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Green Fluorescent Protein-reporter Mammalian One-hybrid System for Identifying Novel Transcriptional Modulators for Human p14^{ARF} Tumor Suppressor Gene

Hye Jin Lee, Dong Hwa Yang, Tae Hee Yim, Byung Kirl Rhee, Jung-Wook Kim, Jungwoon Lee, Jin Bae Gim, and Jungho Kim*

Department of Life Science, College of Natural Sciences, Sogang University, Seoul 121-743, Korea

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To improve conventional yeast one-hybrid screening, we have developed an efficient mammalian one-hybrid system that allows rapid isolation of complementary DNAs which are able to induce human p14^{ARF} tumor suppressor gene. A 1.5 kb promoter region of p14^{ARF} was fused to EGFP to generate ARF promoter-EGFP reporter vector. This reporter plasmid was stably transfected into NIH3T3 cells for generation of reporter cell line. When the reporter cell line was infected with E2F-1 together with excess amounts of empty vector, the cells that received the positive modulator were readily identifiable by green fluorescence using FACS. The GFP-positive cells were cloned directly from the cultured cells and expanded in bulk culture. The genomic DNAs from GFP-positive cells were prepared and the cDNA insert in integrated retroviral genome was recovered by PCR using primers annealing to the retroviral vector sequences flanking the insert-cloning site. This system should be useful for efficient screening of expression cDNA libraries in mammalian cells to identify novel upstream regulators for specific genes by one-hybrid interaction.

For several decades, DNA-binding protein has been intensively studied because of their involvement in several cellular processes including gene expression and DNA repair. One of promising approaches to obtain cDNA clones that encode DNA binding proteins is yeast one-hybrid interaction screening (Luo et al., 1996; Wang and Reed, 1993; Wilson et al., 1991). This analysis has naturally led to the detection and isolation of novel proteins that bind to a known target sequence element. The one-hybrid system is also applied to the anaylsis of DNA-protein interactions mediating transcription under *in vivo* conditions (Luo et al., 1996; Wang and Reed, 1993; Wilson et al., 1991).

Like the related yeast two-hybrid system, yeast one-hybrid is also based on the bimolecular structure of eukaryotic transcription factors, consisting of a transcription activation domain and a DNA binding domain. Tandem copies of putative transcription regulatory DNA elements are cloned upstream of a reporter gene in a reporter vector. The second component of the yeast one-hybrid system is the so-called target vector, a cDNA library vector, expressing a fusion protein of a constant activation domain and a variable DNA binding domain, encoded by the respective cDNA. Yeast cells are transformed with both vector components and interaction between the hybrid protein and target DNA sequence in the reporter vector can be detected by reporter gene expression (Sieweke, 2000). In 1993, the yeast one-hybrid assay was first used to clone the gene encoding the transcription factor OLF-1 (Wang and Reed, 1993). This method has since been used to obtain genes encoding several other transcription factors, including REST and ORC-6 (Gstaiger et al., 1995; Lehming et al., 1994; Li and Herskowitz, 1993; Luo et al., 1996; Strubin et al., 1995).

However, the one-hybrid screening system using yeast cells has some limitations. Some of interactions between mammalian proteins and target DNA sequences may not occur in the yeast milieu because of possible lack of associating factors or protein modifications (such as signal-induced phosphorylation) or correct protein folding. Therefore, it seemed desirable to develop an improved screening system with mammalian cells to identify binding proteins that are difficult to detect by the yeast system. To improve the conventional yeast one-hybrid system, we developed a green fluorescent protein-reporter mammalian one-hybrid system. We selected human p14^{ARF} gene (Weber et al., 2000) to test whether the mammalian one-hybrid system works properly in identifying novel DNA binding proteins that recognize regulatory sequence elements. A promoter region of p14^{ARF} was fused to enhanced

* To whom correspondence should be addressed.
Tel: 82-2-705-8461, Fax: 82-2-716-2092
E-mail: jkim@sogang.ac.kr

green fluorescent protein (EGFP), and stably integrated into a genomic DNA region of the reporter cell line. In this paper, we introduce mammalian one-hybrid screening approach that should be useful for isolating novel genes encoding proteins that bind to a target, *cis*-acting regulatory element, in mammalian system.

