INTRODUCTION OF PURIFIED GENES INTO MAMMALIAN CELLS

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I. INTRODUCTION

A number of advances in mammalian genetics, especially human genetics, have been made during the last two decades. Gene transfer methods have made an important contribution in these developments. First among these methods is somatic cell hybridization. The advent of cell hybridization methods together with several important properties such hybrid cells possess have contributed to the rapid expansion of the human genetic map, generation of monoclonal antibodies, and the study of mammalian gene expression. Though some hybrid cells lose chromosomes of one of the parental type, it is not possible to predetermine the amount and type of genetic information retained by the cells. The use of microcell-mediated fusion[1] and chromosome-mediated transfer[2] permit introduction of partial genetic information into cells. However, these methods do not permit control over the quantity of DNA introduced into cells.

The advent of recombinant DNA techniques has made it possible to isolate a variety of genes from mammalian and nonmammalian sources. These sequences can be readily subjected to physical analysis by constructing detailed restriction enzyme maps and by obtaining the complete nucleotide sequences where necessary. The functional analysis of these genes is facilitated by the development of methods to transfer them into intact cells. The combination of in vitro manipulation of DNA and its introduction into intact cells is making possible the identification of *cis*-acting elements that are necessary for gene expression and regulation. Newer developments in this area are contributing towards understanding the molecular basis for such processes as development and differentiation and neoplastic transformation.

As in many successful scientific endeavors, the DNA-mediated gene transfer systems have been made possible through the interaction of several disciplines and the gene transfer systems in turn are helping to bring a large number of previously distinct disciplines together. The aim of this article is to provide an introduction to the methodologies of DNA-mediated gene transfer, the fate of the DNA in the recipient cell, and the parameters that affect the introduction and expression of these sequences in the new environment. We have chosen a number of articles to illustrate the points and substantiate the statements, but this list is not intended to be exhaustive. Omissions of specific articles are not intentional. In addition, we

have not discussed the various aspects in the newly developing and expanding role of gene transfer in answering questions about the basis of a variety of mammalian malignancies. For recent reviews in this area, see Varmus[3] and Cooper.[4]

II. METHODS OF INTRODUCING GENES

The various methods that are available to introduce genes into mammalian cells can be classified into natural and artificial systems. Infection of cells with intact viruses with the subsequent entry of viral genome into the cell can be considered a natural way of gene introduction. The viruses may have DNA or RNA as their genetic information. In the case of DNA viruses, if the cell type is permissive, the viral DNA undergoes replication and new virus particles are synthesized, eventually resulting in cell death. If the cell is nonpermissive, the viral sequence may become integrated into the host cell DNA and transform the host cell. In cases of several RNA viruses, the RNA is converted into a double-stranded DNA which becomes integrated into the host cell and could then be expressed as part of the host genome. The integration process may lead to cellular transformation and/or continuous production of transcripts which are packaged and released into the surroundings. Because of the relatively small size of most of these viral genomes, they are obliged to utilize the cellular machinery for transcription and translation and effect a variety of changes in the host cells through mechanisms which are not yet clearly defined. These features have made viruses attractive for detailed molecular biological analysis leading to the definition of sequences which are responsible for the various functions. This knowledge, in turn, has led to the development of vector systems in which portions of the viral genomes can be effectively used to introduce purified genes into mammalian cells. Aspects of such systems are dealt with in detail later in this article.

In addition to the natural systems there are a number of artificial systems to introduce genes into mammalian cells. These methods in turn can be divided into direct or indirect methods of introducing DNA. Microinjection of purified DNA into the nuclei of somatic cells or embryos can be considerd a direct method. Anderson et al.[5] and Capecchi[6] have microinjected somatic cells with purified DNA and permitted them to grow in the absence or presence of a selection system and the clonally derived population of cells analyzed for the presence and expression of the foreign genes. Other investigators have injected DNA into the pronuclei of fertilized mouse embryos and reimplanted them into pseudopregnant mice to continue development in vivo and give rise to newborn mice.[7-13]

The indirect methods of introducing DNA involve packaging the DNA in a variety of forms and presenting it to cultured cells. The most widely used of these techniques is the calcium-phoshate coprecipitation of DNA. Bachetti and Graham[14] have shown that infectivity of adenoviral DNA is enhanced if the DNA is mixed with calcium and phosphate. This technique was later used by Wigler et al.[15] and Maitland and McDougall[16] to show that specific restriction enzyme fragments of herpes simplex viral (HSV) DNA are capable of conferring the TK^+ phenotype to mouse cells deficient in this gene. Several variations of this procedure are now available, but the basic protocol involves mixing DNA with calcium chloride and a phosphate buffer to form a very fine precipitate. This minicrystalline precipitate is presented to cultured cells. Though this method was originally developed for use with naked DNA, chromatin as well as DNA packaged in bacteriophage λ can be used to transfect cells.[17,18] The cells used for transfection are usually adapted for growth in monolayer, but methods to transfect cells growing in suspension are also available.[19,20] An alternative method for presenting DNA to cells involves use of DEAE dextran.[21] Though this method results in good transient expression, it has not been found to yield stable transfectants.[22]

Since most of the purified DNA sequences are maintained in bacteria, it is useful to have methods to directly introduce the plasmid sequences into mammalian cells without prior

purification. Shaffner[23] has reported that fusion of bacterial protoplasts with intact cells would result in gene transfer. Recent reports[24,25] have shown this method to be very efficient, yielding stable transfection frequencies approaching 10%. Yet another method of gene transfer which has been used to a limited extent is the incorporation of DNA into liposomes which are fused with intact cells.[26,27] The choice of the method to be used in a specific experiment would depend upon its success with specific cell types. $Ca\text{-}Po_4$ coprecipitations and DEAE dextran-mediated transfer do not require extensive preparation, whereas liposome-mediated transfer or protoplast fusion require prior preparation. However, the nature of the cell type and the experimental situation may call for a specific approach.

Different cell types seem to have different efficiencies with which they are capable of being transfected. Despite these differences a variety of cell types have been successfully used for transfection. These include transformed cells and normal cells and cells which grow in monolayer or in suspension. The basis for the differences in the ability of cells to take up and express DNA is not understood, but Corsaro and Pearson[28,29] report clonal variability of transfection among mouse L cells.

III. FACTORS THAT AFFECT EFFICIENCY OF GENE TRANSFER

Several factors seem to play a role in the efficiency with which a gene can be transferred into mammalian cells. The most important of these are the nature of the recipient cell, the nature of the gene that is transferred, and the method of gene transfer. In the direct transfer methods, physical constraints such as the size of the cell and the size of the nucleus may play an important role in the ease with which DNA can be introduced. Capecchi[6] has studied the effects of SV40 DNA sequences on the transfection efficiency in microinjection experiments and found them to have an enhancing effect. Whether it is the origin of replication or the transcription enhancer sequences that are responsible for this positive effect is not known.

