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Isolation of the gene encoding the human T-lymphocyte differentiation antigen Leu-2 (T8) by gene transfer and cDNA subtraction

(amplification/fluorescence-activated cell-sorter/transfectants/cell-surface antigens)

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ABSTRACT **We report the isolation of genomic and cDNA clones encoding the human T-lymphocyte cell-surface differentiation antigen, Leu-2 (T8), by use of a combination of transfection, fluorescence-activated cell-sorting, and subtractive cDNA hybridization. We constructed a cDNA library with mRNA from a mouse L-cell transfectant in which the human *Leu-2* gene is expressed and amplified. We identified *Leu-2* cDNA clones by screening with a selected cDNA probe from a second amplified Leu-2 transfectant. This probe contained cDNA species not removed by hybridization with L-cell mRNA. A *Leu-2* cDNA clone was used to isolate a genomic clone. Transfection with DNA from this clone resulted in a high number of Leu-2 transfectants. This approach can be used to clone genes coding for other cell-surface molecules.**

Leu-2 (synonym, T8) is expressed by most T cells with cytotoxic or suppressor function. The molecule appears to be composed of multimers of a 32-kDa and a 45-kDa polypeptide on thymocytes, and of a 32-kDa polypeptide on peripheral blood lymphocytes (PBL) (1–3). A possible role for the Leu-2 antigen in target-cell recognition is suggested by observations that monoclonal antibodies against Leu-2 block killing activity of many cytotoxic T cells, which recognize foreign antigen in association with a class I major-histocompatibility-complex molecule (4–7). The same is true in the mouse, where antibody against Lyt-2, the proposed homologue of Leu-2 (2), shows the same effect (8–10).

Progress in understanding the structure, function, and regulation of Leu-2 and other differentiation antigens would be aided by obtaining clones of the structural genes for these molecules. Because of the small amounts and the slow turnover rates of these proteins on T cells, the abundance of the corresponding mRNAs is expected to be very low. Conventional recombinant DNA cloning methods, which start with mRNA purification or protein sequence determinations, could be quite difficult. For that reason, we used DNA-mediated gene transfer to cotransfect mouse L(TK$^-$) cells with total cellular DNA and a plasmid containing the thymidine kinase (*TK*) gene to obtain mouse L-cell transfectants that had incorporated and were stably expressing genes for T-cell differentiation antigens. Transfectants expressing Leu-2 or other antigens were selected by a fluorescence-activated cell-sorter (FACS) after they were stained with specific fluorescein-conjugated monoclonal antibodies.

Originally, we planned to clone the transferred gene by one of the previously described methods for isolating DNA from transfected cells (12–18). However, we took advantage of the availability of Leu-2 transformants in which the *Leu-2* gene was highly amplified. Some 25–50% of the Leu-2 transfectants spontaneously amplified the *Leu-2* gene (19). We had been able to select highly amplified transfectant cell lines producing large amounts of Leu-2 glycoprotein by sorting the most intensely stained cells with the FACS, growing these cells, and repeating the process multiple times (19). The high levels of surface expression suggested that there would be correspondingly increased amounts of Leu-2 mRNA in these amplified lines. In this work, we constructed a cDNA library from one amplified transformant and screened the library with a selected cDNA probe made with mRNA from a second amplified transformant and subtracted by hybridization with RNA from recipient cells. This approach is highly specific, as evidenced by the fact that all of the positive clones contained *Leu-2* sequences, and is an attractive alternative to some of the other schemes for cloning transferred genes.

