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Immunology

Stable transformation of mouse L cells for human membrane T-cell differentiation antigens, HLA and β_2-microglobulin: Selection by fluorescence-activated cell sorting

(gene cloning/Leu-1, Leu-2 transformants)

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ABSTRACT **We isolated stable transformants of mouse L cells expressing human cell surface differentiation antigens by using immunofluorescence with monoclonal antibodies and selection with a fluorescence-activated cell sorter (FACS). Mouse L cells (TK^-) were cotransformed with human cellular DNA and the herpes simplex virus thymidine kinase (TK) gene. TK^+ transformants were first selected. The TK^+ populations were stained with various fluorescent antibodies to membrane antigens, and positive cells were sorted and cloned by using a FACS. Transformants for HLA class I antigens, for β_2-microglobulin, and for the T-cell differentiation antigens Leu-1 and Leu-2 were isolated. The frequency of antigen transformants among the TK^+ transformants was about 0.5×10^{-3}. The sizes of the HLA, Leu-1, and Leu-2 molecules expressed by the transformants were the same as those of the proteins present on DNA-donor cells.**

Gene transformation of mammalian cells has been a useful tool for cloning certain genes (1, 2) and for analysis of transferred genes and their products (3, 4). However, an appropriate selection system is needed to transfer genes with total cellular DNA because the frequency of transfer of single-copy genes is quite low, $1–10 \times 10^{-7}$ (5, 6). Biochemical and morphological selection systems have been widely used to identify and clone oncogenes and genes coding for certain enzymes (7–10).

Recently, Chang *et al.* (11) showed that transient expression of two human differentiation antigens can be detected in mouse L cells (a transformed fibroblast line) after transformation with human cellular DNA. The antigens My-1 (granulocyte specific) and OKT3 (T-cell specific) were detected with fluorescent monoclonal antibodies and a fluorescence microscope. However, expression was lost after 72 hr so that established procedures for gene cloning of transfected genes and certain kinds of genetic analysis are not feasible because stable transformants are required.

In this paper, we report the isolation of stable transformants of mouse L cells expressing the human T-cell differentiation antigens Leu-1 and Leu-2 after transformation with human T-cell DNA. Leu-1, also called OKT1, is expressed on all T lymphocytes (12) and a subpopulation of B lymphocytes (13) while Leu-2, also called OKT5 (-T8), is expressed on a subset of T cells that have suppressor or cytotoxic function. Leu-2 appears to play a role in recognition of foreign antigen by cytotoxic T cells (14).

To identify and select transformants for cell surface antigens, we used fluorescein-conjugated monoclonal antibodies and a fluorescence-activated cell sorter (FACS). The sorter has been used previously to isolate cells at frequencies of 10^{-7} (15, 16). To enrich for transformants in general, we cotransformed mouse L cells with total cellular DNA and with the herpes simplex thymidine kinase (TK) gene and selected for TK^+ transformed cells by growth in hypoxanthine/aminopterin/thymidine (Hyp/Apt/Thd) medium. Initially, HLA class I and human β_2-microglobulin (β_2m) transformants were selected to establish procedures for selecting rare transformants from populations of TK^+ transformed cells. Later, selection for transformants expressing T-cell differentiation antigens was imposed. The frequency of antigen transformants for both Leu-1 and Leu-2 antigens among the TK^+ transformants was about 0.5×10^{-3}. This frequency is high enough so that appropriately stained Leu transformants can be detected by the FACS and directly cloned into microtiter wells. FACS selection of transformants for cell surface antigens should be generally applicable.

Because the transformants are stable, the differentiation antigens produced by them can be analyzed biochemically and compared with the corresponding molecules in T cells. The Leu-1 molecule is a 70-kilodalton (kDal) polypeptide and the Leu-2 molecule is a disulfide-linked heterodimer of 75–80 kDal (12). Molecules specifically immunoprecipitated from several of the Leu-1 and Leu-2 transformants had the same polypeptide chain compositions and apparent molecular masses as the corresponding molecules on the human donor cells. Two chains of the Leu-2 molecule were present. Thus, these antigens appear to be synthesized by the mouse L cells identically to the DNA-donor T cells.

