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Expression of DNA transferred into mammalian cells

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Abstract. There are several methods to introduce purified DNA into mammalian cells. These include microinjection into the nuclei of the recipient cells and complexing the DNA with facilitating agents such as calcium-phosphate. After it enters the nucleus, the DNA is expressed in a large proportion of the cells. This expression is dependent upon *cis*-acting sequences that are recognized by the mammalian transcriptional and translational machinery. In a smaller proportion of cells, the exogenous DNA becomes covalently integrated into the host cell DNA at random sites. Non-selectable genes can be introduced into mammalian cells by ligating them to a selectable marker or mixing the DNA with carrier DNA containing a selectable marker. The DNA that is introduced into mammalian cells can be rescued for examination and analysis. These gene transfer methods have already proven to be useful in identification of sequences which are necessary for normal gene expression as well as gene regulation. In addition a number of genes have been isolated using gene transfer methods. DNA mediated gene transfer holds much promise to target genes to specific sites in the chromosomes, to understand mechanisms of mammalian development and cell differentiation and is expected to provide a method to produce important and novel gene products that may be used for diagnostic and therapeutic purposes.

Keywords. DNA transfer; gene expression; gene regulation; mammalian cell genetics.

Introduction

A number of methods are currently available to introduce genetic information from one mammalian cell to another. Prominent among these methods is somatic cell hybridization (Kucherlapati and Ruddle, 1975). Intact cells from the same species or two different species can be readily fused with the aid of polyethylene glycol and proliferating hybrid cells can be generated. Such cell hybridization methods are extensively used to construct mammalian genetic maps, to study gene regulation at the cellular level and to produce monoclonal antibodies. Interspecies hybrids usually segregate the chromosomes of one of the parental types thus generating a series of hybrids each of which carries a subset of genetic information from the segregating parent. Though this feature is very useful and indeed forms the basis for gene mapping,

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Abbreviations used: TK, Thymidine kinase; UV, ultra-violet; HSV, Herpes simplex virus; LTR, long terminal repeat; DHFR, dihydrofolate reductase; MTX, methotrexate; CAT, chloramphenicol acetyl transferase; XPRT, Xanthine phosphoribosyl transferase; BPV, bovine papilloma viral; MMTV, mouse mammary tumor virus; MT, metallothionein; MEL, murine erythroleukemia; GaK, galactokinase.

it is not possible to control the level of chromosome segregation. Microcell and chromosome mediated gene transfer permit introduction of partial genetic information from one cell into another but these methods also do not provide the opportunity to predetermine the amount and type of genetic information that can be introduced.

The advent of recombinant DNA technology now permits the isolation of virtually any gene sequence. Structural analysis of these DNA sequences can be readily performed and detailed restriction enzyme maps and nucleotide sequences (when necessary) can be obtained. A functional analysis of these DNA segments is facilitated by introducing them into mammalian cells. These DNA mediated gene transfer systems have become powerful tools to study mammalian gene expression and regulation in their normal host environment. In addition, these techniques are being used to initiate studies of mammalian development and differentiation. The methods also promise a rich future in which it may be possible to replace genes in mammalian cells and embryos with the attendant possibility of gene replacement therapy.

In this article we will describe the various methodologies of DNA mediated gene transfer, parameters that affect the introduction and expression of these genes, use of these methods to isolate DNA sequences and finally some perspectives on the future use of these methodologies. The references we provide are not intended to be exhaustive but have been chosen to illustrate specific aspects of the systems. In addition, there is a large body of literature on the use of the DNA transfer systems to study aspects of malignancy in mammalian cells which is not included in this article. For recent reviews on this subject please see (Varmus 1982, Cooper, 1982).

Methods of gene transfer

The feasibility of introducing DNA into bacterial cells by a variety of natural and artificial methods has enhanced the ability to conduct genetic analysis of several prokaryotic organisms. A similar development of gene transfer techniques in mammalian cells has been achieved over the past few years. As in the case of bacterial systems, the methods of gene transfer can be classified into natural and artificial systems. The most common natural method of introduction of foreign DNA is achieved by infection of cells with intact viruses. If the cell type is permissive the viral DNA undergoes replication and new virus particles are synthesized causing eventual cell death. The viral DNA gets integrated into the host cell DNA and expressed as part of the host genome in case of nonpermissive cell type. The viral genomes utilize the host cell machinery for transcription and translation and cause a variety of changes in the host cells through mechanisms which are not completely understood. The viral genomes are now being used as vectors to introduce purified genes into mammalian cells. This aspect would be discussed later.

One set of these viral transfection methods had an important impact on future developments in gene transfer experiments. Munyon *et al.* (1971) have infected mouse L-cells deficient in the pyrimidine salvage pathway enzyme thymidine kinase (TK) with ultra-violet (UV) inactivated Herpes simplex virus (HSV) type 1. This DNA virus contains a gene for thymidine kinase. They were able to show that the cells can acquire and express the viral TK gene. Similar experiments were conducted by Davidson *et al.*

(1973). These observations paved the way for future use of the TK gene transfer system in DNA transfection studies.

The artificial methods of gene transfer can be classified into direct and indirect methods. The direct methods involve injection of the appropriate DNA into the nucleus of the recipient cell and the indirect methods usually involve the complexing of DNA with facilitating agents and presenting this complex to cells.

Direct methods

Based upon a microinjection procedure originally developed by Diacumakos *et al.* (1970), Anderson *et al.* (1980) have microinjected purified DNA into the nuclei of mouse L-cells. In these experiments, Anderson and colleagues used a cloned fragment of HSV DNA containing the TK gene. TK^+ cells were isolated by the use of the HAT selection system (Szybalska and Szybalski, 1962). The microinjection procedure was found to be quite efficient in that one cell in twenty was stably transformed. The fact that these cells could grow stably in the selective medium indicated that they have acquired and are expressing the exogenously introduced gene. Capecchi (1980) has carefully examined the factors that influence the stable acquisition of DNA by microinjection procedures. He observed that transformation efficiency was relatively insensitive to DNA concentration and did not depend on the presence of non specific carrier DNA. He has also noted that the presence of SV40 DNA replication origin sequences enhanced the stable transformation efficiencies. It is now known that this is most probably due to the presence of transcriptional enhancer signals associated with the early promoter of SY40 which is located in the 'ori' region of this viral genome.

The most extensive and widespread use of microinjection is currently in introducing purified DNA sequences into mammalian embryos. Gordon and Ruddle (1981) have developed a method to microinject DNA into the pronucleus of a fertilized mouse oocyte. The injected embryos are transferred to the uterus of a pseudopregnant female. In a number of cases the implanted embryos develop into complete mice, several of which were found to contain the injected DNA sequences. This method is gaining widespread use and promises to provide a method to follow the expression of a defined gene through all stages of development and differentiation. Results obtained from these experiments will be discussed later.

Indirect methods

Among the indirect methods of gene transfer the most commonly used sytem is that of coprecipitation of DNA with calcium phosphate. Though a method of introducing naked DNA into cultured mammalian cells was described by Szybalska and Szybalski (1962), the widespread use of DNA transfection techniques had to await future developments. In 1973 Graham and Vander Eb developed a method to increase the infectivity of Adenovirus DNA by complexing it with calcium phosphate. In this method, the DNA is coprecipitated with calcium phosphate and the resulting suspension is then added to cells. The cells were allowed to incubate for variable periods of time following which the precipitate is removed and the cells subjected to growth and analysis. The widespread use of this method of DNA transfer followed the demonstration that purified DNA from HSV can confer a TK^+ phenotype to $LMTK^-$ cells

(Wigler *et al.,* 1977; Maitland and McDougall, 1977). These two groups have also shown that HSV DNA digested with certain restriction endonucleases loses its ability to confer a TK^+ phenotype while other enzymes failed to do so. Knowledge of enzymes which failed to destroy the TK activity in HSV1 DNA permitted isolation of a 3·4 kb Bam HI fragment which alone was capable of conferring the TK^+ phenotype to TK^- cells. This was the first gene that was isolated by the use of DNA transfection techniques. Different groups of workers have since used this method to successfully introduce different genes into different recipient cell lines.

