Genome analysis

a practical approach

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Preface

Methodologies used to analyse the genomes of prokaryotes and eukaryotes are advancing very rapidly as it has been realized that there is a resolution gap between what can be viewed under the microscope and what molecular techniques can resolve. Obviously, a valuable goal is to obtain the complete sequence of the genome of interest although even this will not supply all the answers to the control of gene expression. This volume aims to present current successful strategies which are being applied to various organisms to construct physical maps in order to identify and analyse the arrangement and function of genes. These techniques are being used to move from linked markers to candidate genes in several human monogenic disorders. Such advances in the isolation of important disease loci have been paralleled by impressive developments in the sensitivity of tests used to detect and analyse human mutations. The style of presentation of methods in this book should enable any research or diagnostic laboratory to apply them to their particular systems. Finally, two chapters are also included describing new probes and approaches for the localization of human disorders whose analysis has so far eluded the molecular geneticist.

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CHAPTER 1

Techniques for mammalian genome transfer

P.N.GOODFELLOW, C.A.PRITCHARD and G.S.BANTING

1. INTRODUCTION

All genome transfer experiments have two separate components: a method for transferring genetic material from donor to recipient cell and a selection method for isolating recipient cells that have received donor material. Most transfer methods result in between 1 in 10^3 and 1 in 10^7 ($10^{-3}-10^{-7}$) recipient cells receiving the desired material, consequently very powerful selection techniques are required and frequently the selection is the limiting component in a genome transfer experiment.

The first genome transfers were performed by whole cell fusion (1). This technology was applied to the study of differentiation and the tumour phenotype, however, the most successful applications have been in human gene mapping (2) and the production of monoclonal antibodies (3). If a rodent cell is fused with a human cell, the resultant interspecific hybrid spontaneously loses human chromosomes (4). The chromosome loss is essentially random and this allows the construction of hybrid cell lines containing different human chromosome contributions. The correlation between the presence in a hybrid of a human chromosome and the presence of a human genetic marker is the basis of human gene mapping by somatic cell genetics. Of the 1300 human genes assigned to specific chromosomes over one third have been mapped by somatic cell genetic methods (5). In contrast to the chromosomal instability of interspecific hybrids, the loss of chromosomes from intraspecific hybrids is modest (6). The fusion of a mouse myeloma with mouse spleen cells produces stable hybrid cell lines with the immortalized growth characteristics of the myeloma parent and the antibody secreting properties of the spleen cells. The *in vitro* production of monoclonal antibodies has revolutionized antibody-based research as well as diagnostic, therapeutic and industrial methods.

The chromosomal instability of interspecific hybrids and the chromosomal complexity of tetraploid intraspecific hybrids has limited the genetic analysis of complex phenotypes by somatic cell genetics. Microcell-mediated gene transfer (MMGT) was developed to allow the transfer of single chromosomes between somatic cells (7). This method has proved to be technically difficult, nevertheless MMGT has been used to construct hybrids for gene mapping and analysis of differentiation and cancer. The latter experiments have provided conclusive evidence for the existence of specific *trans*-acting regulators of the differentiated phenotype (8) and have confirmed the recessive properties of the Wilm's tumour gene (9).

Although whole cell fusion and MMGT have been exploited for chromosomal localization of genes, subchromosomal localization by these techniques has been restricted

by the availability of translocation and deletion chromosomes. Two experimental solutions to this problem have been attempted: chromosome-mediated gene transfer (CMGT) (10) and irradiation and fusion gene transfer (IFGT) (11). In CMGT purified mitotic chromosomes are added to recipient cells in the presence of calcium phosphate. The recipient cells incorporate fragments of donor chromosomes into their genomes. Application of suitable selection systems allows the isolation of hybrids containing donor fragments of interest. Unfortunately, the chromosome fragments are frequently rearranged and, in some cases, there is clear evidence for non-random retention of sequences from the centromeric region (12). These observations preclude the use of CMGT as a mapping technique, nevertheless CMGT has proved to be useful for the enrichment of specific chromosomal regions as part of cloning strategies employing reverse genetics. Goss and Harris (11) were the first to demonstrate that fragments of the human X chromosome, present in γ-irradiated human cells, could be rescued by fusion of the donor cells to rodent recipient cells. In a recent re-investigation of IFGT, we have found that the fragments generated are rearranged and show evidence for the same centromeric selection observed with CMGT (unpublished observations).