Materials and Methods

Materials and general methods

Restriction endonucleases, calf intestinal alkaline phosphatase, the Klenow fragment of DNA polymerase I, and T4 DNA ligase were purchased from New England Biolabs. Preparation of plasmid DNA, restriction enzyme digestion, agarose gel electrophoresis of DNA, DNA ligation, and bacterial transformations were carried out using standard methods (Sambrook and Russell, 2001).

Reporter plasmid construction

pEGFP-N1 plasmid (Clontech) was digested with Ase I and Xho I, blunted with Klenow enzyme, and ligated to remove CMV promoter region. The ARF promoter-EGFP reporter vector was constructed by cloning a 1.5 kb fragment of the promoter region of $p14^{ARF}$ from pKR21A-4 plasmid (kindly provided by Dr. Keith Robertson, EGRC/NCI/NIH) into promoter-less pEGFP-N1. The complete insert of the reporter vector was sequenced to exclude the presence of unexpected mutations in the promoter region.

Generation of ARF promoter-EGFP reporter cell line

NIH3T3 cells were purchased from American Type Culture Collections and maintained in DMEM supplemented with 10% heat-inactivated fetal calf serum (GIBCO-BRL), penicillin, and streptomycin in a humidified atmosphere of 5% CO_2 at 37℃. The ARF promoter-EGFP reporter cell line was generated by Lipofectamin (GIBCO-BRL) transfection of NIH3T3 cells with ARF promoter-EFGP reporter plasmid. Transfected cells were selected and cloned in medium with Geneticin (GIBCO-BRL) at 600 µg/mL.

Retroviral infections, flow cytometry, and retrovirus rescue

High-titer ecotropic retroviruses were generated by transient transfection using the Phoenix retrovirus packaging system (G. Nolan, Stanford University) as described previously (Serrano et al., 1997). Twenty-four h after transfection, the virus supernatants were collected and immediately used to infect ARF promoter-EGFP reporter cell lines. Forty-eight h after the infections were initiated, cells were trypsinized, suspended at 10^7 cells/mL in DMEM containing 10% FBS, and analyzed on a FACScan cytometer (Becton-Dickinson).

Rescued retroviral supernatants were generated by lipofection of PCL-eco vector (Inder Verma, Salk Institute) into isolated positive monoclonal reporter cell lines. Supernatants were collected 48 h post-transfection and immediately used for infection.

Isolation of cDNA insert

From each positive monoclonal clone, genomic DNAs were isolated by using standard procedure (Laird et al., 1991) and analyzed further by polymerase chain reactions (PCRs) with retrovirus-specific primers (5′ AGCCCTCACTCCTTCTCTAG 3′ and 5′ ATGGCGTT-ACTTAAGCTAGCTTGCCAAACCTAC 3′) and DNA sequencing to isolate and identify inserted cDNA, respectively.

Results and Discussion

A newly designed one-hybrid system using mammalian cells

To overcome limitations we designed a novel one-hybrid system using mammalian cell. New reporter cell lines having a sequence of a specific target element upstream of the reporter gene were prepared to conduct this assay. Instead of *HIS3* gene used in the yeast one-hybrid system, EGFP was selected as a reporter gene because it is easy to isolate EGFP positive mammalian cells using FACS. The green fluorescent protein (GFP) reporter system has many advantages. GFP of the jellyfish *A. victoria* is activated *in vivo* by an energy transfer via Ca^{2+}-stimulation of the photoprotein aequorin (Crameri et al., 1996). The blue light generated by aequorin excites GFP and results in emission of green light. GFP itself consists of 238 amino acids and is synthesized as an apoprotein in which post-translational formation of the chromophore occurs in an O_2-dependent manner independent of any other gene products (Chalfie et al., 1994; Cubitt et al., 1995). It maximally absorbs light at 395 nm and has an emission peak at 509 nm. The nonsubstrate requirement for GFP activity makes this protein an attractive reporter for gene expression studies. In addition to the non-invasiveness of GFP detection, the protein is very stable, non-toxic, and resistant to photobleaching. These properties make GFP an available alternative to traditional reporter genes such as luciferase or CAT, which require a substrate for their detection. Several modifications of the wild-type GFP cDNA have been engineered with optimized codon usage, improved fluorescence activity, and red- or blue-shifted variants with altered excitation maxima intended for fluorescence microscopy (Cubitt et al., 1995).