DNA complexed with polycations such as poly L-ornithine (Farber *et al.,* 1975) or DEAE Dextran (Pagano, 1970; Farber *et al.,* 1975) is also used to transfect cells. These methods are generally used to transfect viral DNA sequences and have proven to be useful in increasing both uptake and expression of foreign DNA sequences. Milman and Herzberg (1981) have, however, reported that L-cells transfected in this fashion show a high level of transient expression but failed to yield stable transformants. This aspect needs further investigation.

Schaefer-Ridder *et al.* (1982) have used liposomes as gene carriers. Stable transformation of mouse L-cells deficient in thymidine kinase was achieved by liposome-mediated transfer of a recombinant plasmid carrying the thymidine kinase gene. Ten per cent of the recipient cells expressed thymidine kinase activity. Human HGPRT gene was transferred into HGPRT deficient mouse cells with a frequency of approximately 1×10^{-5} using lipochromosomes (Mukherjee *et al.,* 1978; Hoffman *et al.,* 1981).

Schaffner (1980) has demonstrated that cloned genes can be transferred from bacteria to monkey cells directly by fusion of lysozyme-treated bacteria (protoplasts) with monkey cells in the presence of polyethylene glycol. Later other groups of workers (Sandri-Goldin *et al.,* 1981; DeSaint Vincent *et al.,* 1981) used this method to successfully introduce foreign genes into mammalian cells. In optimal conditions, transfer by fusion seems to be 10 to 20 fold more efficient than DNA transfection by $Ca–PO_4$ coprecipitation technique (Rassoulzadegan *et al.,* 1982).

It is known that not all mammalian cells respond equally well to each of these methods of gene transfer. It has been found that some cells are quite resistant to $Ca–PO_4$ mediated gene transfer while they are more easily transferable by protoplast fusion. A possible disadvantage with the protoplast fusion is that in addition to the desired gene the bacterial DNA will also be introduced into the mammalian cells.

A number of alternative methods to circumvent the problems with each of the above methods are being examined. One method that is of promise is electroporation. Zimmerman and Vienken (1982) and Neuman *et al.* (1982) have shown that cell fusion can be achieved quite efficiently if a high voltage of electricity is discharged into a solution containing the appropriate mixture of cells. This method is modified where naked DNA is mixed with the desired recipient cells and pulsed with high voltage electricity. This method is now said to be quite useful in transfecting cells which are known to be resistant to transfection by other methods (Potter, H. and Leder, P., personal communication).

Choice of the methods of gene transfer depends on the nature of DNA to be introduced. Use of these methods requires a knowledge of the nature of cell types, nature and physical state of the DNA to be transferred and the experimental conditions. Thus several factors play a role in the efficiency with which a gene can be transferred.

Factors that affect efficiency of gene transfer

Different methods of gene transfer are suited for different purposes. Each of the methods has limitations and advantages. Knowledge about these features would greatly facilitate the appropriate choice of cell type and gene transfer method.

In cases where whole viruses are used, the recipient cell type plays a very important role. Not all cells are susceptible to the viral infection. For example, the DNA tumor virus SV40 can infect a number of mammalian cells (*e.g.*, monkey, human and mouse) but other viruses have a more limited host range. It is also important to note that the fate of the viral nucleic acid, once it enters the cell is also dependent on the host. SV40 can replicate in monkey cells resulting in eventual cell death while mouse and human cells are nonpermissive for replication of the viral DNA.

The microinjection method is well suited for embryos and several cell types that grow attached to a substratum where the target cell nucleus is easily visible. Cells that grow in suspension are not extremely suitable for microinjection. In general, if the number of target cells is limiting, microinjection may prove most appropriate. In this direct method, physical constraints such as size of the cell and size of the nucleus play an important role in the ease with which DNA can be introduced.

Methods that involve the preparation of protoplasts yeild a very high number of transfectants but such a method obviously involves prior preparation. Similarly, liposome mediated transfer also involves manufacture of liposomes and incorporation of the DNA before they can be used.

The calcium-phosphate co-precipitation of DNA and the DEAE-dextran packaging of DNA are among the most widely used methods of DNA transfer. This is probably due to the ease with which these facilitating agents can be used. A variety of factors seem to influence the efficiency with which cells can be transfected with the aid of these agents.

One of the most important factors that affect the efficiency of transfer of genes into new cellular environments is the nature of the recipient cell line. Colbere-Garapin *et al.* (1981) demonstrated that following transfection with kanamycin resistance gene linked to the HSV1 TK promoter region, several mammalian cell lines (murine, simian and human) become resistant to G-418, an analogue of kanamycin. But the efficiency of transfer of this drug resistance was different in different cell lines. Graf *et al.* (1979) have also shown that transformation frequency is critically dependent on the particular line used as recipient. Mouse L-cells have been widely used as recipients for gene transfer. Although only limited information is available, it appears that L-cells act as good recipients while in cells like Chinese hamster ovary cells, the frequencies of DNA transfer are 1/10 those for L-cells (Srinivasan and Lewis, 1980).

Cells of the same species and even cells from a single clone show differences in the efficiency, of gene transfer (Corsaro and Pearson 1981a,b). The transformation efficiency for TK was found to vary 10-20-fold among different subclones of the LM TK-mouse fibroblast cell line.

Specific features of recipient cell membrane also play an important role in the transformation frequency. Lewis *et al.* (1980) and Lowy *et al.* (1978) have shown that post transfection shock of cells with DMSO enhanced the efficiency of DNA transfer by calcium-phosphate precipitation method. The optimum concentrations of DMSO

and time of exposure are different for different cell types and has to be determined for each cell line. Such DMSO shocks are now routinely used by different laboratories.

Graham and Vander Eb (1973) found that the efficiency of transfection by calcium chloride technique could be enhanced by the use of carrier DNA. Our own studies (Noonan, K. and Kucherlapati, R. S., unpublished results), indicated that transfection of HSV TK gene can be enhanced 10-fold by using salmon sperm DNA as carrier. It has been shown by Perucho *et al.* (1980b) also that transformation is much less efficient when carrier DNA is omitted. It is clear, however, that there is no absolute need for carrier DNA (Linnenbach *et al.,* 1980; Huttner *et al.,* 1981). It has been shown that selectable genes introduced with a carrier DNA form a high molecular weight molecule termed a transgenome (Ruddle, 1979,1980; Scangos *et al.,* 1981) or Pekalosome (Perucho *et al.,* 1980b). The selectable gene becomes associated with the carrier DNA to form the transgenome and this association has been directly demonstrated by Perucho *et al.* (1980b). Transgenomes are initially unstable and are lost from the population. After variable generations these transgenomes become stable and are found to be associated with the recipient cell chromosome (Scangos *et al.,* 1981).

Several experimental results address the questions about the possible roles of carrier DNA in transfection. One of the possible roles of carrier DNA is that it provides functional replication origin to the selectable DNA permitting replication of the complex until it is integrated into the host genome. This view is supported by the observation that eukaryotic DNA acts as a better carrier than prokaryotic DNA. Studies carried out by Capecchi (1980) which showed that SV40 containing 'ori' region increased the efficiency of transformation 100-fold supports this view. It is also possible that carrier DNA contains some enhancer sequences like several viruses (Gruss *et al.,* 1981; Benoist and Chambon, 1981) and these sequences enhance transfection efficiencies when selectable genes become ligated to carrier DNA in the proximity of such sequences.

