

# Chapter 91

## Transfection for lymphocyte cell surface antigens

PAULA KAVATHAS & L. A. HERZENBERG

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Recombinant DNA technology provides opportunities for studying the immune system in new ways. DNA-mediated gene transfer is one technique that has already proved to be very useful. Transfer of cloned genes coding for products with immunologic relevance such as MHC antigens or immunoglobulin has provided important information on structure, function, and gene regulation. However, there are many lymphocyte-specific products, such as cell surface differentiation antigens, for which the genes have not been cloned.

Transfection has been a valuable step in cloning oncogenes and 'housekeeping' enzymes. It can also be valuable as the first of several steps in isolating genes for cell surface molecules. The authors demonstrated that mouse L (TK<sup>-</sup>) cells could stably express a variety of mouse and human cell surface lymphocyte antigens after co-transfection with total cellular DNA and the plasmid pBR322 containing herpes simplex thymidine kinase [1]. Transfectants for cell surface antigens can be detected and isolated by fluorescence activated cell sorting after staining cells directly by monoclonal antibody or indirectly with ligands bound to the molecule followed by fluorescent monoclonal antibodies to the ligand. Transfectants expressing a lymphocyte differentiation antigen can be used for studying the particular antigen or for cloning the gene coding for the antigen. This article will focus on transfection of cells with total cellular DNA and its applications in immunology and cell biology.

### History and introduction

Experimental transfer of foreign genes into recipient cells using naked DNA was originally performed in bacterial systems and is now widely used in mammalian systems. In 1968, McCutchan & Pagano [3] demonstrated that purified SV40 DNA could be reproducibly introduced into mammalian cells using

DEAE-dextran as a facilitator. In 1973, Graham & van der Eb [4] made the important finding that naked adenovirus DNA could be taken up much more efficiently by mammalian cells as a calcium phosphate precipitate than with the DEAE-dextran technique. This work was expanded upon by others [5-7] who showed that transfer, stable integration, and expression of single copy eukaryotic genes was regularly possible using total cellular DNA as donor.

The basic process by which DNA-mediated gene transfer (transfection) is accomplished is beginning to be understood. First, foreign DNA containing a gene of interest is introduced into recipient cells which lack expression of the gene of interest. Most of the DNA is rapidly degraded without ever reaching the nucleus [8]. DNA which reaches the nucleus may be transcribed so that transient expression may be observed for certain genes [9], usually after 48 h. Some of the DNA fragments reaching the nucleus may also undergo recombinations [10] or alterations such as mutation [11,12]. For stable expression to occur, the DNA must be integrated into a chromosome. Integration appears to occur after ligation of fragments of DNA into a single concatamer, which is then integrated into an apparently random chromosomal site [13,14]. In transfectants, about  $\frac{1}{1000}$  of the donor DNA is stably integrated into each recipient cell [13,14]. For human DNA transfected into mouse cells this represents about  $3 \times 10^6$  base pairs or  $3 \times 10^3$  kbp of DNA. Much of this DNA may not be stably expressed. Loss of expression can result from gene loss [15], modifications such as methylation [16,17], changes in chromatin structure [18] or other regulatory events.

The success of transfection for cell surface antigens with cellular DNA is based upon the key observation by Wigler *et al.* [15] that if the donor DNA was composed of two purified genes, a selectable gene and a non-selectable gene present in excess (1000-fold molar excess), then transfectants expressing the

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selected marker frequently expressed the non-selected gene. In other words, the co-transfection frequency was remarkably high. This indicates that a subpopulation of cells in the culture is competent to take up and express the foreign DNA efficiently. Thus to increase the frequency of transfectants for single copy genes from frequencies of  $10^{-6}$ - $10^{-7}$  to  $10^{-3}$ - $10^{-4}$  using total cellular DNA, recipient mouse L (TK<sup>-</sup>) cells are co-transfected with a mixed calcium phosphate precipitate of total cellular DNA and a plasmid containing the thymidine kinase gene. Transfectants expressing the TK marker are selected for by growth in HAT medium (see below), and then cells expressing the cellular gene product are selected. Because of the initial enrichment for 'competent' cells by TK selection, a greater proportion of cells are expressing the cellular gene product than before TK selection. However, competence is not a heritable trait since cells expressing foreign gene products are not more efficiently transfected than non-transfected cells [15].

