Chapter 23

# Amplification and Molecular Cloning of Transfected Genes

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## 1. Introduction

The transfection of genes into recipient cells is a very exciting tool because of the diversity of problems that can be attacked and because of the elegance of the answers. For instance, transfer of cloned MHC or immunoglobulin genes has provided important knowledge of the structure, function, and regulation of these genes. Many important genes have been cloned by transfecting with total cellular DNA, selecting transfectants expressing the gene, and then isolating the transferred gene by one of several methods. The identification and cloning of cellular oncogenes, a major breakthrough in the past few years, was an achievement that may not have been possible or have occurred as swiftly without our ability to transfer DNA into mammalian cells and isolate transfectants.

Monoclonal antibodies are extremely useful for selection of transfectants expressing genes coding for cell surface antigens or receptors. Transfectants expressing a variety of mouse and human cell surface molecules can be isolated after staining recipient cells with fluorescein-conjugated monoclonal antibodies and sorting positive cells using a fluorescence activated cell sorter (detailed below). These cell surface molecules include lymphocyte differentiation antigens such as the human T-cell antigen Leu-2 (T8);[2] other cell surface antigens are transferrin receptor,[3,4] and an antigen expressed on human chronic lymphocytic leukemia cells.[5] Genes that code for several of these cell surface antigens can be then isolated from transfected cells.[4,6] It is also possible to select transfectants expressing other types of gene products using monoclonal antibodies and visual selection (see Section 4.3 below).

In this chapter, the process of gene transfer, methods for transfer of DNA into cells, and selection procedures for transfectants will be described. Applications of the technique for gene cloning, for gene amplification, and for analyzing cell surface antigens will also be discussed.

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## 2. Movement of DNA from Outside the Cell into the Nucleus: Basic Process

The basic process by which gene transfer is accomplished using total cellular DNA is partially understood. After the DNA is taken up by the recipient cells, most of the DNA is degraded and never reaches the nucleus.[7] DNA that reaches the nucleus may be transiently expressed; however, for stable expression, transfected DNA must be incorporated into the host genome. If the DNA can replicate autonomously, as does bovine papillomavirus DNA[8] or an Epstein–Barr virus plasmid,[9] then integration is not required for stable expression. Transfected DNA fragments that are incorporated into the chromosome are ligated together as a concatamer (also called pekalasome or transgenome) and integrated into an apparently random chromosomal site.[10,11] Rarely does integration of a transferred sequence into homologous site in the host genome occur.[12] Recombination between fragments in the nucleus can occur before integration,[13] as may alterations such as mutation.[14] For human DNA transfected into mouse L cells about $1/1000$ of the genome is incorporated, or about $3 \times 10^3$ kb.[10,11] DNA that is incorporated may not be stably expressed. Loss of expression can result from gene loss[1,15] or modulation of expression due to changes in methylation,[16,17] chromatin structure,[18] or other regulatory events.

## 3. Methods for Transferring DNA into Cells

Various methods exist for transferring DNA into cells.[19] Purified high-molecular-weight DNA (also called "naked" DNA) can be added to cells as a calcium phosphate precipitate,[1,20] as a polyethylene glycol precipitate,[21] by electroporation in high electric fields,[22,23] or by microinjection.[24] Calcium phosphate precipitation has been most widely used with naked DNA and can also be used with chromatin as well as with DNA packaged as bacteriophage.[25] Alternatively, the DNA is enclosed within membrane vesicles, such as bacterial protoplasts[26,27] or liposomes,[28] which are then fused to cells. Viral vectors, such as bacteriophage, or retroviruses,[29,30] which carry foreign genes can transport those genes into cells. When transient expression is to be assayed, naked DNA can be added with DEAE dextran.[31]

The method of choice depends on a variety of factors. Different cell types are transformed with different efficiencies, depending on the method for introducing the DNA. The size of a cell's nucleus and its membrane properties influence the efficiency of transfer by microinjection. Some methods work better with cloned genes than with total cellular DNA. Because cloned genes are often maintained in bacterial cells as part of plasmid vectors, the method of fusing bacterial protoplasts with recipient cells was developed as a convenient method for introducing a cloned gene into a cell.

