5	13.4	17.0
Clone B5	19.6	8.9	7.7
Clone D8	24.7	32.4	21.9
Clone D12	26.9	15.1	22.9
Clone E11	28.5	24.9	21.5
Clone E12	14.0	29.1	10.5
Clone G7	8.6	10.5	9.4
Clone H1	26.5	14.8	24.2
Clone H3	17.6	17.8	15.9
Clone H9	15.0	8.7	18.8

Cell Culture, Electrotransfections and MACS Selection

FDCW2 cells were maintained at 37°C, 5% CO₂ in OPTI-MEM[™] I medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 8% fetal bovine serum (FBS) and 8% Wehi3B-cell-conditioned medium (W3CM) as a source of Interleukin-3 (IL-3). Electrotransfections were performed as detailed previously (16). Following elec-

trotransfections, 1 × 10⁷ expanded cells were subjected to selection using anti-CD4 conjugated microbeads (Milenyi Biotec). Cells were collected by centrifugation (800× *g* for 10 min) and were resuspended in 10 mL of degassed phosphate-buffered saline (PBS) (2.7 mM KCl, 140 mM NaCl, 1.5 mM KH₂PO₄, 8 mM Na₂HPO₄·7H₂O, pH 7.2) containing 5 mM Na₂EDTA. Cells then were collected, resuspended in 320 µL of PBS, 5 mM Na₂EDTA, 0.5%

Figure 1. pIRES-CD4t and pIRES-CD4t-*luc* dicistronic expression vectors. pIRES-CD4t was constructed by cloning a CD4t cDNA from pBS-CD4t (see Materials and Methods) into pIRES_{1neo} (originally described as pCIN4; Reference 3). Contained in pIRES-CD4t are a CMV major immediate early enhancer/promoter (CMV P/E), a synthetic intron known to enhance mRNA stability (IVS), an MCS, a 585-bp EMCV IRES, the above 3'-positioned CD4t cDNA and a polyadenylation signal sequence from a bovine growth hormone cDNA (omitted in this schematic are an ampicillin-resistance gene and an *E. coli* origin of replication). In CD4t, the start codon is located 102 nt downstream of AUG 10 within the EMCV IRES. pIRES-CD4t-*luc* was constructed by cloning a luciferase cDNA from pSP-*luc+* into *EcoRV* and *EcoRI* sites within the MCS of pIRES-CD4t. Restriction sites that were destroyed during blunt-ended ligation reactions are indexed by open diamonds (◊). Two unique restriction sites, *NruI* and *XhoI*, available for linearization of the vector are in bold.

Figure 2. CD4t marker expression and luciferase activity is coupled in MACS-selected FDC2-pIRES-CD4t-luc cells. FDCW2 cells electrotransfected with pIRES-CD4t-luc were selected by four rounds of MACS to yield populations MACS 1-4. Each population of cells was analyzed by flow cytometry using a PE-conjugated α -CD4 MAb (RPA-T4). Each panel shows histograms of the number of gated fluorescent events (y-axis) vs. PE fluorescence intensity (x-axis). In each histogram, percentages of PE-positive (PE+) cells (C gate) are indexed in the upper right corner. For each MACS population, luciferase activity is indexed (RLU). Results shown are representative of three independent experiments.

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BSA and were incubated with 80 μ L of MACSelect 4 Microbeads (Miltenyi Biotec) for 15 min at 10°C. The total sample volume then was adjusted to 2 mL using PBS, 5 mM Na₂EDTA, 0.5% BSA and was loaded onto an MS⁺/RS⁺ column within the magnetic field of a miniMACS Separation Unit (Miltenyi Biotec). The flow-through volume was collected and was reappplied to the column. The column then was washed 6 $\times$ with 500 μ L of PBS, 5 mM Na₂EDTA and 0.5% BSA. Bound cells were eluted in 1 mL of PBS, 5 mM Na₂ EDTA and 0.5% BSA, collected, resuspended in 3 mL of OPTI-MEM I medium, 8% FBS, 8% W3CM and cultured for 2 days in a 6-well Falcon[®] tissue culture plate (Becton Dickinson Labware, Bedford, MA, USA) in the presence of penicillin (100 U/mL), streptomycin (100 μ g/mL) and amphotericin B (0.25 μ g/mL).

