Preface

The chemokines family of small proteins are involved in numerous biological processes ranging from hematopoiesis, angiogenesis, and basal leukocyte trafficking to the extravasation and tissue infiltration of leukocytes in response to inflammatory agents, tissue damage, and bacterial or viral infection. Chemokines exert their effects through a family of seven G-protein coupled transmembrane receptors. Worldwide interest in the chemokine field surged dramatically early in 1996, with the finding that certain chemokine receptors were the elusive coreceptors, required along with CD4, for HIV infection.

Today, though over 40 human chemokines have been described, the number of chemokine receptors lags behind—only 17 human chemokine receptors have been identified so far. What has emerged over the years is that most chemokine receptors bind several distinct ligands, and indeed the majority of chemokines are able to bind to multiple chemokine receptors, explaining to some extent the apparent disparity in the numbers of chemokines and receptors. Yet in spite of the apparent redundancy in chemokine/chemokine receptor interactions, it is clear that in vivo, spatial, temporal, and indeed cell- and tissue-specific expression of both chemokines and their receptors are important factors in determining the precise nature of cellular infiltrates in physiological and pathological processes.

Understanding chemokines and chemokine receptor biology requires a melange of research activities in many different disciplines including molecular biology, protein chemistry, and immunology. The aim of *Chemokine Protocols* is to make readily available, in one volume, the principal techniques used by leading researchers in the chemokine field. We have attempted to cover all aspects of chemokine biology ranging from the cloning and characterization of chemokines and their receptors, through to the use of animal models to study chemokine function in vivo. Each chapter also includes relevant background information, as well as providing a useful bibliography for more detailed analysis, making the study of chemokines accessible at all levels of experience.

The editors would like to thank Ms. Nadine Huber for her excellent assistance with the preparation of the manuscripts, and Serono Pharmaceutical Research Institute for covering the cost of the color artwork. We would also thank Dr. Manuel Peitsch and Dr. Jeff Shaw for molecular modeling.

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Cloning of Novel Chemokines Using a Signal Sequence Trap Method

Toshio Imai

1. Introduction

Precursors of most secreted and cell surface molecules carry signal sequences at their amino termini. The method coined as signal sequence trap ***(1–3)*** takes advantage of the presence of N-terminal signal sequences in most precursor forms of secretory proteins and transmembrane proteins, which are necessary for the proper orientation of the N-terminal of mature forms inside endoplasmic reticulum and exocytotic vesicles. This method enables to selectively clone cDNA species encoding intercellular signal-transducing molecules without biologic assays. In this chapter, an efficient signal sequence trap method based on an Epstein-Barr virus shuttle vector is described ***(2)***.

5' portion-enriched cDNAs are synthesized using the 5' rapid amplification of *c*DNA ends (RACE) technique ***(4)*** as follows. First-strand cDNAs are synthesized from poly(A)+ RNA by using random hexanucleotide primers and reverse transcriptase. After mRNA templates are destroyed by alkali-treatment, an anchor oligo(dC) sequence is added to the 3' ends of cDNAs by using terminal deoxynucleotidyl transferase and dCTP. The second-strand DNA is synthesized by priming with an anchor primer which contains a *Sal*I site and an oligo(dG) tail. The double-stranded DNAs are sonicated to prepare short fragments. After blunting with T4 DNA polymerase, adapters are ligated to the fragments. DNA fragments are separated by agarose gel electrophoresis, and fragments consisting of 300–600 bp are recovered. The fragments that contained 5' noncoding regions and partial coding regions are then amplified by PCR using primers complementary to a portion of the anchor primer and the ligated adapter. Amplified DNA fragments are digested with *Sal*I and *Xba*I, separated by gel electrophoresis, and recovered from agarose gels.

