MOLECULAR AND CELLULAR BIOLOGY, July 1988, p. 2837–2847
0270-7306/88/072837-11$02.00/0

Vol. 8, No. 7

Epstein-Barr Virus Shuttle Vector for Stable Episomal Replication of cDNA Expression Libraries in Human Cells

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Received 27 January 1988/Accepted 29 March 1988

Efficient transfection and expression of cDNA libraries in human cells has been achieved with an Epstein-Barr virus-based subcloning vector (EBO-pcD). The plasmid vector contains a resistance marker for hygromycin B to permit selection for transformed cells. The Epstein-Barr virus origin for plasmid replication (*oriP*) and the Epstein-Barr virus nuclear antigen gene have also been incorporated into the vector to ensure that the plasmids are maintained stably and extrachromosomally. Human lymphoblastoid cells can be stably transformed at high efficiency (10 to 15%) by such plasmids, thereby permitting the ready isolation of 10^6 to 10^7 independent transformants. Consequently, entire high-complexity EBO-pcD expression libraries can be introduced into these cells. Furthermore, since EBO-pcD plasmids are maintained as episomes at two to eight copies per cell, intact cDNA clones can be readily isolated from transformants and recovered by propagation in *Escherichia coli*. By using such vectors, human cells have been stably transformed with EBO-pcD-*hprt* to express hypoxanthine-guanine phosphoribosyltransferase and with EBO-pcD-*Leu-2* to express the human T-cell surface marker Leu-2 (CD8). Reconstruction experiments with mixtures of EBO-pcD plasmids demonstrated that one clone of EBO-pcD-*hprt* per 10^6 total clones or one clone of EBO-pcD-*Leu-2* per 2×10^4 total clones can be recovered intact from the transformed cells. The ability to directly select for expression of very rare EBO-pcD clones and to then recover these episomes should make it possible to clone certain genes where hybridization and immunological screening methods are not applicable but where a phenotype can be scored or selected in human cell lines.

There are presently only a few general strategies for cloning a eucaryotic gene. In a very limited number of cases, the mRNA of the gene is so highly expressed in a tissue or cell that it may be physically purified and converted into a relatively pure cDNA probe or cDNA clone (2, 8, 15, 23, 29). However, the vast majority of mammalian mRNAs are of such low abundance that this direct approach cannot usually be applied. For a few low-abundance mRNAs, conditions for differential expression allow the generation of a cDNA probe enriched for the sought-after gene. This type of approach has been used to clone several developmentally regulated genes (7, 43) as well as the genes for human fibroblast interferon (39), the T-cell receptor (14, 44), and the δ-opioid receptor (Law et al., Proc. Natl. Acad. Sci. USA, in press). In situations where neither RNA nor enriched cDNA is available but the protein product of the gene is abundant, the following approach has often been successful (4, 12, 28–30, 38–40): the protein is purified, the partial amino acid sequence is determined, and oligonucleotide probe(s) based upon the peptide sequence are synthesized and then used to screen an appropriate library for a clone that hybridizes with the probe(s). However, this approach is of limited utility when the protein product is not known, very rare, or unavailable in sufficient quantity and purity to sequence. Furthermore, if the purified protein has a blocked amino terminus or if oligonucleotides with requisite uniqueness cannot be synthesized, then this approach may be unsuccessful. A recently developed procedure employs expression of a partial cDNA clone of the gene product as a β-galactosidase fusion product in *Escherichia coli* followed by screening of recombinant clones with an antibody specific for the protein of interest (47). This approach may be utilized in the absence of any nucleic acid or protein sequence data. However, it requires a preexisting monospecific antibody and that the antigenic determinants recognized by the antibody be expressed in the context of a β-galactosidase hybrid protein expressed in *E. coli*.

There is a strategy for gene cloning which relies solely upon expression of a cDNA copy of the gene of interest in mammalian cells (32). This approach does not rely upon the availability of purified protein, protein or nucleic acid sequence data, or specific antibodies. Rather, this method relies on the ability to select or screen for the functional expression of the gene in mammalian cells. Previously, methods were described for the synthesis of full-length cDNAs in the pcD vector system designed for efficient expression in mammalian cells (31, 32). Subsequently, a lambda vector was developed which led to efficient transduction and integration of pcD cDNA clones in certain mammalian cells (33). The limitations of this approach are the requirement for full-length (or nearly full-length) cDNA clones for expression, the necessity of obtaining sufficient mammalian cell transformants to provide a full representation of the cDNA library, and the need to recover the integrated cDNA sequence from the mammalian genome. The latter two difficulties have been alleviated by the use of a subcloning vector (EBO-pcD) for cDNA expression libraries which allows the high efficiency transformation of human cells with an entire pcD cDNA library. Furthermore, this EBO subcloned pcD library replicates as autonomous episomes in Epstein-Barr virus (EBV)-transformed recipient cells and thereby simplifies the recovery of desired cDNA clones.

Sugden and co-workers have determined the EBV ele-

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ments required for replication of DNAs as episomes in EBV-transformed cells (35, 37, 45, 46). They have described a *cis*-acting element (*oriP*) necessary for plasmid replication in certain mammalian cells known to express the EBV nuclear antigen (EBNA-1). We have incorporated *oriP*, the EBNA-1 gene, and a drug resistance selection marker (hygromycin phosphotransferase) into a subcloning vector designed to allow pcD expression libraries to be maintained as stably replicating plasmids in certain mammalian cells. In this paper we describe this EBV-based subcloning vector (EBO-pcD) and its use in isolating clones for a cell surface marker (Leu-2 [CD8]) and for the enzyme hypoxanthine-guanine phosphoribosyltransferase (HPRT). The ability to directly select for expression of very rare EBO-pcD clones and to recover these plasmids should facilitate attempts to clone cell surface markers, receptors, and required enzymes that are present at 1 to 50 copies within an EBO-pcD library of 10^6 clones.

MATERIALS AND METHODS

Cells, bacteria, and enzymes. EBV-transformed HPRT-deficient human lymphoblastoid UC729-6 cells (UC cells) (10) were obtained from Ronald Levy (Stanford) and maintained in RPMI 1640 medium containing penicillin, streptomycin, and 10% fetal calf serum in 5% CO_2–95% air at 37°C. Transformation-competent *E. coli* DH5 was obtained from Bethesda Research laboratories, Inc. (Gaithersburg, Md.), and *E. coli* MC1061 (3) was made competent as described previously (26). All restriction enzymes and T4 DNA ligase were obtained from New England Biolabs, Inc. (Beverly, Mass.), calf intestine alkaline phosphatase was from Boehringer Mannheim Biochemicals (Indianapolis, Ind.), and terminal transferase was from P-L Biochemicals, Inc. (Milwaukee, Wis.). All enzymes were used under conditions recommended by the purveyors.

Construction of cDNA libraries. cDNA libraries were prepared with the pcD expression plasmid of Okayama and Berg as previously described (31, 32). The preparation of poly(dT)-tailed primer and oligo(dG)-tailed linker plasmids were altered from the previously described procedure to ensure that only 50 to 70 deoxythymidylate (dT) or 10 to 12 deoxyguanylate (dG) residues would be incorporated. The primer reaction mixture (40 μl) contained 100 μg of *Kpn*I endonuclease-digested pcDV1 DNA (91 pmol of DNA termini), 140 mM sodium cacodylate, 30 mM Tris hydrochloride (pH 6.8), 1 mM $CoCl_2$, 0.1 mM dithiothreitol, 156 μM [^{3}H]dTTP, and 80 U (3 μl) of terminal deoxynucleotidyl transferase. The linker reaction mixture (40 μl) contained 100 μg of *Pst*I endonuclease-digested pL DNA, (200 pmol termini), 140 mM sodium cacodylate, 30 mM Tris hydrochloride (pH 6.8), 1 mM $CoCl_2$, 0.1 mM dithiothreitol, 75 mM [^{3}H]dGTP, and 50 U of terminal deoxynucleotidyl transferase. After 15 min at 37°C the reactions were temporarily stopped by cooling to 0°C, and the length of the tails was determined by trichloroacetic acid precipitation. If primer tail length was greater than 50 dT residues, the reaction was terminated by the addition of sodium dodecyl sulfate; otherwise the reaction was continued by further incubation at 37°C for 5 to 10 min or until 50 dT residues were incorporated per end. If the linker tail length was greater than 10 dG residues the reaction was terminated; otherwise it was continued by further incubation at 37°C until 10 to 12 dG residues were incorporated per end. The reaction conditions are such that 100% incorporation of dTTP into poly(dT)-tailed pcDV yields a tail of 70 dT residues and 100% incorporation of dGTP into oligo(dG)-tailed pL yields a tail of 15 dG residues. These tailing conditions ensure that one cannot overshoot the desired tail length for the linker or primer plasmids. The purification of tailed linker and primer DNAs was as described previously (31, 32).