In order to transfect cells with total DNA, two kinds of methods are required: (1) methods to introduce DNA into cells, and (2) selection methods to isolate transfectants which occur at low frequencies. Transferring genes into mammalian cells is accomplished by a variety of methods. These include: (i) addition of DNA as a calcium phosphate precipitate [4,15,19], (ii) addition of DNA via electrophoresis [20,55], (iii) membrane vesicle fusion with protoplasts [21,22] or liposomes [23], (iv) transfection with viral vectors [24,56], (v) micro-injection [25], and (vi) DEAE-dextran [3]. The most common method for transfection with total cellular DNA is its addition as a calcium phosphate precipitate. The method of choice may depend on the particular recipient cell.

Because stable transfection is a low frequency event, a good selective system is required to find cells expressing the foreign gene. Examples of selective systems are given below.

### *Biochemical selection of wild type from enzyme-deficient mutants*

For example, mutants lacking thymidine kinase (TK) activity (selected by growth in BrdU) will survive in HAT (hypoxanthine 15  $\mu$ g/ml, aminopterin 1  $\mu$ g/ml, thymidine 5  $\mu$ g/ml) medium only if they acquire the TK gene. This is a widely used selective procedure that works as follows: when the pathway of *de novo* synthesis of purine nucleotides and thymidylate synthesis is blocked by aminopterin, thymidine kinase is required to utilize exogenous thymidine and convert it to thymidine monophosphate. Only TK<sup>+</sup> transfectants will survive in HAT medium.

### *Dominant biochemical selection*

These methods have the advantage that normal cells without any particular mutation can be used, thus avoiding the sometimes difficult search for deficiency mutants. Three types of dominant selection exist for mammalian cells. (1) GPT selection [26]: The *E. coli* enzyme xanthine-guanine phosphoribosyltransferase (GPT) resembles animal cell HPRT. However, in contrast to HPRT, it can use xanthine as a purine source and is not inhibited by mycophenolic acid. Therefore, when the normal purine metabolic pathway is inhibited by the aminopterin in HAT, the hypoxanthine is substituted by xanthine in HAT medium and mycophenolic acid is included in the medium to block HPRT activity. Cells transfected with the *E. coli* GPT gene will be the only ones to grow. (2) NEO selection [27]: The neomycin-resistance gene codes for aminoglycoside phosphotransferase that phosphorylates and thus inactivates neomycin and its analogue G418, which blocks ribosome function. Although eukaryotic cells are insensitive to neomycin, they are sensitive to G418 unless they acquire the neo gene. Thus the neo gene can be used for co-transfectant selection. Cell density appears to be important for optimum NEO selection. (3) HPH selection [56]: The hygromycin-B-phosphotransferase (HPH) gene confers resistance to hygromycin B. Cell lines should be pretested to see whether the NEO, GPT or HPH selection is better.

### *Colony morphology*

This is the common method used for selecting cells transfected with an oncogene. NIH 3T3 cells transfected for an oncogene are recognized because they grow piled on top of each other forming a visible raised focus on a monolayer of normal 3T3 cells.