METHODS

Cell Lines. TK^- mouse L cells were originally established by Kit *et al.* (17). They were grown in Dulbecco's modified Eagle's medium/10% fetal calf serum containing penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells were collected either by incubating them at 37°C for 5 min in 0.25% trypsin (GIBCO) or by rinsing the dishes with phosphate-buffered saline (P_i/NaCl) and adding warm (37°C) P_i/NaCl/0.6 mM Na_2EDTA for 5 min at 37°C. Cells were dislodged by vigorous pipetting.

The JM line was established from a patient with acute lymphoblastic leukemia of T-cell origin and was made available by J. Minowada (Roswell Park Memorial Institute, Buffalo, NY). Its HLA type is A3, A25, B7, B37, C4, and it also expresses the T-cell antigens Leu-1–Leu-6 but does not have detectable surface HLA-DR.

Reagents. The fluorescein-conjugated mouse anti-human Leu reagents were obtained from Becton Dickinson Monoclonal

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Abbreviations: FACS, fluorescence-activated cell sorter; TK, thymidine kinase; Hyp/Apt/Thd, hypoxanthine/aminopterin/thymidine; β_2m, β_2-microglobulin; kDal, kilodalton(s); P_i/NaCl, phosphate-buffered saline; kb, kilobase(s).

Antibody Center (Mountain View, CA). Monoclonal antibodies W6/32 and BBM1 were provided by Frances Brodsky and Peter Parham (Stanford University). Reagents were centrifuged at 100,000 × *g* for 10 min in an Airfuge before use.

Immunofluorescence Staining and FACS Analysis. Cells were collected in P_i/NaCl/EDTA and chilled on ice. For sorting, one million cells were stained with saturating amounts of antibody for 30 min at 0°C in 100 μl of staining medium [biotin-free RMPI 1640 medium (Irvine Scientific)/10 mM Hepes, pH 7.4/0.1% NaN_3/2% heat-inactivated serum]. Cells were washed with staining medium and stained with fluorescein-conjugated goat anti-mouse Ig for 30 min. Propidium iodide (Calbiochem–Behring, La Jolla, CA) 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 microtiter plates (Dynatech Laboratory, Alexandria, VA) containing 5×10^5 cells per 0.1-ml well were used.

Cells were analyzed and sorted on a FACS II (Becton Dickinson FACS Systems, Mountain View, CA) modified to include a logarithmic amplifier and a direct cloning attachment (18). A 540-nm shortpass filter (Ditric Optics, Marlboro, MA) was used to reduce autofluorescence of the L 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.

For fluorescence microscope work, 1 to 2×10^5 cells were plated in a 30-mm dish containing a glass coverslip. After 2 to 3 days of growth, the dishes were rinsed with staining medium, 10 μl of antibody solution was added, a coverslip was placed on top, and the dish was covered with moist filter paper to prevent evaporation. After 30 min, the cells were washed and incubated with second-step antibody as above. After washing, the cells were dried, fixed in 95% ethanol for 10 min, and dried. Two drops of 0.1 M Tris base, pH 9.5/glycerol, 2:9 (vol/vol), was placed on a clean glass slide and the coverslip was placed on top with the cells facing down. A fluorescence microscope with 488-nm excitation from an argon laser was used.

Preparation of Cellular DNA. Cells were collected in P_i/NaCl/EDTA as described above. The DNA was prepared as described by Wigler *et al.* (19). DNA was treated with previously boiled RNase at 20 mg/ml for 50 min at 37°C and then for 10 min at 65°C. Extraction as above was repeated. The DNA was dialyzed into 10 mM Tris·HCl/1 mM EDTA, pH 7.9, with four changes of buffer.

Transformation. The calcium phosphate precipitation method of Graham and van der Eb (20) as modified by Wigler *et al.* (19) was followed except that cells were incubated with DNA (20 μg of cellular DNA and 1 μg of plasmid DNA per 10^6 cells) overnight according to Graham *et al.* (21). Hyp/Apt/Thd was added the following day and every 3 to 4 days thereafter. Colonies were stained with methylene blue as described (22).