pcD-cDNA expression libraries were constructed from poly(A)$^+$ mRNA purified from P2F and J107 clonal mouse L cell lines expressing transfected Leu-2 (CD8) on the cell surface (19, 20). Poly(A)$^+$ mRNA (5 μg) was denatured in 9 μl of deionized water at 65°C for 3 min, cooled to room temperature, and mixed with 2 μg of dT-tailed primer, buffer (10 mM Tris hydrochloride [pH 8.35], 6 mM $MgCl_2$, 30 mM KCl), 2 mM deoxynucleotide triphosphates, and 50 U of avian myeloblastosis virus reverse transcriptase in a final volume of 40 μl. First-strand cDNA was synthesized by incubating at 42°C for 60 min; the reaction was terminated by adding 2 μl of 20% sodium dodecyl sulfate. The reaction mixture was made 2 M in ammonium acetate and extracted twice with phenol-chloroform; then the aqueous phase was precipitated at room temperature by the addition of 2.5 volumes of ethanol. The precipitated RNA-DNA complex was suspended in 2.5 M ammonium acetate and reprecipitated at room temperature by the addition of 2.5 volumes of ethanol. The ammonium acetate-ethanol precipitation was repeated, followed by precipitation from 0.25 M sodium acetate with 2.5 volumes of ethanol at −20°C. Then, 10 to 15 deoxycytidylate (dC) residues were added to the first cDNA strand with terminal deoxynucleotidyl transferase. The product was cleaved with *Hin*dIII endonuclease (40 U) by overnight (12 to 14 h) incubation at 37°C and then annealed to 2 μg of dG-tailed linker DNA. After annealing, cyclization, and synthesis of second-strand cDNA, the reaction mixture was used to transform competent *E. coli* MC1061. The libraries, selected on ampicillin-containing medium, contained 4×10^6 to 5×10^6 independent transformants. A portion was grown to saturating density (overnight), and plasmid DNA representing the entire library was recovered by two cycles of centrifugation in ethidium bromide-cesium chloride gradients (34). Total library DNA (10 μg) was digested with 50 U of *Sfi*I restriction endonuclease for 5 h at 50°C and then fractionated by electrophoresis in 0.8% agarose gels. The gels were sliced to separate regions corresponding to plasmids containing cDNA inserts of 0 to 500 base pairs, 500 base pairs to 2.0 kilobase pairs (kb), 2.0 to 2.6 kb, 2.6 to 3.7 kb, 3.7 to 4.4 kb, 4.4 to 6.4 kb, and greater than 6.4 kb. DNA was recovered from each slice by electroelution. To make size-fractionated pcD sublibraries, the recovered DNAs were cyclized by ligation and introduced into competent *E. coli* MC1061. To make size-fractionated EBO-pcD sublibraries, the recovered DNAs were ligated to EBO DNA (see below) and then introduced into competent *E. coli*.

Plasmids. pSV2-*neo* (36) is a simian virus 40 (SV40)-based mammalian vector expressing the bacterial Tn*5* gene neomycin phosphotransferase (6). pcD-*hprt* (18) is the cDNA expression vector containing a cDNA copy of the *hprt* gene. pcD-*Leu-2* contains a cDNA copy of the T-cell surface marker Leu-2 (CD8) (1). pcD-*Leu-2* was isolated from a pcD-cDNA expression library (pcD-J107, see above). The pcD-J107 size-fractionated sublibrary corresponding to cDNA inserts of 2.0 to 2.6 kb was plated onto Luria broth (LB) plates with ampicillin (50 μg/ml), transferred onto nitrocellulose disks, and amplified overnight at 37°C on LB containing ampicillin and chloramphenicol (10 μg/ml). An oligonucleotide probe specific for the known partial *Leu-2* sequence was 5′ end labeled with [γ-^{32}P]ATP and polynu-

cleotide kinase under standard conditions (27). This labeled oligonucleotide probe was used to screen nitrocellulose-lift colonies for *Leu-2*-specific clones. From 8,000 screened colonies, 24 positive clones were identified. cDNA inserts ranged from 1.8 to 2.4 kb. The largest clones were screened for a full-length *Leu-2* clone by expression in COS cells (11). Of four clones tested, one was positive for expression; this clone was designated pcD-*Leu-2.1*. Another clone (pcD-*Leu-2.14*) had a more extensive 5′ untranslated region yet did not lead to cell-surface Leu-2 expression due to an alternate splicing form. A recombinant clone was constructed with the pcD-*Leu-2.14* 5′ region upstream of the *Aat*II restriction site and the pcD-*Leu-2.1* 3′ region downstream of this site. This clone was designated pcD-*Leu-2* and was used in the EBO-pcD-*Leu-2* construction. Cell surface Leu-2 expression was comparable in COS cells transfected with pcD-*Leu-2* or with pcD-*Leu-2.1*.

Construction of EBO. To convert pcD-cDNA expression libraries (pcD-X) into a form which could be directly selected and maintained as episomes in mammalian cells, an additional segment of DNA (EBO) was incorporated into pcD clones and pcD libraries. This DNA segment encodes a dominant-acting hygromycin phosphotransferase (*hph*) marker which confers resistance to hygromycin B in mammalian cells (A. Smith, D. Strehlow, A. Miyajima, and P. Berg, unpublished results). It also contains the EBV origin for plasmid replication (*oriP*) and the EBNA gene to ensure replication as episomes in human or simian cells, even if the cell line is not EBNA positive. The EBO DNA segment was constructed as a closed circular molecule with a specific *Sfi*I restriction site complementary to the unique *Sfi*I restriction site present within the SV40 regulatory region of pcD clones or pcD libraries. The *Sfi*I restriction site ($GGCCN_5GGCC$) is relatively rare, because its sequence has eight specified base pairs. Furthermore, since cleavage of the site occurs in staggered fashion within the five unspecified central nucleotides, each *Sfi*I cohesive end can only join in a head-to-tail orientation.

To construct the EBO DNA segment, the 7.4-kb *Bam*HI restriction fragment of p220-1 (a derivative of p201 [46]) containing EBV *oriP*, EBNA-1, and *hph* was cyclized by T4 DNA ligase promoted self-ligation at a DNA concentration of 2 μg/ml and then cleaved with *Sfi*I endonuclease. This permuted the order of gene markers and generated a unique 7.4-kb *Sfi*I restriction fragment. This fragment was ligated to the phosphatase-treated *Sfi*I site of pcD-*Leu-2*, pcD-*hprt*, or pSV2-*neo* to produce EBO-pcD-*Leu-2*, EBO-pcD-*hprt*, or EBO-pSV2-*neo*, respectively. The structure of EBO-pcD-X, where X is any cDNA, is shown in Fig. 1. *Sfi*I endonuclease cleavage of pcD or pSV2 DNAs was as follows: 10 μg of DNA was digested with 50 U of *Sfi*I endonuclease for 5 h at 50°C under Paraffin oil in 50 mM NaCl, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 100 μg of bovine serum albumin per ml, and 10 mM Tris hydrochloride (pH 7.8). The cleaved DNA was chilled on ice and made alkaline by the addition of 50 mM glycine (pH 9.4), 10 U of calf intestine alkaline phosphatase was added, and the mixture was incubated at 37°C for 30 min. The reaction was terminated by the addition of sodium dodecyl sulfate to 1.0%, followed by phenol-chloroform extraction and ethanol precipitation.