Although many different cell types are capable of being transfected, the efficiencies vary widely. Some transformed cells, such as mouse L cells,[32] NIH 3T3 cells, and a mouse melanoma line.[33] are transformed at high efficiencies,

with frequencies ranging from about 1.0 to 0.1% after transfection with a cloned gene. Normal cells, such as fibroblasts, can also be transformed, although the efficiency is usually less. Therefore, experiments using total cellular DNA usually rely on highly efficient cells as recipients. By contrast, transfer of a cloned gene into a cell line of interest, such as that of an immunoglobulin gene into a hybridoma or myeloma line, can be accomplished even if the frequency of transfer is low.[34,35]

## 4. Methods of Selection

Because only a small proportion of cells in a given population are competent to take up and express foreign DNA, there must be schemes for selecting transfectants. For cloned genes this is not a problem. A cloned gene can be cotransferred with a gene for which a good selective system exists. A high proportion, in some cases greater than 80%, of transfectants expressing the selected gene also express the cotransferred gene.[1] Alternatively, the cloned gene can be ligated to the selectable gene before transfection. However, for genes that are not cloned, specific selective systems must exist.

### 4.1. Selection for Complementation of Prototrophic Genes in Mutant Recipient Cells

Initial work in gene transfer relied on the HAT selection method to transfer genes coding for herpes simplex[36,37] or mammalian thymidine kinase[38] into mouse L cells that were deficient in thymidine kinase and were not known to revert. Genes coding for hypoxanthine phosphoribosyltransferase (HPRT) and adenine phosphoribosyltransferase (APRT) could be transferred to mutant cell lines and transfectants similarly selected using HAT medium.[39]

A problem with this approach is that in order to serve as recipients, cells must lack expression of the particular gene product. In cases where a cell line does not have an appropriate mutation, cells can be mutagenized and selection for mutant cells imposed. The revertant frequency, however, must be less than the transfection frequency.

### 4.2. Dominant Biochemical Selection

Cells expressing a transferred gene normally found in a different species can sometimes be selected. Cells with a particular mutation are not required. Two bacterial genes used for this purpose are: (1) neomycin resistance gene (NEO) coding for the aminoglycoside phosphotransferase that phosphorylates and thus inactivates neomycin and its analogue G418, which block ribosome function,[40] and (2) xanthine-guanine phosphoribosyltransferase (GPT), which

utilizes xanthine as a purine source and is not inhibited by mycophenolic acid, in contrast to animal cell HPRT.[41] Cells grown in medium containing mycophenolic acid, aminopterin, xanthine, and thymidine will only survive if they are expressing a transfected GPT gene. A more comprehensive list of selection schemes can be found in Kucherlapati and Skoultchi.[42]

### 4.3. Visual Selection

Alterations in a visible phenotype can be identified with a microscope. This type of selection was used for isolating transfectants of cellular oncogenes. 3T3 cells transfected with DNA from certain tumor lines formed foci after expressing the cellular oncogene.[43] Besides morphological alterations, histochemical or other cytological procedures can distinguish between cells that have acquired a particular product and those that have not. Unfortunately, the cells are often killed in the process of characterization. However, if the principle of sib selection (see below) is applied, this procedure may be a feasible approach for isolating certain kinds of transfectants.