Another possible role of carrier DNA is that it is providing sites of integration into the host genome by homologous recombination. It is possible that integration of transgenome occurs as a result of recombination between unique or reiterated sequences present in carrier DNA and homologous sequences present in the recipient cell genome. This view can be supported by some of our experimental results which shows that carrier effects can be mimicked if plasmid carrying a Chinese hamster Alu family of repeated sequence is used without any additional carrier DNA (Kucherlapati, R., Jelinek, W., Krauter, K. and Leinwano, L., unpublished results). Further experiments are necessary to prove that association of transgenome with the recipient cell genome is the result of homologous recombination events.

Besides all these factors so far discussed, another factor affecting the efficiency of gene transfer is the nature of the DNA introduced. It has been found that linearized plasmid DNA is five times more efficient in transferring the TK^+ phenotype to mouse cells than its circular counterpart (Colbere-Garapin *et al.,* 1979). It is now known that mammalian somatic cells are quite efficient at end-to-end joining of DNA segments and that linear DNA molecules are more recombinogenic (Wilson *et al.,* 1982; Subramanian, 1979). This knowledge may provide the basis for higher transformation frequency with linearized plasmid.

Thus efficiency of gene transfer depends on a number of factors and elucidation of

the different factors will provide the opportunity to devise methods for high frequency of transfer of any gene into any mammalian cell type.

Fate of the DNA in the recipient cells

We have already documented the fact that mammalian cells are able to take up exogenously added DNA and express genes included on that DNA. Different DNA-mediated gene transfer methods proved to be useful in introducing foreign DNA into mammalian cells. Several investigators have made attempts to study the fate of the exogeneous DNA in the recipient cell.

The most commonly used method of gene transfer is co-precipitation of DNA with calcium phosphate. DNA forms a tight complex with calcium phosphate and becomes resistant to nucleases present in serum or added externally. Under optimal conditions, all of the recipient cells take up CaPi-DNA complex but ultimately only a fraction of the cells have detectable CaPi-DNA complex in the nucleus (Loyter *et al.,* 1982). The exact mechanism by which DNA makes its way into the nucleus is not clear. It can be suggested from the results that the movement of the DNA from the cytoplasm to the nucleus constitutes the most significant barrier to gene transfer. To increase the efficiency of gene transfer it is necessary to facilitate entry of DNA from cytoplasm to the nucleus and no expression is possible if it is injected into the cytoplasm by microinjection procedure (Capecchi, 1980). After entering the nucleus several possible fates may await the foreign DNA. The transforming element may persist within the nucleus as an autonomously replicating extrachromosomal unit or it is stably integrated into a host chromosome.

The possibility that the transforming DNA may exist as an autonomously replicating extrachromosomal element depends on the nature of the vector used. For example, vectors containing SV40 replication origin and an intact *A* gene can replicate autonomously in monkey cells (Hamer *et al.,* 1979). A number of genes have been cloned *in vitro* with SV40 vectors and propagated successfully as autonomously replicating molecules in cultured monkey kidney cells. Similarly vectors containing polyoma sequences replicate autonomously in mouse cells (Colbere-Garapin *et al.,* 1981). Foreign DNA linked to bovine papilloma viral DNA also persists as an extrachromosomal element in several mammalian cells (Dimaio *et al.,* 1982). If the DNA is unintegrated it may not segregate equally into the progeny cells after each cell division and may result in the loss of the phenotype among a certain percentage of cells in the population. It is also possible that integrated DNA is ex*cis*ed during early stages of the development of the cell line causing instability in the phenotypic expression of the transferred gene. These views are supported by results reported by Pellicer *et al.,* (1980); Ostrander *et al.* (1982); Davies *et al.* (1982). The autonomous replication pattern of any DNA in recipient cells can be deduced by isolating low molecular weight DNA from the cells.

Different laboratories studied the fate of exogenous sequences in the recipient cells and found that foreign DNA is stably integrated into the host DNA. The integrated DNA can be isolated as high molecular weight form of DNA and can be detected by appropriate restriction enzyme digestion and hybridization using the foreign DNA as

probe. Pellicer *et al.* (1978) examined the fate of the TK gene in several independent clones after tranfecting mutant mouse cells (LTK^-) deficient in thymidine kinase with BamHI restriction endonuclease cleaved HSV1 DNA. Examination of the clones showed the TK gene is present in all cells at a frequency of one copy per chromosomal complement and is stably integrated in the DNA of all the transformants. The integration is not site specific and occurs at different regions of the recipient cell DNA.

When a selectable gene is transferred with carrier DNA, cells which incorporate selectable markers are also likely to incorporate sequences from the carrier DNA. As discussed before, the selectable gene becomes associated with carrier DNA to form a high molecular weight molecule termed a transgenome. This transgenome is maintained stably and after a few generations is found to be integrated into the recipient cell chromosomes (Scangos *et al.,* 1981). This has been demonstrated directly by Perucho *et al.* (1980b) using salmon DNA as carrier DNA for the HSV TK gene. They showed that host cell ligates incorporated DNA into a large concatameric structure which is finally integrated in a stable fashion within the high molecular weight nuclear DNA of the host cell. The studies by different groups of workers (Huttner *et al.,* 1979; Wigler *et al.,* 1980) indicated that virtually any purified sequence can be introduced into mammalian cells by cotransformation with a selectable gene. The exogenous DNA integrates into the host genome and the selection of the selectable gene is necessary for the maintenance of nonselectable sequences. Pellicer *et al.* (1980) have shown that in buffalo rat liver cells cotransformed with a growth hormone gene (hGH), the foreign gene is inserted into particular chromosomes and the site of insertion is invariant within a given cell line. Different lines, however, contain the transforming element on different chromosomes; the site of insertion is not restricted to a unique chromosome. It is apparent from these experiments that TK or hGH sequences are not directing the site of insertion. In another set of experiments (Robins *et al.,* 1981) a series of rat liver cell lines co-transformed with a variant human growth hormone gene were examined by *in situ* hybridization; it was found that in most of the cell lines the contransformed sequences reside in a chromosome of the host cell and each line revealed a different site of integration for the transforming sequences. After integration into the host genome the foreign DNA may undergo some rearrangements. We (Hwang and Kucherlapati, 1983) studied the organization of integrated SV40 sequences in an uncloned population of a transformed human fibroblast cell line and found that following the initial integration event, viral as well as the flanking host DNA sequences become unstable and are subject to deletions and rearrangements. This instability is for a short period after which the integration of SV40 is stable and maintained for a number of generations.

The fact that the foreign DNA sequences seem to integrate at random sites has raised the issue of the ability of mammalian cells to mediate homologous recombination. Several earlier experiments to detect homologous recombination have failed to yield positive results (Rosenstraus and Chasin, 1978; Tarrant and Holliday, 1977). This question has been reinvestigated by the gene transfer methods. The general strategy of many of these experiments is to introduce two mutant, non complementing, selectable genes into mammalian cells and select for events which result in reconstruction of an intact gene by homologous recombination. DeSaint Vincent and Wahl (1983) analyzed the fate of two recombinant plasmids containing overlapping fragments of a cloned Syrian hamster CAD gene introduced into Chinese hamster cells. They found that the

mammalian cells catalyzed homologous recombination between the two plasmids resulting in the reconstruction of an intact gene. The recombined gene sequences however integrated into the host chromosome at random sites. Results obtained by Folger *et al.* (1982), Small and Scangos (1983), Shapira *et al.* (1983), Miller and Temin (1983) have also shown that mammalian cells have the ability to mediate homologous recombination between exogenously introduced plasmids. We have recently shown that homologous recombination between plasmid molecules can be enhanced in mammalian cells if one of the plasmids is linearized by introducing a double-stranded cut within the region of homology (Kucherlapati *et al.,* 1984).

