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TRANSFECTION AND CLONING OF GENES FOR MEMBRANE ANTIGENS USING THE FACS

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In order to facilitate cloning of genes for cell surface molecules, we cotransfected LTK$^-$ mouse fibroblasts with thymidine kinase (TK) genes and total human or mouse DNA. TK$^+$ cells, selected by growth in HAT medium, were stained with fluorochrome conjugated monoclonal antibodies or other fluorescent ligands which bind to one or another membrane differentiation antigen or receptor. We isolated fluorescent transfectants expressing these molecules using a fluorescence activated cell sorter (FACS). For some antigens, spontaneous gene amplification occurred. By repeated cycles of FACS sorting and regrowth we obtained high expressing clones. We then isolated *c*DNA and genomic clones using selected *c*DNA probes to screen phage with *c*DNA inserts. DNA from virtually any tissue source transfected equally well for the various molecules except for DNA from a trophoblast derived choriocarcinoma cell line which did not transfect for Leu-2.

Key words: **Cotransfection, FACS, Gene amplification, Leu-2, Differentiation antigens.**

INTRODUCTION

The development of the whole field of immuno-flow-cytometry has depended upon the existence of specific glycoprotein cell surface molecules which are sufficiently different antigenically so that monoclonal antibodies can be made which react with certain marker molecules on one functional type of cell and with other such molecules on another functional cell type. The fortunate development of hybridoma technology for generating monoclonal antibodies,[1] coming just as the development of fluorescence activated cell sorter or flow fluorescence technology was reaching a high level of technical reliability, allowed this field to flourish. (For a recent review, see Ref 2.) Although some of the antigens detected by these antibodies have been studied biochemically, very little is known about their primary (amino acid sequence) structure. This is because, with very few exceptions, such as membrane immunoglobulins and products of the MHC loci, they are present on the surface in rather small amounts and usually turn over metabolically quite slowly.

Despite this, we and others have postulated homologies between molecules found on the surfaces of human cells and murine cells.[3] For example, we have proposed the following homologies based in part on their distribution on functional cell types: (1) the Lyt-2,3 molecule, which marks suppressor and cytotoxic cells in the mouse, is homologous to the human Leu-2 (T8) molecule, which marks suppressor and cytotoxic cells in the human; (2) Ly-1, which is present on all T-cells in the mouse, albeit in higher density on helper cells than on suppressor or cytotoxic cells, and which is found on a subpopulation of B-cells,[4] is homologous to the Leu-1 (T1) molecule. Homologies have also been proposed for murine L3T4 and human Leu-3 (T4),[5] and for Leu-4 (T3), which is associated with the T-cell antigen receptor molecule, in the human, and an unnamed molecule in the mouse associated with the murine T-cell receptor.[6]

In addition to identifying these molecules by their distributions on functional cell types, studies of the polypeptide chain compositions and approximate mol. wts have supported the homology assignments that we made.[3] Nevertheless, a geneticist's definition of homology is based upon evolutionary relationships, most easily defined by amino acid sequence and nucleic acid sequence comparisons. To facilitate sequence studies on these molecules, we have embarked on a program of isolating and cloning the genes which code for these molecules since sequencing can be much less laborious at the nucleic acid level than at the protein level.

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COTRANSFECTION APPROACH TO CLONING

Since these molecules are not very abundant and turn over relatively slowly, we expect the level of corresponding *m*RNA also to be rather low. Thus, we decided to use an approach to gene cloning which did not depend upon isolating enough antigen to determine an amino acid sequence and using this sequence to make corresponding nucleic acid probes for screening a library. Although this is a powerful technique for gene cloning, we were afraid it would be too difficult to use for many of the cell surface antigens we were interested in. Instead, we decided to use another approach, one that has proved to be successful for isolating genes coding for housekeeping enzymes and a number of oncogenes. This is the approach of DNA mediated gene transfer or DNA transformation or transfection.[7] In our case, the plan was to cotransfect total cellular DNA with a suitable selectable marker, thymidine kinase (TK), using a good recipient cell line, LTK^- mouse transformed fibroblasts deficient in this enzyme. After selection for TK^+ transfectants in HAT medium, we would stain with one or another monoclonal antibody or ligand coupled to a fluorochrome and use the fluorescence activated cell sorter (FACS) to isolate specific cell surface molecule transfectants. Then we expected that one or another kind of 'marker rescue' method could be used to isolate the gene coding for the transferred antigen. As it turned out, our original idea was a good one in that it led to our being able to clone the gene for one cell surface molecule and being well advanced in efforts to clone several other molecules using the transfection approach. However, the details of how we isolated and cloned the genes had to be changed in the light of actual experimentation. This is an example of where the idea was good, not because it was totally correct, but because it suggested useful experiments, which, helped by the hand of serendipity, led to a successful outcome.