### 4.4. Dominant Selection with Monoclonal Antibodies or Ligands

Transfectants expressing a receptor or a cell surface antigen that is not normally expressed by the cells can be selected by staining cells with a monoclonal antibody or a ligand coupled to a fluorochrome. Positive cells are readily detected with a fluorescence activated cell sorter (FACS) if they occur at frequencies of greater than $10^{-4}$. Positive cells occurring at a lower frequency can be enriched by several orders of magnitude by a first passage through the FACS. When the culture is sufficiently enriched, positive cells are sorted individually into wells of microtiter plates using the FACS autocloning device. For transfection of mouse and human lymphocyte antigens, cellular DNA from expressing[2] or nonexpressing tissue or cell lines[44] can be cotransferred with the thymidine kinase gene into mouse L cells and $TK^+$ transfectants selected by growth in HAT medium. This procedure enriches for cells competent to incoporate and express foreign DNA. For most antigens, transfectants expressing a cell surface antigen occur at a frequency of about $10^{-3}$ of the $TK^+$ cells. For large genes the frequency is usually reduced tenfold.[37]

## 5. Cloning Genes

A powerful approach for gene cloning is the transfer of genes into recipient cells by DNA-mediated gene transfer and then isolating the transferred gene by one of several methods. There are many attractive aspects of this approach. First, the success of the approach does not depend on the level of a specific mRNA in a cell. In fact, most genes cloned by this approach encode rare mRNA.

Second, a number of recipient cells (e.g., mouse L cells) that will efficiently incoroporate and express a wide variety of different genes exist. Third, there are multiple ways for isolating the transferred gene (see below) once transfectants are obtained. Fourth, transfection can be used to confirm that a candidate clone is the gene of interest. A genomic clone containing the complete gene or several clones containing parts of the gene should be capable of transfecting for the gene of interest.[4] Fifth, unknown genes responsible for a certain cell phenotype may possibly be cloned by transfection. For instance, a human gene involved in DNA repair was recently cloned from transfectants obtained by first transforming UV-light-sensitive CHO cells with total cellular human DNA and selecting for cells that were no longer sensitive.[45]

A variety of approaches for isolating transferred genes have been developed. Natural linkage of a gene to an identifiable sequence such as a repetitive sequence or linkage by recombinant DNA techniques with a biologically selectable marker allow rescue of the gene. A more recent method relies on cDNA subtraction whereby cDNA made from mRNA of a transfectant is subtracted with mRNA from recipient cells. Since transfectants and recipient cells express the same gene products except for expression of transferred genes, the subtracted cDNA should correspond to those genes. Alternatively, gene libraries can be screened for the gene of interest by transfecting cells and assaying for expression of the gene product. The following describes the methods in greater detail.

## 5.1. Methods of Isolating Transferred Genes

### 5.1.1. Plasmid or SupF Rescue

The rescue method was first used by Perucho *et al.*[46] to isolate the chicken thymidine kinase gene. Chicken DNA was digested with an enzyme that did not cleave inside the TK gene and the fragments ligated into pBR322 to construct a plasmid library. After transfection with DNA from the pBR322 plasmid library, $TK^+$ transfectants were selected. After a second round of transfection to eliminate pBR322 sequences not linked to TK, the DNA from a secondary transfectant was digested with an enzyme that only cut into the plasmid and the DNA was ligated under cyclization conditions. The circular molecules were transferred to *Escherichia coli* and selected for a drug resistance marker carried by the plasmid. The rescued plasmids were then tested for ability to confer the TK phenotype to mouse cells. A variation on the procedure by Lowy *et al.*[47] involved construction of a genomic library from the secondary transfectant and screening it with labeled pBR322 as a probe.

Goldfarb *et al.*[48] developed an alternative strategy because of limitations with plasmid rescue (i.e., insert size). DNA from a human bladder carcinoma cell line was first digested with an enzyme that did not cleave the gene of interest. Restriction fragments were separated by size and fragments in the fraction containing the gene (see Section 5.3) were ligated to a bacterial SupF

gene. After several rounds of transfection, a phage library was constructed with DNA from a secondary transfectant and a bacterium with a suppressible mutation was infected. Only phage carrying the *Sup*F gene would form plaques. A transforming gene was isolated because of its location next to the *Sup*F gene.