From: *Methods in Molecular Biology, vol. 138: Chemokine Protocols*
Edited by: A. E. I. Proudfoot, T. N. C. Wells, and C. A. Power © Humana Press Inc., Totowa, NJ

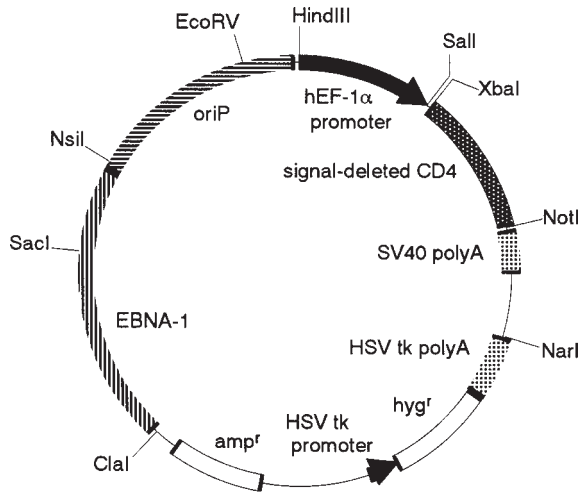

Fig. 1. Schematic diagram of the signal sequence trap vector pDREF-CD4ST. pDREF-CD4ST contains the EF-1α promoter, signal sequence-deleted CD4, the hygromycin resistant gene for selection (hygr), the EBNA-1 gene, and the EBV origin for episomal replication (oriP). 5' terminal-enriched cDNAs are inserted between *Sal*I and *Xba*I sites and expressed as fusion proteins with signal sequence-deleted CD4.

An expression library is constructed by inserting 5' portion-enriched cDNAs into the *Sal*I and *Xba*I sites upstream of signal sequence-deleted CD4 cDNA in an Epstein-Barr virus trapping vector, pDREF-CD4ST (**Fig. 1**) ***(2)***. This plasmid vector allows highly efficient transformation of human lymphoblastoid Raji cells with full representation of an expression library. Exogenous signal sequences allow CD4 fusion proteins to be expressed on the cell surface if cloned in frame. The advantage of this stable expression system is that cells positive for the reporter protein (CD4) can be enriched repeatedly by cell sorting. Furthermore, Epstein-Barr virus shuttle vector that is maintained as episomes can be readily isolated from transformants ***(5,6)***.

After electroporation into Raji cells, CD4 antigen-positive cells are enriched by repeated cell sorting and plasmids are recovered in *Escherichia coli*. Individual plasmids are retransfected and clones that induce expression of CD4 antigen on the cell surface were identified. Positive clones are sequenced and their deduced amino acid sequences are examined for the presence of a signal peptide. If a stretch of hydrophobic amino acids is detected immediately

following the initiator methionine ***(7)***, their putative protein products are analyzed by using computer program designated to search for signal peptides ***(8)***.