METHODS

cDNA Library. RNA was extracted from exponentially growing cells by the guanidinium thiocyanate method (20). Poly(A)$^+$ RNA was selected by oligo(dT)-cellulose chromatography (21). Single-stranded cDNA was synthesized from poly(A)$^+$ J10 RNA by oligo(dT) priming using avian myeloblastosis virus reverse transcriptase. Double-stranded cDNA was synthesized by "self-priming" using the Klenow fragment of *Escherichia coli* DNA polymerase I. The resulting "hairpin loop" was cleaved by nuclease S1 digestion. The double-stranded cDNA was treated with *Eco*RI methylase, and flush ends were achieved with the Klenow fragment of DNA polymerase I. *Eco*RI linkers were added and digested with *Eco*RI, and the material was then size-fractionated on a Sephacryl S-1000 (Pharmacia) column. cDNAs [0.7–3 kilobase pairs (kbp)] were inserted into the imm434 *Eco*RI insertion vector λgt10 (22). The ligated DNA was packaged and the phage was grown in *E. coli* C600 Hfl. The cDNA library contained 3×10^5 clones.

cDNA Subtracted Probe. The procedure used for preparing ^{32}P-labeled selected cDNA probes from P2F RNA is similar in principle to those of Alt *et al.* (23), Timberlake (24), and Davis *et al.* (25). ^{32}P-labeled cDNA was synthesized from poly(A)$^+$ RNA by use of avian myeloblastosis virus reverse transcriptase in the presence of actinomycin D. The cDNA was hybridized to an equivalent R_0t [initial concentration of nucleic acid (moles of nucleotide/liter) × time (sec)] of ≈1000 with a 20-fold mass excess of poly(A)$^+$ L-cell RNA. RNA·cDNA hybrids were removed by chromatography on

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Abbreviations: TK, thymidine kinase; FACS, fluorescence-activated cell-sorter; PBL, peripheral blood lymphocytes; kb, kilobase(s); kbp, kilobase pair(s).

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hydroxyapatite. The single-stranded cDNA was concentrated, desalted on a Sephadex G-50 column, precipitated, resuspended, and used for a second round of hybridization to L-cell mRNA. Hydroxyapatite-purified single-stranded cDNA was then used directly as a probe. Starting with 5 μg of poly(A)$^+$ RNA, we obtained ≈10^6 cpm (representing 2% of the ^{32}P-labeled cDNA initially synthesized) in the effluent of the second hydroxyapatite column.

RNA Blots. RNAs were prepared by the guanidinium thiocyanate method (20). Poly(A)$^+$ RNA was obtained by chromatography on oligo(dT)-cellulose (21). RNA samples in 50% formamide/2.2 M formaldehyde/20 mM 3-(*N*-morpholino)propanesulfonic acid (Mops)/5 mM sodium acetate/1 mM EDTA, pH 7.0, were denatured by heating for 5 min at 70°C, chilled, and electrophoresed through a 1.2% agarose gel containing 2.2 M formaldehyde and ethidium bromide (6.25 μg/ml) in 20 mM Mops/5 mM sodium acetate/1 mM EDTA, pH 7.0. The electrophoresed RNA samples were transferred to a nitrocellulose filter according to the procedure of Thomas (26). The blot was hybridized to the nick-translated (>10^8 cpm/μg) 1.2-kbp insert of a *Leu-2* cDNA clone for 18 hr at 42°C in 40% formamide/0.6 M NaCl/0.06 M sodium citrate/0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin/0.1% NaDodSO$_4$/10% dextran sulfate/20 mM Tris Cl, pH 7.6, containing denatured herring-sperm DNA at 100 μg/ml. The filter was washed three times at room temperature with 0.3 M NaCl/0.03 M sodium citrate, pH 7.0/0.05% NaDodSO$_4$ and twice at 52°C with 15 mM NaCl/1.5 mM sodium citrate, pH 7.0/0.05% NaDodSO$_4$ and then exposed to XAR-5 film for 16 hr with an intensifying screen.

Transfection and Cell Staining. The L(TK$^-$) cells were transfected with 18 μg of L-cell carrier DNA and 1 μg of pBR322 containing the chicken *TK* gene per 10^6 cells by a standard calcium phosphate precipitation procedure (27, 28). In addition, some of the dishes received 1 μg of pBR322 containing the putative *Leu-2* genomic clone. TK$^+$ cells were selected by growth in hypoxanthine/aminopterin/thymidine medium. After 2 weeks, cells were stained with fluorescein-conjugated anti-Leu-2a monoclonal antibody followed by fluorescein-conjugated goat anti-mouse antibody (11).