### 5.1.2. Search

When DNA from one species is transfected into recipient cells from another species, a repetitive sequence linked to the gene can serve as a marker for gene isolation.[49] There is sufficient divergence between species in repetitive DNA sequences so that most repetitive DNA hybridizes specifically to DNA of the same species. Because the human genome contains at least 300,000 interspersed repetitive sequences, many, though not all, human genes will be closely linked to one or more copies of a repetitive sequence.

The general strategy is to isolate DNA from primary transfectants and retransfect recipient cells for the gene of interest. If human DNA is transferred into mouse cells, then after two or three cycles of transfection the only human DNA likely to be present is the gene of interest and possibly closely linked sequences. Southern blots are then performed with total genomic DNA from a number of independent secondary transfectants and hybridized with $^{32}$P-labeled human repetitive or total DNA as a probe. If common bands are observed, then it is likely that the gene of interest contains a repetitive sequence or is closely linked to such a sequence. In some cases a common band may be observed in most but not all transfectants.[50] Since digestion and linkage of transferred DNA occurs (as described above), a new restriction fragment containing the gene may be generated.

### 5.1.3. Sib Selection

A chicken B cell lymphoma (Blym)-transforming gene[51] and a gene for a cell surface antigen on human chronic lymphocytic leukemia cells[5] were isolated using this procedure. Essentially, a phage library was constructed with DNA from the transfected cells. DNA from pools of phage were tested for the ability to transfect for the gene of interest. If a pool was positive, it was subdivided such that only one or a few subpools were likely to contain the positive phage and the incidence of the phage in that pool would be increased compared with the initial pool. This procedure was continued until a pure phage was isolated. This procedure may require long periods of time, although transfecting and monitoring transient expression may be carried out when the positive phage is sufficiently enriched. In addition, if there is sufficient differential replication between the positive phage and other phage, dilution of the positive phage can occur.

### 5.1.4. cDNA Subtraction

Because transfectants theoretically contain the same mRNA species as recipient cells as well as mRNA transcribed from the transferred genes, highly

specific "subtracted" cDNA probes can be made for identifying transferred genes.[52,53] To make such probes cDNA made from mRNA of transfected cells is depleted of shared sequences by hybridization to mRNA of the recipient cells. Hydroxyapatite columns are used to separate the unhybridizable cDNA which would theoretically correspond to mRNA transcribed from the transferred genes. The "subtracted" cDNA probe can be used for screening cDNA or genomic libraries.

Since primary transfectants typically incorporate about 0.1% of the foreign DNA, the cDNAs specific to the transfectants may correspond to multiple transferred genes if they are expressed. To isolate a specific cDNA corresponding to the gene of interest one of the following approaches can be taken: (1) Selected cDNA made with a secondary transfectant should be quite specific, since the secondary transfectant will only differ from recipient cells by expressing the selected gene as well as possibly linked sequences. (2) Because transfectants only incoporate a small amount of total cellular DNA, several independent transfectants are unlikely to have incorporated the same genes except for the selected gene and perhaps closely linked sequences. Therefore, cDNA clones in a library that hybridize with two subtracted cDNA probes made from different primary transfectants should contain sequences corresponding to the gene of interest. (3) To clone the gene coding for the human T-cell antigen Leu-2,[2] a cDNA library was constructed from a Leu-2 transfectant in which the Leu-2 gene was amplified. Leu-2 cDNA clones were identified by screening with a "subtracted" cDNA probe made from an independent, amplified Leu-2 transfectant.

This technique is probably one of the best for isolating transferred genes. Fewer steps are required compared with some of the other methods. Isolation of genes of any species is possible even when, for instance, a mouse gene has been transferred into a mouse recipient cell. In addition, there is a good chance of success since it is unlikely that the recipient cell will contain an mRNA species highly homologous to the mRNA transcribed from the transferred gene.

