Experimental Hematology 24:324–329 (1996)
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Experimental Hematology

Applications of retrovirus-mediated expression cloning

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(Received 2 October 1995; revised 26 October 1995; accepted 1 November 1995)

Abstract

We have recently established a novel expression cloning system using retroviral vectors. The system is based on a high-efficiency packaging cell line, BOSC23, and a simplified retroviral vector, pBabeX, carrying no selection marker. cDNA libraries, constructed in the pBabeX vector, are transiently transfected into BOSC23 cells. The supernatant contains more than 3×10^6/mL, which would cover large complexities of cDNA libraries. The retrovirus stock gave 100% infection efficiency in NIH3T3 cells and 5–40% infection efficiency in various hematopoietic cell lines. In contrast to the conventional expression cloning system, in which it is necessary to transfect cDNA libraries transiently into particular cell types such as COS cells, retrovirus-mediated expression cloning allows us to transduce cDNAs into a wide variety of cell types. This method therefore makes it possible to select cells expressing a cDNA of interest by various functional assays. When combined with polymerase chain reaction (PCR)-driven random mutagenesis, this system is also useful in searching for mutations of various molecules that will result in alterations of their functions.

Key words: Packaging cell line—Retroviral vector—PCR—Random mutagenesis

Introduction

Vectors for cDNA expression in mammalian cells were developed based on the SV40 replication origin and the early gene promoter more than 10 years ago [1]. Since then, this expression system has been successfully used in isolating a variety of genes, including cDNA for cytokines [2,3], cell-surface antigens [4–6], cytokine receptors [7–11], and transcription factors [12]. SV40-ori-bearing plasmids are replicated and amplified in COS cells [13] expressing SV40 large T antigens [1], allowing the plasmids to be recovered by transformation into *Escherichia coli*. The amplification of the plasmid also increases expression of the cDNA in the plasmid. This expression system, however, has limitations due to its dependence on SV40 T antigen. In contrast to transient transfection systems, retroviral gene transfer efficiently delivers genes stably into a wide range of target cells and is expected to overcome the limitations of the conventional expression system. Therefore, it would be ideal if retrovirus can be used for expression cloning. Several groups have recently published works which involve expression cloning using retroviruses [14–16]. All these methods utilized NIH3T3-based packaging cell lines, and two groups used neomycin selection to establish transfected packaging cells. However, it would be difficult to cover large complexities of cDNA libraries using NIH3T3-based packaging cell lines because of the low titers of virus produced by transient transfection of these cells. Moreover, the frequency of each cDNA in the library may change during the drug selection of stable packaging cell lines, and thus the resulting retrovirus stock may not represent the original cDNA library.

To overcome these limitations, we developed a method to efficiently screen cDNA libraries in retroviral vectors (Fig. 1) [17]. In this method, cDNA libraries are constructed in a simplified retroviral vector, pBabeX (Fig. 2A). The retrovirus stock, which is representative of the cDNA library, is produced using a transient retrovirus packaging cell line, BOSC23 [18], with modifications [17]. The supernatants of the packaging cell line, containing high-titer retroviruses ($>3\times10^6$ pfu/mL), are then used to infect hematopoietic cells, and infected cells are selected for expression of the cDNA of interest. We have recently demonstrated that relatively rare cDNAs encoding the human CD2 antigen ($1/10^4$) and the human interleukin-3 receptor α subunit (IL-3Rα) ($1/10^5$) can be easily and effectively isolated by this method [17]. In this paper, we describe the results from some of our expression cloning projects and discuss possible applications using the high-efficiency retrovirus-mediated gene transfer. In addition, we also demonstrate development of a new retrovirus vector, pMX, which gives us more efficient infection and higher expression levels in various cells than the pBabeX vector.