In the indirect transfer systems, a number of factors can be expected to have effects on DNA transfer. Not all cells are equally efficient for gene transfer. Not only do different cells from the same or different species exhibit different DNA transfer efficiencies, but even clones of a single cell line may show variability.[28,29] Specific features of the recipient cell membranes must also play an important role. Early transfection conducted experiments were conduced by incubating the cells in the calcium-phosphate coprecipitate for variable periods of time, but studies by Lewis et al.[30] and Lowy et al.[31] have shown that a posttransfection shock with DMSO results in enhanced gene transfer. Such DMSO and glycerol shocks are now routinely used in many laboratories. A caution in this regard is that not all cells respond equally to these shocks and it is necessary to empirically determine the concentration (usually 10 to 20%) of these reagents and time (usually 1 to 2 min) of exposure that are optimal for each cell type.

Most of the early transfection experiments were conducted with a small amount of selectable DNA mixed with a large excess of salmon sperm, calf thymus, or homologous cell DNA as carrier and were believed to be essential for gene transfer. Later experiments clearly demonstrated that there is no absolute need for carrier DNA in DNA transfer systems.[32-34] Our own studies[35] on the effects of carrier DNA indicated that transfection of HSV TK gene is enhanced tenfold in the presence of salmon sperm DNA as the carrier as compared to experiments in which no carrier DNA was used. The role of the carrier DNA in transfections was much speculated. At least two different hypotheses for the role of carrier DNA in transfections can be proposed. These are based upon the knowledge that the selectable DNA becomes covalently ligated to the carrier DNA and the complex becomes integrated into the host cell DNA (see next section). Based on this knowledge, it is possible to conceive that the carrier DNA may provide functional replication origins which would permit replication

of the complex until it becomes integrated into the host DNA. Though there is no direct proof of this hypothesis, the fact that bacterial DNA is a relatively poor carrier lends support to it. The result that SV40 sequences including the "ori" region seem to increase the efficiency with which stable transformants can be obtained by microinjection[6] also supports this view. It is known that several viruses (e.g., SV40, murine retroviruses) contain an approximately 70 bp repeated sequence which acts as an enhancer of transcription.[36,37] It is possible that eukaryotic genes or DNA sequences may also contain such sequences and when the selectable gene becomes ligated to the carrier DNA in the proximity of such a region, its transcription level may be altered. Such an altered transcription may result in enhanced transfection efficiencies.

Another possible role for the carrier DNA is that it may provide several targets for integration into the host cell DNA. This view is supported by some of our experimental results.[38] We have observed that the carrier effects can be mimicked if we used a plasmid carrying a Chinese hamster Alu family of repeated sequences without additional carrier DNA. To prove that this is indeed the role of carrier DNA requires demonstration that recombination is mediated through the repeated DNA sequences.

Experiments to deduce the effects of linear vs. circular DNA have also been conducted. Colbere-Garapin et al.[39] have noted that linearized plasmid DNA is five times more efficient in transferring the TK^+ phenotype to mouse cells than its circular counterpart. The facts that loose ends of DNA molecules are more recombinogenic and that there is a tendency for linear molecules with flush ends or single-stranded tails to readily join end to end[40,41] provide a basis for this observation. Recent evidence (see next section) suggests that homologous recombination can occur in mammalian somatic cells. As such it is clear that the mammalian cell carries appropriate recombination and repair machinery which will be used in the foreign DNA integration. Efforts to determine the role of agents, which are known to increase chromosome breakage, sister chromatid exchange, and DNA damage and repair, in the efficiency of gene transfer are underway.

IV. FATE OF THE DNA IN THE RECIPIENT CELL

A number of studies dealing with the uptake and fate of the DNA in the recipient cell have been conducted. Loyter et al.[42] have examined the need for facilitators of DNA transfer such as calcium phosphate and DEAE dextran. When naked DNA was presented to cultured cells in medium containing serum, the DNA was rapidly degraded. When the DNA was complexed with calcium phosphate it was protected from degradation. They have also shown that the DNA facilitator complex is taken up as a unit by the cell. The uptake was dependent upon the concentration of DNA and the pH of the buffer. The exact mechanism by which the DNA present in the cytoplasm finds its way into the nucleus is not understood, but the fact that a large proportion of cells exhibit transient expression of the exogenously introduced genes[22] indicates that the DNA is translocated to the nucleus where it is expressed. All of these steps are, of course, eliminated when the DNA is directly microinjected into the nucleus of the recipient cell. After the DNA enters the nucleus, several possible fates may await it. These aspects were investigated by several different methods.

A. Phenotype Stability

The DNA that enters the nucleus may remain unintegrated; it may also replicate autonomously. Alternatively it may become covalently integrated into the host cell DNA. An indirect way to distinguish between these possibilities is to measure the stability of the phenotype in the absence of selection. If the DNA is unintegrated it may not segregate equally into the progeny cells after each cell division. Such an improper segregation results in the loss of the phenotype among a certain percentage of cells in the population. A typical

experiment to test this possibility is to grow transfectant cells in nonselective media and examine their relative plating or cloning efficiencies in nonselective and selective media at various time periods. Several investigators found that there is variability among cell lines in this respect.[43-45] A general picture that emerges from several of these studies is that DNA, after it enters the nucleus, is expressed in a large number of recipient cells. The DNA remains unintegrated, at least in some instances, showing a high degree of instability and in a few cases becomes stably integrated into the host cell DNA. In addition to the studies mentioned above, results from Pellicer et al.[46] and Milman and Hertzberg[22] support this view. It has to be added, however, that the instability of gene expression need not result exclusively from nonintegration of DNA. It is possible that integrated DNA is excised during the early stages of the development of the cell line at a higher rate or the chromosome bearing the DNA may be lost. It is also possible that the DNA sequences remain integrated throughout and it is the modulation of gene expression that causes the instability. Evidence to support these views was presented by Pellicer et al.,[46] Ostrander et al.,[47] and Davies et al.[48]

B. Molecular Determination of the Fate of the DNA

The fate of the DNA in the cell, whether it is integrated or unintegrated, can be more directly determined by the use of restriction enzyme digestion followed by blot hybridization[49] of the transfected cell DNA.