RESULTS

We used two highly amplified cell lines derived from primary Leu-2 transfectants, J10 and P2F. The J10 and P2F transfectants were isolated after transfection with DNA from a human T-cell lymphoma (JM) or from human placenta, respectively. We constructed a cDNA library starting with poly(A)$^+$ mRNA from the amplified J10 cells and screened the library with a selected cDNA probe made from mRNA from the amplified P2F transfectant, as follows: ^{32}P-labeled cDNA reverse-transcribed from mRNA of P2F cells was depleted of L-cell sequences by hybridization in solution with L-cell mRNA and passage over a hydroxyapatite column to remove cDNA·mRNA hybrids (25). After two cycles of hybridization and hydroxyapatite separation, the probe hybridized detectably to 0.7% of 8500 phage plaques in the cDNA library (as did a selected cDNA probe reverse-transcribed from J10 mRNA) (Fig. 1). We expected that the major common cDNA species between the P2F probe and the J10 cDNA library would be cDNAs corresponding to *Leu-2* and *TK* (used for cotransfection). cDNA species of human genes closely linked to *Leu-2*, of nondepleted mouse sequences, or of pBR322 might also be present. Approximately 80% of 60 hybridizing plaques were positive when retested. We isolated DNA from phage stocks prepared from 9 of these plaques.

The cDNA inserts from all 9 candidate clones cross-hybridized (data not shown). The insert of each clone was nick-translated and hybridized to Southern blots of genomic DNA from the two Leu-2 transfectants P2F and J10 and from L cells. The DNA was digested with *Bam*HI since this enzyme did not cleave within the gene, as determined by DNA-mediated gene transfer (unpublished results). After autoradiography, a band of ≈14 kbp was seen for J10 and P2F DNA (Fig. 2). An additional band of >23 kbp was present with P2F DNA. No bands were visible with L-cell DNA. None of the hybridizing fragments represented *TK* DNA; a Southern blot with P2F and J10 DNA hybridized to a cloned *TK* gene probe resulted in a totally different pattern (data not shown).

The cDNA probes also hybridized to a 14-kbp *Bam*HI fragment on Southern blots made with DNA from a T-cell