Construction of EBO-pcD libraries. pcD library DNAs were digested to completion with *Sfi*I endonuclease and treated with calf intestine alkaline phosphatase (as described above for pcD plasmids). The 7.4-kb EBO fragment was isolated from *Sfi*I endonuclease-digested EBO-pSV2-*neo* and ligated to *Sfi*I endonuclease-linearized, phosphatase-treated pcD-P2F DNA. The EBO piece was also ligated into *Sfi*I endonuclease-cleaved, phosphatase-treated, size-fractionated pcD-P2F DNA to generate EBO-pcD sublibraries.

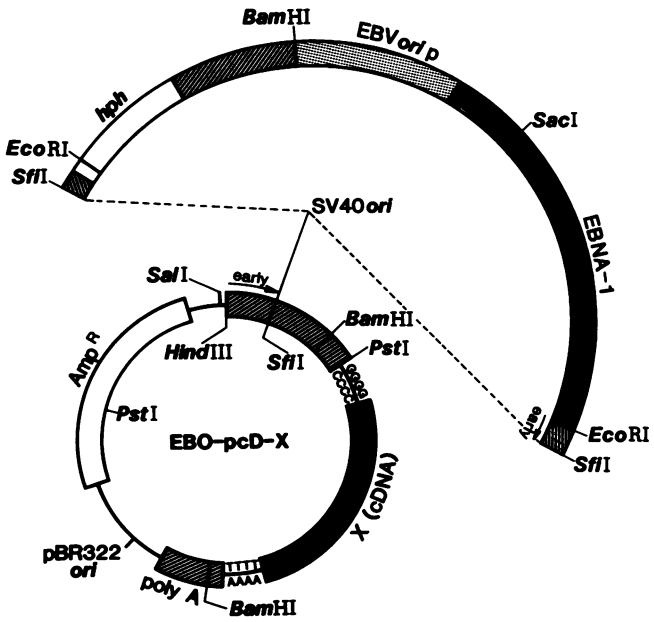

FIG. 1. General structure and component parts of the EBO-pcD-cDNA plasmid. X symbolizes the cDNA inserts. The inner circle depicts the pcD-cDNA plasmid which has been described in detail elsewhere (32); the structure is summarized here to facilitate the presentation in this paper. The pcD-X plasmid can be linearized at the indicated unique *Sfi*I site and ligated to the *Sfi*I-linearized EBO subcloning vector for episomal expression in human lymphoblastoid cells. The principle elements of the EBO-pcD vector DNA are as follows: the segment containing the SV40 origin of DNA replication (*ori*) and the early region promoter oriented in the clockwise direction joined to a segment containing the junctions of the 19S and 16S SV40 late-region pre-mRNA intervening sequences (hatched area at top of inner circle); the various cDNA segments plus the flanking deoxyguanidylate-deoxycytidilate and deoxyadenylate-deoxythymidylate bridges to the vector produced in the cloning operation, where X symbolizes the particular cDNA insert (solid black area); a fragment containing the SV40 late-region polyadenylation signal (poly A) (hatched area at bottom of inner circle); the segment containing the pBR322 β-lactamase gene (open area of inner circle) and the origin of replication (pBR322 *ori*) (thin line of inner circle); the semicircular arc above the inner pcD circle depicts the EBO subcloning vector which contains the SV40 regulatory region including *ori*, the early-region promoter oriented in the clockwise direction (hatched area at left extreme of arc); the hygromycin phosphotransferase gene (*hph*) (open area of arc); the SV40 small t-antigen intervening sequence and polyadenylation signal (hatched area centrally placed within arc); the EBV origin for plasmid replication (*ori* P) (stippled area); the EBNA-1 gene (gray area). The SV40 late-region promoter is oriented in the counterclockwise direction (hatched area at right extreme of arc) to transcribe the EBNA-1 gene. The relevant restriction sites referred to in this work are the two *Sfi*I sites at the junctions between the EBO subcloning vector and the pcD-cDNA plasmid, the three *Bam*HI sites, the unique *Hin*dIII site of pcD, and the unique *Sac*I site of EBO.

Transfection of human lymphoblastoid cells. UC cells (5×10^7) in the logarithmic growth phase were pelleted, washed with phosphate-buffered saline with 0.5 mM Mg^{2+}–0.9 mM Ca^{2+}, and then suspended in 1.0 ml of 1× HBS (20 mM [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.05], 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM

dextrose), 100 to 300 μg of EBO-pcD DNA, and 200 to 400 μg of sheared salmon sperm DNA (total of 500 μg of DNA per ml). Cells and DNA in HBS were kept at room temperature for 10 to 15 min and then electroporated with a capacitor discharge of 250 V and 1,028 μF. The transfected cells were kept at room temperature for an additional 10 to 15 min and then diluted into RPMI 1640 at 5×10^5 cells per ml. Cell survival was between 20 and 40%. Selection for hygromycin B-resistant cells was begun 48 h posttransfection with hygromycin B (Eli Lilly & Co.) at 100 μg/ml for 2 days, 150 μg/ml for 3 days, and then 200 μg/ml thereafter. Selection in HAT medium (25) was initiated at 48 h posttransfection.

Fluorescence-activated cell sorter analysis and sorting. Transfected UC cells to be analyzed or sorted were collected in $NaPO_4$-NaCl-EDTA and chilled on ice. For sorting, 5×10^6 cells were stained with saturating amounts of fluorescein-conjugated anti-Leu-2a antibody (Becton Dickinson Antibody Center, Mountain View, Calif.) for 30 min at 0°C in 250 μl of staining medium (biotin-free RPMI 1640 medium (Irvine Scientific)–10 mM HEPES (pH 7.4–0.1% NaN_3–2% heat-inactivated serum). Propidium iodide (Calbiochem-Behring Corp., La Jolla, Calif.) was added to a final concentration of 1 μM. After 5 min, the cells were centrifuged and washed with staining medium. For routine analysis, round-bottomed polypropylene microdilution plates (Dynatech Laboratories, Inc., Alexandria, Va.) containing 5×10^5 cells per 0.1-ml well were used.

Cells were analyzed and sorted on a fluorescence-activated cell sorter (FACS) (FACS II; Becton Dickinson) modified to include a logarithmic amplifier and a direct cloning attachment. A 540-nm short-pass filter (Ditric Optics, Marlboro, Mass.) was used to reduce autofluorescence of the UC cells relative to fluorescein fluorescence. A second detector receiving light above 580 nm was used to detect propidium iodide. The signals from this detector were electronically balanced to subtract any signal due to fluorescein.

Transient expression of Leu-2 on the cell surface was measured at 48 h posttransfection. Stable expression of Leu-2 was measured at 2 weeks or more posttransfection. To isolate cells expressing Leu-2 on their surface, the top 0.5 to 1.0% of fluorescent cells was physically sorted from the general population, grown in nonselective medium for 24 h, grown under the stepwise hygromycin B selection (as described above for the electroporated cells), and sorted. Generally, three to four successive sorts were sufficient to greatly enriched for cells expressing Leu-2.

Southern transfer of DNA. DNA was prepared from cells by the method of Wigler et al. (41), digested with *Bam*HI, *Hin*dIII, *Asp*718, or *Sac*I restriction endonuclease as indicated, and then electrophoresed in 0.8% agarose gels. The electrophoresed DNA was transferred to Gene Screen Plus (Du Pont Co., Wilmington, Del.) nylon membranes and hybridized with ^{32}P-labeled, random hexamer-primed probes (9). Specific hybridizations for *Leu-2*, *hprt*, and *neo* utilized gel-purified coding region probes derived from pcD-*Leu-2*, pcD-*hprt*, and pSV2-*neo*, respectively. Hybridization, probe stripping, and rehybridization were as suggested by the manufacturer of Gene Screen Plus (Du Pont). Hybridized bands were visualized by autoradiography aided by intensifying screens (24).