In addition to the homologous recombination events, foreign DNA is also subject to non homologous recombination events. Subramanian (1979) has shown that linear molecules tend to join end-to-end irrespective of the nature of the ends. More extensive results of this type have been provided by Wilson and colleagues (Wake and Wilson, 1979, 1980; Wilson *et al.,* 1982).

All of these results indicate that perhaps the DNA introduced into mammalian cells is highly recombinogenic and can undergo homologous or non homologous recombination events forming concatemeric molecules or transgenomes. This DNA then integrates into the host cell chromosome by mechanisms which are currently not well understood.

Vectors for introduction of DNA

The development of DNA-mediated gene transfer systems permits the introduction of a number of DNA sequences into mammalian cells. As a consequence, a variety of DNA vectors have been developed which will propagate and express covalently linked genes in different recipient cell types.

The transfer of foreign genes need not depend upon the expression of the gene of interest in the recipient cells. Rather, one may ensure its presence by introducing such a nonselectable DNA sequence together with a selectable marker into recipient cells. A frequently used selectable marker is the herpes virus TK gene. This system has been chosen because TK^+ phenotype can be efficiently selected over a TK^- background by utilizing growth conditions in which the pyrimidine salvage pathway enzyme, thymidine kinase, is necessary for survival. There exist cell lines deficient in TK with low rates of spontaneous reversion to the TK^+ phenotype which can be used as recipients. The viral protein is well characterized and is readily distinguishable from the cellular enzyme. Maitland and McDougall (1977) and Wigler *et al.* (1977) have shown that purified DNA from HSV types I or II can confer the TK^+ phenotype to TK^- cells when the DNA was presented to the cells as calcium phosphate coprecipitate. Several groups of investigators (Wigler *et al.,* 1977; Minson *et al.,* 1978) have shown that the functional HSV TK gene is located within a 3·4 kb DNA fragment that can be isolated from the HSV genome by Bam H1 restriction enzyme digestion and can be used to stably transfect TK^- cells to the TK^+ genotype. The 3·4 kb Bam HI fragment has been cloned into the prokaryotic plasmid pBR322 and the cloned TK gene retains the capacity to transfect TK^- cells (Colbere-Garapin *et al.,* 1979; Enquist *et al.,* 1980; McKnight *et al.,* 1979; McKnight, 1980).

Colbere-Garapin *et al.* (1981) have used TK gene to construct a vector in which the

promoter region of the HSV type ITK gene has been linked to the gene coding for the aminoglycoside 3′-phosphotransferase coded for by the Tn5 transposon. This enzyme makes the cells resistant to the antibiotic G-418, which is otherwise toxic to the cells. They found that several mammalian cell lines (murine, simian and human) became resistant to G-418 after transfection with this recombinant plasmid and in this case it can be shown that transcription is initiated using the TK promoter.

Palmiter *et al.* (1982) used the structural gene of herpes virus thymidine kinase in constructing a fusion plasmid pMK by fusion with mouse metallothionein 1-promoter regulator region. pMK was introduced into mouse embryos by microinjection followed by reinsertion of the eggs into foster mothers. pMK sequences were detected in 15 % of the mice and seven of them showed high level of viral thymidine kinase in liver. In this case, TK gene was expressed using the mouse metallothionein promoter.

We have constructed a chimeric plasmid containing a DNA fragment from the genome of Moloney murine leukemia virus including the viral LTR and covalently linked HSVI-TK gene whose promoter was removed. This hybrid DNA structure was introduced into TK^- mouse cells and was found to express TK^+ phenotype. We were able to show that transcription of the gene is initiated in MOMLV LTR (Gilboa *et al.,* 1982).

In the cases described above, the ability to introduce the foreign DNA is dependent upon the lack or mutant activity of a native gene. This problem has been circumvented by the use of gene sequences which are easy to assay or which provide a dominant selectable system.

Dihydrofolate reductase (DHFR) gene is such a dominant selectable marker and presence of this gene in a mutant form or high copy number confers resistance to high levels of the folate antagonist methotrexate (MTX) (Christman *et al.,* 1982; Schimke *et al.,* 1978; Wigler *et al.,* 1980). Murray *et al.* (1983) constructed a biologically active DHFR chimera by placing transcriptional promoter of the Harvey sarcoma virus long terminal repeat at the 5′-end of a DHFR cDNA. This chimera was dominant acting and was able to confer a methotrexate-resistant phenotype on wild type NIH 3T3 cells. The use of this gene or other more convenient drug resistance markers such as the bacterial gpt gene (Mulligan and Berg, 1980) may permit transfer and expression of virtually any genetic element into a variety of new cellular environments.

The use of bacterial genes which do not have a eukaryotic counterpart has become an important alternative method to transfer genes into mammalian cells. The bacterial genes that are commonly used for this purpose are chloremphenicol acetyl transferase (CAT), Xanthine phosphoribosyl transferase (XPRT or gpt) and the bacterial neo gene. The CAT gene product can convert chloremphenicol to its acetylated products each of which can be readily distinguished by thin layer chromatography. Gorman *et al.* (1982) have constructed plasmids in which the CAT gene was placed under the transcriptional control of SV40 or RSV promoters. The CAT gene, though not selectable, can be easily assayed and has become an important tool in functional assays for promoters and other *cis* acting regulatory sequences.

Mulligan and Berg (1980) constructed chimeric plasmids in which the bacterial gpt gene was placed under the control of the transcriptional signals of SV40. This plasmid can be introduced into mammalian cells and its expression can be selected for by the use of mycophenolic acid and xanthine. When this plasmid is introduced into $HPRT^-$ cells,

medium containing HAT can be used to select for the gpt gene expression. The basic vector into which the gpt gene was introduced was used by Southern and Berg (1982) to obtain bacterial *neo* gene expression plasmid. This gene when expressed in mammalian cells confers the cells resistance to the aminoglycoside G418. All of these SV40 vectors are quite extensively used for gene transfer purposes.

Different DNA and RNA tumor viruses are good candidates for use as vectors for the introduction of foreign DNA into animal cells because many of these viruses have been studied extensively and they could either replicate to give progeny or integrate their DNA into host chromosome depending on recipient cells. Since the viral genome includes strong promoters, it is possible to ensure efficient expression of the foreign DNA carried on it and hence efficient production of foreign gene products.

Two approaches have been exploited to utilize viral nucleic acid sequences as vectors. The first is to make recombinants between viral DNA and a piece of foreign DNA and then to pass the hybrid molecule through permissive cells in the presence of helper virus to produce encapsidated virions containing the recombinant DNA. Alternatively, recombinants between virus and foreign DNA can be constructed and used directly for transfection. A number of different gene transfer vectors are now available. Each of these vectors have different properties which make them useful in different cell types. The nature of some of the vectors and their specialized properties are described below.