Genomic DNA can be transferred by adding purified DNA to recipient cells in DNA-mediated gene transfer (DMGT). This technique is also an important method for testing gene function in mammalian cells. Three main methods for DMGT have been developed: calcium phosphate precipitation (13), electroporation (14) retroviral vector-mediated transfer (15) and microinjection (16). It is generally assumed that for each of these techniques the size of the DNA fragment transferred intact is limited. This assumption has not been rigorously tested for all the methods but it is not expected that fragments greater than 100 kb will be routinely transferable. In addition to its use in testing gene function, DMGT has been used for the random incorporation of selectable genes into the genome (17) and the cloning of selectable genes by expression (18).

In this chapter we describe methods for the transfer of genomic DNA by whole cell fusion, MMGT, CMGT and DMGT. General reviews, which explore the biology and applications of these techniques can be found elsewhere (19–24).

2. GENERAL CONSIDERATIONS

2.1 Good husbandry

All cells cultured *in vitro* should be maintained and passaged under conditions which maximize cell viability. This self-evident edict is especially true of cells to be used in experimental manipulations. Most genome transfer experiments involve exposure to potentially toxic chemicals [e.g. polyethylene glycol (PEG)] and cells in poor condition are more likely to be killed.

Transfer techniques that require isolation of clones will be limited by the plating efficiency of the recipient cells. Consequently, all cell lines to be used as recipients should be checked for plating efficiency under the conditions of the experiment. Cell lines with plating efficiencies of less than 10% should not be used except *in extremis*.

All of the manipulations described in this chapter should be carried out using *sterile* techniques and reagents. All solutions should be made with tissue culture grade reagents and double-distilled water. It is assumed that the reader will be familiar with standard tissue culture and cell cloning methods.

2.2 Selection

It is outside the scope of this chapter to present details of selection methods; however, it should be stressed that without an appropriate selection method it is pointless to attempt a genome transfer experiment. Selection methods are designed to confer a growth advantage on the desired cell. The commonly used methods can be divided into four different categories.

2.2.1 *Biochemical selection based on endogenous genes*

The most widespread example of this approach is the hypoxanthine, aminopterin, thymidine (HAT) selection methods of Szybalski *et al.* (25). In the presence of aminopterin (or the closely related drug methotrexate) *de novo* synthesis of DNA precursors is inhibited. Cells that lack the enzyme thymidine kinase (TK) cannot utilize exogenous thymidine and die in the presence of aminopterin. Similarly, cells that lack hypoxanthine phosphoribosyl transferase (HPRT) cannot utilize hypoxanthine and also die in the presence of aminopterin. The somatic cell hybrid produced by fusing a TK^-, $HPRT^+$ with a TK^+ $HPRT^-$ will be both $HPRT^+$ and TK^+. This hybrid cell will grow in the presence of aminopterin if hypoxanthine and thymidine are supplied (HAT medium). A common variant of the HAT theme is 'half selection' where one cellular partner does not grow in culture and the other is HPRT or TK deficient (26). A large number of complementation systems have been developed for somatic cell genetics; particularly numerous are those based on the auxotrophy/prototrophy of hamster cell lines (27). The problem with this approach is that it requires the construction of suitable mutant recipient cells.

In view of the general importance of the HAT selection method we have presented the specific details of making HAT medium in *Table 1*.

Table 1. Preparation of HAT medium.

HAT medium is made by adding 1 ml of solution 1 and 1 ml of solution 2 to 98 ml of growth medium.

Solution 1: methotrexate (also known as amethopterin)

1. Add 0.045 g to 10 ml of double-distilled water.
2. Add 1.0 M sodium hydroxide until the methotrexate dissolves.
3. Add 10 ml of double-distilled water.
4. Adjust the pH to between 7.5 and 7.8 with 1 M HCl.
5. Make up to 100 ml and filter sterilize.
6. Store the stocks frozen.

Solution 2: hypoxanthine and thymidine

1. Add 0.14 g of hypoxanthine to 30 ml of double-distilled water.
2. Add 1 M sodium hydroxide until the hypoxanthine dissolves.
3. Adjust the pH to 10.0 with 1 M HCl.
4. Add 0.039 g of thymidine to 35 ml of double-distilled water.
5. Combine the hypoxanthine and thymidine solutions and adjust to 100 ml using double-distilled water.
6. Filter sterilize and store frozen (−20°C).

2.2.2 *Biochemical selection of exogenous genes*

This approach avoids the need to isolate specific mutants by exploiting bacterial genes that confer selective advantage when expressed in mammalian cells (28). Plasmid and retroviral expression vectors have been created in which the bacterial genes have been given mammalian promoters, splice sites and polyadenylation signals. Introduction of the bacterial genes into mammalian cells by transfection or infection results in random integration into the recipient genome. Examples of genes that provide a dominant selective advantage to mammalian cells include the *Escherichia coli gpt* gene which allows cells to utilize xanthine as a precursor for purine synthesis (28) and the *neo* gene which confers resistance to the mammalian-toxic antibiotic G418 (29). The major disadvantage with this approach is the random integration; however, recent advances in targeting by homologous recombination may allow site-directed integration (30).