Materials and methods

A packaging cell line BOSC23

An ecotropic retrovirus packaging cell line BOSC23 (ATCC CRL 11554) [18] and an amphotropic cell line Bing (ATCC CRL 11270) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and the appropriate concentrations of GPT selection reagents as indicated by the manufacturer (Specialty Media, Lavallette, NJ). The cells were transferred to DMEM/10% FCS without GPT selection reagents 2 days before transfection.

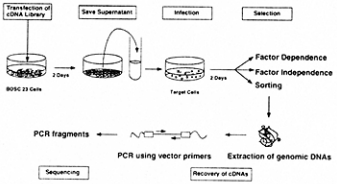

Fig. 1. Procedure of expression cloning using retrovirus cDNA libraries.

Retroviral vectors and constructs

As described before [17], we modified the pBabe-puro vector [19] to develop the pBabeX vector by deleting the SV40 promoter and the puromycin-resistance gene (*Cla* I–*Sal* I fragment) and inserting the multicloning polylinkers with a stuffer to make the vector convenient for cDNA library construction (Fig. 2A). We recently noticed that the MFG vector (Rich Mulligan, Somatix, unpublished) gave us more efficient infection in several cell lines than the pBabeX vector, although the basic structures of the two vectors, both derived from Mo-MuLV, are similar. The MFG vector, however, is not appropriate for cDNA library construction, because its relatively large size will limit the length of the inserts and, more critically, the start codon of cDNA has to be matched to that of the retroviral gag sequence of the vector. One of the characteristics of the MFG vector is its large packaging signal, which is believed to be important for generating high-titer retroviruses. Therefore, we replaced the packaging signal of the pBabeX vector with that of the MFG vector as follows, and designated this fusion vector pMX (Fig. 2B). First, the sequence from the 5′ LTR to the *Nco* I site of the MFG vector was amplified by PCR using a 5′ primer with *Nsi* I site on the end and 3′ primer with *Bam*HI site on the end, which disrupted the start codon of the gag sequence. The purified PCR fragment was then digested with *Nsi* I and *Bam*HI restriction enzyme and ligated between the *Pst* I and the *Bam*HI sites of the pBabeX (Fig. 2B). To construct the test vector carrying a cDNA for the human interleukin-3 receptor α subunit (hIL-3Rα), the cDNA insert was excised from the pME-DUK-1 vector [11] and inserted into the *Eco*RI–*Not* I sites of pBabeX vector (designated pBabeX-DUK) or the pMX vector (designated pMX-DUK).

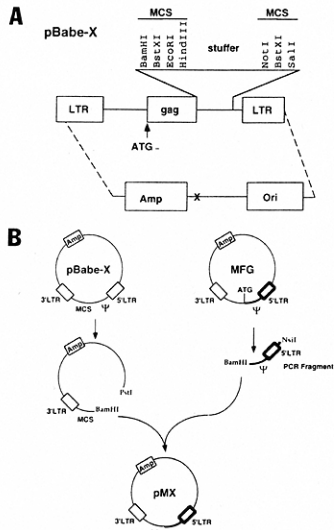

Fig. 2. Structures of the pBabeX and pMX vectors. The pBabeX vector **(A)** was constructed from the pBabe-puro vector [19], as described [17]. The pMX vector **(B)** is a fusion vector of the pBabeX vector and the MFG vector. The sequence derived from the MFG vector is indicated as a bold line including the 5′ long terminal repeat (LTR) and the large packaging signal including a part of the gag sequence. Y = packaging signal; MCS = multicloning site; Amp = ampicilin-resistant gene; Ori = replication origin of pUC.