SELECTION OF TRANSFECTANTS

In Fig. 1, we show the general scheme for isolation of transfectants.[8] DNA extracted from almost any human or murine tissue or cell line mixed with chicken or herpes simplex virus TK is precipitated with calcium phosphate and sprinkled on monolayers of LTK^- cells. After 48 h, the cells are washed and HAT medium added. After 2 weeks incubation, we have about 1000 TK^+ colonies per million cells plated. These cells are suspended, mixed with fluorochrome conjugated monoclonal antibodies or ligands (or in some cases with unconjugated antibodies or ligands followed by fluorochrome conjugated second step reagents) and analyzed and sorted using the FACS. Positive cells are enriched and finally cloned using the FACS. The lists in Table 1 show that we successfully transfected for a wide variety of human and mouse cell surface molecules using this technique. The only limitation seems to be that transfection for only one gene at a time is required. The frequency of antigen transfectants is in the range of 1 per 1000 among TK^+ cells. Thus, if two genes have to be simultaneously transfected to produce a surface molecule, we expect to have only one stained cell per million TK^+ cells or per 10^{12} originally treated cells. This is too large a number of cells to conveniently try to transfect. Nevertheless, we have successfully transfected for some two chain

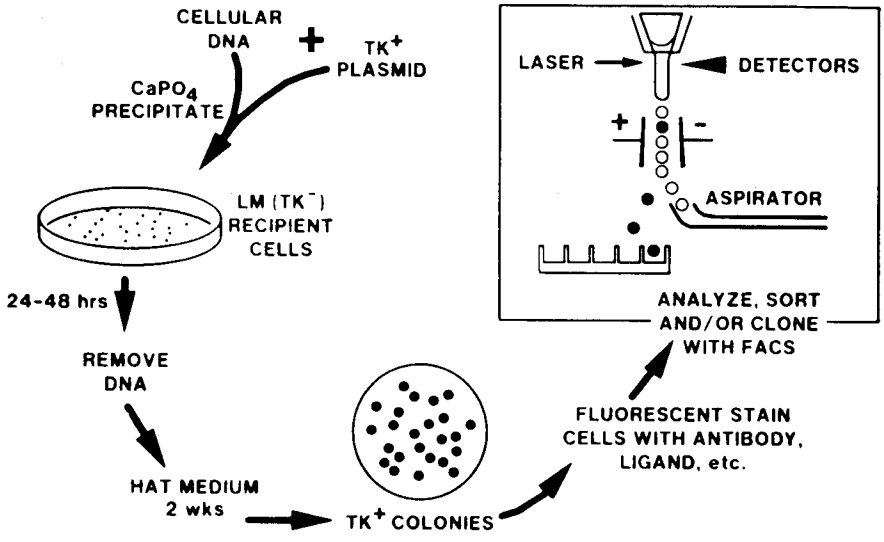

Fig. 1. Selection of transformants.

Table 1. Molecules successfully cotransfected into LTK$^-$ mouse fibroblasts

B-Cell differentiation antigens	T-Cell differentiation antigens	Other membrane molecules
BLA-1	Ly-1	HLA
BLA-2	Lyt-2	H-2d
(30-F1)	T200	β2m
ThB	Thy-1	Human transferrin receptor
	L3T4a	Human NGF receptor
	Human Leu-1	Murine Fc receptor (2.4G2)
	Human Leu-2 (124-40)	

molecules. But, in these cases, the recipient cell provides one polypeptide chain which can complement with a chain coded for by the input DNA. For example, murine beta 2-microglobulin (β2m) can associate with human HLA heavy chains to make complete cell surface molecules. Similarly, murine H-2 heavy chains can complement human β2m. One surprise was that we could readily transfect for Leu-2 or Lyt-2 despite the fact that these are often found on thymocytes or T-cells as heterodimers.[9] In such transfectants, we found that they formed homodimeric molecules when they did not have the other chain to associate with.