2. Materials

2.1. Synthesis of 5' Portion-Enriched cDNA

1. For first-strand cDNA synthesis: SUPERSCRIPT II RT reverse transcriptase (200 U/µL) supplemented with 5X RT buffer (250 m*M* Tris-HCl, pH 8.3, 375 m*M* KCl, 15m*M* $MgCl_2$) (Gibco-BRL), random hexamers (50 ng/µL), 0.1 *M* DTT, 10 m*M* dNTP mix, and DEPC-treated water. These solutions should be prepared as RNase-free. We utilize solutions supplemented with cDNA synthesis kit (Gibco-BRL)
2. For mRNA degradation; 6 *N* NaOH, 6 *N* acetic acid
3. Terminal deoxinucleotidyl transferase (TdT) (15 U/µL) supplemented with 5X TdT buffer (200 m*M* potassium cacodylate, pH 7.2, 10 m*M* $CoCl_2$, 1 m*M* DTT) (Gibco-BRL).
4. For second-strand DNA synthesis: Oligo-dG anchor primer (5'-CTACTA CTACTAGGCCACGCGTCGACTAGTAC(G)16-3'), *E. coli*. DNA polymerase I (10 U/µL) (Gibco-BRL), 10X Pol I buffer (100 m*M* Tris-HCl, pH 7.5, 100 m*M* $MgCl_2$, 100 m*M* DTT), and T4 DNA polymerase (10 U/µL) (Gibco-BRL).
5. For Adapter ligation: T4 DNA ligase (1 U/µL) supplemented with T4 DNA ligase buffer (Gibco-BRL), Uni-Amp Adaptor, *Xba*I (Clontech, Palo Alto, CA).
6. For purification of cDNA fragments from agarose gels: PCR Prep Kit (Promega).
7. For PCR: Taq DNA polymerase I (5 U/µL) supplemented with 10X PCR buffer (200 m*M* Tris-HCl, pH 8.4, 500 m*M* KCl) and 25 mM $MgCl_2$ (Takara), UAP1 Primer (5'-CCTCTGAAGGTTCCAGAATCGATAG-3'), UAP2 primer (5'-CTA CTACTACTAGGCCACGCGTCGACTAGTAC-3')
8. For restriction enzyme digestion: 10X T buffer (T4 DNA polymerase buffer): 330 m*M* Tris-acetate, pH 7.9, 660 m*M* K-acetate, 100 m*M* Mg-acetate, 5 m*M* DTT and 200 m*M* spermidine (Sigma). Restriction enzymes; *Sal*I and *Xba*I (Takara).
9. Other solutions: TE (10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA); Phenol/CIAA saturated with TE; 2 m*M* dCTP; 7.5 *M* NH_4OAc; EtOH; 70% EtOH, and sterile water.

2.2. Construction Signal Sequence Trap Library

1. Signal sequence trapping vector: pDREF-CD4ST vector contains EF-1 alpha promotor ***(9)***, signal sequence-deleted CD4 gene, hygromycin resistance gene, EBV oriP, EBNA-1 gene, and ampicillin resistance gene. This vector can be distributed upon request.
2. For preparation of vector fragment: Chromaspin TE-1000 (Clontech).
3. For electroporation: ELECTROMAX DH10B competent cells with pUC119 control plasmid (Gibco-BRL). We use Gene Pulser (Bio-Rad, Richmond, CA) equipped with Pulse Controller and *E. coli* Pulser cuvet with 0.1-cm gap (Bio-Rad) for electroporation of *E. coli*.

4. Primers used for analysis of insert DNA: EF seq F primer (5'-CCTCAGA CAGTGGTTCAAAG-3' and CD4 ST seq R primer (5'-TGTACAGGTCA GTTCCACTG-3')
5. Other solutions: yeast tRNA (1 μg/μL), S.O.C. medium (Gibco-BRL), LB medium, 1.2% agar-LB plate containing 100 μg/mL ampicillin.

2.3. Signal Sequence Trap

1. Monoclonal antibody (mAb) to human CD4: OKT4 (ATCC) and FITC-Leu3a (Becton Dickinson).
2. Sheep antimouse IgG-coated magnetic beads (Dynabeads M450, Dynal #11001).
3. Magnetic separator (Advanced Magnetics, Cambridge, MA).

3. Methods

3.1. Synthesis of 5′ Portion-Enriched cDNA

3.1.1. First-Strand cDNA Synthesis

1. Add 3 μL (150 ng) of random hexamers to a sterile 1.5-mL microcentrifuge tube. Add 5 μg of poly(A)$^+$ RNA, and DEPC-treated water to a total vol 10 μL. Heat the mixture to 70°C for 10 min, and quick-chill on ice. Spin down the reaction mixture, and add the following: 4 μL 5X RT buffer; 2 μL 0.1 *M* DTT; 1 μL 10 m*M* dNTP mix.
2. Incubate at 37°C for 2 min. Add 3 μL of SUPERSCRIPT II (200 U/μL). Incubate at 37°C for 1 h, then place on ice. Spin down the reaction mixture.