cDNA library construction

Poly(A)$^+$ RNA was prepared from various cells using FAST-TRACK (Invitrogen). Both unidirectional and bidirectional cDNA libraries were constructed using a cDNA synthesis kit (Pharmacia) or SuperScript Plasmid System (Gibco-BRL), according to the manufacturer's suggestions, with modifications [17]. Briefly, cDNA was synthesized using an oligo dT primer or an oligo dT primer containing *Not* I site at 3′ end for bidirectional or unidirectional cDNA libraries, respectively. For bidirectional libraries, *Bst*XI adapters (Invitrogen) were added to cDNAs, and cDNAs with *Bst*XI adapters were then ligated into *Bst*XI sites of the pBabeX or the pMX vector. For unidirectional libraries, *Eco*RI adapters (Pharmacia) were added to cDNAs, followed by digestion with *Not* I. The resulting cDNAs with *Eco*RI adapters on 5′ end and exposed *Not* I sites on the 3′ end were ligated into *Eco*RI and *Not* I sites of the pBabeX or the pMX vector.

Production of retrovirus stock and infection of recombinant retroviruses to various cell lines

We used the Lipofectamine (Gibco-BRL) procedure to generate high-titer retrovirus stock, and after 2 days the culture supernatant was used for infection of various cell types as described [17]. To monitor the retrovirus titer, we used the

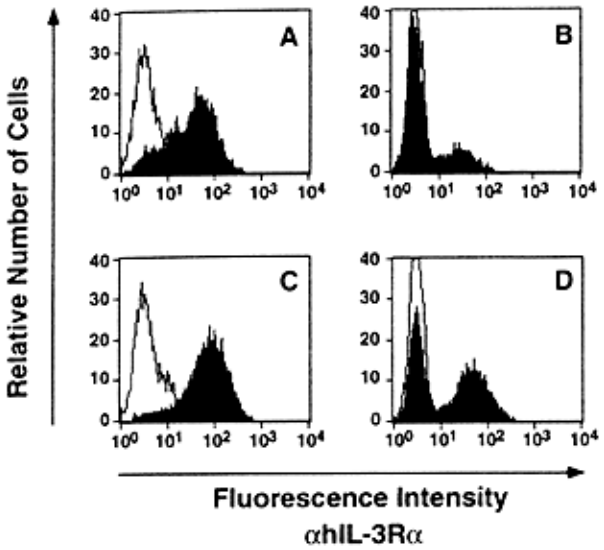

Fig. 3. Infection efficiency of pBabeX or pMX retroviruses. NIH3T3 cells **(A, C)** and Ba/F3 cells **(B, D)** were infected with the pBabeX-DUK virus **(A, B)** or the pMX-DUK virus **(C, D)** and tested for the expression of hIL-3Rα by FACS analysis.

test constructs pBabeX-DUK and pMX-DUK carrying the cDNA for hIL-3Rα. For infection of NIH3T3 cells, 2×10^5 cells were seeded into 60-mm plates the night before infection and incubated with 1.5 mL virus stock for 6–8 hours in the presence of 8 μg/mL polybrene. Then, 1.5 mL fresh DMEM/10% FCS was added to the culture and the incubation continued. The medium containing retroviruses was changed to fresh DMEM/10% FCS 24 hours after the beginning of the infection. After another 24 hours, the cells were removed from the plates after 5-minute incubation in phosphate-buffered saline (PBS)/2mM EDTA at 37°C, stained with anti-IL-3Rα monoclonal antibody (N3A) [20], and subjected to fluorescence-activated cell sorter (FACS) analysis to detect the expression of the introduced hIL-3Rα subunit.

For infection of hematopoietic cell lines, 2×10^5 cells were incubated with 0.5 mL virus stock containing 10 μg/mL polybrene and, if necessary, the cytokine required to support cell growth (mIL-3). After 8 hours, 0.5 mL fresh growth medium was added to the culture and the infection period was extended another 16 hours. Twenty-four hours after infection, cells were washed, re-fed with growth medium, and allowed to grow 1 more day before being subjected to FACS analysis to detect the expression of the transduced hIL-3Rα subunit. Infection of hematopoietic cells with retrovirus stocks derived from cDNA libraries was performed in general as described above.