Immunoprecipitation and $NaDodSO_4$ Gel Electrophoresis. We followed methods used previously for lactoperoxidase catalyzed ^{125}I labeling and detergent lysis (23, 24). [^{35}S]Methionine labeling, immunoprecipitation, and $NaDodSO_4$ gel electrophoresis were carried out as described by Jones (24) and Parham and Ploegh (25).

RESULTS

DNA was isolated from the human T-cell line JM, which expresses HLA class I molecules and the T-cell differentiation antigens Leu-1, Leu-2, Leu-3, and Leu-4 (26), also known as (OK)T1, T5 (or T8), T4, and T3, respectively (27). Using the calcium phosphate precipitation method, we cotransformed mouse L (TK^-) cells with this DNA and the herpes simplex virus *TK* gene on plasmid pBR322. TK^+ transformants were selected by growth in Hyp/Apt/Thd medium. After 3 wk, there were roughly 2×10^3 colonies per dish per 10^6 cells initially plated.

HLA and β_2m Transformant Isolation. To test our ability to isolate transformants coding for cell surface antigens by using the FACS, we first screened for transformants expressing HLA class I molecules and human β_2m. We expected these antigens to be expressed in L cells because the homologous antigens H-2 and β_2m are expressed by the L cells. In addition, monoclonal antibodies such as W6/32 and BBM1, which detect a framework (monomorphic) determinant on the HLA heavy chain (28) or on human β_2m (29), respectively, are not affected by association of the human HLA heavy chain with mouse β_2m or the mouse H-2 heavy chain with human β_2m, as shown by work with somatic cell hybrids (30). Samples of 10^6 cells from nine dishes were allowed to react with W6/32 or with BBM1 for the first step and with fluorescein-conjugated goat anti-mouse antibody as the second step. Viable cells were then analyzed with the FACS. Because dead cells pick up fluorescent proteins nonspecifically, we eliminated all such cells from analysis by including (in the last wash of the cells prior to passage through the FACS) the red nucleic-acid-intercalating dye propidium iodide, which penetrates only cells with damaged membranes and thus stains dead cells red (31). Red propidium iodide-stained cells were then electronically gated out. It is important to exclude the unavoidable few percent of dead cells when detecting and sorting stained cells occurring at a low frequency.

The distributions of staining intensities observed with the L-cell and the TK^+ transformant populations were the same; however, a low frequency of cells that stained above background was seen in the transformed populations (Fig. 1). These clearly stained cells, about 0.05% of the total, were sorted aseptically. The FACS-enriched cells were grown to a population of 1–10 × 10^5 cells and then restained and reanalyzed on the FACS. In most cases, a distinct population of fluorescent cells stained clearly above background were observed (Fig. 1). Samples having <0.5% cells stained above background were resorted, grown, and reanalyzed to confirm that they were indeed positive. Of the nine dishes, eight were positive for HLA and eight were positive for human β_2m. The dish lacking detectable HLA transformants was different from the one lacking β_2m. According to the Poisson distribution, the frequency of transformants for either antigen would be an average of at least two per dish. In subsequent experiments, we usually found transformants on every dish.

Having established the presence of putative transformants, we then cloned cells from each dish with the FACS; under aseptic conditions, cells stained with either anti-HLA or anti-β_2m were sorted into the wells of microtiter plates. The majority of clones that grew expressed the antigen selected for. None of five HLA transformants expressed human β_2m and none of four β_2m transformants expressed HLA class I molecules as determined with fluorescent antibody. Some clones analyzed were negative or had both negative and positive cells. We recloned the brightest W6/32-stained cells from one clone with a mixed population and obtained a stable HLA-expressing line. This line and another independent HLA transformant have been growing for 10 months in normal medium and have not reverted.