Experimental strategy

Fig. 1 describes the schematic drawing of reporter cell line for the mammalian one-hybrid system. Briefly, a 1.5 kb promoter region in human $p14^{ARF}$ tumor suppressor gene was chosen as a target regulatory element.

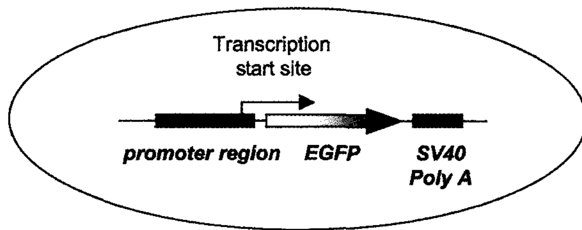

Fig. 1. Diagram of the ARF promoter-EGFP reporter cell line. A 1.5 kb of human p14^{ARF} promoter region was linked at 5′ of EGFP cDNA to generate a mammalian one-hybrid reporter construct.

Recombinant plasmid was constructed to express EGFP in response to p14^{ARF} gene activation. To generate EGFP reporter cell lines, NIH3T3 cells were transfected with ARF promoter-EGFP reporter plasmid and selected in medium containing G418.

The overall strategy for the mammalian one-hybrid screening is presented in Fig. 2. In order to produce infectious virus particles that carry the gene of interest, expression cDNA libraries are transfected into packaging cell lines that harbor a source of the viral proteins that are required in *trans* for virus production. To identify genes that are able to modulate the p14^{ARF} expression, retroviral cDNA library is introduced into ARF promoter-EGFP reporter cell lines and positive cells are analysed by flow cytometry. Target cells displaying the EGFP signals are isolated and the gene is subsequently recovered by PCR using genomic DNA from the positive cells.

Evaluation of the mammalian one-hybrid system

For the present approach to work, activation of the reporter gene transcription must be dependent on protein-DNA complex formation mediated by interaction between novel transcription modulators and the promoter DNA. Human p14^{ARF} gene was selected to test whether the mammalian one-hybrid system is viable for identifying novel DNA binding proteins that recognize regulatory sequence in promoter region. p14^{ARF} is a tumor suppressor gene that, when overexpressed, can cause cell cycle arrest in both the G1 and G2 phases of the cell cycle (Quelle et al., 1995).

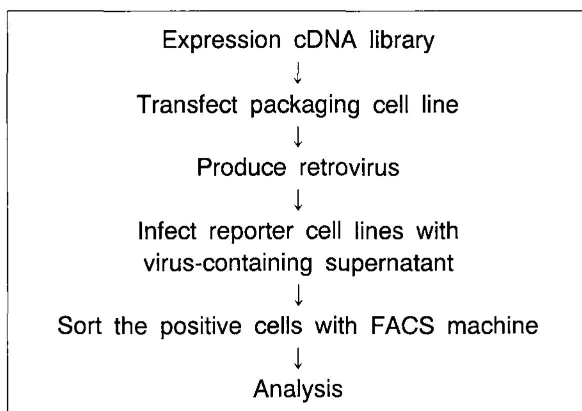

Fig. 2. Strategy for mammalian one-hybrid screening using expression cDNA libraries. The p14^{ARF} promoter-EGFP reporter cells are infected by expression retroviral cDNA libraries and the positive cells are sorted using FAC. After expansion to mass culture, genomic DNA is isolated and subjected to cDNA rescue by PCR. See Results and Discussion for general description.