Sequencing of the integrated retroviruses

To recover retrovirally transduced cDNAs, genomic DNA was isolated from each clone that acquired the phenotype of interest, and cDNA segments were amplified from small amounts (10 ng) of genomic DNAs by PCR using retroviral vector primers. The PCR reaction was run for 35 cycles (1 minute at 94°C, 2 minutes at 58°C, and 3 minutes at 72°C)

Table 1. Infection efficiency of pBabeX and pMX vectors in various cells

Target cells	Cell type	Infection efficiency (%) pBabeX	pMX
Ba/F3	pro B	13.4	50.5
FDC-P1	myeloid	2.1	21.2
DA1a	myeloid	9.4	28.2
LG3	myeloid	3.1	8.3
LGM3	myeloid	3.4	11.8
OTT-1	myeloid	7.6	19.0
B6Mast[a]	mast cells	1.0	12.0
P815	mastocytoma	0.4	10.8
BW5147	thymoma	2.7	100.0

[a]A mast cell line established from bone marrow cells of B6 mice in the presence of IL-3 and stem cell factor (SCF) (Ichihara et al., unpublished).

using *Taq* polymerase (Perkin-Elmer Cetus) and HotWax Magnesium Beads (Invitrogen). For longer cDNAs, we sometimes used the Long Accurate PCR kit (Takara, Kyoto, Japan). The resulting PCR fragment was purified using Qiaex (Qiagen) and sequenced directly using Taq DyeDeoxy Terminator (Applied Biosystems) on an Applied Biosystems model 373A sequencer. When necessary, PCR fragments were cloned into the TA vector (Invitrogen) and then sequenced.

Results

Improved infection efficiency

Efficiencies of the pBabeX vector and the pMX vector were compared using test constructs carrying hIL-3Rα cDNA (pBabeX-DUK and pMX-DUK). In NIH3T3 cells, both constructs resulted in 100% infection, but the expression level was moderately higher in pMX-infected cells, as assessed by fluorescence intensity using the N3A monoclonal antibody (mAb) [20] (Fig. 3). The pMX vector gave much higher infection efficiency compared to the pBabeX vector in all hematopoietic cell lines tested (Fig. 3 and Table 1). In particular, while the pBabeX vector did not work efficiently in BW5147 (a mouse thymoma cell line), the pMX gave 100% infection efficiency in this cell line. Recently, we have reported two model experiments of expression cloning using retrovirus infection in which the cDNAs for the human CD2 and hIL-3Rα were cloned from cDNA libraries derived from human T cell clones and human IL-3-dependent TF-1 cells, respectively [17]. In these experiments, we observed 10–30% infection efficiency of the pBabeX vector in mouse IL-3-dependent Ba/F3 cells. Obviously, increased efficiency of retroviral infection will make the expression cloning system more powerful because we will be able to screen cDNA libraries more efficiently and choose a wider range of target cells. However, efficient infection of replication-defective retroviruses usually results in multiple integrations in individual cells. Therefore, it would be required to examine multiple integrations for the phenotype of interest. In our experience, with infection efficiencies below 20%, most cells harbor only one copy of the retrovirus and multiple integrations are observed in less than 10% of the infected cells. When the infection efficiency