Biosynthetically labeled antigens ([^{35}S]methionine) precipitated with W6/32 from two of the HLA transformants were analyzed by two-dimensional gel electrophoresis. Each transformant produced a different subset of spots at molecular mass of 45 kDal with isoelectric points within the range for the human parent JM line (Fig. 2). No spots were observed with the mouse L cells (not shown). The variety of subsets of spots indicates that

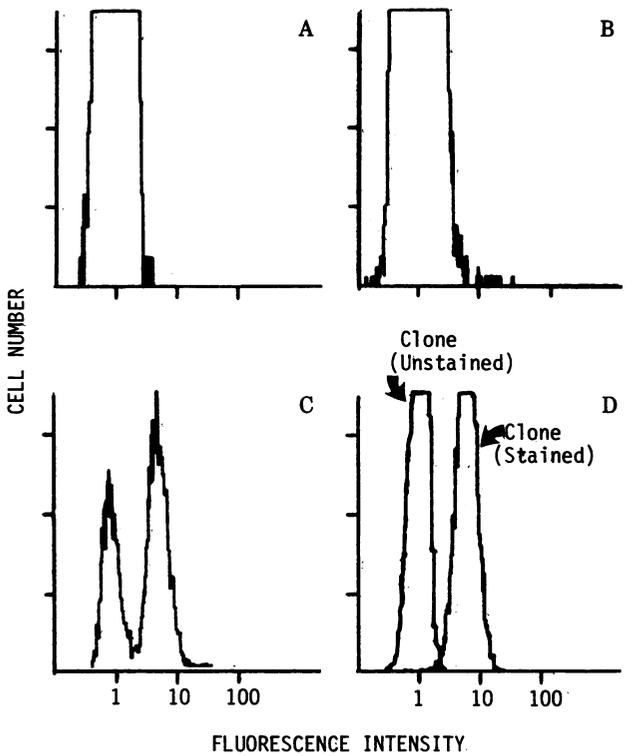

FIG. 1. FACS fluorescence-intensity histograms at different points during isolation of HLA transformants. Cells were stained with W6/32, which detects a framework determinant on class I heavy chains, and then with fluorescein-conjugated goat anti-mouse Ig. In contrast to the L cells (*A*), the TK^+ transformants had a few brightly stained cells above background (*B*). These bright cells were sorted and grown and then reanalyzed, and an enriched population was seen (*C*). Cells from the brighter stained peak were placed individually in microculture wells, grown, and reanalyzed. (*D*) A single cloned population of brightly stained cells representing an HLA transformant stained with fluorescein-conjugated goat anti-mouse Ig alone or after exposure to W6/32 and goat anti-mouse Ig. A modified FACS II fitted with a logarithmic amplifier was used for the analysis.

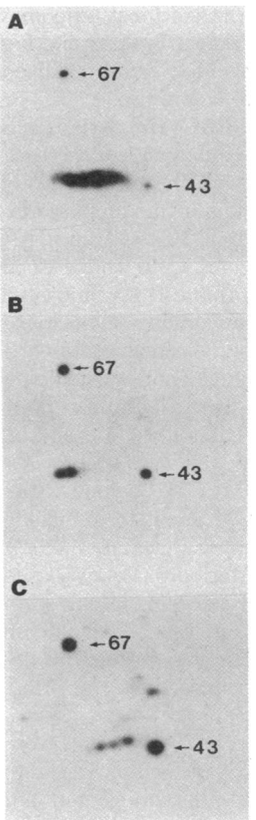

FIG. 2. Two-dimensional gel electrophoresis of the HLA class I heavy chain on HLA transformants. A detergent lysate from [^{35}S]methionine-labeled cells was treated with W6/32, and the immunoprecipitate was suspended in $NaDodSO_4$ sample buffer/2-mercaptoethanol and electrophoresed. The first dimension was a charge-separation step; right side, acidic; left side, basic. The second dimension was a size-separation step in 10% $NaDodSO_4$/polyacrylamide using bovine serum albumin (67 kDal, pI 4.9) and ovalbumin (43 kDal, pI 4.7) as markers. They were detected by staining with Coomassie brilliant blue and marked with ink containing [^{35}S]methionine so that they could be detected on the film. (*A*) JM leukemia line. (*B*) J2a, one HLA transformant. (*C*) J3a, another HLA transformant. Portions of the fluorograms are shown.

the two transformants are probably transformed by different alleles.