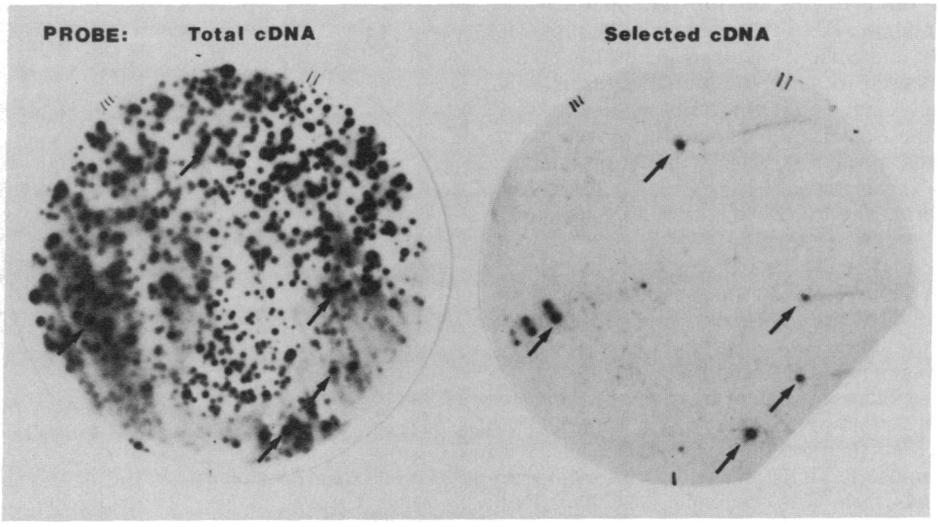

FIG. 1. Autoradiogram of replica filters of the J10 cDNA library hybridized to ^{32}P-labeled P2F unselected cDNA (*Left*) and selected cDNA (*Right*). Hybridization was at 65°C overnight in 5× NaCl/P$_i$/EDTA (1× is 0.15 M NaCl/0.05 M sodium phosphate/1 mM EDTA)/10% dextran sulfate/poly(A) (50 μg/ml)/0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin/salmon sperm DNA (100 μg/ml). Filters were washed twice in 2× NaCl/P$_i$/EDTA/0.1% NaDodSO$_4$ at room temperature for 30 min and then once in 0.4× NaCl/P$_i$/EDTA/0.1% NaDodSO$_4$ at 65°C. Autoradiography was at −70°C for 24 hr using Kodak XAR-5 film and an intensifying screen. Of 1500 plaques on the original plate, ≈80% were positive with the unselected probe and 0.7% were positive with the selected probe. Arrows show that colonies hybridizing with the selected probe also hybridized with the unselected probe.

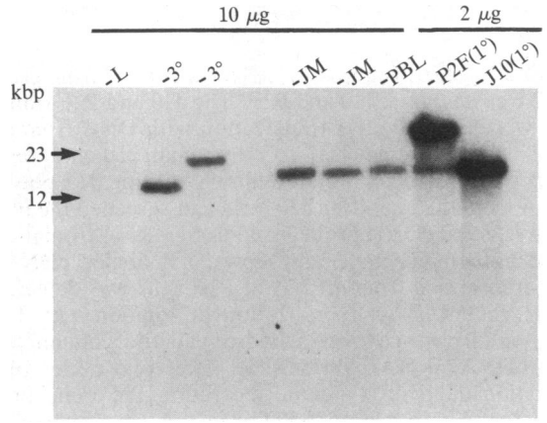

FIG. 2. Autoradiogram of Southern blot showing that a cDNA insert from a candidate phage clone corresponds to a human gene that is amplified in the *Leu-2*-amplified transfectants. Ten micrograms of DNA from mouse L cells (L), each of two tertiary Leu-2 transfectants (3°), a human T cell lymphoma (JM), and human PBL, and 2 μg of DNA from each of two *Leu-2*-amplified primary transfectants, P2F and J10, were digested to completion with *Bam*HI, electrophoresed through a 0.75% agarose gel, transferred to nitrocellulose filter paper, hybridized with a ^{32}P-labeled 1.7-kbp cDNA insert in 50% formamide/5× NaCl/P_i/EDTA/0.1% polyvinylpyrrolidone/0.1% Ficoll/0.1% bovine serum albumin/0.1% NaDodSO$_4$/salmon sperm DNA (100 μg/ml)/poly(A) (50 μg/ml) at 42°C, washed twice at room temperature with 2× NaCl/P_i/EDTA containing 0.1% NaDodSO$_4$ and twice at 65°C with 0.2× NaCl/P_i/EDTA containing 0.1% NaDodSO$_4$, and then exposed to XAR-5 film with a DuPont Lightning Plus intensifying screen.

lymphoma, JM, and from PBL (Fig. 2). Furthermore, the bands detected with J10 and P2F DNA were highly amplified relative to those from JM and PBL DNA (Fig. 2). The presence of two amplified bands with P2F DNA probably represents a gene rearrangement that occurs during gene amplification.

In support of the conclusion that the cloned cDNAs correspond to the *Leu-2* gene, we showed that the cloned cDNA hybridized to a single band on Southern blots of DNA from tertiary Leu-2 transfectants. This hybridization provides strong evidence that the cloned cDNAs encode Leu-2, because after two or three cycles of transfection through mouse cells the only human DNA that is likely to be present is the gene of interest and closely linked sequences. In addition, since the DNA used to make the secondary transfectants was completely digested with *Bam*HI, linked sequences outside of the *Bam*HI sites should have been lost. The hybridizing bands were either slightly larger or smaller than 14 kbp (Fig. 2). This is not surprising since alterations in DNA may occur during the cycles of transfection through mouse L cells.