It is now clear that the fate of the foreign DNA is dependent upon the nature of the vector used. For example, vectors containing SV40 replication origin and an intact A gene can replicate autonomously in monkey cells.[50] SV40 vectors without the A gene can replicate autonomously in monkey cells which produce SV40 T-ag in a constitutive fashion (Cos cells[51]). The same vectors integrate into the host cell DNA if the cell is nonpermissive for SV40 replication (e.g., mouse cells). Similarly, appropriate vectors containing polyoma sequences replicate autonomously in mouse cells[52] and some of the bovine papilloma vectors replicate autonomously in several mammalian cells.[53] This autonomous replication pattern can be deduced by isolating low molecular DNA from the cells[54] and showing that the fraction contained DNA identical to the exogenously introduced DNA. O'Hare[55] provided convincing evidence in this regard by showing that the DNA isolated from mouse cells transfected by polyoma vectors has a different modification pattern than the corresponding DNA isolated from bacteria. Appropriate restriction enzyme digestion of the total cellular DNA and blot hybridization would also reveal unit-size supercoiled DNA molecules, if the DNA replicates autonomously.

Integration of the donor DNA into host cell DNA was inferred when it was found to be in high molecular weight form.[56] This view had to be slightly modified in view of later experimental results. Most of the initial transfection experiments were conducted by mixing the selectable genes with excess carrier DNA. Detailed examination of the selectable gene in these transfectants revealed that the selectable DNA is integrated into the carrier DNA and this complex in turn integrated into the host cell DNA. Evidence for integration of the selectable DNA into carrier DNA was obtained by Perucho et al.[43] and Scangos et al.[44] When a selectable gene such as HSV TK was mixed with excess of a variety of nonselectable genes (e.g., ϕX174,[57] globin genes [58,59]) and carrier DNA, a large proportion of the TK^+ transfectant cell lines also contained the nonselectable genes. The frequency of this cotransfer was in some cases greater than 80%. Wigler et al.[57] have shown that this is not due to a differential competence of the recipient cells. Perucho et al.[43] have noted that when TK^- revertants were obtained, these cells also lost some of the nonselectable sequences. They have shown that the blot-hybridization patterns of several derivative cell lines, though different, could be derived from a single original pattern. From these and other studies the following picture has emerged. It seems that if a cell has acquired several copies of the same or different DNA molecules, these molecules tend to undergo recombination or become

catenated by some unknown cellular mechanism. This catenated DNA then becomes integrated into the host cell DNA. The size of this high molecular weight DNA complex, referred to as pekelosomes[43] or transgenome,[44] is estimated to be at least 100 kb.

Direct evidence for the formation of DNA complexes is provided by a number of investigators. Subramanian[41] has shown that linear SV40 molecules become circularized after entering mammalian somatic cells and that this ligation can occur between blunt ends or with 5′ or 3′ overhangs of single-stranded DNA. More recently Wilson et al.[40] have shown that DNA molecules tend to join end to end in a nonspecific fashion. Anderson et al.,[5] who injected two different circular plasmid molecules into mouse L cells, have shown that recombination occurred between the two input molecules. In all these cases the recombination or end-to-end joining was nonspecific. Evidence for homologous recombination is presented by three groups of investigators. Folger et al.[60] have injected circular and linear plasmid molecules into somatic cells and found that in almost all cases the molecules form head-to-tail tandem arrays. These investigators conclude that both circular and linear molecules are coopted into concatemer formation by homologous recombination. de St. Vincent and Wahl.[61] have shown that homologous recombination occurs in mammalian cells by simultaneously introducing two plasmids, each of which contained a deleted multifunctional hamster gene called the CAD gene. Selecting for expression of this gene they have shown that CAD^+ recombinants arise with functional gene activity. Small and Scangos[62] also report that it is possible to obtain a functional HSV TK gene when two molecules with nonoverlapping deletions are introduced into TK^- cells and selected in HAT medium. Though there are some discrepancies between these various results, it is clear that somatic mammalian cells do have the mechanisms to mediate homologous recombination and end-to-end joining of related or unrelated molecules.

Direct evidence for the integration of the DNA complex into mammalian cells was obtained by Robins and colleagues.[63] They have introduced human growth hormone genes into rodent cells and showed by *in situ* hybridization methods that the complex is integrated at a specific site on one of the chromosomes. The chromosome bearing the foreign sequences was different in different independent cell lines. This evidence indicated that there is no specificity, at least at the gross level, for integration of foreign DNA into the recipient cell chromosomes. These data are similar to those obtained from studies of viral integration sites.[64,65]

When no carrier DNA was used in the transfection experiments, it can be shown that the DNA integrated into host cell DNA by the fact that it is found in the high molecular weight fraction.[32-34] Truly definitive information about integration sites in the host chromosomal DNA can be obtained by isolating the junction fragments and analyzing them. Such experiments were done with some virally transformed cells (SV40 and adenovirus,[66,67]) in which the foreign DNA sequences were shown to be integrated into host cell DNA. Since there is no reason to believe that different cellular mechanisms would operate on viral and nonviral DNA sequences, as far as integration is concerned, it can be concluded that DNA introduced into mammalian cells, in the absence of carrier, integrates into host chromosomes.

It is intriguing that interactions between two exogenously introduced molecules may be mediated by sequence homology, while those between the exogenous and endogenous sequences are more random and not based on extensive sequence homology. It is possible that exogenously introduced molecules are in close physical proximity resulting in greater ease with which homologous sequences can be found, whereas the large size of the host cell genome precludes such interactions based on homology. But the fact that somatic cells have the enzymatic machinery to mediate homologous recombination provides additional experimental strategies to determine the frequency with which homologous recombination of exogenously introduced sequences with the resident genes occurs in somatic cells. If such recombination is detected, it may be possible to enhance it with the aid of agents known to induce recombination.

V. VECTORS FOR INTRODUCTION OF DNA

Because of the large variety of cell types that would serve as recipients and the large class of DNA molecules which can act as donors, it is necessary to have a diverse set of vectors for gene transfer. All of the vectors share the general property that they usually carry genes which can be readily expressed in mammalian cells and whose expression can be selected for or at least easily assayed. The first among these vectors to be developed utilized the HSV TK gene.