Isolation of Transformants for T-Cell Differentiation Antigens. Having established that transformants for cell surface antigens could be selected by the FACS, we thawed TK^+ transformed cells from different dishes in the same experiment in which the HLA transformants had been isolated. These were screened for expression of the T-cell differentiation antigens Leu-1, Leu-2, Leu-3, and Leu-4. We grew the cells in tissue culture dishes containing glass coverslips for 2 to 3 days. The cells on the coverslips were stained with fluorescent antibodies, fixed, and examined for fluorescence with a fluorescence microscope. Specifically stained cells occurred in small apparently clonal groups and had a speckled appearance due to patching. The appearance of clustering was an important criterion for determining that a sample contained transformants.

The $Leu\text{-}1^+$ and $Leu\text{-}2^+$ transformants detected by microscope analysis were confirmed by immunofluorescence analysis with the FACS. The frequency of stained positive cells among the TK^+ transformants was $\approx 1/2{,}000$ for each antigen by FACS analysis, similar to the frequency we had found for HLA and for β_2m. The brightest 0.5–0.1% of the cells were sorted, grown, and cloned. From dishes with transformants, 20% or more of the clones were positive for either antigen. Transformant clones were initially maintained in Hyp/Apt/Thd medium and remained homogeneously staining. We isolated five independent primary transformants from different dishes for Leu-1 and similarly for Leu-2. We subsequently isolated Leu-2 secondary transformants from mouse L cells cotransformed with the *TK* gene and cellular DNA from three different primary transformants by directly cloning the brightest cells after TK^+ colonies had grown sufficiently. All the Leu-2 transformants were screened with anti-Leu-2a and also reacted with anti-Leu-2b, which detects a second determinant on the same molecule. So far, we have not found Leu-3- or Leu-4-stained cells. We discuss reasons for this below.

The molecular masses of the Leu-1 and Leu-2 expressed on several transformants tested are the same as those of the Leu-1 and Leu-2 on cells that normally express the antigen. We labeled membrane proteins by the lactoperoxidase method with ^{125}I, solubilized the membranes with Nonidet P-40, immunoprecipitated the antigen, and analyzed the precipitates by $NaDodSO_4$ gel electrophoresis under reducing conditions for Leu-1 and nonreducing conditions for Leu-2. The size of the Leu-1 molecule precipitated from two transformants was identical to that of the Leu-1 precipitated from the JM lymphoid line (Fig. 3). No Leu-1 molecule was precipitated from the untransformed mouse L cells. The Leu-2 molecule precipitated with either anti-Leu-2a or anti-Leu-2b is a dimer of similar size to the molecule on the JM line (Fig. 4). Under reducing conditions, the dimer band disappeared and at least one of the subunit polypeptides appeared (data not shown).

The relative amounts of specific staining of different transformant clones varied. The mean staining intensity of different $Leu\text{-}1^+$ transformants ranged from 79 to 204 fluorescein units. Although exact quantitation of antigen cannot be obtained by indirect immunofluorescence, such large differences between clones, with much smaller spreads within clonal populations, suggest different amounts of surface antigen per cell in the different clones. The light-scattering profiles, which are useful for approximation of size, were similar for all but a few transform-