Isolation of episomal DNA. Low-molecular-weight DNA was recovered from 10^7 UC cells in the logarithmic growth phase by a modification of the Hirt method (16). Cells were pelleted, washed, lysed in 0.6% sodium dodecyl sulfate–1 M NaCl, then stored at 4°C for 24 to 48 h. High-molecular-weight DNA was removed by centrifugation at 17,000 × *g* for 45 min at 4°C. The supernatant containing the low-molecular-weight DNA was digested with proteinase K for 1 h at 65°C and then extracted sequentially with phenol, phenol-chloroform, and butanol. DNA was precipitated by the addition of ammonium acetate to 2.5 M and 2 volumes of ethanol followed by storage at −20°C for 24 to 48 h. The DNA was centrifuged at 17,000 × *g* for 45 min at 4°C; DNA pellets were washed with 80% ethanol, dried, and suspended in 50 μl of TE (10 mM Tris, 1 mM EDTA [pH 7.5]).

Transformation of *E. coli*. Transformation of *E. coli* DH5 was as recommended by the vendor (Bethesda Research Laboratories). Low-molecular-weight DNA (10 μl; approximately 100 pg of DNA from 2×10^6 UC cells) was added to 100 μl of competent cells; the mixture was kept at 0°C for 30 min, heated to 42°C for 45 s, and cooled to 0°C for 2 min. Then 0.9 ml of SOC medium (13) was added, and the mixture was shaken at 225 rpm at 37°C for 1 h. The transformed bacteria were grown on LB plates containing ampicillin (50 μg/μl). Transformations yielded between 5 and 100 bacterial colonies from 10 μl of DNA. Individual bacterial colonies were picked and grown overnight in 2-ml liquid cultures containing LB-ampicillin. Plasmid DNAs were prepared by a modification of the boiling method of Holmes and Quigley (17). A 1-ml sample of the overnight culture was centrifuged in an Eppendorf tube for 1 min, the supernatant was aspirated, and the bacterial pellet was suspended in 0.4 ml of ice-cold buffer (8% sucrose, 0.5% Triton X-100, 50 mM EDTA, 10 mM Tris hydrochloride [pH 8.0]) containing 0.67 mg of fresh lysozyme per ml. The mixture was heated at 100°C for 40 s and centrifuged at room temperature for 10 min. Then the pellet was removed with a sterile toothpick, 100 μl of 10 M ammonium acetate was added to the supernatant, and 2.5 volumes (1.0 ml) of ethanol was added; the mixture was vortexed briefly and then set for 5 min at room temperature to precipitate the DNA. The precipitate was spun at room temperature for 10 min, washed with cold 80% ethanol, air dried, and then suspended in 50 μl of TE.

RESULTS

The utility of pcD expression libraries has been increased by introduction of a DNA segment (EBO) that enables pcD-cDNA plasmids to replicate autonomously and to be selected in mammalian cells (see Materials and Methods) (Fig. 1). The EBO segment contains the gene encoding hygromycin phosphotransferase (*hph*) expressed by the SV40 early region promoter (A. Smith, D. Strehlow, A. Miyajima, and P. Berg, unpublished results), the EBV origin for plasmid replication (*oriP*) (35, 37, 45), and the EBNA-1 gene required for *trans*-activation of *oriP* replication (46). The EBO prefix denotes the presence of the *hph*, *oriP*, and EBNA elements in the basic pSV2 or pcD clones or libraries. EBO clones (EBO-pcD-*Leu-2*, EBO-pcD-*hprt*, and EBO-pSV2-*neo*) and EBO-pcD-cDNA expression libraries (EBO-pcD-P2F and EBO-pcD-J107) can be introduced into EBV-transformed human lymphoblasts with high efficiencies and be maintained as autonomously replicating episomes in these cells by selection for *hph* expression.

EBO-pcD-*Leu-2* expression. UC human lymphoblastoid cells (5×10^7) were transfected with 100 μg of EBO-pcD-*Leu-2* DNA by electroporation. Transient expression of Leu-2 on the cell surface was detected at 48 h posttransfection by staining cells with fluorescein-conjugated anti-Leu-2a antibody. About 20% of the cells survived the electroporation, and about 33% of the survivors expressed Leu-2 on the

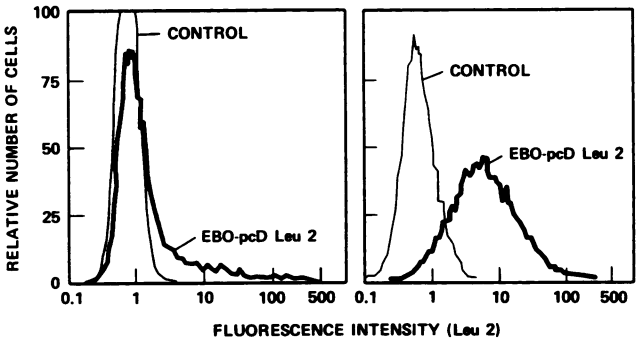

FIG. 2. FACS fluorescence intensity histograms. UC cells transfected with EBO-pcD-*Leu-2* were stained with fluorescein-conjugated anti-Leu-2a antibody, which detects Leu-2 (CD8) on the cell surface. The cells in the left panel were stained with antibody at 48 h posttransfection. The cells in the right panel were stained after 2 weeks in medium containing hygromycin B. Control cells (thin line) were transfected with EBO-pSV2-*neo*. Note that fluorescence intensity is shown on a logarithmic scale.

cell surface after 48 h (Fig. 2). Selection for hygromycin B resistance was begun at 48 h posttransfection; after 2 to 3 weeks a pure population of hygromycin-resistant cells expressing Leu-2 was obtained (Fig. 2). At 3 weeks posttransfection, 15% of those cells which had survived the electroporation were transformed to hygromycin resistance; i.e., from 5×10^7 UC cells transfected with EBO-pcD-Leu-2 about 1.5×10^6 hygromycin-resistant, Leu-2-expressing cells were obtained.

To simulate attempts to clone a cell surface marker by expression from an EBO-pcD library, transfections were performed with mixtures of EBO-pcD-*Leu-2* and EBO-pSV2-*neo* DNAs in the following ratios: 1:20, 1:200, 1:2,000, 1:20,000. For each of these mixtures 100 μg of DNA was electroporated into 5×10^7 UC cells, and then transient (48 h posttransfection) and stable (2 weeks posttransfection) expression of cell surface Leu-2 was monitored with the FACS. Cells expressing Leu-2 could be detected at 48 h postelectroporation for all the transfections (Fig. 3). After

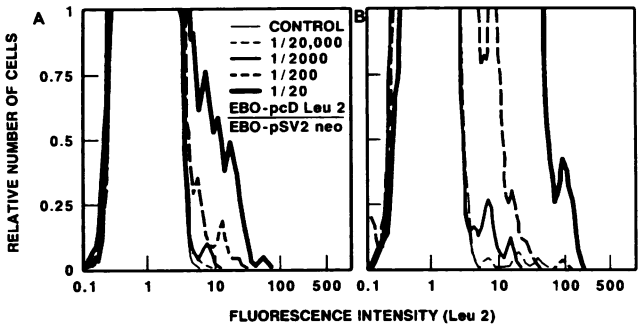

FIG. 3. FACS fluorescence intensity histograms. UC cells transfected with DNA mixtures of EBO-pcD-*Leu-2* and EBO-pSV2-*neo*. A constant amount of total plasmid DNA (100 μg) was introduced by electroporation into UC cells. The ratios of EBO-pcD-*Leu-2* to EBO-pSV2-*neo* ranged from 1:20 to 1:20,000 as indicated. At 48 h posttransfection (A) or 2 weeks posttransfection in medium containing hygromycin B (B) cells were stained as in Fig. 2. Control cells (thin line) were transfected with EBO-pSV2-*neo*. Note that only a small percentage of the total cells is displayed so that the most highly fluorescent cells may be more readily detected.