### *Selection with monoclonal antibodies or other ligands*

Transfectants expressing foreign cell surface molecules are detected as antigens with fluorescein-conjugated monoclonal antibodies (McAb) or as receptors with directly or indirectly fluorescent ligands. Stained cells occurring with frequencies of  $< 10^{-4}$  are readily detected and isolated with a fluorescence activated cell sorter (FACS). Cells at lower frequency can be enriched by several orders of magnitude in a first passage through the FACS. Positive cells can then be sorted individually into the wells of microtitre plates: so called 'sorter cloning' or 'autocloning'. There are alternative methods which can be used to isolate antigen transfectants but each must be individually developed for the specific monoclonal antibody. For

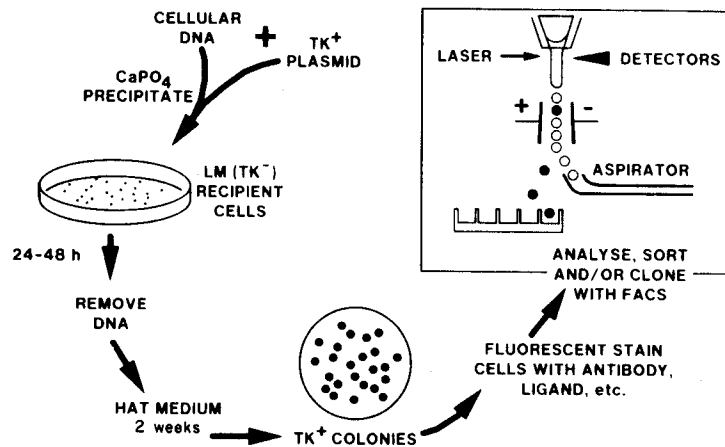


Fig. 91.1. Selection of transformants.

example, solid-phase immunoaffinity methods [28], which can be excellent for depletion of cells bearing a particular antigen, are often difficult to use for enrichment of rare cells with a particular surface molecule. Recently, human red blood cells coated with antibody were successfully used [57].

The following methods will detail co-transfection of mouse L(TK<sup>-</sup>) cells with cellular DNA and a TK plasmid using the calcium phosphate precipitation method. L(TK<sup>-</sup>) cells efficiently take up foreign DNA. The line is derived from mouse strain C3H fibroblasts that were transformed *in vitro* with methylcholanthrene. TK<sup>-</sup> mutants were selected by growth in the thymidine analogue 5-bromodeoxyuridine, and revertants have never been isolated [29]. Because the cell line has been in culture for many years, L cells from different sources may have some differences.

**Isolation of cellular DNA** (modified after Wigler *et al.* [15])

**Materials**

Sterile polypropylene tubes are used throughout.  
 TEN buffer: 150 mM-NaCl, 10 mM-Tris-HCl, pH 7.8, 1 mM-EDTA.  
 Proteinase K solution: 0.4 mg/ml proteinase K (Merck) in TEN with 0.4% SDS. Incubate at 37 °C for at least 10 min to predigest contaminating RNase and DNase.  
 Water-saturated distilled phenol is equilibrated with 100 mM-Tris (pH 8.0) on the day of extraction. Wear impermeable gloves whenever working with phenol.  
 RNase A stock: 10 mg/ml, in 10 mM-Tris: 1 mM-EDTA (TE) (boiled for 15 min).

**Procedure**

- 1 Collect the cells by aspirating medium, rinsing with PBS, and adding PBS/EDTA (0.6 mM-EDTA). Use vigorous pipetting to collect.
- 2 Count the cells and then centrifuge.
- 3 Aspirate and resuspend in TEN (about 5 ml/10<sup>8</sup> cells).
- 4 Add an equal volume of proteinase K solution. Cells will lyse.
- 5 Incubate for 15 min at 65 °C, then 37 °C for 5–10 h or overnight. The solution should appear clear; otherwise, add more proteinase K.
- 6 Add enough 2 M-Tris so that the final concentration is 100 mM (pH 8.0).
- 7 Add an equal volume of phenol. Rotate to mix for 10–20 min.
- 8 Centrifuge to separate the aqueous from the phenol phase (2000 g, 10 min.)
- 9 Remove the upper aqueous phase to a clean tube (or remove phenol).
- 10 Add equal volume of ½ phenol and ½ chloroform/isoamyl alcohol (24:1).
- 11 Rotate the tube for 10 min; centrifuge for 10 min.
- 12 Remove the upper aqueous phase to a clean tube.
- 13 Add an equal volume of chloroform/isoamyl alcohol (24:1). Rotate the tube for 10 min.
- 14 Centrifuge for 5 min. The interphase should be clear.
- 15 Collect the aqueous phase. Add sodium acetate solution to 0.3 M. Add 2 vols. ethanol and invert.
- 16 A precipitate will form immediately which can be pulled out with a hooked glass pipette or with plastic pipettes used like chopsticks. Otherwise, centrifuge for 5 min at 6000 rev./min in Sorvall.
- 17 Wash pellet in 95% ethanol or dip precipitate in 95% ethanol. Allow to dry.