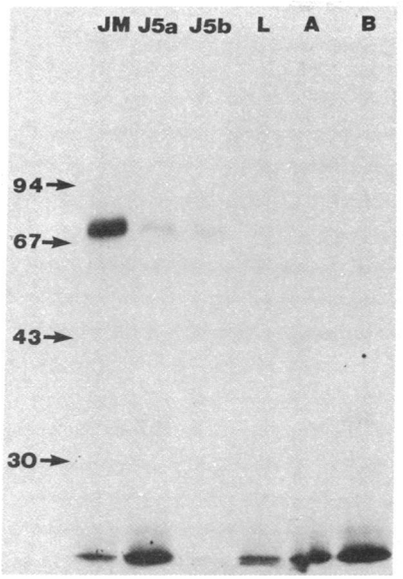

FIG. 3. Immunoprecipitation of Leu-1 from surface ^{125}I-labeled mouse L cells, the human T-cell leukemia line JM, and Leu-1 transformants J5a and J5b. The immunoprecipitated protein was electrophoresed in NaDodSO$_4$/10% polyacrylamide gels under reducing conditions. J5a and J5b were isolated from the same dish of TK$^+$ transformants. Lanes A and B: precipitation of J5a and J5b with *Staphylococcus aureus* protein A alone. Numbers on the left are size markers (kDal).

ants, which exhibited somewhat more light scattering. Cells having the same antigen density but different sizes will differ in absolute amount of antigen, but the differences we observe cannot be explained by this alone.

We stained the Leu-2$^+$ transformants directly with fluorescein-conjugated anti-Leu-2a antibody. Three stained about the same (within a factor of 2). However, a fourth Leu-2$^+$ transformant clone, J10, had a very broad distribution of fluorescence intensities and its mean intensity was 7 times that of the three other transformants (Fig. 5). When this clone was grown in the absence of Hyp/Apt/Thd medium, a duller or nonstaining population appeared that increased proportionately with time. When the cells were returned to Hyp/Apt/Thd medium and grown for several months, ≈20% of the cells were dull or nonstaining. Even when the 0.001% brightest cells were cloned,

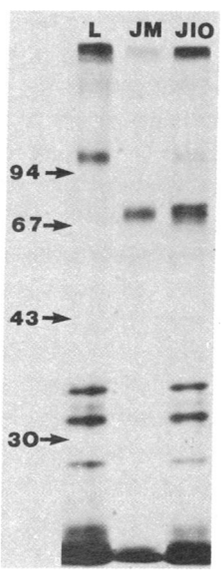

FIG. 4. Immunoprecipitation of Leu-2 from surface ^{125}I-labeled mouse L cells, the human T-cell leukemia line JM, and Leu-2 transformant J10. The immunoprecipitated protein was electrophoresed on NaDodSO$_4$/10% polyacrylamide gels under nonreducing conditions. The band corresponding to Leu-2 between the size markers at 67 and 94 kDal is present only in transformant J10. Other bands present in lanes L and J10 are background bands and could also be precipitated by *Staphylococcus aureus* protein A alone. Numbers on the left are size markers (kDal).

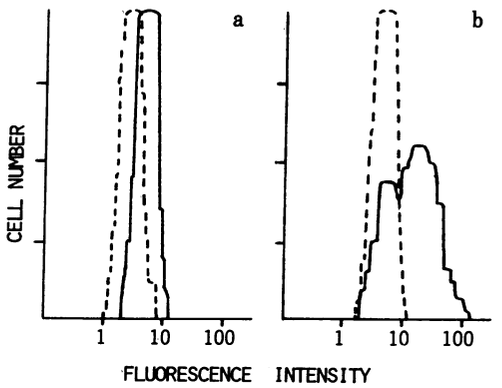

FIG. 5. Variability of Leu-2 staining on independent Leu-2$^+$ transformant clones. Leu-2$^+$ transformants maintained in selective medium were stained with fluorescein-conjugated anti-Leu-2 antibody (——) or with medium alone (---) and analyzed on the FACS. Transformant J6 (*A*) had a homogeneous staining pattern whereas transformant J10 (*B*) was more brightly stained and had a heterogeneous pattern.

the staining patterns of the six clones were still heterogeneous. To determine whether we could select a population of cells that had increased expression, we sorted the brightest 0.5% of the cells, grew the cells, and resorted them. After three rounds of sorting, we obtained a population of cells whose mean staining is 5 times brighter than the brightest J10 cells assayed by direct staining. These properties may be due to multiple gene copies of Leu-2 because amplification of transferred genes can occur (32–34).