### 5.1.5. Shuttle Vectors and Cosmid Rescue

Cosmids can serve as shuttle vectors between bacteria and mammalian cells since they contain selectable markers for both bacteria and mammalian cells.[54,55] To clone a gene, a cosmid library is constructed, mammalian cells are transfected with DNA from the library, and transfectants are selected. The cosmid DNA is recovered by *in vitro* packaging of the cellular DNA from the transfectant. Cosmid DNA that is packaged is used to transform bacteria. Bacteria now containing the gene of interest are then identified.

One advantage of cosmids is that up to 45 kb of DNA can be inserted per cosmid. Cloning of the gene coding for human thymidine kinase was accomplished by this method.[55] One potential problem is that some cosmids are not always rescued from the mammalian cell by *in vitro* packaging. While cosmids are useful for transferring large genes or closely linked genes to study gene

expression, the generality of the approach for gene cloning has yet to be established.

### 5.2. Transfection with cDNA Expression Vectors

Instead of transfecting cells with DNA from a genomic library and isolating the gene of interest by sib selection, it may be possible in the future to transfect with DNA from a cDNA library constructed in an expression vector. A plasmid vector described by Okayama and Berg[56] contains the simian virus 40 (SV40) early region promoter, a modified SV40 late region intron, and a polyadenylation signal such that cDNA inserts can be transcribed and processed in mammalian cells. Rare cells expressing the gene of interest could then be selected. So far this has not been an approach for cloning genes. However, expression of cloned cDNA in mammalian cells has been used to confirm the identity of specific cDNAs and to isolate full-length cDNA clones.[57]

### 5.3. Transfection as an Aid in Gene Cloning

Transfection of a gene can provide important information about the gene. For instance, the sensitivity to cleavage by various restriction endonucleases can be determined by transfecting with digested DNA. If no transfectants are obtained, it can be presumed that an endonuclease cuts into the gene. For restriction endonucleases that do not cleave the gene, the size of the restriction fragment containing a complete gene can be determined by fractionating the DNA on gradients and transfecting with the different fractions.

Transfectants can also be useful in helping to identify whether a candidate clone obtained from cDNA or genomic libraries codes for the gene of interest. For instance, if a candidate clone is isolated from a library and is presumed to encode the cell surface antigen for the mouse T-cell antigen Lyt-2, then on Northern blots, the labeled DNA from the clone should hybridize with mRNA from independent Lyt-2 transfectants but not with mRNA from L cells. Hybridization with a number of transfectants would be strong evidence that the candidate clone coded for Lyt-2. The screening of transfectants should be useful for characterizing clones that are isolated from cDNA-selected libraries.

## 6. Amplification

Gene amplification is one way in which cells can increase expression of a gene product. During normal development, amplification of rRNA genes in amphibian oocytes or chorion genes in *Drosophila* egg chambers occurs to provide abundant amounts of the gene products. Although there are no examples of gene amplification occurring during mammalian cell development, gene amplification occurs in somatic cell lines and in tumor cells. Examples

of amplification of specific genes can be found in (1) cells resistant to various drugs (*in vivo* or *in vitro*), (2) tumors such as neuroblastomas or tumors with translocated chromosomes, and (3) transfected cells. Several excellent reviews on gene amplification have recently been written.[58,59] In this chapter, the focus will be on gene amplification occurring after transfection. Such amplification events can be used to (1) study the process of gene amplification, (2) amplify genes that are not normally amplifiable, and (3) improve methods for gene cloning.

Amplification of some genes coding for cell surface antigens can occur spontaneously after transfection and cells with increased expression can be selected using a fluorescence activated cell sorter (FACS). Transfectants expressing a foreign cell surface antigen are first selected by staining with fluorescein-conjugated monoclonal antibodies and sorting and/or cloning with the FACS. If gene amplification has occurred, the staining pattern will be more heterogeneous and the mean fluorescence will be greater than for transfectants in which amplification has not occurred.[60] To select for cells with increased expression, the most brightly staining cells are sorted with the FACS. The cells are grown and the process repeated. Highly amplified mouse L-cell transfectant lines expressing the human T-lymphocyte glycoprotein Leu-2(T8) are obtained in this way.[60] The same is true for transfectants expressing the mouse homologue of Leu-2, Lyt-2, and the nerve growth factor receptor.