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- 18 Dissolve in TE.
- 19 Add RNase to 20  $\mu\text{g}/\text{ml}$  and incubate at 37 °C for at least 1 h.
- 20 To remove RNase, add proteinase K and SDS as above. This step may be omitted.
- 21 Repeat steps 7–18. Alternatively, repeat steps 7–14 and dialyse DNA against four changes of TE, pH 8.0.

##### Notes and recommendations

The yield is about 10  $\mu\text{g}/10^6$  cells. To help get DNA into suspension, rotate overnight at 37 °C. Check DNA on a 0.5% agarose gel to make sure the DNA is high molecular weight (> 40 kbp). Some investigators do not remove RNA.

##### Isolating DNA from tissue (modified after Blattner *et al.* [30])

##### Materials

Liquid nitrogen.  
STE buffer: 1% sarkosyl, 10 mM-Tris, pH 8.0, 0.1 mM-EDTA (about 25 ml/g of tissue).

##### Procedure

- 1 Rinse the tissue in physiological buffer and weigh, if yield determination is desired.
- 2 Immediately freeze in liquid nitrogen.
- 3 Pulverize the tissue using a mortar and pestle. The mortar should be sitting in a liquid nitrogen bath, and the pestle should be precooled in liquid nitrogen before use.
- 4 Add the pulverized frozen tissue slowly to warm (55 °C) STE buffer with 10 mg of proteinase K while stirring at 50–55 °C.
- 5 Continue to stir the mixture for 1 h at 50–55 °C.
- 6 If the tissue is not completely dissolved, add additional proteinase K and stir for an additional hour.
- 7 Add RNase to reach a final concentration of 10  $\mu\text{g}/\text{ml}$  and incubate the mixture in a 55 °C water bath overnight. Phenol-extract material at least three times with redistilled phenol equilibrated with STE buffer.
- 9 Extract four times with chloroform:isoamyl alcohol (24:1).
- 10 Band in cesium chloride (1.29 g is added per ml extract) using a Beckman SW50.1 rotor for 60 h at 42000 rev./min, 20 °C.
- 11 Recover DNA by inserting a 20  $\mu\text{l}$  siliconized micropipette (attached to tubing at one end) to the bottom of the tube and pump out the solution with a peristaltic pump. Collect fractions and measure OD to

detect DNA. DNA can also be detected by viscosity changes.

12 Dialyse against four times one litre of DNA dialysis buffer (Tris/EDTA). Concentrate with a Speedvac.

##### Transfection [15,19]

##### Recipient cells

- 1 Collect exponentially growing or just confluent cells by aspirating the medium, rinsing the dish with 0.25% trypsin in medium without serum, and incubating with 1 ml 0.25% trypsin solution per 100 mm dish at 37 °C for 5 min. Add medium with serum and vigorously pipette with a Pasteur pipette to dislodge the cells. The medium is Dulbecco's modified MEM with 10% FCS. Alpha-MEM with 10% serum is also satisfactory.
- 2 Put  $0.5\text{--}1 \times 10^6$  cells in 9.0 ml medium in 100 mm Petri dishes. Spread the cells evenly over the surface by gentle tilting. Do not swirl; otherwise, cells will move to the centre.
- 3 About 24 h later, when the cells have attached, transfect with DNA precipitate.