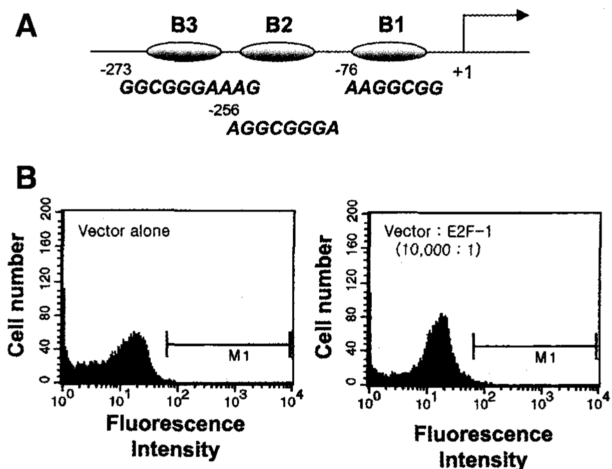

Fig. 3. Schematic presentation of putative E2F-1 binding sites in human p14^{ARF} promoter and flow cytometric profiles of reporter cell lines infected with retroviruses. A, The positions of three E2F-1 binding sites in the human p14^{ARF} proximal promoter region. Potential E2F-1 binding sites are denote as B1, B2, and B3. The transcription start site is indicated by a bent arrow and is defined as position +1. B, Primary FACS profiles of ARF promoter-EGFP reporter cell lines infected with insert-free vector (left panel) or mixture of pLIB-based retroviruses carrying E2F-1 cDNA and without an insert (1:10^4 ratio, right panel). Fluorescence intensity is shown in logarithmic scale on axis X and cell counts are shown in linear scale on axis Y.

E2F-1 was chosen as a model system for cloning cDNAs for upstream modulator proteins. E2F-1 is a known p14^{ARF} modulator and is characterized as a critical component of normal cell cycle regulator. In addition, it has been shown that the p14^{ARF} promoter was found to be highly responsive to E2F-1 as has been observed previously at the RNA level (DeGregori et al., 1997). As shown in Fig. 3A, the proximal region of human p14^{ARF} promoter contains at least three putative E2F-1 binding sites.

Infection of NIH3T3 reporter cell lines with the retroviral supernatants produced by transient transfection resulted in the transfer of functional viruses to about 98% of the cells, as measured by EGFP expression (data not shown). To test whether representative transfer of mixed cDNA populations could be accomplished, we set up a reconstruction/competition experiment with a lacZ-encoding virus vector and a neomycin-resistance gene-encoding virus vector. The results indicated that we could readily detect a neomycine-resistant retrovirus at a ratio of 1 in 10^7 after transfection of Phoenix cells with a mixture of pBabePuro-Neo and pBabePuro-LacZ (data not shown).

We therefore proceeded mammalian one-hybrid screening with pLIB-E2F-1. To evaluate the ability to

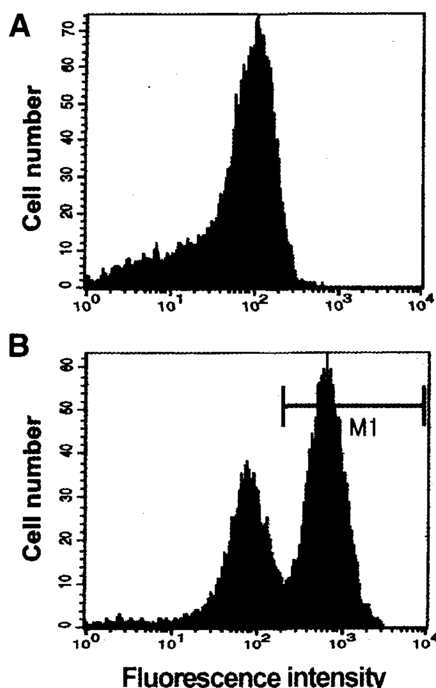

Fig. 4. Secondary FACS profiles of expanded fraction. A, FACS profile of control ARF promoter-EGFP reporter cell line. B, Secondary FACS analysis of positive fraction of ARF promoter-reporter EGFP reporter cell lines infected with retroviral mixture of empty vector and E2F cDNA after expansion to mass culture. M1 portion was re-collected and cultured in DMEM medium for further characterization. Horizontal bar defines the area corresponding to cells collected during the second sorting. Green fluorescence intensity is shown in logarithmic scale on axis X, cell counts are shown in linear scale on axis Y.