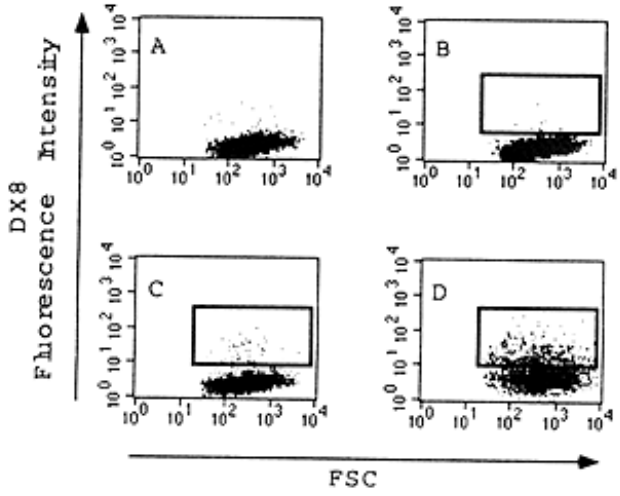

Fig. 4. Cloning of a cDNA for DX8 antigens using retrovirus-mediated expression system. Murine Ba/F3 cells transduced with a human T cell cDNA library were selected by sorting for expression of DX8 antigen. **A.** Control staining of the library-infected Ba/F3 cells using an isotype control. **B.** Staining of the library-infected Ba/F3 cells using the DX8 antibody. **C.** DX8 staining of the Ba/F3 cells after the first sorting. **D.** DX8 mAb staining of the Ba/F3 cells after the second sorting. Boxed regions indicate DX8-positive cells; the cells in the bold boxes were sorted in **B, C,** and **D.**

increases above 30%, we frequently observe multiple integrations of retroviruses per cell. Using conditions yielding 100% infection efficiency, most cells harbor multiple integrations (data not shown). Therefore, we chose either pBabeX or pMX vector in Ba/F3 cells, depending on each experimental requirement. Because the pBabeX and pMX vectors use the same promoter (LTR of Mo-MLV), the difference in the expression level of hIL-3Rα in the infected Ba/F3 cells (Fig. 3 and data not shown) probably reflects the difference in the number of integrated retroviruses.

Expression cloning of cell-surface antigens

As we demonstrated previously, we cloned a cDNA for human CD2 in a model experiment using the retrovirus-mediated expression cloning system [17]. We have recently succeeded in cloning of a cDNA encoding a human leukocyte surface molecule recognized by a mAb called DX8. DX8 mAb recognizes a molecule that is expressed widely in the immune system including T cells, NK cells, B cells, and monocytes and seems to be involved in immune responses (AS, LLL, JP, unpublished). We constructed a cDNA library from a polyclonal human T cell line and infected murine Ba/F3 cells with a retrovirus stock derived from the library. After two cycles of FACS cell sorting for cells reacting with the DX8 mAb, 35% of the Ba/F3 cells were found to express the DX8 antigen (Fig. 4). Twelve clones were isolated from the positive fraction by single-cell sorting, and 11 of them were found to express the DX8 antigen stably (data not shown). Genomic DNAs extracted from two of the 11 DX8-positive Ba/F3 clones were subjected to PCR using the retrovirus vector primers, and a common 0.6-kbp band was

Table 2. Application of retrovirus-mediated expression cloning system

Target molecules	Cloning strategy
Surface antigen	FACS sorting
Cytokine	Autocrine proliferation
Receptor	Factor-dependent growth or survival
Signaling molecule	Complementation
Metastasis-inducing gene	Metastasis in vivo
Differentiation-inducing gene	Differentiation
Activated form of receptors, kinases, and transcription factors	PCR-driven random mutagenesis

detected. The sequence of the PCR fragment was identical to the sequence of the cDNA for human CDw52, a previously characterized PI-linked glycoprotein [21].