##### Forming DNA precipitate

The order of addition of components, pH of buffers/medium and concentrations of DNA, etc. are important.

##### Materials (all sterile)

TE buffer: 1 mM-Tris (pH 7.9), 0.1 mM-EDTA.  
CaCl solution: filter sterilized 2 M stock (Mallinckrodt; not all brands work).  
2 $\times$  HBS buffer: autoclave 280 mM-NaCl, 50 mM-HEPES, 1.5 mM-sodium phosphate (equal amounts of mono- and dibasic) pH.  $7.1 \pm 0.05$ . Check pH before use. Store in a stoppered bottle at room temperature or frozen. Several stocks made at different pH values can be tried for optimal transfection frequency.

##### Transfection for one dish of cells

- 1 Dilute 20  $\mu\text{g}$  of DNA with TE buffer so that the concentration is 20  $\mu\text{g}/0.42$  ml.
- 2 Add 60  $\mu\text{l}$  of CaCl solution and flick the tube.
- 3 In a separate tube have 0.48 ml 2 $\times$  HBS.
- 4 Blow air vigorously through HBS solution using a sterile cotton plugged pipette attached to a pipette gun. Add DNA/CaCl solution dropwise.
- 5 Initially the solution looks cloudy. After 20–30 min a white precipitate will form. Mix and add 5–10 drops

in a dispersed array around the dish of L cells. Tilt the dish to distribute over all the cells.

6 After 24 h, aspirate and add fresh medium.

7 Twenty-four hours later, aspirate and add HAT medium.

8 After a further 1–2 days, refeed with HAT. Refeed every 3–4 days thereafter. Initially, when there is a lot of cell death, feed the dishes more frequently.

9 Colonies are visible after 7–14 days. Expect about 1000 colonies. Some of these will be satellite colonies that form when dividing cells round up and move and resettle away from the parent colony. Adding medium gently helps to avoid satellites.

#### Notes and recommendations

The amount of plasmid DNA carrying the selectable marker such as the herpes simplex virus thymidine kinase gene (HSVTK) should be experimentally determined by titring different amounts with carrier DNA. Anywhere from 0.1 to 1.0  $\mu\text{g}$  may be needed to yield about 1000 HAT-resistant TK<sup>+</sup> colonies per dish.

Overnight incubation of the DNA precipitate on mouse L cells gives numbers of colonies similar to those obtained with shorter periods of incubation followed by treatments such as dimethyl sulphoxide, glycerol or sucrose [19]. However, such treatments may be helpful with other cell types.

After DNA purification, the size of DNA fragments is usually about 50–100 kbp. The DNA is usually resuspended at a concentration of 500  $\mu\text{g}/\text{ml}$ . If the DNA at this concentration is very viscous then shearing the DNA by drawing through a 22-gauge needle with a sterile syringe may improve transfection frequency.

The source of DNA can be from expressing or non-expressing tissue or cells in most cases [2].

Transfection with either phage DNA or intact phage particles can be performed. Phage DNA alone inhibits transfection so that usually it is mixed with carrier (mammalian) DNA at a ratio that is minimally inhibitory [31], usually 1:20; efficient transfection of mouse L cells with phage particles is possible although the conditions for transfection differ [32].

#### Staining with fluorescent monoclonal antibodies

##### Materials

Phosphate-buffered saline (PBS) with 0.6 mM-EDTA. Antibody: centrifuge, preferably in an airfuge (Beckman for 10 min) or an Eppendorf centrifuge. Staining medium (SM): RPMI 1640 medium (without biotin or phenol red, Irvine Scientific), 10 mM-HEPES, pH 7.4, 0.1% sodium azide, 1–3% complement-free serum.