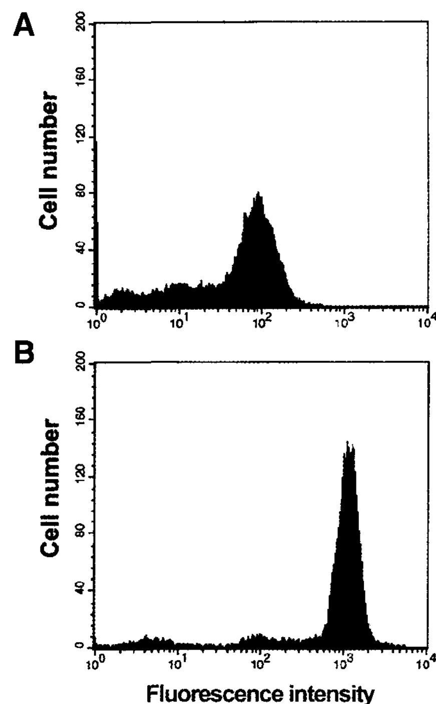

Fig. 5. Flow cytometry of a representative monoclonal cell line. The plot of ARF promoter-EGFP reporter cell line (A) and one of positive monoclonal cells isolated from M1 portion in secondary FACS (B). The isolated monoclonal cells show the strong fluorescence of EGFP. Fluorescence intensity is shown in logarithmic scale on axis X and cell counts are shown in linear scale on axis Y.

recover an E2F-1 cDNA from a mixture containing excessive amounts of other cDNA expression plasmids, retroviral E2F-1 cDNA was mixed with LIB retroviral vector DNA. The ratio of pLIB retroviral vector to pLIB-E2F-1 cDNA was 10^4. Transient transfection of retroviral packaging cell lines was used to produce a mixture of retroviruses. With calcium phosphate-mediated transfection protocol combined with chloroquine, titers of about 10^7~10^8 infectious retroviruses per ml, as determined by puromycin resistance, were obtained with the Phoenix packaging cell line and pLIB-puro (data not shown).

The reporter cell lines were subsequently infected with retroviruses, subjected to cell sorting, and GFP positive portions (M1) were collected (Fig. 3B). The sorted cells (2×10^3 cells out of total 1×10^7 cells) were expanded for 10 d in a bulk culture and reanalyzed by FACS (Fig. 4). 60.3% of the re-sorted cells were positive for GFP (compare Fig. 4B to Fig. 4A). These cells were subjected to a third round of sorting and the sorted cells were again expanded in bulk culture. These cells were nearly 100% positive for GFP (data not shown).

The GFP-positive cells were then subjected to single-cell sorting, and 20 subclones were isolated. The sorted single clones were expanded in 96-well plate and subjected to further characterization. As shown in Fig. 5, one of representative single clone cell line showed strong green fluorescence intensity compared to the parental reporter cell line (compare Fig. 5B to Fig. 5A). To perform an unbiased test for cell lines that show strong green fluorescence signal, the infected replication defective retroviruses were rescued with PCL-eco plasmid. For this experiment, isolated single clones were transiently transfected with PCL-eco rescue plasmid. After 48 h, the supernatant was collected and added to fresh reporter cell lines. These cells were then analyzed for the expression of GFP by flow cytometry. As shown in Fig. 6, 54.6% of reporter cells were positive for GFP after infection with rescued retroviruses from isolated single positive clone. Genomic DNAs extracted from three representative subclones gave rise to a common band after PCR amplification using retroviral vector primers (Fig. 7). These PCR products were sequenced and were confirmed to be identical to the human E2F-1 cDNA (data not shown).