3.1.2. RNA Hydrolysis

1. Add 1.5 μL of 6 *N* NaOH to the 20 μL of cDNA and incubate at 70°C for 20 min to hydryze mRNA. Add 1.5 μL of 6 *N* acetic acid and 127 μL of TE.
2. Add 150 μL of Phenol/CIAA, vortex thoroughly, and centrifuge at room temperature for 5 min at 10,000*g*. Carefully remove 140 μL of the upper aqueous layer, and transfer it to a new 1.5-mL microcentrifuge tube.
3. Add 70 μL of 7.5 *M* NH4OAc, followed by 500 μL of absolute EtOH (–20°C). Incubate at –20°C for 30 min. Centrifuge at room temperature for 10 min at 10,000*g*.
4. Remove the supernatant, and overlay the pellet with 0.5 mL of 70% EtOH (–20°C). Centrifuge at room temperature for 2 min at 10,000*g*, and remove the supernatant.
5. Dry the cDNA at 37°C for 10 min to evaporate residual ethanol, and dissolve the cDNA in 16.5 μL of sterile H_2O.

3.1.3. TdT Tailing of cDNA

1. Add the following to a new 0.5-mL microcentrifuge tube: 16.5 μL cDNA sample; 5.0 μL 5X TdT buffer; 2.5 μL 2 m*M* dCTP.
2. Incubate at 94°C for 3 min, and quick-chill on ice. Spin down the reaction mixture.

3. Add 1 µL of TdT (15 U/µL) and incubate at 37°C for 10 min. Heat inactivate the TdT at 70°C for 10 min. Spin down the reaction mixture and place on ice.

3.1.4. Second-Strand cDNA Synthesis

1. Add 1 µL (500 ng) of Oligo-dG anchor primer to the TdT reaction mixture.
2. Incubate at 70°C for 10 min, and quick-chill on ice. Spin down the reaction mixture, and add the following: 100 µL sterile H_2O; 15 µL 10X Pol I buffer; 5 µL 10 m*M* dNTP mix; 5 µL *E. coli* DNA polymerase I (10 U/µL);
3. Incubate at 16°C for 2 h, then place on ice. Spin down the reaction mixture.

3.1.5. Fragmentation of Second-Strand cDNA

1. Sonicate the cDNA for 30 s in ice-water, chill on ice for 30 s, and spin down the reaction mixture. Repeat sonication for 5 times.
2. Incubate at 16°C for 5 min and add 2 µL of T4 DNA polymerase (5 U/µL). Incubate at 16°C for 5 min, then place on ice.
3. Add 150 µL of Phenol/ CIAA, vortex thoroughly, and centrifuge at room temperature for 5 min at 10,000*g*. Carefully remove 140 µL of the upper aqueous layer, and transfer it to a new 1.5-mL microcentrifuge tube.
4. Add 70 µL of 7.5 *M* NH_4OAc, followed by 500 µL of absolute EtOH (–20°C). Incubate at –20°C for 30 min. Centrifuge at room temperature for 10 min at 10,000*g*.
5. Remove the supernatant, and overlay the pellet with 0.5 mL of 70% EtOH (–20°C). Centrifuge at room temperature for 2 min at 10,000*g*, and remove the supernatant. Dry the cDNA at 37°C for 10 min to evaporate residual ethanol.

3.1.6. Adaptor Ligation

1. Add the following to cDNA pellet on ice: 5 µL sterile H_2O; 4 µL 5X T4 DNA ligase buffer; 10 µL Uni-Amp Xba I adaptor (2 µ*M*);1 µL T4 DNA ligase (1 U/µL).
2. Incubate at 16°C overnight.
3. Incubate at 70°C for 10 min, and quick-chill on ice. Spin down the reaction mixture.
4. Electrophorese on a 2% low melting agarose gel.
5. Recover the cDNA fragments ranging in size from 300–600 bp by PCR Prep Kit (Promega).
6. Elute the fragments with 50 µL of TE.