10  $\times$  Propidium iodide (PI) (Calbiochem) solution: 10  $\mu\text{M}$ -PI in PBS. Store frozen.

##### Procedure

1 Collect cells by aspirating medium, rinsing with PBS, and adding PBS/EDTA at room temperature. Dislodge cells by vigorous pipetting. (Collecting the cells with trypsin could destroy some molecules.)

Carry out the following at 4 °C:

2 Place 10<sup>6</sup> cells, in a centrifuge, resuspend in 50  $\mu\text{l}$  SM and add antibody. Normally saturating amounts of antibody are used for staining. Since only rare cells will express the transfected antigen, the amount of antibody can usually be reduced to  $\frac{1}{2}$ – $\frac{1}{4}$  of the saturating concentration.

3 Incubate for 20–30 min on ice.

4 Add PI to concentration 1  $\times$  and incubate for 5 min longer.

5 Wash cells once, twice to remove PI, and resuspend in 0.5 ml SM. Pass cells through sterile nylon mesh (TEKTO, Inc. HD-3-85) to remove clumps. Keep on ice.

6 If a second-step antibody is used, wash cells to remove first antibody and stain as per first antibody. Do not add the PI until the end of the second incubation.

##### Notes and recommendations

Dead cells pick up fluorescent proteins non-specifically. Such cells are eliminated from analysis and sorting by staining with PI, which penetrates only cells with damaged membranes and intercalates in DNA rendering the cells brightly red fluorescent [33]. Red PI-stained cells are then electronically gated out. This is very important for sorting rare cells.

For most antigens studied by the authors, the frequency of transfectants is about 1 per 1000 of the TK<sup>+</sup> transfectants. Therefore cells representing about 1000–2000 colonies for each sample should be pooled so that the sample will probably contain a transfectant. Since colony sizes differ, a transfectant from a small colony may be present only at a frequency of 10<sup>-4</sup>. Nevertheless, cells occurring at such frequencies are readily detected and recovered by FACS sorting. Only transfectants arising from colonies on separate dishes should be considered independent.

Since the amount of antigen on transfectant cells may be low and the autofluorescence of L cells is high, it is important to optimize staining and FACS discrimination between L cells and stained transfectants. A convenient way to increase staining brightness is to use two fluorescent antibodies. The authors often use a fluorescein-conjugated monoclonal antibody as a

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first-step reagent and a fluorescein-conjugated goat anti-mouse antibody as a second-step reagent. Alternatively, they use two McAb recognizing different epitopes on the same molecule. They have, however, isolated transfectants for an antigen using a single fluorescein-conjugated monoclonal antibody. (See other chapters on fluorochrome conjugation, staining and FACS operation.)

All reagents should show negligible staining of L cells. For example, if a reagent falsely stains 1% of the L cells, then the 0.1% of truly positive cells will be diluted tenfold among cells sorted at this threshold. If no other reagent is available, steps can be taken to improve its quality. Fluorescein/protein (F/P) ratios, absorption of DEAE, and reagent purification are described (see Chapters 31 and 109).

To select for multiple antigen transfectants simultaneously, cells may be stained with a mixture of monoclonal antibodies. The brightest staining cells are sorted, grown, and reanalysed. If positive cells are detected, cell samples stained with individual reagents can then be sorted.

Once transfectants stained for the antigen of interest are isolated, immunoprecipitation and SDS-PAGE or functional tests can confirm that the molecule is indeed present.

There are a number of possible explanations for failure to detect an antigen transfectant: (1) products of two unlinked genes are required for expression of the antigen; (2) staining reagents are not adequate for detecting low level of expression; and (3) frequencies of transfection can vary over a tenfold range [7] so that inadequate members of independent TK<sup>+</sup> colonies are screened.