Combination of mammalian one-hybrid screening and expression cloning

In summary, we have demonstrated a mammalian one-hybrid approach that should be applicable to interacting screening between target DNA sequence and regulatory protein. The interaction between transcription

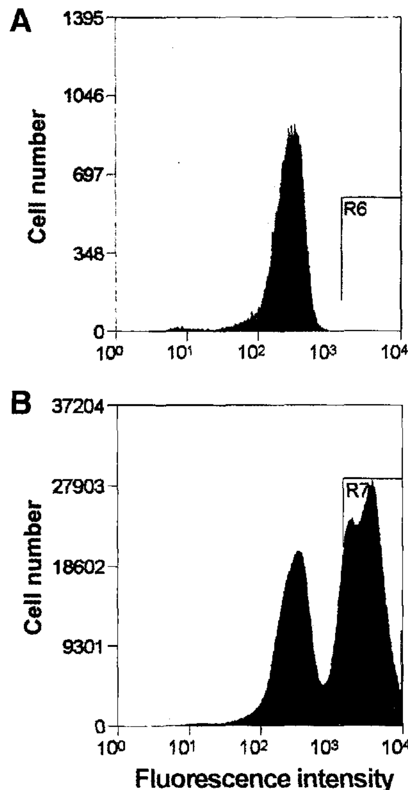

Fig. 6. Result of a retroviral rescue experiment in which ARF promoter EGFP reporter cell lines were infected with rescued retroviral supernatants from a control reporter cell line (A) or a positive monoclonal clone isolated from M1 portion after mobilizing retroviruses (B). The mobilized retroviruses were obtained by transfecting PCL-eco rescue plasmid. Fluorescence intensity is shown in logarithmic scale on axis X and cell counts are shown in linear scale on axis Y.

factor and target sequence was detected by expression of the EGFP reporter gene integrated stably into the chromosome of a mammalian cell line. The prey cDNA sequence was readily isolated by PCR of genomic DNA from the EGFP positive monoclonal cells by using vector annealing primers.

In addition, we have also applied expression cloning that is another powerful tool with which to isolate a cDNA of interest when a phenotypic function of a protein is known but its amino acid sequence is not. The use of retroviral vectors for expression cloning has several advantages over traditional methods. Recent advances in viral packaging systems ensure that virtually any mitotic cell type can be transduced with efficiencies approaching 100%. The copy number of individual cDNA expression cassettes can be easily controlled by varying the multiplicity of infection (MOI). Thus, populations of infected cells may be generated in which greater than 90% of the cells are transduced with 1-5 individual cDNAs per cell, greatly reducing the time and labor of isolating the gene of interest. Thus, our approach should prove to be an efficient method to perform mammalian one-hybrid assays for the purpose of interacting screening.

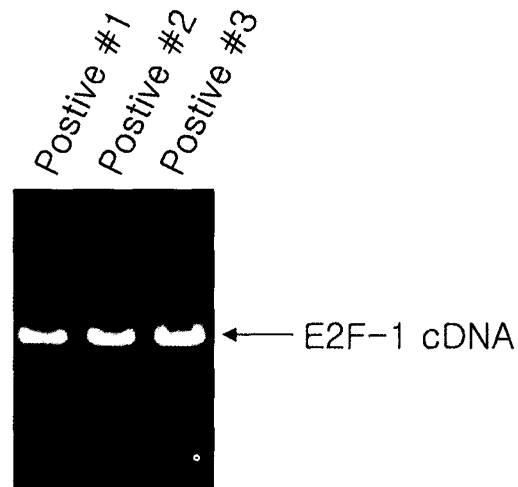

Fig. 7. PCR amplifications of E2F-1 cDNA from the positive monoclonal cell lines. PCR products were fractionated on a 0.8% agarose gel. Three representative experiments are shown.

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