EXPRESSION OF TRANSFECTED GENES FOR DIFFERENTIATION ANTIGENS BY L-CELLS

Many have wondered how it is possible that differentiation antigens normally expressed only on certain specialized cell types can be expressed so readily on fibroblast cell surfaces. What this must mean, we believe, is that expression of these molecules, which might or might not represent a special class, is not restricted in L-cells (mouse fibroblasts). This may be because donor DNA in a TK$^+$ transfectant is found all in a single long stretch of DNA generated by ligation of the input DNA fragments along with the expressed TK gene. Whatever regulates expression of TK could also be sufficient for expression of most, or at least some, of the cotransfected genes. In fact, estimates of the frequency with which single copy (human) genes are present in TK transfected cells using our protocol are in the order of 1 per 1000 cells. Thus, the approximately 1 per 1000 TK$^+$ cells expressing any particular antigen indicates, within our error of estimation of perhaps 2–4 fold, that every human antigen gene present is initially expressed. (This is in contrast to the findings of Morrison and Oi, partly in this laboratory[10] that cloned immunoglobulin genes need tissue-specific enhancer elements to be expressed and are not expressed in L-cells when transfected in plasmid vectors. Whether this apparent paradox is due to some difference in the cotransfection system we are using or some other unrecognized experimental difference remains to be seen.)

GENE AMPLIFICATION FOR LEU-2

Our first attempt to clone a gene from these transfectants was for the structural gene of Leu-2. We unsuccessfully tried several types of marker rescue approaches. However, we have nevertheless been successful in cloning Leu-2 (Kavathas P, Sukhatme V P, Herzenberg L A, Parnes J R: Isolation of the gene coding for the human T lymphocyte differentiation antigen Leu-2 (T8) by gene transfer and *c*DNA subtraction. *Proc Natl Acad Sci USA* (in press)). In Fig. 2A we show fluorescence histograms of one kind of Leu-2 transfectant isolated; as can be seen, the fluorescence distributions with anti-Leu-2 are rather tight. In Fig. 2B we depict another kind of fluorescence histogram we observed in from 25 to 50% of Leu-2 transfectant clones.

You notice the distribution appears bimodal and the distribution for the brighter peak is much broader. This latter type of distribution suggested a certain degree of instability, similar to the kinds of distribution one would expect if gene amplification were occurring (e.g. Ref. 11). Therefore, we decided to sort the brightest fraction of 1% of Leu-2 staining cells using the FACS, regrow these, re-sort the brightest cells again and do this for several cycles. What we obtained were cells that were some 500 times brighter staining for Leu-2 than the donor T-cell line, which provided DNA for the original transfection, and the distribution still was

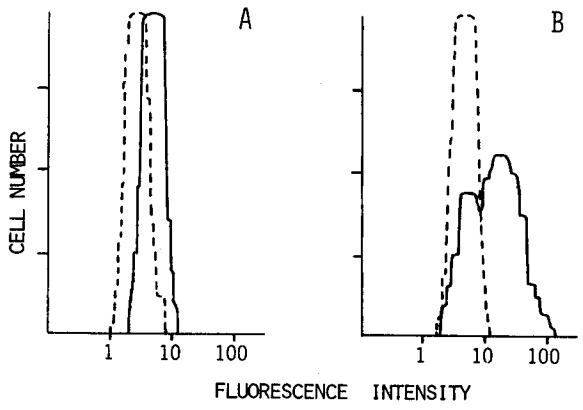

Fig. 2. FACS fluorescence histograms for Leu-2 transfectants. (A) Stable type; (B) Unstable amplified type.

heterogeneous. Preparation of metaphase spreads from these bright cells revealed the presence of numerous double minute (DM) chromosome fragments. These lack centromeres and therefore cannot line up on the mitotic spindle and be equally distributed at cell division. Rather, the daughter cells receive larger or smaller numbers of DMs. Apparently, the Leu-2 genes are present on these DMs, and by sorting for the brighter cells we have sorted for increased numbers of DMs. We estimate each DM to have a minimum of 200 kilobase pairs (Kb) of donor (human) DNA including, of course, one or a few Leu-2 genes. Thus, the gene amplification observed is due to selection for increased numbers of double minutes carrying and expressing the Leu-2 gene.

SELECTED *c*DNA PROBES FOR GENE CLONING

Because of this lucky finding that a large number of Leu-2 transfectants spontaneously amplify the number of Leu-2 copies expressed, we expected to find large amounts of Leu-2 *m*RNA in the amplified transfectants. This presented another, perhaps easier, and more generalizable strategy for cloning the Leu-2 gene. Our successful approach involved isolating *m*RNA from two different amplificants and making *c*DNA from each of these. Then, a phage library was made containing *c*DNA inserts from one of these amplificants. Then a *c*DNA subtracted probe was made starting with P^{32}-labeled *c*DNA from the second amplificant and 'subtracting' with *m*RNA from recipient L-cells to enrich for *c*DNA corresponding to human genes. This is illustrated in Fig. 3. *c*DNA species corresponding to L-cell *m*RNA species form double stranded hybrid molecules which are retained on hydroxylapatite columns while *c*DNA species representing donor transfected DNA remain single stranded and come out in the effluent of such columns. The labeled effluent *c*DNA is then used to screen the *c*DNA library made from the other amplified transfectant. The positive *c*DNA clones thus obtained were used to screen a genomic library and a candidate genomic clone was isolated and confirmed by being able to transfect L-cells at high frequency for Leu-2. Thus, we obtained *c*DNA and genomic clones for this cell surface molecule. Characterization and sequencing is now underway.