3.1.7. PCR Amplification of cDNA fragments

1. Add the following to a 0.5-mL microcentrifuge tube on ice: 5 µL cDNA fragments; 5 µL 10X PCR buffer; 3 µL 25 m*M* $MgCl_2$; 1 µL 10 m*M* dNTP mix; 1 µL UAP1 primer (10 µ*M*); 1 µL UAP2 primer (10 µ*M*); 33.5 µL Sterile H_2O.
2. Incubate at 94°C for 3 min and add 1 µL of Taq DNA polymerase (5 U/µL).
3. Perform 25 to 30 cycles of PCR amplification: Denature, 94°C for 30 s, Anneal, 60°C for 1 min; Extension, 72°C for 2 min. Incubate at 72°C for 5 min following the last cycle of PCR, then maintain at 4°C.

3.1.8. Digestion of the Amplified Fragments with Restriction Enzymes

1. Electrophorese amplified fragments on a 2% low melting agarose gel.
2. Recover the cDNA fragments ranging in size from 300–600 bp by PCR Prep Kit (Promega).
3. Elute the fragments with 50 µL of sterile H_2O.
4. Add the following: 50 µL cDNA fragments; 7 µL 10X T buffer; 7 µL 200 m*M* Spermidine; 3 µL *Sal*I; 3 µL *Xba*I.
5. Incubate at 37°C for 1 h.
6. Electrophorese on a 2% low melting agarose gel.
7. Recover the cDNA fragments ranging in size from 300–600 bp by PCR Prep Kit.
8. Elute the fragments with 50 µL of TE.

3.2. Construction Signal Sequence Trap Library

3.2.1. Preparation of the pDREF-CD4ST Vector

1. Add the following: 10 µg pDREF-CD4ST; 7.5 µL10X T buffer; 7.5 µL 200 m*M* Spermidine; Sterile H_2O to 75 µL.
2. Add 2.5 µL of *Sal*I and incubate at 37°C for 1 h. Add 2.5 µL of *Xba*I and incubate at 37°C for 1 h.
3. Purify the vector by Chromaspin TE-1000 (Clontech) spun column.
4. Add 10 µL of 10X T buffer and 10 µL of 200 m*M* Spermidine to the elute. Add 2.5 µL of *Xba*I and incubate at 37°C for 1 h. Add 2.5 µL of *Sal*I and incubate at 37°C for 1 h.
5. Purify the vector by Chromaspin TE-1000 (Clontech) spun coloumn. Analyze 5 µL of the elute on a 0.8% agarose gel.

3.2.2. Ligation of the cDNA Fragments to the pDREF-CD4ST Vector

1. Add the following to 0.5-mL microcentrifuge tubes at room temperature:

cDNA	Vector 5X ligase buffer	H_2O	T4 DNA ligase
8 µL	150 ng 4 µL	to 19 µL	1 µL
2 µL	150 ng 4 µL	to 19 µL	1 µL
0.5 µL	150 ng 4 µL	to 19 µL	1 µL
0 µL	150 ng 4 µL	to 19 µL	1 µL

2. Incubate for 3 h at room temperature.
3. Add 5 µL of yeast tRNA (1 µg/µL) and 12.5 µL of 7.5 *M* NH_4OAc.
4. Add 37.5 µL of Phenol/CIAA, vortex thoroughly, and centrifuge at room temperature for 5 min at 10,000*g*. Carefully remove 35 µL of the upper aqueous layer, and transfer it to a 0.5-mL microcentrifuge tube.
5. Add 70 µL of absolute EtOH (–20°C). Incubate at –20°C for 30 min. Centrifuge at room temperature for 10 min at 10,000*g*.

6. Remove the supernatant, and overlay the pellet with 0.5 mL of 70% EtOH (–20°C). Centrifuge at room temperature for 2 min at 10,000*g*, and remove the supernatant. Dry the cDNA at 37°C for 10 min to evaporate residual ethanol.
7. Dissolve ligated cDNA in 5 µL of sterile H_2O.