Flow Cytometry, FACS and Luciferase Assays

In assays of CD4t expression, cells were labeled with a phycoerythrin (PE)-conjugated anti-CD4 monoclonal antibody (MAb) (PE-RPA-T4; Pharmingen, San Diego, CA, USA) and were analyzed by flow cytometry. Specifically, cells (1×10^7) were incubated at 4°C for 10 min, collected by centrifugation at 200 $\times$ g for 10 min and washed at 4°C in PBS and 0.05% BSA. Cells then were collected at 1000 $\times$ g for 5 min, resuspended in 1 mL PBS, 0.05% BSA containing 200 μ g of mouse γ -globulins (Cohn fraction II and III; Sigma, St. Louis, MO, USA) and incubated at 4°C for 15 min. PE-RPA-T4 antibody (0.5 μ g) was added to 100 μ L of resuspended cells (1×10^6 cells), and samples were incubated at

Figure 3. Isolation of FDCW2-pIRES-CD4t-luc cells by FACS. MACS 2 cells (see Figure 2) were stained using optimized conditions with PE-RPA-T4 and positive (vs. negative) populations of cells were isolated by FACS (FACS-negative vs. -positive, lower panels). In parallel, MACS 2 cells were subjected to one round of MACS yielding MACS 3 cells (upper right panel). Shown are histograms of the number of gated fluorescent events (y-axis) vs. the level of PE fluorescence intensity (x-axis) for each FACS- or MACS-selected line. Percentages of CD4t-positive cells (gate C) are indexed in upper right corners of each histogram.

4°C for 60 min. Cells then were diluted with 1 mL of PBS, 0.05% BSA, collected (1000× *g* for 5 min), resuspended in 500 µL of PBS, 0.05% BSA and held at 4°C. Flow cytometric analyses were performed using an EPICS® XL system (15-mW argon-ion laser, 488 nm) and Coulter® XL-2 software (both from Beckman Coulter, Fullerton, CA, USA). PE-conjugated calibration particles (Catalog No. RCP 30-5; Sphero-tech, Libertyville, IL, USA) were used to calculate the average number of PE-RPA-T4 molecules on the surface of transfected cells. FACS was performed using an EPICS Elite system (15-mW argon-ion laser, 488 nm; Beckman Coulter), and PE-positive vs. -negative populations were gated and collected. Each population was washed in OPTI-MEM I medium, cultured for 24 h in OPTI-MEM I medium, 8% FBS, 8% W3CM supplemented with penicillin (200 U/mL), streptomycin (200 µg/mL) and amphotericin B (0.5 µg/mL) (Sigma) and expanded. In luciferase assays, cell lysates were prepared by collecting cells (3×10^6) at 200× *g* for 10 min, washing cells in 10 mL of PBS and preparing lysates in 60 µL of 1% Triton® X-100, 2 mM 1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid, 2 mM dithiothreitol (DTT), 10% glycerol, 25 mM Tris-phosphate, pH 7.8 (Catalog No. E153A; Promega). To ensure lysis, samples were mixed by vortex mixing for 15 min at 23°C, and cleared lysates were prepared by centrifugation at 8000× *g* for 10 min. In luciferase assays, 20 µL of lysate were added to 100 µL of 20 mM tricine, 1.07 mM (MgCO₃)₄Mg(OH)₂·5H₂O, 2.67 mM MgSO₄, 0.1 mM EDTA, 33.3 mM DTT, 270 µM coenzyme A, 470 µM luciferin, 530 µM ATP (Catalog No. E1483; Promega). To control for possible differences in cell number or lysis, lysates were assayed for protein content (BCA Protein Assay; Pierce Chemical, Rockford, IL, USA), and values were used to normalize luciferase activities.