About 25–50% of the Leu-2 transfectants spontaneously amplified the Leu-2 gene, but spontaneous amplification for six or seven other antigen transfectants was not observed. It may be that some genes have structural features or sequences that increase their propensity for undergoing gene amplification after transfection. Alternatively, amplification of some genes may not be observed because increased expression is lethal or because increased expression is dependent on increased expression of other unlinked gene products. For instance, the MHC class I gene products must couple to β-microglobulin for expression on the cell surface. Surface expression of MHC class I genes would be limited by the amount of β-microglobulin in the cells.

## 6.1. Analyzing the Process of Gene Amplification

Genes that can undergo gene amplification in tumor lines, such as dihydrofolate reductase (DHFR), can be transferred into recipient cells, and cells with amplified copies selected in order to study mechanisms of gene amplification. Because transferred genes are integrated in different chromosomal locations and become linked to different sequences, the effect on amplification rates can be examined. Wahl *et al.*[61] found that in three-fourths of CAD gene transfectants the genes were amplified with about the same frequency as observed for the endogenous gene, while those in the remaining transfectant were amplified at a frequency 100 times higher. Examination of linked sequences may provide information on why the frequency was so much greater in the one transfectant.

### 6.2. Amplification of Normally Nonamplifiable Genes

Selection for amplification of some genes is not possible nor readily feasible. However, by cotransformation with an amplifiable gene such as DHFR, amplification of the nonselected gene can be obtained.[62] Two genes become physically linked after cotransformation. When selection for DHFR amplification is imposed, DHFR gene copies will increase, as will linked sequences. In mammalian cells the amount of DNA coamplified with each gene is large, up to 3000 kb. Therefore, sequences that become linked after transfection will generally be coamplified. 3T3 cells making large amounts of hepatitis B surface antigen can be isolated after cotransformation with the DHFR gene and then selecting for amplification of DHFR.[63] Alternatively, it is possible to insert genes in amplifiable vectors that contain an amplifiable unit.

### 6.3. Applications for Gene Cloning

Methods for isolating transferred genes would be facilitated if amplification of the gene of interest could be obtained. Cloning of the gene for the human T-cell antigen Leu-2 was aided because of the availability of highly amplified Leu-2 transfectants.[60] In cases where spontaneous amplification may not occur frequently, it still may be possible to obtain amplification for the gene of interest. As described above, an amplifiable gene could be cotransferred with total cellular DNA into recipient cells. After antigen transfectants are selected the cells could be grown in a selective medium to select for amplification of the amplifiable gene. Because the gene of interest has been linked to the amplifiable gene during concatamer formation after transfection, there is a good probability that the linked gene will be coamplified. This approach, originally proposed by Wigler *et al.*,[62] seems promising.

## 7. Analyzing Cell Surface Antigens

Numerous types of experiments can be performed involving DNA-mediated gene transfer. Some examples are: (1) studying the regulation of gene expression by modifying a cloned gene, transferring the gene into recipient cells, and monitoring expression; (2) structural or functional studies using cells transfected with modified genes; (3) defining the function of cell surface antigens by testing for functional properties acquired by transfectants expressing the antigen and (4) identification of the protein products of cloned genes.

Identification of the products of cloned major histocompatibility genes was possible using gene transfer.[64] For instance, because there are numerous homologous MHC genes in a cell, it is difficult to identify which genes code for the different MHC cell surface antigens. Transfecting with an MHC gene and analyzing the protein product can provide this information. Alternatively, mouse cells transfected with a human gene encoding a cell surface antigen can be used as immunogens in the mouse strain from which the recipient cells

were derived in order to generate other specific antibodies to the human cell surface antigen.

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