Autonomous replication vectors

SV40 is one of the most commonly used viral vector for introducing foreign DNA sequences into mammalian cells. SV40 is particularly attractive as a transducing vector for various reasons. The viral genome consists of a single, small, covalently closed circular DNA molecule whose entire nucleotide sequence has been determined (Reddy *et al.,* 1978; Fiers, 1978); the genomic regions responsible for the various viral functions have been accurately located with respect to the detailed physical map of the DNA (Reddy *et al.,* 1978). The genome can be divided into early and late regions; the early region is expressed throughout the lytic cycle and encodes the *T* antigens which are responsible for malignant transformation of nonpermissive cells as well as for initiation of viral DNA replication in permissive cells. The late region encodes the viral structural proteins and in between the early and late regions there is a DNA sequence containing the origin of viral DNA replication. In COS cells (Gluzman, 1982) any piece of DNA containing a SV40 origin of replication will replicate autonomously because the cells produce SV40 *T* antigen in a constitutive fashion. A number of genes have been cloned *in vitro* with SV40 vectors and propagated successfully as extra-chromosomal elements in cultured monkey kidney cells. For example Hamer *et al.* (1979) inserted rabbit *β*-globin complimentary DNA (cDNA) into SV40 DNA in place of the gene coding for the virus major capsid protein VPI. The recombinant genome multiplied efficiently in CV1 monkey kidney cell cultures and was transcribed to yield cytoplasmic, poly-adenylated hybrid mRNAs containing the *β*-globin coding sequence. Cells propagating the recombinant plasmid produced substantial quantities of rabbit *β*-globin poly-peptide. Other genes which have been cloned *in vitro* with SV40 vectors and introduced in monkey cells include segments of phage DNA (Ganem *et al.,* 1976). *Escherichia coli* DNA coding for tRNA Tyr (Hamer, 1977), and guanine phosphoribosyl transferase (Mulligan and Berg, 1981).

Bovine papilloma viral (BPV) DNA has been found to replicate autonomously in susceptible mouse cells (Law *et al.,* 1981); no integration of BPV- 1 genome in the host chromosome was detected when mouse C127 cells were transformed with BPV type I virions. Sarver *et al.* (1981) demonstrated that a novel eukaryotic vector derived from the transforming region of BPV is highly effective for introducing foreign genes into mammalian cells. They constructed a DNA hybrid molecule BPV_{69T}-rI containing the transforming region of BPV DNA and the rat preproinsulin gene I (rI1) and used it to transform mouse cells. DNA hybridization analysis revealed the presence of multiple unintegrated copies of hybrid DNA molecules with the BPV1 DNA segment and the rIl gene covalently linked in selected transformed cell lines. BPV DNA has been suggested as potential vector because BPV transformed cells contain multiple copies (10 to 20 per cell) of the viral DNA which exist as plasmid molecules and they are efficient in inducing transformed foci in susceptible mouse cells (Howley *et al.,* 1980; Lowy *et al.,* 1980a). Since BPV DNA does not integrate into host genome, the physical contiguity of the foreign DNA segment should be preserved.

DiMaio *et al.* (1982) have shown that BPV vectors can be propagated as a plasmid in both bacterial cells and mouse cells. They constructed a BPV-derived recombinant plasmid composed of a subgenomic transforming fragment of BPV DNA, a deletion derivative of pBR322 and a 7·6 kilobase fragment of DNA from the human *β*-globin gene and found that this plasmid propagated as an extrachromosomal element in both mouse and bacterial cells with a copy number of about 10–30 per cell. The ability of BPV plasmids to act as shuttle vectors between bacterial and mammalian cells provides a rapid means of recovering and analyzing foreign genes introduced into mammalian cells.

Polyoma virus replicates autonomously in mouse cells but integrates into the host cell DNA if the host is a nonmouse cell. O'Hare (1981) has utilized a polyoma vector successfully for the introduction and expression of the bacterial neomycin gene in mouse cells. This vector can be used to transfer any foreign gene as extrachromosomal element into mouse cells.

Double or multiple vectors

Some vectors can be constructed in such a way that they would permit expression of two or more genes. SV40 and retroviral vectors can be used for this purpose. SV40 has two sets of genes and it is possible to delete one and to replace it with foreign DNA and introduced into the mammalian cells along with helper virus to provide essential viral functions. The viral DNA would provide the promoter, poly A addition signal and the splice signals when necessary.

Retrovirus vectors are now drawing more attention because they possess several advantages. They can be readily introduced into cultured cells as well as laboratory animals and it efficiently integrates into the chromosomes of the host cell. Up to 7·5 kb of nonretroviral coding information can be packaged and so more than one gene can be packaged into the vector. Retroviruses have a genome from which a single primary transcript is made which is processed in two alternative forms yielding two separate mRNAs each coding for a different protein. This characteristic of the virus can be utilized in using it as a double vector. The viral gene can be replaced with any foreign

DNA sequence for expression in mammalian cells. It has been shown by Joyner *et al.* (1982) that long terminal repeats (LTRs) of a murine retrovirus can activate expression of heterologous gene coding sequence from which a functional promoter region has been deleted. Gilboa *et al.* (1982) constructed hybrid DNA containing 0·9 kb DNA fragment from the genome of Moloney murine leukemia virus, including LTR and HSV I TK gene whose promoter was previously removed. The hybrid DNA was expressed when introduced into the chromosome of TK^- mouse cells and expression of the TK gene was found to be dependent on functions provided in *cis* by the viral DNA fragment. In a second series of experiments Gilboa (1983) replaced the neo viral *gag* and *env* genes with HSVTK and bacterial neo genes and both of the genes were expressed.

Regulatable vectors

Different viral sequences may respond to different regulatory signals and this feature can be utilized to construct vectors containing the sequences which can be regulated and hence the expression of the gene cloned into the vector can be regulated after introduction into the recipient cells.

Huang *et al.* (1981) have used a chimeric plasmid in which the foreign gene is ligated to a mouse mammary tumor virus promoter. They have shown that the level of the foreign gene expression can be regulated by glucocorticoids and the sequences necessary for hormonal control are specified by the viral genome carrying the foreign gene and probably map within the viral LTR. They constructed chimeric molecules between p21 transforming gene of Harvey murine sarcoma virus and DNA containing the long terminal repeat of mouse mammary tumor virus (MMTV) and transfected NIH3T3 cells with the hybrid DNA. The levels of p21 RNA and protein in the transformants were found to be regulated by physiological concentrations of dexamethasone, a synthetic glucocorticoid. In addition, the transcripts were shown to originate at the normal MMTV transcription initiation site. Lee *et al.* (1981) have provided evidence that MMTV LTR contains sequences which are sufficient for glucocorticoid regulation of gene expression. As additional knowledge about these regulatory sequences become available, it will be possible to construct vectors with more defined functions.

Another sequence which provides for regulation of gene expression is the mouse metallothionein (MT)-I promoter. The MT gene expression can be induced by heavy metals. It has been shown that expression of HSV thymidine kinase gene joined to mouse MT-1 -promoter is regulated by cadmium when it is either transfected into mouse L-cells (Mayo *et al.,* 1982) or injected into mouse eggs (Brinster *et al.,* 1982).

Chao *et al.* (1983) have shown that some specific DNA sequences within or flanking the β-globin genes, are involved in the activation of globin genes in murine erthyroleukemia (MEL) cells by chemical inducers such as dimethyl sulphoxide. MEL cells were transfected with hybrid mouse-human β-globin gene as well as intact human β-globin gene and those genes were regulated appropriately during differentiation of MEL cells in culture. The addition of chemical inducers to the co-transformed cells resulted in a 5 to 50 fold increase in the level of mRNA transcribed from the exogenous globin gene and experiments indicated that induction of hybrid mRNA resulted at least

in part from the increase in the rate of globin gene transcription and the induction appeared to be specific for globin genes within an erythroid cell.

Thus, understanding of the DNA sequences involved in gene regulation can be utilized in the construction of the vectors containing those sequences for efficiently introducing and regulating genes in different mammalian cell types.

Amplifiable Vectors

There are several genes which are amplified in mammalian cells and one of these well characterized genes is the DHFR gene. This gene is dominant and amplifiable in wild type mouse cells.

Murray *et al.* (1983) constructed a recombinant DNA plasmid in which the transcriptional promoter of the Harvey sarcoma virus long terminal repeat was at the 5′-end of a mouse DHFR cDNA. This chimeric sequence mediated MTX resistance when introduced into mouse 3T3 cells and was amplified when the MTX resistant transfectants were selected to grow in increasing concentrations of MTX. Mouse DHFR gene linked to MMTV promoter is also expressed and amplified in response to the addition of MTX (Lee *et al.,* 1981). Another well characterized amplifiable gene is the multifunctional hamster gene referred to as the CAD gene which shows amplification in response to the drug PALA.

