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Amplification of a gene coding for human T-cell differentiation antigen

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Using previously isolated mouse L-cell transferents for the human T-cell differentiation antigen Leu-2, we now report the first example of spontaneous gene amplification for membrane antigens. The Leu-2 (or T8)[1,2] antigen is normally expressed on T lymphocytes that have cytotoxic or suppressor functions. Cells of a Leu-2 transfected clone were stained with fluorescein-tagged monoclonal anti-Leu-2, and the brightest 0.1–0.3% of cells were viably separated using a fluorescence activated cell sorter (FACS). After growth of these selected cells, sorting and regrowth was repeated six times, resulting in a population of cells that, compared with the starting population, stains 40 times brighter for Leu-2 and whose DNA transforms 20 times more efficiently for Leu-2. In addition, these cells have 10- to 50-fold amplified human DNA sequences and numerous double minute chromosome fragments, a common indicator of gene amplication in mouse cells.

Following co-transfection of mouse L cells (TK^-) with the herpes simplex thymidine kinase (TK) gene and total human DNA from JM, a human T lymphoma line, we selected Leu-2^+ and other human lymphocyte membrane antigen transferents by FACS sorting[3]. We found the frequency of transferents for Leu-2 to be about 10^{-3} of the TK^+ cells selected in hypoxanthine–aminopterin–thymidine (HAT) medium. Although most cloned transferents had narrow ranges of antigen density per cell, one of the first four Leu-2^+ transferents found was strikingly more variable in the amount of Leu-2 antigen per cell. Further, this transferent, J10, had a mean Leu-2 staining per cell that was seven times greater than that of the other transferents[3].

To investigate whether the increased Leu-2 expression on the tranfected cells was due to gene amplification, we sorted the brightest 0.3% of the cells aseptically with the FACS. These cells were grown, reanalysed, and the brightest cells again sorted and regrown. Each round took about 3 weeks. After six rounds of selection the mean fluorescence of the cells was about 40 times greater than that of the original cloned transferent J10 (Fig. 1); the mean brightness approximately doubled with each sort. Curiously, we found no further increase in mean fluorescence after more rounds of sorting. Further increases in Leu-2 expression may not have been possible because the surface was saturated with Leu-2 (The amount of Leu-2 antigen per cell matches the levels of H-2 antigen on these cells.) Increased Leu-2 expression was specific to Leu-2 since the amount of H-2 per cell remained unchanged throughout the sorting selections.

The presence of double minute (DM) chromosomes is characteristic of mouse cells that have amplified sequences[4,5]. DMs are self-replicating acentric chromosomal fragments that are distributed unequally in daughter cells at mitosis. We examined metaphase spreads of J10-6 cells (the J10 transferent after six cycles of sorting) and could clearly see DMs in most spreads (Fig. 2). Variation from <10 to >100 in the number of DMs per spread was noted, which is like other counts of DMs in amplified mouse cell lines[5].

Because a Leu-2 gene probe does not yet exist for quantitation of Leu-2 gene copies by molecular hybridization, we used a biological assay, quantitative transfection, to test whether the Leu-2 gene number had increased. We found the transfection frequency of Leu-2 into mouse L cells to be roughly 15–20 times higher per μg of DNA using DNA from the amplified line J10-6, than the frequency using JM or J10 DNA (Table 1). We next titred J10-6 DNA in a transfection experiment with different amounts of carrier DNA. When 1 μg of J10-6 DNA was used with 20 μg carrier (L cell) DNA per dish, Leu-2^+ transferents could be detected on every dish (four dishes per

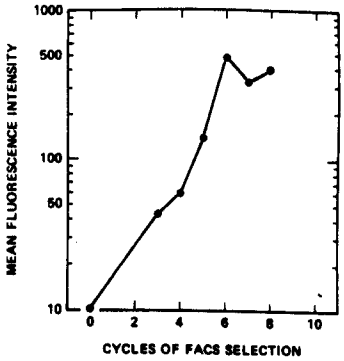

Fig. 1 Mean fluorescence of Leu-2^+ transferent, J10, after each round of sorting the brightest cells. Starting with a cloned transferent J10, the cells were stained with fluorescein-conjugated anti-Leu-2a monoclonal antibody and the brightest 0.3% of the cells were sorted aseptically by the FACS. The cells were regrown, stained, analysed and resorted as described elsewhere except that propidium iodide was not used. The amount of anti-Leu-2a was sufficient for maximum staining. The mean fluorescence is calculated as the geometric mean of the distribution and expressed relative to 1.83 μm green fluorescent beads (Polysciences Inc.) which were used as a standard of 100 fluorescence units. As the fluorescence of the cells increased the voltage of the photomultiplier was reduced or a neutral density filter was inserted.