The presence of a gene coding for TK in HSV was shown by Munyon and colleagues[68,69] and Davidson et al.[70] They have shown that TK^- mouse cells can be infected by HSV and such cells become TK^+. They have also shown that TK^- cells can be stably transfected to a TK^+ phenotype by UV-inactivated HSV and that the TK present in these cells is of the viral origin. Maitland and McDougall[16] and Wigler and colleagues[15] have shown that purified DNA from HSV types I or II can confer the TK^+ phenotype to TK^- cells, if the DNA was presented as a calcium phosphate coprecipitate. They have also shown that the HSV DNA digested with some restriction endonucleases loses its ability to transfer the TK^+ phenotype, while the same DNA digested with other enzymes is unaffected in its ability to transfer the TK phenotype. Such restriction enzyme inactivation profiles serve as signatures for each gene and are useful in their initial characterizations. Wigler et al.[15] also have shown that specific size fragments resulting from HpaI, KpnI, or Bam HI digestions of HSV I DNA carry the TK gene sequence. These experiments were soon confirmed by other investigators.[71] A 3.4-kb fragment of HSV I DNA was shown to contain the complete coding sequence of the TK gene and was cloned into bacterial vectors.[39,72-74] The portions of the TK coding region and adjacent regions were completely sequenced.[75,76]

The nucleotide sequences which play a functional role in the expression of the TK gene have been analyzed in a series of experiments by McKnight and colleagues.[75,77-79] They have constructed deletion mutants, and generated mutants in which a cluster of bases were substituted at various positions by a method called linker insertion. These various mutants have been tested in the *Xenopus oocyte* transcription system, as well as in transfection experiments into mouse TK^- cells. These experiments enabled identification of several features of the TK gene which play an important role in its expression. This information together with that of the nucleotide sequence data provide a functional map of the TK gene. The TK gene is much like a typical mammalian gene. The transcribed portion of the gene contains untranslated regions at the 5′ and 3′ end and it has a concensus poly A addition signal and it does not contain any introns. There is a proximal transcriptional signal at region -25 which corresponds to the TATA box. Two other regions, one between 47 to 60 bases upstream from the cap site and another between 80 to 105 bases upstream from the cap site also seem to have important roles in proper transcription of the gene. The availability of this information enables the separation of the TK gene into its control or promoter segment and another containing the structural information. This can be conveniently achieved by digesting the fragment with HincII or Bg1II, both of which cut once within the region corresponding to the 5′ untranslated region of the mRNA. The 5′ fragment can be used as a promoter for DNA sequences which lack a functional promoter, but which contain all the other necessary features (e.g., initiation codon, ATG). The structural portion of the gene can be used to test the efficiency of various promoters or putative promoter sequences.

Colbere-Garapin et al.[52] have used the TK gene to construct a vector in which a bacterial gene is efficiently expressed in mammalian cells. They have digested the HSV TK gene with HindII and inserted a fragment from the bacterial transposon Tn 5 carrying the gene conferring resistance to the antibiotic neomycin (neo^r). They have shown that this chimeric plasmid expresses the bacterial gene by transfecting it into mammalian cells. Mammalian cells are normally resistant to neomycin, but are sensitive to one of its derivatives — G418.

Colbere-Garapin et al.[52] have shown that wild type mammalian cells die in the presence of G418, while some cells transfected with the chimeric plasmid described above survive. In this case it can be shown that the transcription is initiated using the TK promoter.

The portion of the TK gene containing the structural information was utilized to test for putative and/or effective promoter sequences. In one series of experiments we have constructed chimeric plasmids containing the left long terminal repeat (LTR) region of Moloney murine leukemia virus along with variable amounts of 3′ non-LTR sequences and ligated them to a BglII-Eco RI fragment of HSV 1 TK containing the TK coding information.[80] We have shown that one such plasmid efficiently confers TK^+ phenotype to mouse TK^- cells. In this case we were able to show that the transcription of the gene is initiated in the MOMLV LTR. A number of other similar plasmids were also constructed and were shown to be functional. These include metallothionein-TK chimeras[81] and other retroviral promoter-TK chimeras.[82]

The basic strategy described above can be used to construct a variety of plasmid vectors, each one designed for specific functions. The construction of such vectors is facilitated from an understanding of the function of DNA sequences of different origins. The identification and functional understanding of various mammalian sequences would eventually permit "custom-built" vectors each designed for specific purposes. It is also possible to "mix and match" various components to alter the properties of these plasmids. Some such vectors, which are referred to as modular vectors, and how they can serve different functions are described below.

A. Autonomous Replication Vectors

There are three different vectors which are currently used as autonomous replication vectors. These use the DNA viral sequences from SV40, polyoma, or bovine papilloma virus (BPV). The genome of SV40 and polyoma are well characterized and complete nucleotide sequences of these genomes are available. Both of these viruses have a sequence which functions as a replication origin and a promoter which is utilized during the early phase of viral infection (early promoter) and another which is functional during the late stages of infection. Hamer and colleagues[50,83-85] have inserted mouse globin genes into the late region of SV40 and transfected them into monkey cells. They were able to show that the mouse genes can be transcribed from the SV40 promoter. The presence of intact early region coding for the T antigen permits replication of these molecules in permissive monkey cells. These molecules can also be packaged into virions, if the size of the substituted genetic material is close to that of the deleted material and if supplied with a defective helper virus. Berg and colleagues[86-89] have constructed a variety of vectors based on SV40 promoters some of which contain the early region intact and others which do not. These vectors utilized the bacterial xanthine phosphoribyl transferase (XPRT) and the bacterial neo^r genes as the selectable markers. The vectors with the "ori" region and a functional T-ag gene can replicate autonomously in monkey cells. The development of monkey cells which are transformed by SV40 and which constitutively express T-ag[51] form excellent hosts for vectors containing the SV40 "ori" region. These same vectors integrate into host cell DNA if the host cells are other than monkey cells.[90]

O'Hare[55] has utilized a polyoma vector for expression of the bacterial $neomycin^r$ gene. Since polyoma virus replication is permitted in mouse cells, they serve as appropriate hosts for autonomous replication in mouse cells. As with SV40 vectors, the polyoma vectors integrate into host cell DNA if the host is a nonmouse cell. Because of the absence of mouse cells comparable to monkey COS cells, autonomous replication of polyoma vectors in mouse cells requires intact T-ag gene on the vector.

The complete nucleotide sequence of the bovine papilloma virus is now available,[91] but the nature of the various genes carried by it is not yet clear. However, the genome is capable of transforming mouse 3T3 cells,[92,93] and vectors based on this viral genome have been

constructed.[53,94] The BPV vectors which carry different genes have been tested, but it is not clear whether all of them are capable of autonomous replication. Further knowledge about the basic organization of the BPV genome and the functions of its various genes is necessary before widespread use of these vectors is possible. Such information should be forthcoming in the near future.

B. Double or Multiple Vectors

Any plasmid that carries a selective gene can form the basis for a double vector. Some of these vectors permit introduction of genes which will be expressed in the foreign environment only if they carry their own regulatory signals. Some of the genes which are introduced by this "piggy back" method are described in a later section. Here we discuss only those vectors which would permit expression of any two genes. SV40 and retroviral vectors can be used for these purposes. As we have described, SV40 has two promoters and it is possible to delete the early as well as late genes and replace them with other DNA sequences with or without their own promoters. The viral DNA would provide the promoter, the splice signals when and if necessary, and the poly A addition signal. Each of the genes in this case would be under the control of a separate promoter. Such double vectors from SV40 have not yet been constructed but it is clearly feasible to do so.