3.2.3. Introduction of Ligated cDNA into E. coli *by Electroporation*

1. Add 1 µL of the ligated cDNA or control plasmid (10 pg of pUC119) to 20 µL of ELECTROMAX DH10B cells. Put the mixture into 0.2-cm Pulser cuvet, and place on ice.
2. Electroporate at 16.6 kV/ cm, 200 ohm and 25 µF.
3. Immediately add 1 mL of S.O.C. medium, and incubate at 37°C for 1 h with vigorous aeration.
4. Plate portions of the cells on LB plate containing 100 µg/mL ampicillin. Plate the equivalent of 10, 1, and 0.1 µL, made by serial dilution into LB medium.
5. Incubate the plates overnight at 37°C, and remaining transformed cells at 4°C overnight.
6. Count the colonies on each plate, calculate the number of colonies that would result from plating the entire 1 mL of cells.
7. Plate at a 5×10^4 colonies per 150 mm LB-Amp plate.
8. Incubate the plates overnight at 37°C, and then store at 4°C.

3.2.4. Analysis of Insert DNA Fragment by PCR Amplification

1. Add the following to 0.5-mL microcentrifuge tubes: 2.5 µL 10X PCR buffer; 2 µL 2.5 m*M* dNTP mix; 100 ng EF seq F primer; 100 ng CD4 ST seq R primer; 0.1 µL Taq DNA polymerase (5 U/µL); sterile H_2O to 25 µL.
2. Pick up single colony into the reaction mixture.
3. Incubate at 94°C for 3 min. Perform 40 cycles of PCR amplification: denature, 94°C for 30 s; annealing, 55°C for 1 min; extension, 72°C for 1 min. Incubate at 72°C for 5 min following the last cycle of PCR, then maintain at 4°C.
4. Analyze 10 µL of the amplified sample on a 2% low melting agarose gel.

3.3. Signal Sequence Trap

3.3.1. Electroporation of a Signal Sequence Trap Library into Raji Cells

1. Maintain Raji cells in RPMI-1640/10% fetal calf syndrom (FCS) in a humidified 37°C, 5% CO_2 incubator. Before the day of electroporation, dilute the culture 3X to ensure that the cells are in logarithmic growth phase.
2. Wash cells with phosphate-buffered saline (PBS) twice at room temperature and suspend the cells at 1×10^7 cells per 500 µL of PBS. Add 500 µL of cell suspension and 20 µL of plasmid to 0.4-cm Pulser cuvet, and incubate at room temperature for 10 min.

3. Electroporate at 270 V-500 μF using Gene Pulser equipped with Capacitance extender (Bio-Rad). Place the cuvet at room temperature for 10 min, transfer the cells to 30 mL RPMI/10% FCS in a 75-cm^2 flask, and culture in a humidified 37°C, 5% CO_2 incubator.
4. Two days after transfection, add hygromycin to a final concentration at 200 μg/mL to obtain stable transformants. When the cells grow nearly confluent, dilute the culture 3–5× by adding RPMI1640/10% FCS/200 μg/mL hygromycin. Continue culture under the selection with 200 μg/mL hygromycin at least for a week and until total cell number becomes over 2×10^7.