experiment). No Leu-2^+ transferents were detected with 1 μg of JM DNA with the same amount of carrier DNA. This indicated that the Leu-2 gene was amplified at least 20-fold.

Analyses of DNA in stable transferents after introduction of specific genes into cells have shown that physically unlinked DNA segments tend to be physically linked in the transferents[6]. We would therefore expect that Leu-2^+ DNA, other human DNA sequences and TK sequences would be ligated together in transferents generated by co-transfection of mouse L cells with human DNA and the TK gene. We found indeed that all human DNA and many or all TK sequences were amplified in the J10-6 cells. Southern blots of DNA from J10, L cells and J10-6 were probed with human repetitive DNA (Fig. 3) or with the TK gene. Bands of the same size that were detected by human repetitive DNA probes in J10 were also present in J10-6 but with greatly increased intensity. We titred the amount of J10-6 DNA on the blot to see how much J10-6 DNA was required for the band intensities to be equal to those obtained with 10 μg of J10 DNA and found that for most bands 0.2–0.3 μg of J10–6 DNA was roughly equivalent to 10 μg of J10 DNA; thus there was about a 30- to 50-fold increase in copy

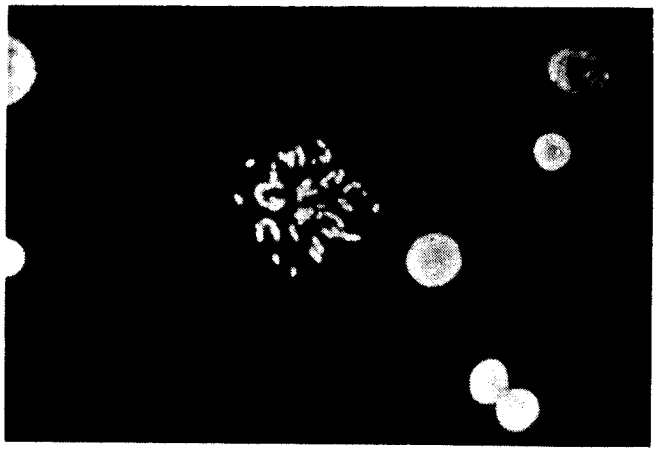

Fig. 2 Metaphase spread of J10-6 cells obtained after six cycles of FACS selection of sorting cells with increased antigen density. Metaphase spreads were stained with Hoechst 33258 stain (1 μg ml^{-1} in phosphate-buffered saline).