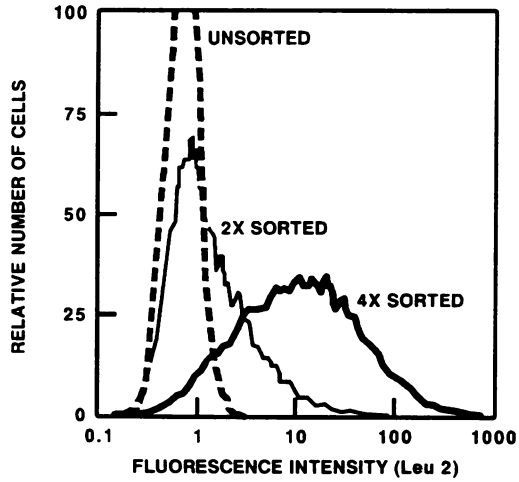

FIG. 4. FACS fluorescence intensity histograms. UC cells transfected with a 1:20,000 mixture of EBO-pcD-*Leu-2* to EBO-pSV2-*neo* were grown in medium containing hygromycin B and analyzed for cell surface Leu-2 expression (as in Fig. 2). During each round of sorting the most highly fluorescing 0.5 to 1.0% of Leu-2-positive cells were isolated from the general population, grown in medium containing hygromycin B, and resorted until a homogeneous population of Leu-2 expressing UC cells was obtained. Histograms of unsorted, twice-sorted, and four-times-sorted cells are shown.

selection in hygromycin there were stable transformants expressing Leu-2 even from the transfection with EBO-pcD-*Leu-2* at the 1:20,000 dilution (Fig. 3).

Repeated cycles of FACS sorting of the Leu-2-positive fraction of the stable transformants from the transfection with the 1:20,000 mixture (Fig. 4) yielded a pure population of Leu-2-positive cells. In experiments not shown here we could detect apparent Leu-2-positive transformants after transfection with dilutions of the EBO-pcD-*Leu-2* plasmid at 1:200,000 or $1:10^6$. However, repeated cycles of FACS sorting failed to enrich for Leu-2 expressing cells from these transformants. Repeated rounds of sorting of cells which had received only EBO-pSV2-*neo* or EBO-pcD-*hprt* yielded no Leu-2-positive cells (data not shown).

EBO-pcD-*Leu-2* DNA copy number and recovery from transfected cells. Total DNA from the Leu-2-positive cells shown in Fig. 4 was digested with *Sac*I or *Hin*dIII endonuclease to linearize the EBO-pcD-*Leu-2* DNA or with *Bam*HI to release the *Leu-2* insert from EBO-pcD-*Leu-2*. Digestion with *Asp*718 (which does not cut EBO-pcD-*Leu-2*) was used to distinguish between high- and low-molecular weight copies of EBO-pcD-*Leu-2*. The restricted DNAs were electrophoresed in agarose gels, transferred to nylon membranes, hybridized with a ^{32}P-labeled *Leu-2*-specific DNA probe, and autoradiographed (Fig. 5). These blots indicate that this cell line contains approximately 10 copies of EBO-pcD-*Leu-2* per cell; 2 copies are maintained as low-molecular weight episomes, whereas 8 are present as high-molecular weight DNA (the possible integration of some copies of EBO-pcD-*Leu-2* is addressed in the Discussion). After the *Leu-2* probe was stripped off, the blots were rehybridized with a ^{32}P-labeled *neo*-specific DNA probe to determine whether the cells also carried any EBO-pSV2-*neo* plasmids. Some EBO-pSV2-*neo* was detected, but at less than 0.1 copy per cell (data not shown).

Low-molecular-weight DNA was isolated from the Leu-2-expressing cells that have been transformed with the 1:20,000 mixture (Fig. 4). Transformation of *E. coli* DH5

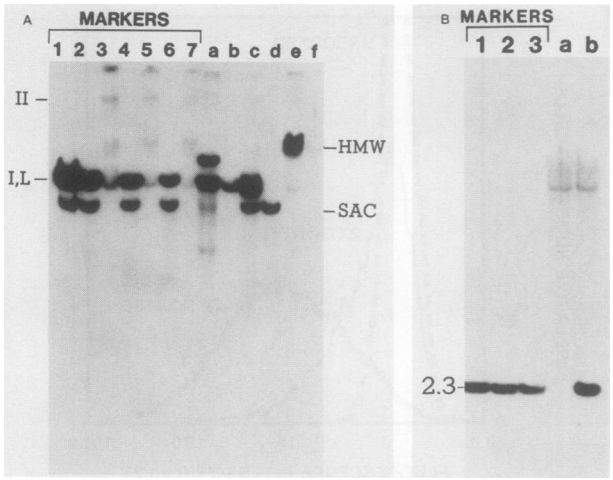

FIG. 5. Analysis of EBO-pcD-*Leu-2* plasmids in UC transformants which had received the EBO-pcD-*Leu-2*/EBO-pSV2-*neo* DNA mixture of 1:20,000 and were then sorted and resorted for Leu-2 expression through four cycles on the FACS (Fig. 4, 4× sorted). DNAs were separated by electrophoresis in 0.8% agarose gels and detected by the method of Southern by using a uniformly ^{32}P-labeled *Leu-2*-specific DNA probe. (A) Total DNA (10 μg) from transfected, Leu-2-sorted UC cells (lanes a, c, and e) or untransfected UC control cells (lanes b, d, and f). Markers containing reconstructions of 16 (lane 1), 8 (lanes 2 and 3), 4 (lanes 4 and 5), or 2 (lanes 6 and 7) EBO-pcD-*Leu-2* and EBO-pSV2-*neo* plasmids per cell were obtained by mixing 640, 320, 160, or 80 pg each of EBO-pcD-*Leu-2* and EBO-pSV2-*neo* plasmid DNAs with 10 μg of genomic DNA from control untransfected UC cells. DNAs were digested with *Sac*I (lanes 1, 2, 4, 6, c, and d), *Asp*718 (lanes 3, 5, 7, e, and f), or *Hin*dIII (lanes a and b). EBO-pcD-*Leu-2* is linearized by digestion with *Hin*dIII or *Sac*I restriction endonucleases. EBO-pcD-*Leu-2* is uncut by *Asp*718 restriction endonuclease. The positions of supercoiled (form I), nicked (form II), and linear (L) EBO-pcD-*Leu-2* are indicated on the left. The positions of high-molecular-weight UC DNA (HMW) and *Sac*I-digested genomic *Leu-2* DNA (SAC) are indicated on the right. (B) Total DNA from transfected, Leu-2-sorted UC cells (lane b) or UC control cells (lane a). Markers (lanes 1 through 3) containing reconstructions of 8, 4, or 2 EBO-pcD-*Leu-2* and EBO-pSV2-*neo* plasmids per cell were obtained as noted for (A). DNAs were digested with *Bam*HI. *Bam*HI digestion of EBO-pcD-*Leu-2* yields a 5.4-kb fragment (vector), a 5.2-kb fragment (vector), and a 2.3-kb fragment (*Leu-2* cDNA insert). The position of the 2.3-kb *Leu-2*-containing fragment is indicated at the left side of the panel.

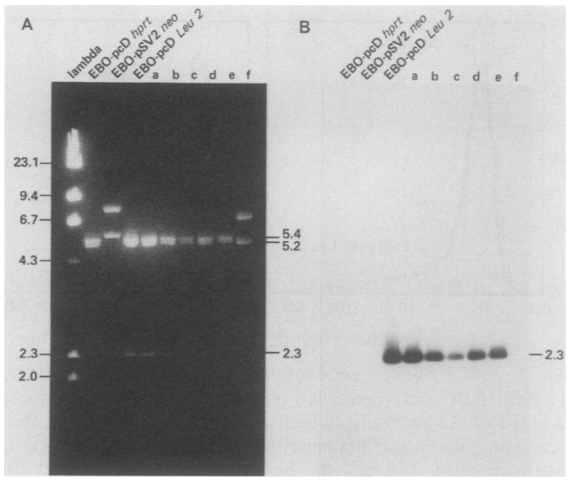

FIG. 6. Analysis of plasmids recovered from UC transformants which had received the EBO-pcD-*Leu-2*/EBO-pSV2-*neo* DNA mixture of 1:20,000 and been sorted and resorted for Leu-2 expression through four cycles in the FACS (Fig. 4, 4× sorted). Low-molecular-weight DNA from 5 × 10^6 of these Leu-2-positive cells was used to transform *E. coli* DH5 cells. Plasmid DNAs from six bacterial clones were digested with *Bam*HI endonuclease, separated by electrophoresis in 0.8% agarose gels, stained with ethidium bromide to visualize the DNAs, transferred, and hybridized. (A) ethidium-stained gel; (B) corresponding Southern blot hybridized with a *Leu-2*-specific probe. Plasmid DNAs from rescued colonies (lanes a through f) or marker DNAs (EBO-pcD-*hprt*, EBO-pSV2-*neo*, and EBO-pcD-*Leu-2*) were restricted with *Bam*HI. The positions and sizes in kilobases of *Hin*dIII-cut lambda DNA are indicated on the left margin. The *Bam*HI fragments from EBO-pcD-*Leu-2* are indicated at the right side of panel A. The position of the 2.3-kb *Bam*HI fragment from EBO-pcD-*Leu-2* which contains *Leu-2*-specific sequences is indicated in the right margin.