2.2.3 *Cell surface antigens*

Antibodies can be used as selective agents for isolating cells with defined cell surface antigen phenotypes (31). A number of different methods are available including the use of the fluorescence-activated cell sorter (FACS), rosetting with antibody-coupled red cells and panning on antibody-coated surface. Selection of antigen-positive cells is generally limited in efficiency and is more correctly described as enrichment. Nevertheless, antibody-based selection has been used with all of the genome transfer methods described here, with the exception of MMGT.

2.2.4 *Activated oncogenes*

Mutated proto-oncogenes, especially members of the *ras* family, can confer growth advantages on mammalian cells and this can be exploited for selection in somatic cell genetics (22).

3. WHOLE CELL FUSION

3.1 Introduction

Cells mixed together will spontaneously fuse, but only with a very low efficiency (1). Fusion rates can be markedly increased by fusogenic agents. Initial experiments used inactivated Sendai virus as the fusogen (32), however, biological variability in different batches of inactivated virus and the cumbersome nature of the virus-promoted fusion protocols has resulted in the widespread use of the chemical fusogen PEG (33). The method presented below can be used for fusing cells of the same or different species. Intraspecific hybrids should be produced with a frequency of between 10^{-3} and 10^{-5}. Interspecific hybrids should be produced with a frequency of between 10^{-5} and 10^{-7}. Cells with similar phenotypes and growth characteristics tend to produce hybrids with a higher frequency.

3.2 Basic method for producing whole cell hybrids by PEG-induced fusion

(i) Prepare solutions listed in *Table 2*.

(ii) Harvest 5×10^6 of each parental cell, transfer to a sterile centrifuge tube with a conical base and spin to a common pellet by centrifugation (1500 *g* for 5 min

Table 2. Stock solutions required for whole cell fusion.

Growth medium	Routinely we use Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS for cell culture, however, the particular growth medium used does not directly affect the fusion protocol.
Growth medium without FCS (or other proteins)	
Selective medium	Growth medium suitable for selecting hybrid cells (e.g. HAT medium for isolating hybrids between TK^- and $HPRT^-$).
50% v/v PEG made up in serum-free medium	5.5 g of PEG 4000 (Baker) and 5 ml of serum-free growth medium are mixed and autoclaved. The final pH should be adjusted to 8.2 and the solution should be pre-warmed to 37°C immediately prior to use.

at room temperature). Wash the pellet once by resuspension and centrifugation in serum-free medium.

(iii) Remove the supernatant and loosen the pellet by tapping the centrifuge tube.

(iv) Gently add 1 ml of pre-warmed 50% v/v PEG (37°C) whilst carefully disturbing the pellet with the tip of the pipette used to add the PEG (see below for a discussion of PEG grade and supplier).

(v) Incubate for 90 sec at 37°C.

(vi) Add 1 ml of serum-free medium drop by drop over a period of 1 min. Carefully mix the contents of the tube by gentle stirring with the pipette used to add the serum-free medium.

(vii) Add 5 ml of serum-free medium over a period of 1–2 min. Mix the contents of the tube by gentle stirring with the pipette.

(viii) Add 10 ml of serum-supplemented medium over a period of 1–2 min. Mix the contents of the tube by gentle stirring with the pipette.

(ix) Centrifuge the cells (1500 *g* for 5 min at room temperature), remove the supernatant and gently resuspend the cells in growth medium by gentle tapping of the tube. Do not pipette the cells up and down to resuspend the pellet. Plate the cells in 10 × 9.0 cm diameter tissue culture plates.

(x) After 24 h change the growth medium to selective growth medium. Change the medium every 3–4 days.

(xi) Colonies should be visible in 14–21 days for intraspecific hybrids and 21–28 days for interspecific hybrids. Occasionally hybrids may take longer to appear and it is worth keeping plates for up to 45 days.

3.3 Trouble-shooting and variant techniques

Some combinations of cells are difficult to fuse, if it proves impossible to produce hybrids, it is worth trying the following:

(i) Vary the ratios of the parental cells over the range 1:10–10:1.

(ii) Vary the PEG solution.

(a) The batch and/or supplier. In our experience a batch that works well for one cell type tends to work for other cells. A batch which is known to induce fusion with a high frequency should be guarded. It has been claimed that

Table 3. A PEG fusion method for attached cells.