The retrovirus vectors provide an attractive alternative method with unique advantages. In general the 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 property can be used to substitute the viral genes with the desired selectable or nonselectable genes of different origin. Several investigators have constructed such vectors utilizing the Moloney murine leukemia virus genome. In one set of experiments Gilboa et al.[80] have tested the efficiency of the LTR to act as a promoter to the HSV TK structural region. Similarly Joyner et al.[95] have shown the feasibility of replacing promoters with viral LTR sequences. In another series of experiments Gilboa[96] removed the viral *gag* and *env* genes and replaced them with HSV TK and bacterial neo^r genes. It was shown that both of the genes inserted into these positions are functional. In addition to those features which provide a functional promoter, splice sites, and a poly A addition signal, the retroviral sequence also contains signals which permit their genomes to be packaged into virions.[97] It is possible to construct vectors containing this packaging signal, and if the vector along with the inserts do not greatly exceed the size of the viral genome can be readily packaged. Since the packaged material is fully processed mRNA, these vectors provide an attractive alternative to synthesize complete cDNA copies of genes for which the corresponding genomic clone is available.

The identification of sequences which act as enhancer sequences and the fact that they act in a species-specific manner[36,37,98-100] make the use of specific retrovirus vectors attractive in some specific transfections.

Different retroviral sequences may respond to different regulatory signals. This feature can be utilized to construct vectors containing genes which are regulated. An example of this type of vector utilizes mouse mammary tumor virus (MMTV). MMTV is known to respond to steroid hormones, and vectors with MMTV promoter have been constructed[101,102] and were shown to be regulated by steroids.

C. Regulatable Vectors

The understanding of DNA sequences which play an important role in gene regulation would result in the availability of a variety of sequences which can be used in the construction of vectors. The use of MMTV DNA to act as a promoter and as a regulatory sequence is already described. Another sequence that has been used for similar purposes is the metallothionien (MT) promoter. This gene which is expressed by many different cell types is

inducible by heavy metal ions (e.g., Cd). The MT promoter has been linked to the HSV TK gene and to the rat growth hormone gene, and was shown to be quite effective in expression of the ligated genes.[81,103] Chao et al.[104] have shown that the presence of the 5′ region of mouse globin genes in chimeras of mouse/human globin genes is inducible by agents such as dimethylsulfoxide when introduced into appropriate mouse erythroleukemia cells. This aspect, dealt with in greater detail later, indicates that it will soon be possible to construct vectors for efficiently introducing and regulating genes into each of a variety of mammalian cell types. If a gene which is constitutively expressed in all cells (e.g., gene coding for an enzyme in the TCA cycle) and which can be induced to produce high levels of its product can be identified and if it has cis-acting DNA sequences which provide this property, it will be possible to develop a vector whose genes can be induced in almost all cell types.

D. Amplifiable Vectors

During the past few years, several genes which are amplified in mammalian cells have been identified. Among these, the dihydrofolate reductase gene (dhfr) which is amplified in response to increasing concentrations of the antimetabolite methotrexate and the multifunctional hamster gene referred to as the CAD gene which shows amplification in response to the drug PALA have been well characterized. Though mouse cellular dhfr gene is quite large, Schimke and colleagues have constructed a number of cDNA clones containing the complete coding sequence for this gene. This cDNA clone was used in the construction of vectors involving the MMTV promoter[102] or the Harvey sarcoma viral promoter.[107] These studies have shown the feasibility of introducing these chimeric plasmids into mammalian cells and have demonstrated that the dhfr gene not only can be expressed, but can be amplified in response to the addition of methotrexate. The CAD gene has also been isolated[25] and can be put to similar use. Identification of the amplification signals and the specific elements which respond to them should help in construction of modular vectors, an element of which would be its amplifiability.

E. Other Signals

Over the past few years information has been accumulated which showed that specific peptide sequences at the amino terminal end of the protein would help its translocation to the cellular membrane and eventual secretion into the surrounding fluid.[108] It is now clear that similar signals may be important in the cellular localization or compartmentalization of proteins. Further definition of these sequences would also help construction of modular vectors whose gene products can be predetermined to be located in a specific cellular compartment, cell membrane, or be secreted. This should prove to be a useful technique to have mammalian cells produce and secrete large quantities of products which are otherwise available in low levels.

It is clear that the availability of new information about DNA sequences which play a role in a variety of processes involved in gene expression can be readily used in constructing gene transfer vectors designed to perform in any given fashion in any desired cell type. The various possible features of such modulor vectors are summarized in Table 1.

VI. TRANSFER OF SELECTABLE AND NONSELECTABLE GENES

The methods of gene transfer have sufficiently advanced to introduce any DNA sequence into mammalian cells. The genes that have been transferred can be classified into selectable and nonselectable categories. The strategies for introducing each of these classes of genes are slightly different and they are considered below. A list of the various genes that have been introduced into mammalian cells is presented in Table 2.

Table 1
SOME SEQUENCES WHICH ARE AVAILABLE FOR CONSTRUCTING MODULAR VECTORS

Nature of sequence	Origin	Function
Promoter	A number of mammalian genes	Permit expression in mammalian cells
	Yeast	Expression in yeast cells
	Bacteria	Expression in bacterial cells
Enhancer sequences	SV40, MSV, and other retrovirus genomes	Efficient expression in specific mammalian cells
Splice signals	SV40, adenovirus, retrovirus	Processing of transcripts
Poly A addition	SV40, adenovirus, retroviruses, and a number of mammalian cells	Needed for proper function of mRNA
Origin of replication	SV40	Autonomous replication in monkey (cos) cells rescue by fusion with monkey cells
	Polyoma	Autonomous replication in mouse cells
	BPV	Autonomous replication in mammalian cells
Amplification signals	Mouse dhfr hamster CAD	Amplification in appropriate media
Packaging signal	Retroviruses	Packaging into virions
Rescue	SUP F	Rescue in SUP F$^-$ bacteria
Regulatory signals	MMTV promoter	Response to steroids
	HSV TK	For induction and possibly cell cycle response
	Metallothionein	Response to heavy metal induction
Selectable genes	Various sources	See Table 2

A. Selectable Genes

Those genes for which a positive selective pressure can be applied would be considered selectable genes. They can in turn be classified into recessive or dominant acting. Recessive genes are those that can be introduced into only those cells which are mutant and lack its expression. The genes coding for TK, adenine phosphoribosyl transferase, and hypoxanthine phosphoribosyl transferase fall into this category. Dominant genes are those that can be introduced into any cells. The methotrexate-resistant dihydrofolate reductase (dhfr) gene can be considered to be in this category.