cells with DNA from about 5 × 10^6 cells yielded six bacterial transformants. Plasmid DNAs from these six bacterial clones were digested with *Bam*HI endonuclease, electrophoresed, transferred, and hybridized with a *Leu-2*-specific probe. Five of the six bacterial clones yielded *Bam*HI restriction endonuclease fragments which comigrated with those from EBO-pcD-*Leu-2* (Fig. 6). Furthermore, the 2.3-kb *Bam*HI restriction fragment from these five bacterial clones hybridized to the *Leu-2*-specific probe (Fig. 6). The sixth clone had presumably undergone a rearrangement, since it did not display a characteristic restriction pattern or hybridize to *Leu-2*- or *neo*-specific probes. Reintroduction of each of the five rescued EBO-pcD-*Leu-2* DNAs into UC cells led to expression of Leu-2 on the cell surface (data not shown).

Isolation of an EBO-pcD-*Leu-2* clone from an EBO-pcD library. A pcD library was constructed from mRNA obtained from a mouse L-cell line which had been transformed with human genomic DNA and then selected for Leu-2 expression by multiple rounds of the FACS (19–21). The EBO segment was inserted into this pcD library (see Materials and Methods). Three EBO-pcD sublibraries containing cDNA inserts from 0.5 to 3.7 kb were individually introduced by electroporation into UC cells, and hygromycin B-resistant transformants were analyzed for cell surface expression of Leu-2. The top 1.0% of Leu-2 fluorescent cells were sorted out, grown in hygromycin B, and resorted. After three or more rounds of sorting, only those cells which had received the sublibrary with 2.6- to 3.7-kb inserts expressed Leu-2. Five rounds of FACS sorting yielded an enriched population of transformants expressing Leu-2 (Fig. 7). Total DNA from these cells was digested with *Bam*HI, electrophoresed, transferred, and hybridized with a *Leu-2*-specific probe (Fig. 8). This blot indicates the presence of about eight copies per cell of an EBO-pcD-*Leu-2*-like plasmid with a *Leu-2* hybridizing cDNA insert of about 2.5 kb (Northern blots indicate that the *Leu-2* mRNA is about 2.5 kb in length [21]). Other blots (data not shown) indicate that eight copies of EBO-pcD-*Leu-2* are present as high-molecular-weight DNA and two copies are maintained as low-molecular-weight DNA (see Discussion). The low-molecular-weight DNA from these cells was used to transform *E. coli* DH5: DNA from 10^7 transformed UC cells yielded nine bacterial colonies. Four of the nine colonies yielded *Bam*HI restriction patterns similar to that of EBO-pcD-*Leu-2*, except that the *Leu-2* hybridizing cDNA insert was 2.5 kb in size (data not shown).

EBO-pcD-*hprt* expression. To test the feasibility of using the EBO-pcD expression vector for cloning a cDNA encod-

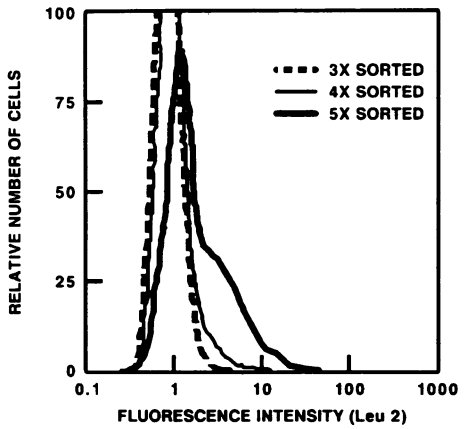

FIG. 7. FACS fluorescence intensity histograms. UC cells were transfected with a size-fractionated EBO-pcD-P2F sublibrary containing cDNA inserts of 2.6 to 3.7 kb. Hygromycin B-resistant transformants were analyzed for cell surface Leu-2 expression, and Leu-2-positive cells were sorted and resorted as described in the legend to Fig. 4. Histograms of cells sorted three, four and five times are displayed.

ing a metabolically required enzyme, we constructed EBO-pcD-*hprt* (Fig. 1). This EBO-pcD clone expresses the human *hprt* gene and complements the HPRT$^-$ defect of UC cells. Transfection of 5×10^7 UC cells with EBO-pcD-*hprt* yielded a hygromycin resistance transformation frequency of 10 to 15% and a HAT resistance transformation frequency of 5 to 10%.

Mixtures at levels of 1:2,000, 1:20,000, 1:200,000, and 1:1,000,000 of EBO-pcD-*hprt* and EBO-pSV2-*neo* DNAs were introduced by electroporation into UC lymphoblastoid cells.

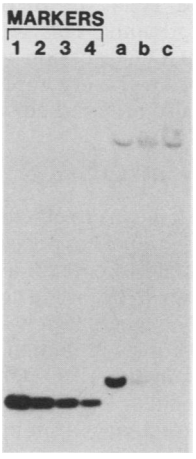

FIG. 8. Analysis of EBO-pcD-*Leu-2* plasmids in UC transformants which had received the EBO-pcD-P2F sublibrary containing cDNA inserts of 2.6 to 3.7 kb and had been sorted for Leu-2 expression as in Fig. 7. Total DNA from transfected UC cells was sorted five (lane a), four (lane b) or three (lane c) times. Markers containing reconstructions of 16 (lane 1), 8 (lane 2), 4 (lane 3), or 2 (lane 4) EBO-pcD-*Leu-2* and EBO-pSV2-*neo* plasmids per cell were obtained by mixing EBO-pcD-*Leu-2* and EBO-pSV2-*neo* plasmid DNAs as described in legend to Fig. 5. DNAs were digested with *Bam*HI, electrophoresed into 0.8% agarose gel, transferred to nylon, probed with a *Leu-2*-specific ^{32}P probe, and autoradiographed. *Bam*HI digestion of EBO-pcD-*Leu-2* yields a 5.4-kb fragment (vector), a 5.2-kb fragment (vector), and a 2.3-kb fragment (*Leu-2* insert).

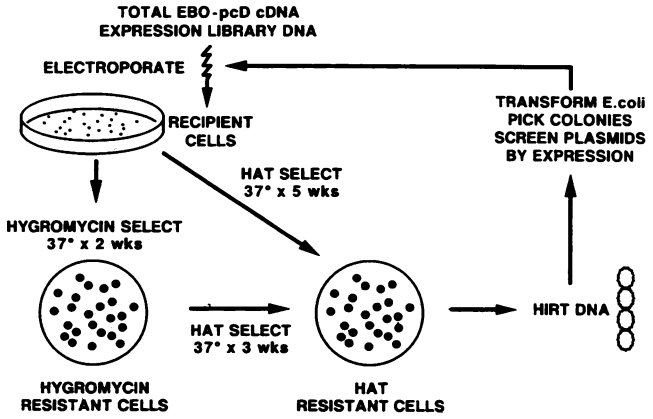

FIG. 9. Diagram of EBO-pcD cDNA expression cloning of a metabolically required enzyme (*hprt*). Reconstructed mixtures of EBO-pcD-*hprt* and EBO-pSV2-*neo* were introduced into UC cells by electroporation. At 48 h posttransfection, cells which survived electroporation were selected in medium containing HAT (5 weeks), or in medium containing hygromycin B (2 weeks) followed by medium containing HAT (3 weeks). Low-molecular-weight DNA isolated from HAT-resistant cells was used to transform *E. coli* DH5 cells. Plasmid DNAs were isolated from the *E. coli* transformants, reintroduced by electroporation into UC cells, which were tested for HAT resistance.