Identification of activating mutations in various molecules

Screening for activating mutations in various molecules is another application of the retrovirus-mediated expression system. Retroviral reverse transcriptase spontaneously introduces mutations at low frequencies, and Yoshimura et al. [23] identified activating mutations of the erythropoietin receptors (EpoR) using "ping-pong amplification" of retroviruses carrying the EpoR cDNA. In ping-pong amplification, retrovirus infection is repeated in the mixture of an ecotropic packaging cell line and an amphotropic cell line, thus accumulating mutations caused by multiple reverse transcription during repeated superinfection between the two packaging lines. On the other hand, we used PCR for introduction of random mutations into a particular sequence. Briefly, a target sequence was amplified by PCR using primers containing *Eco*RI or *Not* I on the 5′ or 3′ end, respectively. The resulting PCR fragments were then cut by *Eco*RI and *Not* I and ligated with *Eco*RI and *Not* I sites of the pBabeX or pMX vector. The mixture of the ligated DNA was amplified in *Escherichia coli*, and plasmid DNA was prepared. Thus, we generated retrovirus vector constructs carrying a target cDNA with random mutations. In other words, these retrovirus constructs are a sort of a mutation library for the particular molecule. In some experiments, we introduced random mutations into a specified portion of cDNAs and ligate the PCR fragment into appropriate sites of the original cDNA after digestion with the enzymes. Under the standard PCR conditions, frequency of mutation ranges from 1/500 to 1/1000 in our hands. The retrovirus stock was then generated by transfecting the DNA into BOSC23 cells and used to infect mIL-3-dependent Ba/F3 cells. To identify activating mutations, factor-independent Ba/F3 cells were selected by growth in the absence of mIL-3. Using this strategy, we have recently found an activating mutation in the transmembrane domain of MPL (thrombopoietin receptor) (Onishi et al., manuscript submitted).

Discussion

We have recently established a retrovirus-mediated expression cloning system [17]. One of the important advantages of this method over the conventional COS cell expression cloning is that a single infection can give rise to cells that stably express

the introduced gene. Therefore, once infected, the cells expressing the surface molecules of interest can be easily selected by multiple FACS sortings, as demonstrated in cloning of a cDNA for the human CD2 [17] and a cDNA encoding a glycoprotein recognized by the DX8 mAb (Fig. 4). The most important advantage of the retrovirus-mediated expression cloning system is that any functional assay can be used for selection of cells with a desired phenotype. One straightforward selection is to select cells by either factor-dependent or -independent growth. We have recently demonstrated that a cDNA for hIL-3Rα could be isolated by hIL-3-dependent growth of murine Ba/F3 cells [17]. This selection was based on our previous observation that mIL-3-dependent Ba/F3 cells became hIL-3-dependent when hIL-3Rα was ectopically expressed [22]. Some cytokine receptors may only stimulate survival or short-term growth in some types of cells. Cells transduced with such receptors may also be isolated by modified selection methods. On the other hand, factor-independent growth can be used to identify signaling molecules that are involved in cell proliferation.

In addition to the applications described above, the retrovirus expression cloning system may be applied to a variety of experiments (Table 2). For example, we can search for a ligand for an orphan receptor as follows. A cDNA library is generated from putative producers of the ligand, retroviruses derived from the library are used to an appropriate factor-dependent cell transduced with the orphan receptor of interest, and the infected cells are selected for growth in the absence of any growth factor. By this approach, factor-independent growth due to autocrine factor production allows selection of cells expressing a ligand for the orphan receptor.

The system can also be used for cloning of signal transduction molecules by complementation of signal transduction pathways. For this experiment, a strategy would be to establish mutated cells that are defective in signaling pathways and to complement a particular defect by introducing a retroviral cDNA library derived from cells that do not have the defect in the signaling pathway. Mutated cells can be established by chemical mutagens or by establishing stable transfectants, expressing mutants of cytokine receptors or various signaling molecules, in which a particular pathway is disrupted by the mutation.

In addition, we can even use in vivo selection such as tumor metastasis to isolate metastasis-inducing genes from tumors with high metastatic activity. In this experiment, cDNA libraries of tumors with high metastatic activity can be screened by infecting cDNA viruses to nonmetastatic tumor variant followed by isolation of metastasis in vivo. In summary, the expression cloning system described here, together with an improved retroviral vector, will make many approaches available for a variety of experiments.

Acknowledgments

The authors thank Dr. Alice Mui for valuable discussions and excellent help. DNAX Research Institute of Molecular and Cellular Biology is supported by Schering-Plough. SK is supported by Toyobo Biotechnology Research Fund and currently the Irvington Institute. GPN is a special Fellow of the Leukemia Society of America and is supported by NIH grant AI35304.

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