### Sorting and cloning

About 0.2–0.5% of the brightest staining cells are sorted aseptically. Most of the sorted cells should be transfectants rather than false-positive L cells; otherwise, the enrichment may be inadequate. After the sorted cells have grown sufficiently (> 50 000 cells), reanalyse them. If a few per cent or greater are now positive, clone them directly with the FACS into microtitre wells. If a FACS cloner is not available, resort the brightest cells until there is sufficient enrichment so that cloning by limiting dilution is feasible. Occasionally several rounds of sorting the brightest cells are required before a positive population is noted. The authors found the best discrimination between fluorescein staining and cell autofluorescence using a 520–560 nm or 515–545 nm band pass filter in the FACS fluorescein channel. At least one-half of the clones obtained after enrichment

appear to be stable. These clones are maintained in HAT medium.

To confirm that a cloned piece of DNA codes for a particular antigen, it might be possible to save time by assaying for transient expression. From 1 to 25% of cells transfected with a cloned HLA gene express HLA on their surface after 48 h [9]. Removing the cells from the dish after 24 h and replating them helps eliminate non-specific staining.

### Generality of transfection

Most genes coding for cell surface antigens appear to be expressed in the mouse L cells. Hsu *et al.* [2] transferred either human or mouse DNA to mouse L cells and screened with a panel of monoclonal antibodies against fifteen different cell surface antigens. Transfectants expressing thirteen of the fifteen antigens were isolated. Human lymphocyte antigens Leu-1, Leu-2 and mouse lymphocyte antigens Lyt 1, Lyt 2, Thy 1, ThB, FcR, L3T4, BLA-1 and BLA-2 are some of the expressed antigens. In addition, these cells express the gene for nerve growth factor receptor. The receptor was detected by staining with purified NGF followed by antibody to NGF [2]. Transfectants for transferrin receptor were also isolated [60].

### Gene amplification

Gene amplification of some transfected genes can occur spontaneously [34] and be selected [35–37]. Spontaneous amplification of the gene coding for the human T cell differentiation antigen Leu-2 (T8) occurs in 25–50% of the Leu-2 transfectants [34]. Cells with greater amplification and thus more antigen on the surface can be selected with fluorescein-conjugated McAb and the FACS by sorting the brightest staining cells, growing the cells, and repeating the process. Spontaneous amplification of a promotor-defective TK gene which was transfected into mouse L cells was reported by Roberts & Axel [35,36]. Variants that grew in HAT medium had many copies of the TK gene; amplified clones occurred at frequencies of 10<sup>-4</sup>. Spontaneous gene amplification frequently appears to occur after transfection for certain cell surface expressed genes (unpublished observations).

To obtain amplification for any cloned gene, Wigler *et al.* [37] described co-transfection of an amplifiable gene such as dihydrofolate reductase (dhfr) with the gene of interest. Then amplification of dhfr, selected for by growth in methotrexate, causes linked sequences to be co-amplified. This approach was used successfully to amplify the gene coding for hepatitis B surface antigen [38]. However, recipient cells which do

not spontaneously mutate to methotrexate resistance at a high frequency are required.

#### **Applications to immunology**

##### *Cloning genes*

Cloning genes using transfection as a first step was initially performed for several 'housekeeping' enzymes and subsequently for cellular oncogenes [39-43]. Recently, the authors cloned the gene coding for the human T lymphocyte antigen Leu-2, synonym T8, using transfection as a first step [44]. The beauty of the transfection approach is that as long as there is a good selective system, genes can be cloned even if the levels of protein and mRNA are quite low in normally expressing cells, as appears to be true for most lymphocyte differentiation antigens.

After transfection for a cell surface antigen is demonstrated, restriction enzymes which do not cut into the gene of interest can be identified. Thus, if antigen transfectants can be isolated using restriction enzyme cut DNA as donor DNA, then the enzyme does not cut into the gene of interest and destroy its activity. The size of the restriction fragment containing the gene of interest can be determined by size fractionating the digested DNA on a sucrose gradient or a gel and transfecting with DNA from the different fractions. The size is estimated from the fraction yielding the most transfectants per microgram of DNA. This information is important for deciding which cloning vector to use for constructing a library since cloning vectors only incorporate certain size fragments.