Survivors of the electroporation were selected in medium containing either hygromycin B or HAT (Fig. 9). HPRT-positive transformants were obtained irrespective of whether cells were selected initially in hygromycin B medium or directly in HAT medium (Tables 1 and 2). The frequency of HAT-resistant cells after initial selection with hygromycin B was approximately half that which would be predicted if each hygromycin-resistant transformant had received and maintained only one EBO-pcD plasmid (Table 2).

Episomal DNA and total genomic DNA were isolated from UC cells which had received the 1:200,000 mixture and been subjected to either the two-step selection (hygromycin followed by HAT) or the one-step selection (HAT alone) (Fig. 9). The DNAs were restricted with *Bam*HI endonuclease to release the *hprt* insert, with *Sac*I endonuclease to linearize the EBO-pcD-*hprt*, or with *Asp*718 (which does not cut EBO-pcD-*hprt*) to distinguish between high- and low-molecular-weight copies of EBO-pcD-*hprt*. Endonuclease-

TABLE 1. Direct HAT-resistance selection of UC lymphoblastoid cells transfected with EBO-pCD-*hprt* and EBO-pSV2-*neo* DNAs mixed at various ratios

Plasmid ratio[a]	HAT-resistant colonies[b] per:		Predicted HAT resistance frequency[c]
	5×10^6 cells	2×10^7 cells	
0:20,000	0	0	0
1:20,000	+	+	3.5×10^{-6}
1:200,000	+	+	3.5×10^{-7}
1:1,000,000	+	+	7.0×10^{-8}

[a] Ratio of EBO-pcD-*hprt* to EBO-pSV2-*neo*. The total amount of mixed plasmid DNA used for transfection was 100 μg per 5×10^7 UC cells.

[b] UC cells (5×10^6 or 2×10^7) that survived electroporation were selected en masse in flasks containing HAT medium.

[c] The expected HAT resistance frequency was calculated based upon the known hygromycin resistance frequency (7.0%) and the ratio of EBO-pCD-*hprt* to EBO-pSV2-*neo* in the transfected DNA.

TABLE 2. HAT resistance selection after preselection in hygromycin B of UC lymphoblastoid cells transfected with EBO-pcD-*hprt* and EBO-pSV2-*neo* DNAs mixed at various ratios

Plasmid ratio[a]	HAT resistance frequency	
	Determined[b]	Predicted[c]
0:20,000	0	0
1:20,000	2×10^{-5}	5×10^{-5}
1:200,000	3×10^{-6}	5×10^{-6}
1:1,000,000	5×10^{-7}	1×10^{-6}

[a] See footnote *a* of Table 1.
[b] Determined by dilutional plating of hygromycin-resistant transformants into HAT medium.
[c] Calculated based upon the ratio of EBO-pcD-*hprt* to EBO-pSV2-*neo* in the transfected DNA, assuming entry of one EBO plasmid per cell.

digested DNAs were electrophoresed, transferred, hybridized with a *hprt*-specific probe, and autoradiographed. In these transformants EBO-pcD-*hprt* was present as an unrearranged extrachromosomal plasmid at between two to eight copies per cell (Fig. 10). The *hprt* probe was stripped off and blots were rehybridized with a *neo*-specific probe and then autoradiographed. In transformants which were directly selected for HAT resistance, EBO-pSV2-*neo* was also present as an unrearranged episome at about 0.1 copy or less per cell (data not shown). In transformants which underwent the two-step selection (hygromycin then HAT), EBO-pSV2-*neo* was also present as an unrearranged episome at about 0.5 copy or less per cell (data not shown). The low-level presence of EBO-pSV2-*neo* presumably reflects passive loss of the *neo* episome during HAT selection, a condition which selects for EBO-pcD-*hprt* but not for EBO-pSV2-*neo*. During 30 to 50 generations in HAT selective medium, UC transformants increased the relative representation of EBO-pcD-*hprt* DNA versus EBO-pSV2-*neo* DNA by a factor of 10^6 to 10^7 (i.e., for the input DNA the ratio of EBO-pcD-*hprt* to EBO-pSV2-*neo* was 1:1,000,000, whereas in the recovered DNA the ratio was between 5:1 and 50:1 [data not shown]).

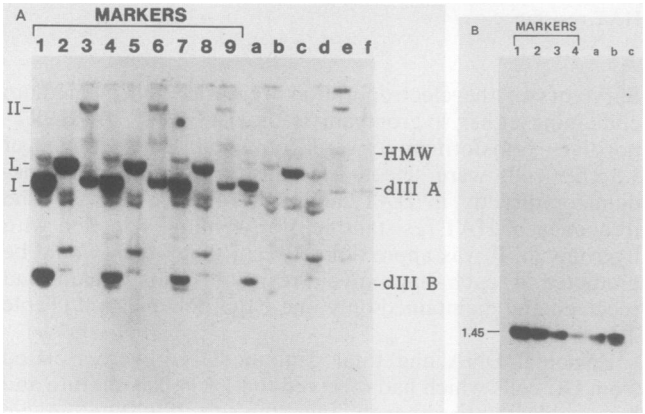

FIG. 10. Analysis of EBO-pcD-*hprt* plasmids in UC transformants which had received the EBO-pcD-*hprt*/EBO-pSV2-*neo* mixture of 1:200,000 and had been selected for HAT resistance directly or after preselection in medium containing hygromycin B (Fig. 9). DNAs were separated by electrophoresis in 0.8% agarose gels and detected by the method of Southern with a uniformly ^{32}P-labeled *hprt*-specific DNA probe. (A) Total DNA from transfected UC cells selected directly in HAT medium (lanes a, c, and e) or untransfected UC control cells (lanes b, d and f). Markers contained reconstructions of 8 (lanes 1 through 3), 4 (lanes 4 through 6), or 2 (lanes 7 through 9) EBO-pcD-*hprt* and EBO-pSV2-*neo* plasmids per cell. DNAs were digested with *Hin*dIII (lanes 1, 4, 7, a, and b). *Sac*I (lanes 2, 5, 8, c, and d), or *Asp*718 (lanes 3, 6, 9, e, and f). EBO-pcD-*hprt* is linearized by *Sac*I, uncut by *Asp*718, and cut into two fragments by *Hin*dIII. The positions of supercoiled (form I), nicked (form II), and linear (L) EBO-pcD-*hprt* are indicated in the left margin. The positions of high-molecular-weight UC DNA (HMW) and the two *Hin*dIII restriction fragments (dIII A and dIII B) of EBO-pcD-*hprt* are indicated on the right. (B) Total DNA from transfected UC cells selected directly in HAT medium (lane a) or preselected in medium containing hygromycin B followed by HAT selection (lane b) or untransfected control UC cells (lane c). Markers (lanes 1 through 4) contained reconstructions of 16, 8, 4, or 2 EBO-pcD-*hprt* and EBO-pSV2-*neo* plasmids per cell. DNAs were digested with *Bam*HI. *Bam*HI digestion of EBO-pcD-*hprt* yields a 5.4-kb fragment (vector), a 5.2-kb fragment (vector) and a 1.45-kb fragment (*hprt* insert). The position of the 1.45-kb *hprt*-containing fragment is indicated at the left.

Episomal DNA isolated from these HAT-resistant transformants was used to transform *E. coli* DH5. Between 20 and 100 bacterial colonies were obtained from the episomal DNA from 2×10^6 HAT-resistant UC transformants. Ten bacterial colonies each were picked from the one-step (HAT) or two-step (hygromycin then HAT) selected samples. Plasmid DNAs were prepared, restricted, and hybridized as above for the episomal DNAs. All 10 rescued colonies from the one-step selection contained plasmid DNAs with a restriction pattern characteristic of EBO-pcD-*hprt* (data not shown); 5 of the 10 rescued colonies from the two-step selection contained plasmid DNA with a restriction pattern characteristic of EBO-pcD-*hprt*, 4 contained plasmid DNA with a restriction pattern characteristic of EBO-pSV2-*neo* DNA, and 1 clone was apparently rearranged and lacked either *hprt* or *neo* sequences (data not shown). Five of the 10 clones rescued by one-step selection and the 5 EBO-pcD-*hprt*-like clones rescued from the two-step selection were reintroduced into UC cells; all 10 of the clones yielded HAT-resistant transformants. This set of experiments indicates that the EBO-pcD expression vector can be used to directly select and recover very rare clones (10^{-6}) which code for a metabolically required enzyme.