RESULTS

To test for coupled expression of CD4t and a cDNA insert, a luciferase gene was cloned into pIRES-CD4t, and

this construct (Figure 1) was transfected into FDCW2 cells. By intent, FDCW2 cells were used based on their low transfection efficiency and the associated challenge in retrieving derived cell lines that express transfected cDNAs at useful, uniform levels. Transfected cells were subjected to four rounds of MACS (populations MACS 1–4) and were assayed for CD4t expression (Figure 2). Three rounds of MACS yielded cells expressing CD4t at high frequencies (91% PE-RPA-T4 antibody positive), while a fourth round of MACS only slightly increased frequencies of CD4t-positive cells (96%).

Absolute levels of CD4t expression were estimated using PE-conjugated calibration particles. In MACS 4 cells, an average of 34 000 PE-RPA-T4 antibodies were bound per cell, and CD4t expression is estimated at 17 000 molecules per cell. This indicates efficient translation of CD4t from pIRES-CD4t-*luc* and predicts that selected cells should uniformly express linked 5' cDNAs at useful levels. To test this, luciferase expression was assayed in polyclonal FDCW2-pIRES-CD4t-*luc* cells and in sublines isolated by dilution cloning (16) of MACS 4 cells. Upon MACS retrieval, luciferase activity in polyclonal populations increased markedly (Figure 2). In addition, luciferase expression in each of ten clonal sub-lines was comparable to that in MACS 4 cells (Table 1). This result confirms tightly coupled expression of marker and inserted cDNAs. To test stability of expression, luciferase activity also was monitored over a test period of three months. Activity remained high in all cells tested in the absence of further selection (Table 1).

Finally, MACS and FACS were compared directly as methods for isolating stably transfected cells. Here, a MACS 2 population of FDCW2-pIRES-CD4t-*luc* cells was used based on the presence of low but detectable frequencies of CD4t-expressing cells (approximately 1%). FACS was applied, and two gated populations of cells (FACS-positive and FACS-negative cells) were recovered. Cells from each population were expanded and analyzed for CD4t and luciferase expression (Figure 3). FACS increased frequencies of CD4t-positive cells to 67%,

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whereas MACS (performed in parallel) increased frequencies to 87%. In addition, FACS-positive and MACS 3 populations each expressed significantly increased levels of luciferase activity compared to the FACS-negative and MACS 2 populations (16.7 and 7.7 vs. 0.5 and 0.4 relative light units [RLU], respectively). Therefore, MACS was at least as efficient as FACS in selecting transfected CD4t-positive cells.

DISCUSSION

pIRES-CD4t has been developed as an expression vector that combines the advantages of dicistronic expression and MACS. Two features that merit discussion are the choice of CD4t as a marker and the nature of the IRES used to direct translation. Studies of CD4 in human immunodeficiency virus (HIV) infection have led to the development of MAbs with high specificities and affinities as conjugated forms for direct use in MACS and FACS (4). Thus, cells transfected with pIRES-CD4t can be efficiently retrieved and analyzed quantitatively using commercially available reagents. The α CD4-conjugated, ferritin-dextran microbeads used in the present study, in fact, have been applied to isolate CD4⁺ T-cells from peripheral blood lymphocytes in clinical applications (19). Since CD4 expression is restricted largely to developing thymocytes and major histocompatibility complex (MHC) II-restricted mature T-lymphocytes (2), this marker can be used in a wide variety of cells and cell lines.

IRES from EMCV was used since eukaryotic initiation factors eIF-2/2B bind to this IRES with high affinity (15), and because this IRES is perhaps best characterized (7,9). In addition, in pIRES-CD4t, this IRES is engineered to repress the expression of inappropriate fusion proteins, and to attenuate 3' cassette translation (the predominant initiation site AUG-11 is deleted, and a 94-bp spacer cassette shifts translational initiation to the Met¹ of CD4t; Reference 14). This modification also attenuates translation of 3' cassettes within dicistronic transcripts and favors high-level translation of 5'-positioned cDNAs (14). Thus, pIRES-CD4t is a unique dicistronic vector that provides

for the coupled expression of heterologous cDNAs and a 3'-positioned CD4 marker cassette. Transfected cells can be isolated directly by either MACS or FACS, and coordinate expression provides for sustained high frequencies of heterologous cDNA expression in selected cells.

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