All these different types of vectors so far discussed proved to be efficient in transferring genes into the mammalian cells. Understanding of the role of different DNA sequences in the expression of the gene will provide additional opportunities to construct vectors for introduction and efficient expression of any foreign gene in any mammalian cell type.

Expression of transfected genes

The expression of DNA sequences introduced into mammalian cells can be inferred by indirect methods such as the selective advantage they may confer on the cells or more directly at the level of RNA and or protein. Such expression assays permit identification of *cis* and *trans* acting sequences that may be necessary for proper gene expression.

Transient gene expression

After transfecting mammalian cells with foreign DNA sequences only a small proportion of cells become stably transfected with it. A significantly greater proportion of the cells may express the foreign genes for short periods of time. Such expression is referred to as transient expression. The proportion of cells expressing the foreign gene was found to be maximum at 48 h and begins to fall at 72 h. Milman and Herzberg (1981) detected the expression of transfected SV40 T-ag and the TK gene 1–3 days after addition of the DNA in 0·1–1 % of the transfected cells. If a simple *in situ* assay for the gene product is available, the transient gene expression systems provide a powerful and rapid method to screen a variety of DNA molecules to direct transcription and translation.

The levels of transient gene expression can be further improved by the use of autonomously replicating high copy number vectors such as those that contain SV40

sequences in COS cells. It may also be possible to increase the levels of transient gene expression by including an appropriate enhancer sequence on the plasmid. For example, the presence of SV40 enhancer sequences present on a plasmid containing rabbit β-globin sequences mediated an increased level of the globin gene expression in human cells (Banerjee *et al.,* 1981).

The rapidity with which transient gene expression assays can be conducted permit screening large numbers of DNA sequences for functionality. For example, it would be possible to test individual members of a multigene family for gene activity. It is known that all members of a multigene family need not be functional. This method has been used to identify the functionality of individual cloned members of the class I genes of the major histocompatibility genes (Barbosa *et al.,* 1982; Goodenow *et al.,* 1982; Singer *et al.,* 1982). The transient expression assays also permit determination of the strength of individual promoter sequences and effects of mutation and DNA deletion and rearrangement on gene expression.

Stable expression

In cases where a selection system is utilized to isolate transfected cells, it can be inferred that the exogenous gene is expressed properly. For example, the expression of HSV TK gene in mouse cells deficient in this enzyme can be inferred by the fact that the transfected cells become resistant to medium containing HAT. Since the recipient cells were not known to revert, it was possible to infer that the HAT resistant phenotype was due to the acquisition and expression of the HSV TK gene. Similarly, cells transfected with the bacterial neo gene or gpt genes which have no cellular counterparts can be presumed to be expressing the foreign gene. But in majority of the cases it is necessary to directly assay for the transcripts and protein products.

Expression of non selectable genes.

As mentioned previously, it is possible to introduce any gene sequence into mammalian cells. This is accomplished by ligated or non ligated gene transfer. In ligated transfer the non-selectable gene is covalantly linked to a selectable gene and the chimera used for gene transfer. In the non ligated transfer method a large excess of the non-selectable gene is mixed with a selectable sequence in the presence of carrier DNA and presented to cells. The different DNA sequences become covalently linked in the cellular environment before integration into the cellular genome. The expression of genes introduced by either of these methods has been studied. In one study (Perucho *et al.,* 1980b), APRT, TK^- L-cells were co-transformed with genomic DNA and HSV TK gene and selected for $APRT^+$ phenotype. All $APRT^+$ transformants were found to contain the TK gene sequences, as determined by blot-hybridization, and found to express the TK gene by a functional assay. In a similar study, a bacterial plasmid containing the SV40 early region was co-transformed into L-cells using the HSV TK gene as the selectable marker. Several of the TK^+ transformants were found to synthesize the SV40 T-antigen.

The exact nature of the transcripts produced by the transfected genes have been examined in several cases. Mantei *et al.* (1979) examined the transcription of globin

genes which were transferred by the covalant ligation method and found that most of them initiated transcription at the proper site. Roginski *et al.* (1983) examined similarly transfected human globin genes and snowed that not only did the transcription initiate at the proper site but the globin mRNAs were properly polyadenylated, processed and exported to the cytoplasm. Wold *et al.* (1979) examined the expression of rabbit globin sequences introduced into mouse L-cells by the non-ligated co-transfer method. Of the six transformants analyzed, one of them showed the correct splicing pattern but 45 nucleotides that are normally present in the normal mature transcript of the *β*-globin sequences were missing. The presence of this aberrant but unique 5′-terminus suggests the possibility of incorrect initiation of the transfected gene. In a separate study a transcript 650 nucleotides longer at the 5′-terminus than the normal mature ovalbumin was detected (Breathnach *et al.,* 1980). Not all genes that are transferred by the non-ligated co-transfer method have aberrant transcription initiation, but it is possible that ligated transfer method is more reliable in yielding accurate transcription initiation and processing of non-selectable genes.

Regulation of gene expression

It was found that the expression of the foreign DNA sequences after introduction into mammalian cells is regulated. The regulatory effects are dependant upon the nature of the gene as well as the nature of the recipient cell. HSV TK gene has been subjected to extensive analysis by McKnight and colleagues (McKnight *et al.,* 1981; McKnight and Kingsbury, 1982). They have shown that in addition to the TATA sequences located at – 30, the CAT sequences located at –80, and additional upstream sequences are important for proper expression of the HSV TK gene.

It is now generally thought that all mammalian genes which contain the proper transcriptional signals will be transcribed in any mammalian cell. This may be true if no specialized transcriptional proteins are needed in this process. It is conceivable that such proteins may be needed for expression. Use of gene sequences which are normally expressed in specific differentiated tissues in transfection studies is needed to unambiguosly answer this question.

The requirement for *trans* acting factors in properly regulated gene expression can be deduced from experiments involving the globin genes. We have shown that human α- and *β*-globin genes can be readily and properly expressed in mouse L-cells (Hsiung *et al.,* 1982; Roginski *et al.,* 1983) though these genes are not regulated in these cells. Similar sequences when introduced into mouse erythroleukemia (MEL) cells not only show expression but can be induced by treatment with dimethyl sulphoxide, an agent known to induce endogenous globin gene expression in the MEL cells.

Mouse MT-1 gene is normally inducible by heavy metals including cadmium and it has been shown that this gene introduced into mouse L-cells can be induced by treatment with cadmium. Deletion mapping of this gene revealed that the minimal sequence of DNA required to elicit the heavy metal inducibility lies within 90 bp of the transcription start site (Mayo *et al.,* 1982). Corces *et al.* (1981) showed that a *Drosophila* heat shock gene can be induced in mammalian cells and the sequences necessary for this response lie within 3·6 kb of the *Drosophila* DNA segment.

Because the transfection method is relatively simple, it is possible to adapt it to assay

for regulatory sequences. The general strategy is to ligate a selectable or easily assayable gene to the putative regulatory sequences and assay the effects of such sequences on the expression of the selectable gene under various conditions. It has been shown that the expression of the p21 transforming gene of Harvey murine sarcoma virus linked to the DNA containing the LTR of MMTV is regulated by physiological concentrations of dexamethasone. Lee *et al.* (1981) observed that the MMTV sequences can drive the expression of a mouse DHFR cDNA sequence and can be regulated by glucocorticoids. All of these examples serve to illustrate the fact that gene transfection techniques permit identification of *cis* acting regulatory sequences. It is to be expected that similar methods may enable identification and isolation of genes which code for *trans* acting products which are necessary for proper regulation of genes.