3.3.2. Isolation of CD4 Positive Cells by Magnetic Cell Sorting

1. Wash cells with PBS/10% FCS twice at room temperature and suspend the cells at 2×10^7 cells/mL of PBS/10% FCS. Add OKT4 mAb to a final concentration of 10 μg/mL and incubate at room temperature for 30 min on a rotator with end-over-end rotation, at 6 to 10 rpm.
2. Wash cells to remove unbound mAb with PBS/10% FCS twice at room temperature and suspend the cells at 2×10^7 cells/mL of PBS/10% FCS.
3. During washing cells, wash magnetic beads as follows. Resuspend the magnetic beads suspension well by shaking the vial thoroughly, immediately transfer the beads suspension (25 μL [10^7 beads] per 10^7 cells) to a 15-mL polypropylene tube. Add 10 mL PBS/10% FCS, agitate, and then pull beads to the side of tube with magnetic separation apparatus. After beads have accumulated adjacent to magnet (> 1 min.), aspirate the fluid. Remove the tube from the magnetic separation apparatus, repeat washing with 10 mL PBS/10% FCS, and resuspend the beads in a volume of PBS/10% FCS equal to original beads suspension taken from the vial.
4. Add 50 μL of washed magnetic beads suspension to 1 mL of cell suspension from step 2 and incubate at room temperature for 30 min on a rotator with end-over-end rotation, at 6 to 10 rpm.
5. Place the tube in magnetic separation apparatus and allow the cells coated with magnetic beads to accumulate to the side of tube adjacent to magnet (> 1 min.). Remove the unbound cells using pasture pipet.
6. Remove the tube from the magnetic separation apparatus, carefully resuspend the cells in 10 mL PBS/10% FCS, and separate the cells coated with magnetic beads as in step 5. Repeat washing at least 3 times.
7. Resuspend cells attached to magnetic beads in RPMI/10% FCS and transfer to a 75-cm^2 flask, and culture in a humidified 37°C, 5% CO_2 incubator.
8. Two days after magnetic cell sorting, add hygromycin to a final concentration at 200 μg/mL to obtain stable transformants. When the cells grow nearly confluent, dilute the culture 3–5× by adding RPMI1640/10% FCS/200 μg/mL hygromycin. Continue culture under the selection with 200 μg/mL hygromycin until total cell number becomes over 2×10^7.
9. Determine the percentage of CD4-positive cells by staining with FITC-Leu3a and FACS.
10. Repeat **steps 1–9** until no further enrichment of CD4-positive cells is obtained.

3.3.3. Rescue of Extrachromosomal DNA from Raji Cells

1. Wash the CD4-positive cells with PBS twice at room temperature and suspend the cells at 5×10^6 cells per mL of PBS.
2. Transfer 1 mL of the cell suspension to a 1.5-mL microcentrifuge tube and centrifuge at 10,000*g* at room temperature for 10 s.
3. Remove the supernatant by vacuum aspiration, add 150 µL of solution I to the tube, and resuspend the pellet by gentle vortex. Incubate at room temperature for 5 min.
4. Add 150 µL of solution II and gently mix by inverting the tube several times. Incubate at room temperature for 5 min. Note that the suspension becomes almost translucent.
5. Add 150 µL of solution III and gently mix by inverting the tube several times. Incubate on ice for 10 min. Note that a white precipitate appears.
6. Centrifuge at 10,000*g* at 4°C for 10 min. During the centrifugation, prepare a new tube containing 0.5 mL of phenol/CIAA.
7. Transfer the supernatant to the tube containing phenol/CIAA, vortex thoroughly, and centrifuge at room temperature for 5 min at 10,000*g*. Carefully remove 400 µL of the upper aqueous layer, and transfer it to a 1.5-mL microcentrifuge tube.
8. Add 800 µL of absolute EtOH (–20°C). Incubate at –20°C for 30 min. Centrifuge at room temperature for 10 min at 10,000*g*.
9. Remove the supernatant by decantation, and overlay the pellet with 1 mL of 70% EtOH (–20°C). Centrifuge at room temperature for 2 min at 10,000*g*, Carefully decant the supernatant and respin the pellet in order to remove the last trace of liquid by pipet. Dry the cDNA at 37°C for 10 min to evaporate residual ethanol.
10. Dissolve the DNA in 5 µL of TE and re-introduce into *E. coli* by electroporation as in **Subheading 3.2.3.**