The availability of mutant cells lacking specific genes and the selective systems which permit isolation of appropriate gene transferants have made possible the development of gene transfer techniques. The early gene transfer experiments utilized mouse L cells deficient in TK as recipient cells and DNA containing the HSV TK gene as the donor. TK$^+$ cells were selected by the use of the HAT selection system. This system proved ideal because the L cells are excellent recipients, they are not known to revert spontaneously to a TK$^+$ phenotype, and the foreign gene product could be easily detected by electrophoretic, immunological, and drug sensitivity profiles. The HSV TK gene and a variety of derivative vectors are still used quite extensively. However, a drawback of the TK gene is that it can be introduced only into mutant cells and obtaining stable TK$^-$ mutants is an arduous task. Alternative genes which can be introduced into any wild type cell are now available. Two of the genes which are widely used are the bacterial neomycin resistance gene and the bacterial xanthine phosphoribosyl transferase genes.[86-89] Both of these genes, because of their bacterial origin, are normally nonfunctional in mammalian cells, but ligation of a mammalian promoter at the 5′ end permits their expression. The development of a drug, G418, which is cleaved by the neo^R gene product and to which mammalian cells are sensitive, makes the neo^R gene-carrying plasmid a universal mammalian vector. Similarly, the bacterial gpt gene can be used as a recessive gene and can be selected for by the HAT medium when introduced into HPRT cells. This gene has the additional advantage that it can also be

Table 2
GENES INTRODUCED INTO MAMMALIAN CELLS BY DNA TRANSFER SYSTEMS

Marker name	Source	Ref.
Selectable genes		
APRT	Hamster	18
APRT	Human	117, 118
β-aspartyl hydroxamate	Hamster	112
CAD	Hamster	25
Colchicine^R	Hamster	114
dhfr	Bacterial	107, 119
dhfr	Mouse	102, 115, 120, 121
hprt	Human	116, 117, 122—124
hprt	Mouse	125
NEO^R	Bacterial	52, 59
OUA^R	Mouse	28, 29, 125
TK	HSV1	15
TK	HSV2	16
TK	Hamster	126, 127
TK	Chicken	128
TK	Human	124, 126, 127
xprt	Bacterial	86—90
Nonselectable genes		
Adenovirus		129
Cell surface antigens	Mouse	130
Differentiation marker	Human	131
ES.D	Hamster	127
GaK	Human	124
Gene 27	HSV	132
Growth hormone	Human	63
	Rat	103, 133
β-galactosidase	Bacterial	134
β-globin	Human	5, 12, 35, 46, 59, 136—138
	Rabbit	9, 12, 58, 139, 140
	Mouse	19, 104
	Mouse-human chimera	
γ-globin	Human	34
	Human	137, 138, 141
H.2	Mouse	142—144
HL-A	Human	145
Hepatitis β-surface antigen		146
Interferon	Human	147—150
Membrane glycoprotein	Hamster	114
α_2-microglobulin	Rat	151
pBR322		57—59
Preproinsulin		152
ØX174		57
Retroviral sequences		11, 82
SV40		32, 153
tRNA	*X. laevis*	154
Tumor cell surface antigen		130
TSTA		155

introduced into wild type cells by using an alternative selective strategy using mycophenolic acid and xanthine.

The use of bacterial genes for gene transfer has several advantages. In several cases the bacterial gene has no counterpart in mammalian cells. This provides the clear-cut advantage of using these genes as universal donors and can be introduced into any cell type. The bacterial gpt, neo, and chloremphenicol acetyl transferase (CAT) genes are examples of this sort. Each of these genes has unique advantages. The natural substrate of the gpt gene is xanthine, though it can also use hypoxanthine as a substrate. The use of agents to inhibit purine synthesis along with provision of xanthine as the sole source of purines make it an excellent selection system. The lack of a true counterpart of this gene in the mammalian cell eliminates the need to generate mutants or concerns about rates of reversion. The neo gene confers bacteria resistance to the antibiotic neomycin. Mammalian cells are not sensitive to this antibiotic, but are killed in the presence of an analog G 418. The functional neo gene is capable of conferring mammalian cells resistance to G 418. The third gene in this category is that coding for CAT. Gorman et al. constructed an SV 40 plasmid vector containing the CAT gene and showed that it is functional in mammalian cells. The CAT gene activity can be readily assayed by its ability to convert labeled chloremphenicol to its acetylated form. The nonacetylated and acetylated forms are readily separable by thin-layer chromatography. Since this assay is simple and rapid it should be possible to use this gene to measure the efficiency of gene transfer into a variety of mammalian cells. The readily detectable activity of this gene would also permit its use in detecting the efficiency of a variety of sequences to act as promoters. Gorman et al. have used this gene for such a purpose and showed that the Rous sarcoma virus long terminal repeat acts as a strong promoter for expression of the CAT gene in a number of different cell types. The availability of CAT antibodies also makes this gene an attractive one for gene transfer. Some of the selectable systems used for DNA-mediated gene transfer are listed in Table 3.

B. Nonselectable Genes

There are two strategies for transferring nonselectable genes into mammalian cells. These are referred to as nonligated or ligated cotransfer. As the terms imply, in one case the nonselectable gene is covalently ligated to a selectable gene and such a chimeric plasmid is presented to cells. The nonselectable gene is reliably introduced into cells by virtue of its ligation to the selectable marker. An alternative to this method is to mix the selectable and nonselectable genes together (the nonselectable gene is usually in excess) and present such a mixture, usually in the presence of carrier, to cells. The cells are selected for expression of one gene and screened for the presence of another. Because of the ready recombination and end-to-end joining of DNA molecules described earlier, there is a good likelihood that a cell would acquire both DNA sequences. As shown in Table 2, both methods permit introduction of nonselectable genes. However, the ligated transfer system affords more control over the process: (1) because it provides a virtual certainty that the second gene can be introduced; (2) since the two genes would be equimolar in proportion, it is possible to select for cells which contain a single copy of each of the genes; and (3) because no carrier DNA is needed, the integrations have to occur directly into the recipient cell genome.