AMPLIFICATION MECHANISMS

There are two interesting sidelines we are pursuing which have developed from our transfection and cloning efforts. The first concerns the amplification.

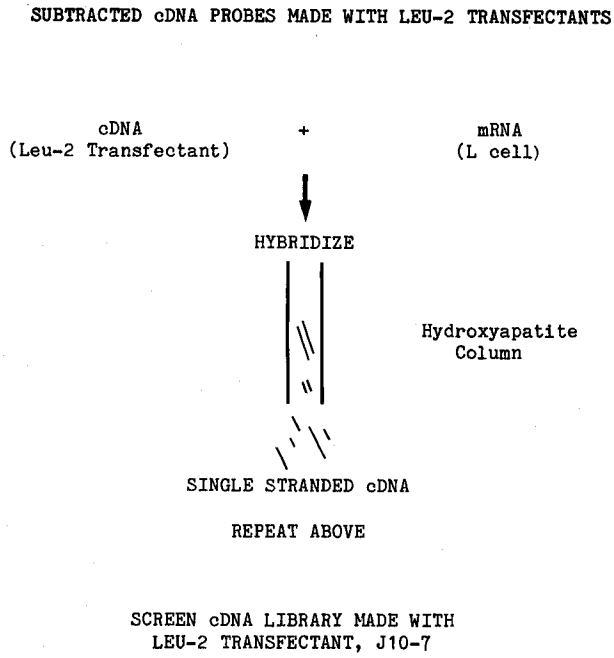

Fig. 3. Protocol for making subtracted *c*DNA probes.

Although we have transfected for about 20 different cell surface molecules of both human and murine origin (see Table 1), it is only Leu-2 and the presumed mouse homolog, Lyt-2, which spontaneously amplified after transfection. Is this due to a linked amplifying element or some structural feature of the gene itself, which is conserved evolutionarily? When we are successful in isolating the Lyt-2 gene and sequencing the two genes, perhaps the explanation for this will emerge. Now, we are initiating experiments to localize the part of the genomic clone responsible for the amplifying property.

Are trophoblast derived cells an exception to the general rule that DNA from any cell will transfect?

Regarding the other interesting sideline, as indicated earlier, it appeared that any human or mouse tissue or cell line could be used as donor DNA to obtain transfectants expressing a particular antigen (Hsu C, Kavathas P, Herzenberg L A: Expression of cell surface differentiation antigens on mouse L-cells transfected with whole genomic DNA from non-expressing cells. Submitted for publication). Thus, placental, kidney, muscle or lymphoid cell DNA transfect equally well. To search for a possible exception, we decided to use DNA from a trophoblast source since some of us had recently shown that trophoblast and certain derived choriocarcinoma cell lines are unique among somatic cells in not synthesizing HLA heavy chain polypeptides or *m*RNA.[12,13] Perhaps these cells, which normally serve as the immunological barrier

between mother and fetus in the placenta, developed some mechanism to inactivate genes for other cell surface molecules. We transfected using DNA from a choriocarcinoma cell line, JAR, which does not express HLA or, of course, Leu-2. No Leu-2 transfectants were found in three separate experiments (Table 2). Transfection for a trophoblast specific cell surface antigen, Trop-1, was found in the usual approximately 1 in 1000 frequency of TK^+ cells. Sperm DNA, which might also have been unable to serve as a source for Leu-2 transfection, was as good at transfecting Leu-2 as any somatic cell. Control leukocyte (PBL) DNA transfection is also shown on Table 2. What could be the reasons for

Table 2. Cotransfection of TK^- L-cells with TK plasmid and DNA from the JAR choriocarcinoma cell line, sperm and blood leukocytes*

	Source of DNA		
Antigen	JAR	Sperm	PBL
Trop-1	12/12†	9/10	8/8
Leu-2	0/12	9/10	6/6

*Cumulative results of three independent experiments.
†Number of positive dishes/total transfected dishes.