Several successful methods for isolating a transfected gene from the recipient genome exist [58,59]. Human genes transfected into mouse cells can be isolated if they are closely linked to a human repetitive DNA sequence [39,45]. Linking donor DNA with a sequence such as the tRNA suppressor gene can also be used to rescue a gene once transfected [40]. Other methods include sib selection and cosmid rescue [41,46]. Newer methods include screening cDNA libraries with cDNA selected probes in which cDNA made from mRNA of a transfectant is depleted of cDNA species in common with non-transfected recipient cells [44]. To prove the DNA codes for the gene of interest, DNA isolated from candidate clones should be capable of transfecting for the gene of interest.

Transfectants can be used to confirm that a clone isolated by another cloning method is correct. Southern blots of DNA from independent antigen transfectants could be hybridized with a probe made from DNA of a candidate clone. If the probe hybridized to

DNA from transfectants and not from DNA of recipient cells, this would be strong evidence that the clone was coding for the gene of interest. For mouse genes transfected into mouse recipient cells, Northern blots instead of Southern blots would need to be done because the DNA fragment of the recipient cells would most likely be the same as the donor DNA fragment.

##### *Assaying unknown genes*

About twenty to thirty different human genes hybridize on Southern blots with certain HLA-specific probes. However, the products coded for by most of the genes are unknown, and it is difficult to know which hybridizing band corresponds to known gene products. To identify what protein product is encoded by the DNA fragment on the blot [9,47], the fragment is isolated (after digesting the DNA with Hind III, which does not appear to cut into HLA genes), and the DNA is co-transfected with a selectable marker into mouse L cells at a ratio of fragment to marker such that most transfectants for the marker will incorporate the non-selected gene. Transfectants can be tested with known antisera to identify the gene product of the fragment. If the transfectants do not react with characterized antisera, transfectants can be used as immunogens in C3H mice (the same strain from which the L cell line was derived). Antiserum absorbed with L cells (to remove tumour related antigens) could be tested on the transfectants and on normal cells to identify the antigen. The same general approach was used to identify specific H-2 alleles [48].

##### *Studies of gene structure, function and regulation*

Essential structural elements of a cell surface antigen and the relevance of structure to function can be studied by transfecting modified genes into recipient cells such as L cells. Transferring a gene for an H-2 antigen in which exons have been deleted or exchanged with an exon from a gene coding for a different H-2 antigen allows determination of regions of the molecule that are required for expression of antigenic determinants, specific functions or expression on the cell surface [49]. Genes can also be modified in non-coding regions to identify regulatory sequences. For instance, treatment of lymphocytes or H-2 transfectants with  $\gamma$ -interferon increases H-2 expression on the cell surface [50]. Modifying H-2 genes, transfecting into L cells and determining the effect of  $\gamma$ -interferon on H-2 expression will help identify regulatory sequences.

Classical means of studying the function of cell surface antigens in immunology has involved (1) blocking studies with antibody, (2) selecting cell

surface mutants and looking for alterations in function, (3) correlating cell function with expression of certain antigens. Transfection provides an alternative method. For instance, L cells that are transfected with an H-2L gene and infected with vesicular stomatitis virus can be killed by MHC-restricted cytotoxic T cells directed against viral antigens in association with MHC antigens [51]. This indicates that the H-2L antigen can serve as a restricting element for viral antigen presentation on the transfectants and presumably then on normal cells as well. Similarly, H-2 IA [52,53] and HLA-DR [54] expression on L cells is being used to study antigen presentation. In addition, an antigen that serves as a receptor can be identified by incubating transfectants with supernatant containing the ligand and looking for depletion of ligand activity. Future potential uses of transfectants beside those mentioned here will surely follow.

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