To prove that the cDNA clones corresponded to *Leu-2*, we used one cDNA clone to isolate a genomic clone that stably transfects L cells with *Leu-2*. To isolate genomic clones, we screened a library we constructed in pBR322 of 12–22 kbp *Bam*HI fragments of J10-amplified DNA. We had previously found that sucrose gradient fractions of *Bam*HI-cut donor DNA corresponding to about 12–18 kbp yielded more Leu-2 transfectants than did smaller-size fractions. We then cotransfected mouse L(TK$^-$) cells with DNA from one of the positive clones from this screening, L-cell carrier DNA, and *TK* plasmid DNA. When the TK$^+$ cells were analyzed on the FACS after staining with anti-Leu-2 antibody, 22% of the cells were positive, whereas none of the control TK$^+$ transfectants were positive (Fig. 3). The transfecting activity of the isolated genomic clone proves that the cDNA and the genomic clones we isolated both encode the Leu-2 antigen.

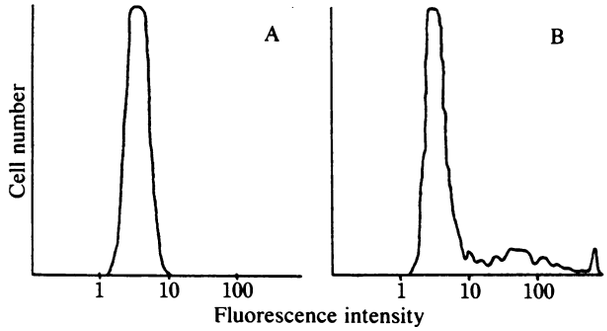

FIG. 3. FACS fluorescence-intensity histograms of *Leu-2* genomic clone and control-transfected mouse L cells. In contrast to L cells that received carrier DNA alone (*A*), ≈22% of the L cells that received DNA from the putative *Leu-2* genomic clone (*B*) were stained with various fluorescence intensities above background.

Using a cloned *TK* probe, we found that the abundance of *TK* cDNA in our library was 0.01%. Thus, it is not surprising that none of the cDNA candidates we isolated from the 8500 plaques screened encoded the *TK* gene.

We then characterized the mRNA for Leu-2 from tissues expressing the antigen on the surface, as detected by immunofluorescence. A single 2.5-kilobase (kb) band was seen on RNA blots from human thymus, JM (a T-cell lymphoma), and the two amplified Leu-2 transfectants, P2F and J10, after hybridization with a ^{32}P-labeled 1.2-kb insert from a *Leu-2* cDNA clone (Fig. 4). The same 2.5-kb band was also present in a blot of membrane-bound mRNA from P2F (data not shown) suggesting that the mRNA for Leu-2 is synthesized on membrane-bound polyribosomes. In contrast, mRNA from human placenta, human B lymphoblastoid line LCL-721 (Epstein–Barr virus-transformed), human erythroleukemic line K562, and mouse L cells showed no hybridization to this probe (Fig. 4). The absence of Leu-2 mRNA in cells that do not express the protein suggests that the regulation of this

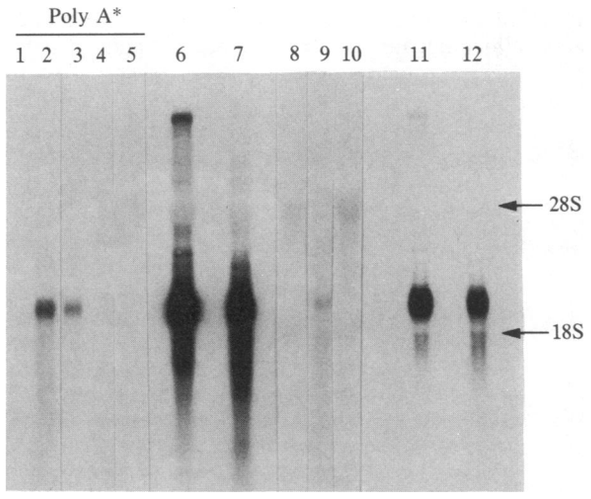

FIG. 4. Autoradiogram of blotted RNA from different sources hybridized to a *Leu-2* cDNA probe. Lanes 1–5 contain (from left to right) 2 μg of poly(A)$^+$ RNA from human placenta, human thymus, JM (a human T-cell lymphoma line expressing Leu-2), K562 (a human erythroleukemic line), and HeLa cells. Lanes 6 and 7 contain 10 μg of total RNA from J10 and P2F cells (two L-cell transfectants expressing Leu-2), respectively. Lanes 8–10 contain (from left) 10 μg of total RNA from L cells, human thymus, and LCL-721 (an Epstein–Barr virus-transformed human B-cell line). Placenta, K562, HeLa, and LCL-721 cells do not express Leu-2 antigen. A less-exposed autoradiogram of lanes 6 and 7 is shown on the right (lanes 11 and 12). The positions of ribosomal RNA size markers are indicated on the far right.