VII. EXPRESSION OF TRANSFERRED GENES IN MAMMALIAN CELLS

A. Expression of Selectable and Nonselectable Genes

The ability of mammalian cells to express foreign genes was well established before the advent of DNA-mediated gene transfer systems. Studies of interspecies cell hybrids and genes transferred from isolated metaphase chromosome clearly indicated that mammalian cells could express genes from other species. All steps needed to produce an active protein

Table 3
SOME SELECTION SYSTEMS USED TO INTRODUCE GENES INTO MAMMALIAN CELLS

Gene	Abbreviation	Selection	Ref.
Adenine phosphoribosyl transferase	APRT	Alanosine adenine	111
β-aspartyl hydroxamate resistance	—	β-aspartyl hydroxamate	112
CAD[a]	CAD	Uridine-deficient medium	113
Colchicine resistance	—	Colchicine	114
Dihydrofolate reductase	dhfr	Methotrexate	115
Hypoxanthine phosphoribosyl transferase	hprt	Hypoxanthine aminopterin and thymidine (HAT)	116
Neomycin resistance[b]	Neo	G418	52
Ouabain resistance[c]	Oua	Ouabain	28, 29
Thymidine kinase	TK	HAT	116
Xanthine phosphoribosyl transferase	gpt	HAT or mycophenolic acid and xanthine	86

[a] CAD is a multifunctional enzyme containing carbamyl phosphate synthetase, aspartate transcarbamylase, and dihydrooratase activities.
[b] The enzyme responsible for this phenotype is aminoglycoside 3′-phosphotransferase type II.
[c] The enzyme responsible for this phenotype is the membrane-associated Na-K ATPase.

gene product could be carried out. This was true not only for genes constitutively expressed by many different cell types (i.e., "housekeeping" functions), but also for specialized proteins produced only in differentiated cells. The demonstration of gene expression after DNA-mediated gene transfer was greatly facilitated by the development in this earlier work of efficient selection systems. The most widely used system combines the HAT selection method[116] with mouse L cells that are deficient in thymidine kinase and which are not known to revert. The expression of the HSV thymidine kinase gene after DNA-mediated transfer was first demonstrated by the isolation of HAT^R, tk^+ colonies in this system.[15,16] HSV tk expression in transfected cells was shown directly by electrophoretic characterization of tk activity. It was also possible to analyze tk gene expressions at the level of mRNA. Cremer et al.[156] have characterized the HSV tk mRNA in virus-infected cells. Measurements of the size of tk mRNA in DNA transfectants by RNA blotting and S1 nuclease mapping have shown that most mRNA transcripts have the proper size.[34,77] The viral origin of tk activity in transfected cells can also be shown by making use of the differences in sensitivity of cellular and viral enzymes to inhibition by the drug acycloguanosine.[127]

As shown in Table 2, a large number of selectable markers have now been expressed in recipient cells after DNA-mediated gene transfer. In many cases the expression of the transferred gene has been verified by studying the RNA transcripts and proteins present in the recipient cells. Expression is not limited to eukaryotic genes. Prokaryotic genes also can be expressed under the control of eukaryotic transcription signals. The bacterial genes for XGPRT,[86-88] dihydrofolate reductase,[119] and aminoglycoside 3′-phosphotransferase type II [APH(3′)-II],[52] which confers resistance to neomycin and its analog G418, are notable because they can be used as dominant selectable markers whose expression can be selected in wild type mammalian cells. The expression of the *Escherichia coli* XGPRT gene was also found to compensate the HGPRT defect in human Lesch-Nyhan cells in HAT medium.[86] Expression of prokaryotic genes in mammalian cells requires their coupling to eukaryotic expression signals. For example, Mulligan and Berg[86] inserted the *E. coli* XGPRT gene between an SV40 promoter 5′ to the gene and RNA splicing sites and a polyadenylation signal on the 3′ side. Studies of the transcripts in transfected cells showed that mRNA specifying the XGPRT enzyme was produced by transcription and processing from the expected sites in the SV40 transcription unit. A similar principle was used for expression

of the bacterial DHFR gene.[119] In this case the eukaryotic transcription unit was constructed by combining the SV40 early transcription promoter with splicing and polyadenylation signals from a rabbit β-globin gene. Other examples of such hybrid vectors combining eukaryotic expression signals and prokaryotic coding sequences include fusions of the bacterial APH(3′)-II gene to either the HSV tk gene[52] or to transcription units contained in cloned retrovirus DNA.[95,96] Although it is possible that prokaryotic genes can be expressed in vectors containing only a eukaryotic transcription promoter, the most efficient expression is probably obtained when splicing and polyadenylation signals are included.[157]

Gene expression after DNA-mediated gene transfer has been observed not only for cloned genes, but also after transfer of total genomic DNA (see Table 2). This observation has been used in several cases as part of the strategy for cloning the genes from total DNA. Recipient cells expressing the foreign gene contain only a small part of the foreign genome which can be reduced further by carrying out secondary transfections with the DNA from primary recipients. The bioassay for the intact gene provided by DNA transfection also can be used to determine its sensitivity to cleavage by various restriction endonucleases. By preparing a recombinant library with a suitable restriction nuclease from the DNA of transfectants and screening that library by either the bioassay and/or hybridization with species-specific probes,[158] it is possible to isolate the gene of interest. A combination of these and other procedures (e.g., plasmid rescue, see below) has been used to isolate several vertebrate genes. The procedure is not limited to genes specifying metabolic enzymes. It has been used to great advantage in the isolation of genes that cause transformation of cultured cells to a malignant phenotype. It can even be used to isolate genes for which a selection scheme is not available. For example, Stanners et al.[130] combined these procedures with fluorescent flow cytometry for identification of transfectant cell populations expressing specific surface antigen to isolate the gene for a human lymphocytic leukemia-associated antigen.

Nonselectable DNA sequences can be introduced into mammalian cells either by the ligated cotransfer method or by nonligated cotransfer discussed above. A compilation of the many different genes or DNA sequences that have been transferred to mammalian cells by these two methods is shown in Table 2. The results of these various studies clearly indicate that expression can occur from genes transferred by either method. In the case of nonligated cotransfers, expression of the nonselectable gene is most readily seen in transfectants containing a large number of copies of that gene. In order to obtain a high proportion of transfectants with at least several copies of the nonselectable gene, it should be present in the transfection mixture at a molar concentration between 10 and 100 times that of the selectable DNA. Under these circumstances, the proportion of transfectants expressing the nonselectable gene can approach 50%.[77] Even more reliable expression of nonselectable genes can be obtained by the ligated cotransfer method. Mantei et al.[139] introduced a rabbit β-globin gene in Ltk$^-$ cells by ligating it to the HSV tk gene and Hsiung et al.[77] carried out a similar study with a human γ-globin gene. Both groups studied a large number of transfectants and found consistent expression of the globin genes into globin mRNA in as many as 90% of transfectant clones. The rare transfectants which were negative for expression probably contained deleted or rearranged globin genes. Ligated cotransfer may result in more frequent expression of the nonselectable gene because integration is more likely to occur in a region of the chromatin that is permissive for gene expression.

Several studies have addressed the issue of fidelity of gene expression after DNA-mediated gene transfer. In the case of selectable markers it is clear that the fidelity of gene expression must be sufficient to produce some amount of the required active gene product. Analysis of the mRNAs produced from a number of selectable and nonselectable genes has generally found them to be of approximately the correct size, properly processed, and polyadenylated and transported to the cytoplasm. However, careful examination of the 5′ ends of the transcripts produced from certain transfected genes has revealed some incorrect termini.