Gene expression affected by position of integration

We have documented the evidence which indicates that in majority of the cases, the exogenously introduced DNA becomes integrated into the host cell DNA and that there is no site specificity for this integration. Since not all of the genome is expressed in any given cell it is to be anticipated that the sites of integration may play an important role in the expression and regulation of the transfected genes. Davidson *et al.* (1973) have described the phenomenon of phenotypic modulation in mouse L-cells transfected with UV inactivated HSV. They observed that the TK^+ cells lose their TK activity without losing their TK gene sequences. This phenotypic switching may occur at very low levels (10^{-6}) or at very high levels (1–10 %, Pellicer *et al.,* 1980). Clough *et al.* (1982) have shown that the low level loss of the TK^+ phenotype is due to hypermethylation of DNA at the CpG sequences and reexpression of the TK gene can be facilitated by treatment of the cells with 5-azacytidine, a compound which causes demethylation of mammalian cellular DNA. Observations made by Christie and Scangos (1982) and Ostrander *et al.* (1982) are also consistent with these results.

Not all of the phenotypic modulation observed in transfectants is the result of changes in methylation pattern. Davies *et al.* (1982) studied the basis for the high frequency switching of the TK phenotype in L-cells transfected with the HSV TK gene. They noted that the high frequency switching is not associated with changes in DNA methylation but with changes in chromatin structure. These results were extended by Roginski *et al.* (1983) who showed that not only TK but other adjacent sequences are also involved in the phenotypic switching.

The fate of genes introduced into mouse embryos by microinjection also support the view that the sites of integration may play an important role in gene expression. A number of investigators have introduced rabbit or human globin genes into mouse embryos (Constantini and Lacy, 1981; Lacy *et al.,* 1983; Stewart *et al.,* 1982). In all these cases the genes showed no expression or expressed at aberrant sites. Rat growth hormone genes introduced into embryos were not expressed but growth hormone gene – metallothionien gene chimeras expressed in liver and other tissues resulting in increased growth of the mice. However, additional experiments utilizing other constituent genes have to be attempted before definitive conclusions, about the factors that are important for tissue specific and developmental regulation of genes introduced into mammalian embryos, can be reached.

Rescue and recovery of transfected genes

Transfection into mammalian cells not only permits introduction of genes but their rescue as well. The rescue and recovery methods that are used depend upon the gene that is introduced into the mammalian cells. The true eukaryotic-prokaryotic shuttle vectors can be readily moved from bacterial cells to mammalian cells and vice versa. An example of this type are the pSV2 vectors described earlier. pSV2NEO (Southern and Berg, 1982) contains a bacterial plasmid, pBR322 carrying the bacterial replication origin and the amp gene. It also contains the neo gene derived from Tn5 which can be expressed in bacterial cells or mammalian cells. This plasmid can replicate autonomously in bacterial cells and Tag^+ monkey cells such as COS cells (Gluzman, 1982). Purified plasmid from bacteria can be introduced into the appropriate monkey cells and after permitting their replication the low mw cellular DNA, containing the plasmid DNA sequences, can be used to transform *E. coli.* Similarly, polyoma vectors can be shuttled between mouse and bacterial cells and BPV vectors can be transferred from appropriate murine cells to bacteria.

The SV40 based vectors can be rescued from their integrated state as well. For example when pSV2 plasmids introduced into mouse or hamster cells they integrate into the host cell genome. If these cells are fused with COS cells, the Tag produced by the COS cells and specific monkey cellular factors will allow DNA replication to be initiated at the SV40 origin. Soon afterwards circular DNA molecules, containing the SV40 sequences and any plasmid or other DNA sequences surrounding them, appear in the nucleus as autonomous plasmids. These can be readily isolated by fractionating low mw DNA by a procedure described by Hirt (1967). Brietman *et al.* (1982) have shown that all of the genetic information carried on SV40 plasmid recombinants can be introduced in mammalian cells and the plasmid can be rescued by fusion with a simian cell line. They introduced the (SV40)-pBR322 recombinant vector pSV2 carrying the gpt gene of *E. coli,* into chinease hamster ovary $HPRT^-$ cells. All gpt transformed cell lines were found to contain one or more insertions of pSV2 sequence exclusively associated with high molecular weight DNA. Upon fusion with COS-1 cells, most gpt-transformed cell lines produced low-molecular weight DNA molecules related to pSV2. This rescued plasmid was tested for ampicillin resistance by transformation in *E. coli* and for gpt by transformation in mouse L-cells and gpt^- *E. coli* to gpt^+ *E. coli.*

Hanahan *et al.* (1980) rescued a bacterial plasmid carrying the early region of SV40 (pOT) stably established in high molecular weight DNA of mouse L-cells by fusion with simian cells.

Our laboratory (Kucherlapati *et al.,* 1984) also used eukaryotic-prokaryotic shuttle vector pSV2Neo to demonstrate that mammalian somatic cells can mediate homologus recombination and the frequency of this recombination can be enhanced by pretreatment of input DNA. Two non-overlapping deletion mutants of pSV2Neo were constructed and introduced into mouse or human cells. The functional pSV2Neo plasmid was rescued from neo resistant colonies by fusion with monkey COS-1 cells which allows ex*cis*ion and amplification of the plasmid. Low molecular weight DNA was isolated from the cells and analyzed by propagating the plasmid in an *E. coli* strain.

Though extremely useful, it has to be noted that the shuttle vector plasmids may suffer rearrangements and mutations as a result of passaging through mammalian cells.

Razzaque *et al.* (1983) have passaged a shuttle vector plasmid containing SV40 sequences and the bacterial galactokinase (Gak) gene in monkey cells. The low MW DNA from these cells was used to transform *E. coli* and tested for Gak activity. They observed that 1 % of the plasmids were defective in Gak after passage through mammalian cells. A similar vector was used by Calos *et al.* (1983) to score for mutations in the *lac i* gene of *E. coli.* The autonomously replicating plasmids were rescued from mammalian cells and propagated in *E. coli* for analysis. Mutants in *lac i* were obtained at frequencies of one to several per cent, compared with spontaneous mutation rate in *E. coli* of less than 10^{-5}. Despite this limitation these and other shuttle vectors should prove extremely useful in analysing a number of different features associated with DNA transfection.

The conventional method of isolating genes involves purification of the appropriate mRNA and constructing a cDNA corresponding to it and using it as a probe to screen genomic libraries. The isolation of mRNA is dependent upon its abundance in a particular cell type or knowledge about the protein product it codes for. The gene transfer system however permits isolation of genes whose products may not have been identified. The gene products should confer a selective advantage on cells carrying them or can be screened easily. Two related schemes which may be termed rescue and screening were successfully used to isolate genes coding for TK and APRT. Perucho *et al.* (1980a) used the bacterial plasmid pBR322 as a vehicle to isolate chicken thymidine kinase gene by rescue strategy. Chicken DNA was first digested with the restriction endonuclease HindIII which does not cleave the TK gene and ligated the resulting DNA to similarly digested *E. coli* plasmid pBR322 coding for ampicillin resistance. This concatenated DNA was used to transform mouse LTK^- cells to TK^+ phenotype. Some transformants were expected to contain chicken TK gene linked to pBR322 sequences and pBR322 sequences residing in the transformants were used to rescue the flanking sequences containing TK gene. Based on this strategy, DNA from the transformant was cleaved with another restriction endonuclease which does not cleave the chicken TK gene and cleaves pBR322 only once, on either side of the single HindIII site of the circular pBR322 molecule. The cleaved DNA ligated in cyclization conditions was used to transform *E. coli* to ampicillin resistance. The rescued plasmids were tested for their ability to transfer TK back into TK^- animal cells. This procedure permitted isolation of the chicken thymidine kinase gene as a 2·2 kilobase HindIII insert in pBR322.