3.3.4. Identification of cDNA Clone Encoding Putative Signal Sequence

1. Pick a single, isolated colony using a sterile toothpick and inoculate them to a 1.5-mL microcentrifuge tube containing 1 mL of LB medium containing 100 µg/mL ampicillin. Place the tube in 37°C shacking incubator and incubate overnight at 250–300 rpm.
2. Centrifuge at 12,000 rpm at room temperature for 1 min, and isolate plasmid as in **steps 3–9** in **Subheading 3.3.3.**, but without phenol/CIAA extraction.
3. Dissolve the plasmid in 25 µL of TE.
4. Reintroduce 20 µL of plasmid into Raji cells by electroporation as in **steps 1–3** in **Subheading 3.3.1.** Use 2.5×10^6 cells per 250 µL of PBS, and electroporate at 270 V-250 µF. Transfer the cells to 5 mL RPMI/10% FCS in a well of a 6-well plate, and culture in a humidified 37°C, 5% CO_2 incubator.
5. Two days after transfection, examine the surface expression of the reporter CD4 antigen by staining with FITC-Leu3a and FACS and identify clones containing with putative signal sequences.

3.4. Analysis of Clones Containing Putative Signal Sequences

1. Determine the length of insert fragments as in **Subheading 3.2.4.** and sequence the fragments longer than 200 bp using with EF seq F primer.
2. Analyze nucletotide sequences and deduced amino acid sequences by computer programs for hydrophathy ***(7)***, prediction of signal sequence ***(8)***, and homology search.
3. If you find an interesting sequence, full-length cDNA can be obtained by using the fragment as a probe. Confirm that recombinant protein is secreted into culture medium from mammalian cells transfected with the full-length cDNA, and then analyze biological and biochemical property of the gene product.

4. Notes

1. To reduce false-positive clones, mRNA should be prepared free from rRNA and genomic DNA as possible. We purify and concentrate mRNA by QuickPrep Micro mRNA purification Kit (Pharmacia) using glycogen as a carrier. Then, contaminated gemonic DNA is digested with RNase-free DNase I (Gibco-BRL).
2. Excess 5' portion-enriched cDNA reduces number of colony and increases the possibility of multiple cDNA inserts into the vector. Thus, optimal concentration of the cDNA inserts must be determined.
3. In our experience, about 10^6 stable transformants were routinely obtained from 10^7 Raji cells. Therefore, in order to obtain a population that represents the full cDNA library, perform one electroporation with 10^7 Raji cells/2 × 10^5 independent *E. coli* colony.
4. Raji cells are weak to exposure to the cold environment. Therefore, we perform all the manipulation at room temperature.
5. MACS and FACS are used instead of magnetic sorting with Dynabeads.
6. After three times of expansion and sorting, CD4 antigen-positive cells were enriched from 0.1% to 44.8% (about 450-fold), in our hands.
7. Out of just 100 randomly selected clones recovered from the transformants after the third sorting, 42 clones were found to direct surface expression of CD4 antigen. To avoid analysis of cDNA fragments encoding hydrophobic sequences in unnatural open reading frames, we only sequenced 36 clones with inserts longer than 200-bp. We found that 22 clones possessed anchor oligo(dC) introduced at 5' ends of mRNA as the tag (61%). Among these 22 clones, at least 10 clones presented 5' sequences with hydrophobic profiles characteristic of signal sequence (45%). In the present experiment, 10 out of 36 trapped clones (28%) contained potential signal sequences, and the rate of trapping signal sequences in the starting *E. coli* clones was thus 10%. The analysis of false-positive clones indicated potential contamination of gemonic DNA fragments and internal fragments of cDNA. These problems would be reduced by treatment of mRNA with DNase and by selective labeling of 5' ends of mRNA by replacing the cap structure with an oligonucleotide ***(10)***.

8. We have obtained signal sequences of transmembrane proteins (CD45, Leu-8, CD6, MGC-24, β2-microglobulin, TCR-ε, granule proteoglycan), secreted proteins (TARC, SCM-1/lymphotactin) ***(2,11)***, and proteins accumulated in intracellular organelles (NAcGlu-1-P-transferase).
9. Recently, signal sequence selection methods in yeast have also been described ***(12,13)***.

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