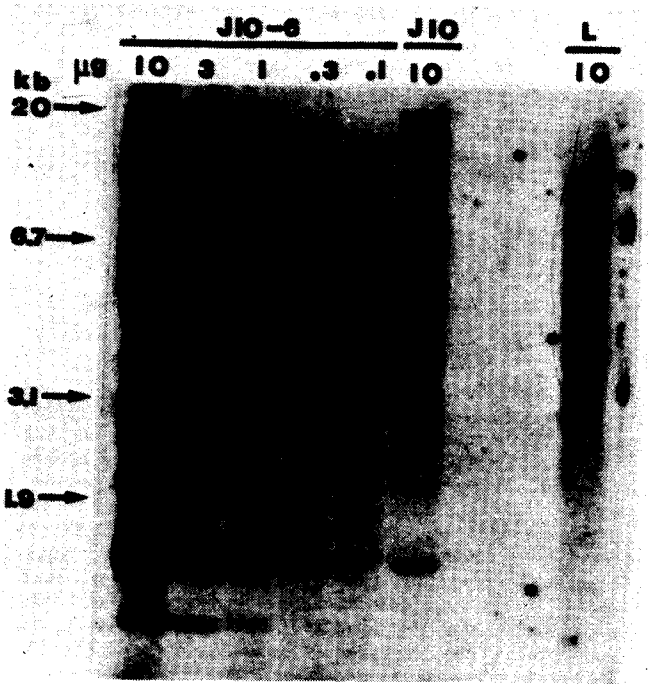

Fig. 3 Southern blot showing amplification of human repetitive sequences in cells with increased expression of Leu-2 on the surface. DNA was digested to completion with restriction endonuclease *Bam*HI, electrophoresed through a 0.75% agarose gel, blot transferred to a nitrocellulose filter, hybridized with ^{32}P-labelled human repetitive DNA[10] (specific activity 2×10^8 c.p.m. μg^{-1}), and exposed to IAR-5 film with a Dupont Lightening Plus intensifying screen. J10 is a cloned Leu-2$^+$ transferant of mouse L cells and J10-6 are J10 cells obtained after sorting the cells with highest antigen density six times. The amount of DNA (in μg) analysed in each lane is indicated below the name of the cell line.

Table 1 Transformation frequency of Leu-2 into mouse L cells

DNA donor cell		No. Leu-2$^+$ Cells* per 10^4 TK$^+$ cells per dish
JM	(T-cell lymphoma)	6, 17, 3, 6
J10	(Leu-2 transformant)	9, 10
J10-6	(J10 cells after six cycles of sorting)	97, 120, 157

Each dish was seeded with 10^6 cells and overlaid with 20 μg total DNA and 1 μg of pBR322 plasmid containing the TK gene. Selection of transferents was as described previously[3].

*Number of cells with fluorescence intensity above background. Background was fluorescence intensity of cells stained with second step antibody alone.

number of the *Alu* marked human sequences detected in J10-6. A few of the higher molecular weight bands, however, were amplified only about 10-fold. Southern blots with TK as a probe showed a similar 30- to 50-fold amplification (data not shown). Thus DNA sequences transfected into the L cells are amplified to approximately the same extent as the increase in Leu-2 expression on the cell surface. The sum of the sizes of the visible human repetitive DNA bands gives us an estimate of the amplified unit of at least 200 kilobase pairs.

Several pieces of evidence lead us to conclude that the structural gene for Leu-2 is amplified in the selected cells expressing high levels of Leu-2: (1) the transfection frequency for Leu-2 using J10-6 DNA is 15–20 times greater than with the same amount of donor JM lymphoma DNA or J10 DNA, (2) double minute chromosomes, known to occur with gene amplification in mouse cells, are present in J10-6 cells, (3) the introduced human or TK DNA integrated in the L-cell genome is amplified 10–50 times based on Southern blots with human repetitive DNA or TK as probes. Spontaneous amplification for the Leu-2 gene in a mouse L-cell transferent may be a fairly common event since at least 4 of 20 independent, FACS isolated, Leu-2 transformed clones showed an increased and unstable pattern of antigen expression. For each of the four clones we were able to select cells with greater antigen expression by FACS selection of the brightest stained cells. Preliminary work indicates that growing cells in HAT medium versus non-HAT medium is more effective for this process.

FACS has been used for selecting cells with other kinds of amplified sequences. Johnston *et al.*[7] selected for amplification of dihydrofolate reductase (DHFR) in CHO cells by multiple cycles of sorting the brightest cells after staining with fluorescent methotrexate, an inhibitor of DHFR. This group obtained cells that had spontaneously amplified the DHFR gene 50-fold. With suitable fluorescent probes, selection for amplification of other genes may be made in a similar manner.

Cells with amplified copies of a gene coding for a cell-surface antigen could be very useful for cloning this gene. Cloning by cDNA methods could be simplified because the levels of mRNA are almost certainly increased in amplified cells. For example, Caskey *et al.*[8] isolated a cDNA clone corresponding to the HPRT gene using a neuroblastoma line with 40-fold amplification. Double minutes could be isolated and used for the production of genomic libraries enriched for amplified sequences[9]. Cells with increased amounts of antigen expression should also make protein purification for structural and functional studies of cell-surface antigens easier.

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