Lowy *et al.* (1980b) adapted an alternative scheme to isolate hamster Aprt gene. This scheme does not rely on the maintenance of intact plasmid function through passage in animal cells. Hamster genomic DNA was cleaved with HindIII which leaves the Aprt gene functionally intact and was ligated to a molar excess of HindIII cleaved plasmid pBR322 DNA. This DNA was used to generate a primary $Aprt^+$ transformant which has integrated multiple plasmid sequences. The DNA from the primary transformant was transferred to recipient $Aprt^-$ cells to construct secondary $Aprt^+$ transformants containing Aprt gene linked to a single plasmid sequence. The DNA from the secondary transformants was subjected to partial cleavage with BamHI. Fractions containing 20 kbp long DNA were collected and used to construct a library of 10^6 independent recombinant phage using charon 4A as the vector. This library of recombinant phage was screened with highly radioactive pBR322 DNA as a hybridization probe. They screened 6×10^5 plaques to identify a single clone containing plasmid sequences which

generated $Aprt^+$ colonies when added to $Aprt^-$ cells with a frequency of one colony per 400 pg/5 × 10^5 cells, which was five fold greater than that obtained with total genomic DNA. Preliminary mapping data localized the functional Aprt gene to an 8-kbp Hind III fragment.

Instead of using pBR322 as DNA tag, suppressor tRNA can be used as vector in the original ligation. If suppressor tRNA is used, DNA from the secondary transformant can be used to construct a genomic library with a λ phage vector that contains an amber mutation in the lysis gene. These phage requires a functional supF gene to complete the lytic cycle. Thus although all the DNA from the secondary transformant can be packaged, phage growth and lysis following transduction into bacteria will occur only with phage that contains supF gene which should be next to the transferred gene. The two experimental designs described should permit the isolation of any gene coding for selectable or identifiable functions for which DNA-mediated gene transfer can be used.

If human DNA is used as the donor and rodent cells are used as recipients, a DNA tag need not be used because of the presence, in the human genome, of *Alu* family of sequences (Schmid and Deininger, 1975) which are repeated several hundred thousand times. These sequences do not readily cross hybridize with any sequences in mouse cells and the probability of finding one or several *Alu* sequences near a given gene is very high. The *Alu* sequences have been used to isolate a human bladder oncogene by a screening procedure. Shih and Weinberg (1982) and Pulciani *et al.* (1982) transferred the human bladder carcinoma oncogene into mouse NIH3T3 cells. After two rounds of transfer, a genomic library was constructed in which the DNA of the transformant carried the human oncogene. The library was screened with human *Alu* sequences and the oncogene was isolated.

Perspectives

Gene transfer methodologies have become important tools of molecular biology in a relatively short period of time. This is due to the relatively simple procedures that can be used to introduce DNA into mammalian cells and the reliable expression of these sequences in the recipient cells. The method has already proved quite useful in identification of *cis* acting elements that are needed for normal gene expression and isolation of a number of new genes whose products can be selected for in mammalian cells. Some additional problems that are currently receiving attention and has further potential are described below.

Gene therapy

The fact that foreign genes introduced into mammalian cells can be expressed raised the possibility of conducting gene therapy. It is known that several human hereditary disorders are the result of single gene defects. In many cases, the normal counterparts of these genes have been isolated and are readily available. If these normal genes could be introduced into cells from patients who suffer from the genetic disorders, it may be possible to provide adequate amount of gene product to alleviate the effects of the disorders. Two potential methods are being tested to reach this goal. The first one

involves the use of retrovirus vectors and the second one uses homologous recombination. As described earlier, mouse ecotropic RNA viruses have been used as vectors for introduction of foreign genes into mouse cells. The development of cell lines which provide the viral coat proteins without any endogenous viral replication permit production of high titers of viruses which carry the viral LTRs and the desired gene. If such viruses are used for infection of novel cell types, the viral RNA will be converted into DNA and integrated into the cellular genome *via* the LTR. Because the LTRs contain potent promoter sequences, the inserted gene should be expressed quite efficiently. Since the viral titers are quite high it is possible to introduce the genetic information virtually into all cells in a population. Before this method can be applied to any human diseases, it is necessary to have cell lines which produce amphotropic coats which will permit introduction of the sequences into human cells. Efforts to develop such cell lines are underway.

Two potential problems of gene therapy by retrovirus mediated gene transfer are (i) the foreign sequences may integrate at a site which is needed for the normal functioning of a cell and (ii) since RNA is delivered to the cell and intervening sequences are removed before RNA is packaged into virions, it is possible to lose any *cis* acting regulatory elements that are located within the introns. The first problem may not prove to be serious because it is unlikely that very many gene insertions would result in abnormal function of the recipient cell. The second problem can be solved by introducing the desired gene in an orientation opposite to that of the viral sequences. Such a construction would prevent splicing and subsequent loss of sequences.

An alternative to using viral vectors for gene therapy is to replace the resident gene with the exogenous gene by a homologous recombination mechanism. It is now clear that somatic mammalian cells are capable of mediating homologous recombination between two exogenously introduced plasmids at very high efficiencies. Several investigators are exploiting this observation to determine if an exogenously introduced plasmid containing a sequence homologous to a cellular gene can recombine in a homologous fashion with the cellular gene. If this is successful and occur at appreciable frequencies, it ought to be possible to obtain gene replacement without altering or affecting any other gene in the genome.

Study of development and differentiation.

The processes of cellular differentiation and development can be considered as a series of steps of gene activation and inactivation. It is believed that these series of steps are mediated by transacting factors acting on DNA sequences of specific genes. If this were the case, gene transfer methods offer a way to identify such sequences. Once identified, *in vitro* modification of such DNA segments and reintroduction into the animal embryo should elicit responses which may be correlated with specific alterations. This strategy assumes that the exogenously introduced sequence would show normal developmental regulation. Though there are no current reports of such regulation, it is anticipated that examples of this nature would be forthcoming in the immediate future.

An alternative possibility offered by the gene transfer system is that an exogenous DNA segment would integrate at a locus which is important for developmental regulation resulting in a recessive lethal phenotype. If this were the case the exogenous

DNA segment can be used as a probe to isolate the developmentally important gene. Indeed Jaenisch *et al.* (1983) recently reported that a retrovirus sequence introduced into the mouse embryo resulted in a developmental lethal mutation. They have been able to identify this locus to be that coding for collagen (Harbers *et al.,* 1984). A similar observation of possible gene inactivation through insertional mutagenesis was reported by Wagner *et al.* (1983).

The general strategy of insertional mutagenesis can be applied not only to study development and differentiation of embroys but also of cultured cells. The only requirement is that the loss of a phenotype can be readily detected or selected.

Mechanisms of mutagenesis

Though a number of agents are known to be mutagenic to mammalian organisms or cells, the exact mechanism by which they cause mutations or their specificity is not well understood. Gene transfer systems may offer a method to study these processes. The feasibility of one strategy has been tested for this purpose. It involved the introduction of a shuttle vectors carrying two genes into a mammalian cell, permit it to undergo several rounds of DNA replication, rescue it in bacteria and examine it for mutations and structural alterations. Unfortunately experimental results from two different laboratories revealed that the shuttle vector plasmids have a tendency to undergo spontaneous mutations and rearrangements at a rather high frequency (Calos *et al.,* 1983; Razzague *et al.,* 1983,1984). However, it is possible that different types of vectors and recipient cells may alleviate this problem. Alternatively, it may be possible to introduce a mutation into a specific site of a specific gene, introduce it into mammalian cells and determine if it is possible to induce its reversion by specific mutagenic agents.

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