DISCUSSION

We have described a means to efficiently transform human lymphoblastoid cells with pcD expression libraries. The pcD vector has been modified to contain a selectable drug resistance marker (*hph*), an EBV origin for plasmid replication (*oriP*), and the EBNA gene. Such EBO-pcD clones are maintained at 2 to 10 copies in human lymphoblastoid cells. Transformants maintain the EBO-pcD episomes stably as long as selection is applied. EBO-pcD has utility as a shuttle vector; selection and recovery from both *E. coli* and human cells are readily achieved.

A high-molecular-weight form of some copies of EBO-pcD-*Leu-2* occurred after four rounds of FACS selection (Fig. 5); this is presumably due to head-to-tail integration or concatamer formation. This seems to be a result of the FACS selection process and not an inherent property of the EBO plasmid. During the third and fourth rounds of FACS selection (Fig. 4), cells expressing an amount of Leu-2 equivalent to that of the topmost 1 to 2% of cells transfected with EBO-pcD-*Leu-2* alone were isolated. This selection procedure may have isolated a rare subpopulation of cells wherein such high expression required plasmid integration or concatamer formation. When UC cells were transfected

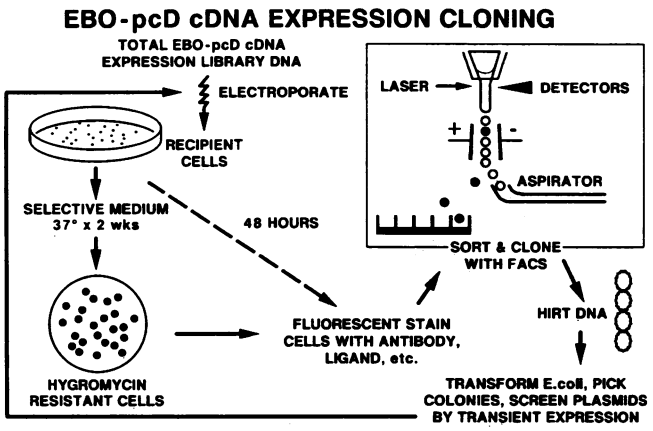

FIG. 11. EBO-pcD-cDNA expression cloning of cell surface receptors (see text for details).

with EBO-pcD-*Leu-2* and continuously selected for hygromycin resistance, but not selected for Leu-2 expression, a uniformly positive population of Leu-2-expressing cells were obtained even after 40 generations. These unsorted cells maintained about 10 copies of EBO-pcD-*Leu-2* per cell, all of which were present as freely replicating monomer plasmids (data not shown).

This vector was developed to improve the pcD expression-cloning system to allow for rapid selection, cloning, and recovery of cDNA clones. A schematic representation of how EBO-pcD-cDNA expression cloning could be utilized to clone cell surface markers and receptors is shown in Fig. 11. The salient features of this approach are as follows. (i) An EBO-pcD library of several million independent bacterial clones is generated with mRNA from a cell line expressing the receptor gene to be cloned. (ii) DNA from the total EBO-pcD-cDNA expression library is introduced by electroporation into a receptor-negative recipient cell line. (iii) Several million independent mammalian cell transformants are selected by growth in medium containing hygromycin B. (iv) Hygromycin-resistant transformants (or unselected cells at 48 h posttransfection) are screened for expression of cell surface receptors by the binding of fluorescent ligand or fluorescent antibody, followed by sorting with the FACS. (v) Repeated cycles of sorting with the FACS are used to isolate those rare transformants expressing cell surface receptor. (vi) EBO-pcD episomes are rescued from receptor positive cells by the Hirt procedure for isolation of low-molecular-weight DNA and used to transform competent *E. coli*. (vii) EBO-pcD plasmids recovered from individual bacterial colonies are screened for the induction of expression of receptors by reintroduction of the DNAs into mammalian cells. This cloning procedure has been used with a fluorescent antibody to clone a cell surface marker cDNA (Leu-2 [CD8]); we are testing its utility in the cloning of mammalian receptor cDNAs by utilizing fluorescent ligands.

The cloning schemes for cell surface markers, cell surface receptors, and metabolically required enzymes utilize the unique capabilities of the EBO-pcD system. The ability to isolate 10^6 to 10^7 EBO-pcD human lymphoblastoid transformants means that the entirety of highly complex pcD expression libraries may be introduced into these cells. Such efficiencies are necessary if cDNAs derived from low-abundance mRNAs present at the level of three to four copies in a typical cell's complement of 1×10^5 to 3×10^5 mRNAs are to be successfully cloned (5, 42).

This method of cloning requires that the cDNA encoding the selectable function be full length (or at least expressible) and that the selected function be encoded by a single cDNA. This is especially important if the cDNA is a copy of a rare or particularly long mRNA. Functions requiring the expression of more than one cDNA are not likely to be detected unless their abundance is such as to ensure that cells will be transfected by the requisite number of plasmids. For an intermediate-sized mRNA such as *Leu-2* (2.3 kb) we found approximately 10% full-length cDNAs (unpublished experiments).

We have included the EBNA gene in our cloning vector so that both the *cis* (*oriP*) and *trans* elements of the EBV replicon are present in the EBO-pcD library. However, with EBNA-positive cell lines, the EBO-pcD-encoded EBNA gene may be unnecessary. Furthermore, cell lines expressing very high levels of EBNA may be superior to UC cells as recipients for transfection and expression of EBO-pcD. Recently, an EBV-derived cosmid vector has been developed which can replicate autonomously in EBNA-positive cells and accommodate ≥35 kb of genomic DNA (22). This raises the possibility (presently untested) of using EBV-based vectors to clone genomic sequences by their expression in transfected mammalian cells.

In contrast to other methods, which require significant preknowledge of the protein of interest to be cloned (e.g., protein purification and peptide sequencing), our approach does not require such data. Indeed, if a mammalian cell function or phenotype can be selected, it may be possible to clone this gene in the absence of any physical data regarding the protein itself. Recently, the pcD expression system has been used to clone one of the opioid receptor genes (Law et al., in press).

The availability of a selectable, stably maintained replicating vector for expression of cDNAs in eucaryotic cells leads to possibilities other than expression cloning. For example, site-directed mutagenesis of EBO-pcD receptor clones followed by introduction into human cells could be used to select mutants with altered binding capabilities. The EBO-pcD vector allows the ready introduction, expression, and recovery of such mutagenized genes. This will allow the types of genetic approaches that have previously only been facile with bacterial, yeast, or viral genes. Other gene targets for this type of directed mutagenesis within a shuttle vector could include required enzymes (e.g., HPRT, Na^+ K^+ ATPase) and polymerases (e.g., α-amanatin-resistant RNA polymerase II).

Finally, we point out the potential utility of EBO-pcD for isolating *trans*-acting regulatory factor cDNAs. For this purpose cell surface markers driven by known *cis* regulatory elements could be transfected into cells which do not normally express that particular surface marker or the specific *cis*-regulated genes. Subsequently, these cells would be transfected with an EBO-pcD expression library containing cDNAs of the *trans*-acting regulatory factor. FACS sorting could be used to identify and recover those cells expressing the *cis*-regulated cell surface marker. Presumably, some of the expressing cells would reflect the *trans*-activation by an EBO-pcD cloned regulatory factor of the *cis*-regulated cell surface marker. Recovery and confirmation of the nature of this EBO-pcD clone would follow as we have described it for EBO-pcD-*Leu-2*.

ACKNOWLEDGMENTS

We thank Bill Sugden for plasmid p220.2 and Hiroshi Hayakawa for plasmid p220-1 and many helpful discussions.

This work was supported by Public Health Service grant GM-13235 from The National Institute of General Medical Sciences. Robert F. Margolskee was the recipient of a fellowship grant from The Jane Coffin Childs Memorial Fund for Medical Research. Paula Kavathas was the recipient of an Argall L. and Anna G. Hill Cancer Research Award.

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