DISCUSSION

Isolation of rare mouse L-cell transformants stably expressing differentiation antigens after transfer with cellular DNA has been accomplished for two T-cell differentiation antigens: Leu-1 (or OKT1) and Leu-2 [or OKT5 (8)]. We first established conditions for isolating rare transformants by isolating HLA and β_2m transformants and determining the frequency of such transformants. β_2m is coded for by a single-copy gene (35) while HLA class I antigens are coded for by at least three genes. After TK$^+$ transformants had been selected for, the frequency of β_2m and HLA transformants was about 0.5×10^{-3}. Cells at that frequency could readily be stained with monoclonal antibodies 2.5 wk from the start of the experiment and directly cloned into microtiter wells using the FACS.

The frequency of transformants found is consistent with results obtained by others. We estimate that about 1 out of 2,000 TK$^+$ transformants expressed a particular cell surface antigen. The size of a human genome is estimated to be 3×10^6 kilobases (kb). Therefore, each transformant should have an average of 1.5×10^3 kb of foreign DNA. Wigler and co-workers (36) estimated by a different method that the average amount of DNA integrated in a transformant is ≈1,000 kb. Robins *et al.* (37) estimated that, in some of their transformants, $\approx 7 \times 10^3$ kb had been incorporated. These estimates are in the same order of magnitude as our estimate. An interesting prediction that follows from this study is that many of the genes incorporated in the 1,000-kb insert might be expressed, especially if the genes were expressed in the DNA donor cells. Thus, if good fluorescent probes are available, isolation of other transformants coding for differentiation antigens should be possible.

Transformants expressing Leu-3a or Leu-4 were not found. Because the DNA donor, the JM lymphoma line, expresses these antigens, it is unlikely that these antigens are not expressed because of methylation of the genes. Possible explanations include the following. (*i*) The staining reagents were not

adequate to detect low levels of antigen expression. (*ii*) The determinant that is recognized by the monoclonal antibody is hidden in the membrane of the L cells. (*iii*) Two unlinked gene products are required for expression of the antigens. (*iv*) These genes cannot be expressed in L cells. (*v*) The frequency of transfer is 1/10th of that of other molecules for which we did get transformants. Lester *et al.* (22) reported at least a 10-fold difference in transfer efficiency between genes coding for human adenosine and hypoxanthine phosphoribosyltransferases. Leu-4 may require two unlinked gene products because immunoprecipitates with anti-Leu-4 antibody give two distinct bands (12). This may explain why Chang *et al.* (11) found only transient expression of OKT3, the equivalent of Leu-4.

Ledbetter *et al.* (12) proposed that the murine homologue of the macromolecule bearing the Leu-2a and Leu-2b determinants is the multimeric Lyt-2, -3 molecule. Lyt-2 and Lyt-3 are on two different polypeptide subunits that are disulfide linked into a heterodimer. Lyt-2 and Lyt-3 are coded for by two closely linked genes that have not been separated by recombination or perhaps by a single gene that may code for a precursor polyprotein. We suggest that two Leu-2 polypeptides are also coded for by a single gene or closely linked genes similarly to Lyt-2, -3. Because the frequency of cotransfer of two genes that are not extremely closely linked is expected to be less than $1/10^3$ (38), the presence of a heterodimer in two independent Leu-2 transformants would be highly improbable if the two chains were coded for by unlinked genes.

Homology between proteins can be definitively established only by comparing either their amino acid sequences or the DNA sequences of the genes. In extrapolating the results of studies of the immune system of the mouse to studies in the human, it is important to know which immune cell differentiation antigens are homologous. The Leu antigens are present in low amounts, so that amino acid sequences are difficult to determine. The isolation of stable transformants for the T-cell differentiation antigens Leu-2 and Leu-1 opens the way for cloning these genes by using transformation as a first step. The DNA sequences of these genes will shed light on Leu–Lyt homologies. Thus, in the near future, the question of Leu-1 and Lyt-1 homology and of Leu-2 and Lyt-2 homology should be answerable.

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