lack of transfection by JAR DNA? One trivial possibility is that JAR has lost (unlikely since JAR has been reported to be hyperdiploid)[14] or mutated the genes for Leu-2. Using Southern blot hybridization, the Leu-2 gene is present in JAR DNA. Although we cannot rule out small mutations or a rearrangement of DNA involving long stretches (greater than about 15Kb), Leu-2 restriction fragments from JAR appear identical or nearly identical to those from PBL using a number of different restriction enzymes including pairs which would reveal some differences in methylcytosine (methylation). Since phenol extracted and proteinase K-treated ('naked') DNA is used for our transfection experiments, some unknown *covalent* DNA modification, methylation in undetected regions (perhaps 5′ to the gene), small mutations or rearrangements, or other undetected DNA changes in structural or possible regulatory genes for Leu-2 must be responsible. At the moment we are attempting to determine if DNA from other choriocarcinoma cell lines are able to transfect for Leu-2. We are also in the process of extending our observations to other cell surface molecules using DNA from JAR and other choriocarcinoma cell lines.

SUMMARY

The main reason for initiating these transfection studies is to facilitate the cloning and study of genes (and their corresponding proteins) encoding cell surface molecules on lymphoid cells with the eventual aim of understanding what functions they have in regulating or carrying out the activities of the immune system. The approach we used is generally applicable. Transfection works for the great majority of cell surface molecules. Subtraction of transfectants *c*DNA with *m*RNA from L-cells is technically straightforward. Gene amplification noted for Leu-2 and Lyt-2 is not necessary for the subtraction *c*DNA approach to be successful. Davis and his collaborators have shown that genes making very low abundance *m*RNA species can be cloned by subtraction.[15] Thus, this whole methodology should help us learn the structures and (hopefully provide leads for learning) functions of the cell surface molecule which distinguish various types of cells in the immune system.

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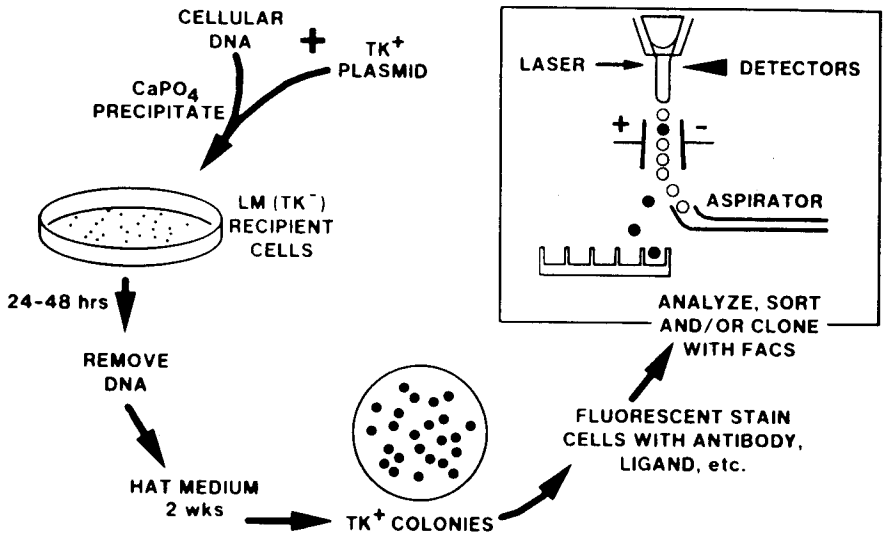

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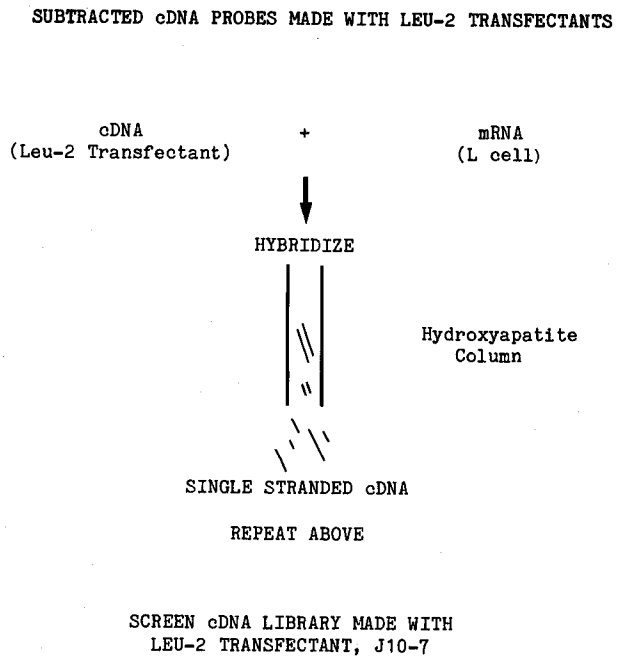

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