1.	Prepare the solutions listed in *Table 2*.
2.	Plate 2.5×10^5 cells of each parental line in a 9.0 cm diameter tissue culture dish (use as many dishes as required).
3.	Culture until the cells are confluent (1 – 2 days).
4.	Remove the medium and wash twice in serum-free medium.
5.	Add 1.5 ml of PEG solution and tilt the plate until the PEG solution has covered the whole surface.
6.	Leave for 90 sec at 37°C.
7.	Remove the PEG solution and gently wash twice with serum-free medium.
8.	Wash with medium containing serum.
9.	Proceed as for steps x and xi in the basic protocol.

poor batches of PEG can be improved by using solutions which do not contain calcium (34).

(b) The molecular weight grade. As a rule the higher the molecular weight the greater the fusogenic activity. However, the higher molecular weight grades are more viscous and more difficult to wash away from the cells. As PEG is toxic, delay in removal may kill cells and reduce the number of hybrids obtained. We have successfully produced hybrids with PEG in the range 1000 – 6000 average molecular weight.

(c) The concentration of PEG. Lower concentrations of PEG are less toxic and less fusogenic.

(iii) Instead of incubating the cells in PEG for 90 sec at 37°C (step v in the basic protocol), centrifuge the cells in PEG for 2 min at 1500 *g* at room temperature; then proceed with steps vi – xi.

If these variations fail to yield hybrids it may be worth attempting alternative fusion protocols. One method, suitable for attached cells, is given in *Table 3*. This method can also be modified for use with an attached cell line to be fused with a suspension cell line. The attached cell line is cultured to subconfluence and treated with PEG (steps 1 – 4, *Table 3*), the suspension cells (5×10^5) are added in a small volume (< 1.0 ml) of serum-free medium and then the plates are centrifuged for 2 min at 1000 *g* using a plate holding rotor. Subsequent treatment follows steps 6 – 8 in *Table 3*. An alternative to centrifugation is to use lectins to agglutinate the attached and non-attached cells.

If, after exhausting these possibilities, success is still elusive, a change of fusogen might help. Two obvious alternatives are Sendai virus and electroporation. We have no experience with using the latter technique, but we are willing to provide, upon request, laboratory protocols for use with Sendai virus.

4. MICROCELL-MEDIATED GENE TRANSFER

4.1 Introduction

A summary of the MMGT strategy is presented in *Figure 1*. Donor cells are blocked in mitosis by treatment with colcemid. After extended treatment the donor cells re-form the nuclear membrane around single or small groups of chromosomes This results in the formation of cells containing multiple micronuclei. Enucleation of the cells by treatment with cytochalasin B and centrifugation produces microcells (micronuclei

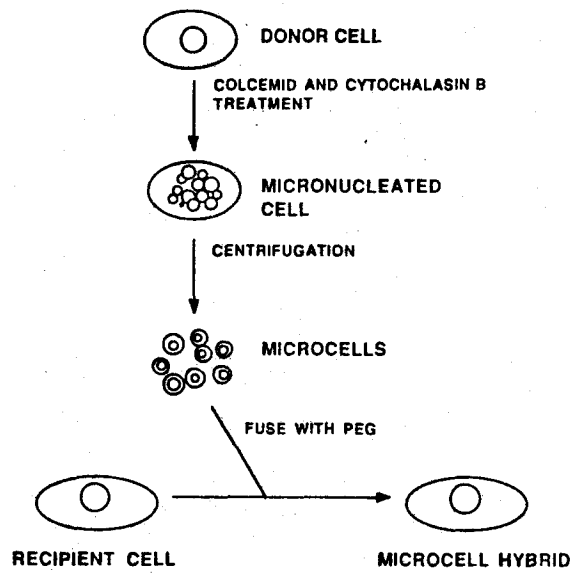

Figure 1. Microcell-mediated gene transfer.

encapsulated in plasma membrane). The microcells are delicate, but can be fused to recipient cells using a slightly modified PEG fusion protocol. Initial experiments suggested that only rodent cells could be induced to produce micronuclei, however, with variation in colcemid dose and length of exposure, most cells will produce micronuclei.

The MMGT procedure is finicky, time-consuming and technically difficult. In our experiments microcell hybrids are produced at a frequency of only $10^{-6}-10^{-7}$ in interspecific fusions.

4.2 Method for MMGT

Day 1

(i) Prepare stock solutions (*Tables 2, 4* and *5*).

(ii) Preparation of donor cells for microcelling. Plate out 2×10^7 cells at 50% confluence and grow overnight in growth medium containing 0.05 μg/ml colcemid. This will induce microcell formation (7). For different cell lines it may be necessary to titrate both the concentration of colcemid (try 0.02–0.10 μg/ml) and length of exposure (try 12–48 h). Micronucleation can be easily scored by microscopic examination using phase contrast optics. In a good experiment about 50% of the cells will be micronucleated. This frequency increases after the cytochalasin B treatment.