gene product is at the level of transcription, although differences in mRNA degradation rates cannot yet be ruled out. The intensity of the Leu-2 mRNA band in the blots from *Leu-2* amplified lines confirms our hypothesis that the increased amount of Leu-2 on the cell surface corresponds to increased Leu-2 mRNA. The additional bands of lesser intensity observed with the amplified mRNA may represent normal or aberrant mRNA precursors or alternative mRNA splicing.

DISCUSSION

The combined use of transfection and selected cDNA probes is a powerful approach to isolating genes for many cell-surface antigens or receptors. Transfectants expressing a variety of mouse and human cell-surface molecules have been isolated by ourselves and others (11, 12, 29–31). The frequency of such L-cell transfectants obtained by using cotransfection with *TK* for primary enrichment is high enough to make FACS selection feasible (11). These transfectants typically incorporate about 0.1% of the human genome (32, 33). Therefore, two primary transfectants generally share few transferred genes besides the selected gene. In addition, once a gene is transferred, its expression may be unstable if there is no selective pressure (34). Therefore, many of the transferred genes may not be expressed after the cells have been passaged. Selected cDNA probes that are highly specific for the gene of interest can be generated because secondary transfectants and probably most primary transfectants express only one or a small number of mRNA species not present in the original L cells.

In support of this assertion is our finding that the frequency of cDNA clones in the library hybridizing with a *TK* probe was about 0.01% even though the *TK* gene was amplified 40-fold in the J10 *Leu-2*-amplified line. Although TK expression was selected for initially, there was no selection for its increased expression during *Leu-2* amplification. In fact, selection may have occurred against cells expressing too much TK because high levels of thymidylic acid are toxic to cells.

In this study, we were able to use transfectants in which the *Leu-2* gene was highly amplified; the abundance of the Leu-2 mRNA sequences in the cDNA library was ≈0.5%. The selected-probe approach should also work for mRNAs of much lower abundance. Hedrick *et al.* (35) showed that a T-lymphocyte selected probe, subtracted with mRNA from B lymphocytes, detected cDNA clones corresponding to very rare mRNA species, such as Thy-1 mRNA. Therefore, even if a gene is not amplified in a transfectant, as long as there is no cross-hybridizing L-cell mRNA, a selected cDNA probe can detect the cDNA clones of interest. Candidate clones can then be analyzed by hybridization with comparative RNA blots of specific transfectant RNA and either irrelevant transfectant or L-cell RNAs and finally confirmed by transfection with isolated genomic clones.

The specificity obtained by our approach of screening a library made with cDNA from one transformant with a selected cDNA probe from a different transformant is greater than that of screening with selected cDNA probes made using closely related tissues. When Hedrick *et al.* (35) used a cDNA probe of T-cell cDNA subtracted with B-cell mRNA, they identified 10 different cDNA clones specific for T cells, and considerably more could exist based on estimates of differences in mRNA species. An independent assay is required to identify a clone of interest. On the other hand, since primary transformants take up about 0.1% of the human genome, two independent primary transformants selected for the gene of interest should not share unlinked transferred genes. Screening a library with a selected probe made from a secondary transfectant or with probes made from two different primary transformants would also be a powerful approach for detecting clones of interest.

It is interesting that the Leu-2 mRNA is 2.5 kb long both in thymocytes and in JM cells. This is substantially longer than the sequence needed to encode the 32-kDa polypeptide found on Leu-2-expressing peripheral blood T cells, thymocytes, JM cells, and the J10 Leu-2 transfectants. Whether the mRNA encodes more than just the 32-kDa polypeptide can now be determined.

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