Using S1 nuclease mapping to study rabbit β-globin transcripts in transfected mouse L cells Wold et al.[58] observed a 5′ terminus about 45 nucleotides downstream from the natural 5′ end. Dierks et al.[140] observed these transcripts in similar experiments, but found that most rabbit β-globin transcripts in L cells had the correct 5′ terminus. It is not clear whether these aberrant transcripts are the result of incorrect initiation or are due to cleavage of correctly initiated chains. A low level of aberrant 5′ termini have also been seen for HSV tk mRNA in L cells.[77] In this case, the trancripts were longer than normal indicating incorrect initiation. Nevertheless, in general the fidelity of transcription of transferred genes appears to be quite high and all evidence points to proper posttranscriptional processing as well.

Studies of gene expression following DNA-mediated transfer have often utilized mouse L cells as recipients. In a number of instances expression was observed for transferred genes that are not normally expressed in these cells. Thus foreign genes for globin, ovalbumin, $\alpha_{2\mu}$-globulin, growth hormone) could be expressed in L cells even though their endogenous mouse counterparts are not expressed. In some cases (e.g., $\alpha_{2\mu}$-globulin, growth hormone) discussed below, hormonal regulation of the foreign gene expression occurred in the absence of endogenous gene expression. These results suggest that transferred genes are not subject to a type of cis-acting regulation that maintains repression of endogenous gene expression. Such regulation could result from specific chromatin structure or other aspects of the developmental history of the genes in these cells. However, it should be noted that L cells have often been used as the "undifferentiated" partner in cell fusion experiments in which extinction of tissue-specific gene expression has been observed. For example, cell fusion of mouse erythroleukemia cells and L cells results in complete extinction of the globin mRNA production (both constitutive and inducible) which is characteristic of the mouse erythroleukemia cells.[159-161] Nevertheless, when cloned globin genes are transferred to L cells they are reliably expressed. Thus it seems that transfected globin genes also are not sensitive to a type of negative, transacting regulation which can influence expression of chromosomal globin gene in cell hybrids.

B. Transient Gene Expression

When a selectable gene is presented to cells by one of the indirect transfer methods such as calcium-phosphate precipitation, only a small proportion of the cells is found to have stably integrated and expressed the gene. In contrast, a much larger proportion (as high as 20%) of stable transfectants can be obtained by direct microinjection of a selectable gene into cell nuclei.[6] This suggests that if DNA is successfully transferred to the nucleus, many cells in a population are capable of expressing the gene. Indeed, it is now clear that integration of DNA is not required for gene expression to occur. When certain cell lines are exposed to DNA and then analyzed by *in situ* methods 24 to 72 hr later, a relatively high proportion of cells in the population was found expressing the gene.[22,46] The proportion of cells expressing the gene depends on the particular cell line used and the sensitivity of the detection method, but it may be as high as 50% or greater.[162] After longer times the proportion of expressing cells decreases. This phenomenon has been termed transient expression.

Because of the rapidity of the procedure, the transient expression assay is being increasingly used to analyze the DNA sequence requirements for gene expression in mammalian cells. Sufficient levels of expression are often obtained to allow analysis of gene product formation as either proteins or RNA transcripts. Where RNA transcripts have been analyzed precisely by S1 nuclease mapping, it appears that the fidelity of transcription is as good as that observed in stable transfectants.[162] The levels of transcription obtainable and the ease of performing transient expression assays are now being improved by several developments. Higher levels of expression can be obtained by utilizing DNA vectors that include portions of the SV40 genome. When these DNAs containing the SV40 replication origin and a functional early region are introduced into monkey cells, increased expression of ligated DNA sequences

can occur through copy number amplification of the DNA which is able to replicate in these cells. Other viral DNA sequences can cause increased transient expression of genes by mechanisms which are not yet understood. Schafner and colleagues[162,163] and others have studied the so-called "enhancer" DNA sequences present in SV40 and polyoma DNAs. These viral DNA fragments cause increased transcription of some but not all genes[138] even when they are located a considerable distance on either side of the transcription initiation site of the gene.

It is now recognized that numerous cellular genes are members of multigene families of related DNA sequences. In certain families only some of the copies may be functional, while others have by various processes acquired features that do not permit their expression (i.e., "pseudogenes"). DNA sequencing has often been used to distinguish among functional and nonfunctional members, but there is no guarantee that a copy identified in this way is indeed functional since we do not yet know all features of genes that are needed for expression. For large multigene families like the major histocompatibility genes, DNA sequencing alone may not serve to assign a particular cloned member to the set of related proteins which may have been characterized with respect to their antigenic determinants or function but not by amino acid sequencing. In both of these situations DNA transfection assays can be useful. Because transient expression assays are relatively easy to perform, they are probably preferred for analysis of multigene families, but stable transfection assay may also be useful in certain cases. Both methods have been used to identify individual cloned members of the major histocompatibility genes.[142,145,164] The expression assays have not yet been used to identify nonfunctional members of multigene families. However, a related application concerns studies of certain naturally occurring mutations in human hemoglobin genes associated with the thalassemia syndromes. Cloned thalassemic genes have been isolated and partially sequenced and specific base changes have been identified, primarily involving RNA splicing defects. Access to nucleated red cells from these patients is often limited, and therefore studies of expression of the mutated genes have been difficult. Several different cloned thalassemic genes have now been studied by DNA transfection assays.[162] The results of these studies have shown that the splicing defects predicted from nucleotide sequencing are indeed present in the mRNA transcripts produced in the transfected cells.

C. Regulated Gene Expression

The ability to introduce defined DNA molecules into mammalian cells provides a powerful tool for investigating the regulation of gene expression and for identifying specific DNA sequences that may play a role in such regulation. When combined with in vitro procedures for modifying recombinant DNA sequences, DNA-mediated gene transfer offers a very attractive alternative to classical genetic and somatic cell genetic approaches to studying gene regulation. A prerequisite for undertaking such experiments is the demonstration of correct, regulated expression of the foreign gene in an appropriate recipient cell. In a few cases, studies of cell hybrids have indicated that regulated expression of a foreign gene can occur when whole chromosomes have been transferred. For example, the introduction of human chromosomes carrying globin genes into mouse erythroleukemia cells (MEL) results in the regulated expression of the human genes.[170-173] The MEL cells are arrested at an early stage in the erythroid differentiation pathway, but they can be induced to complete erythroid maturation in culture by a variety of chemical agents. Human globin genes in the hybrid cells also were found to respond to some of these inducers. Moreover, inducible expression occurs even when the donor human cells have a nonerythroid origin.

There are now several reports demonstrating that regulated gene expression can occur after DNA-mediated gene transfer. Most of these studies have been carried out with fibroblast cells — especially mouse L cells — as recipients. Because these cells have a limited repertoire of regulatory signals to which they can respond, they are expected to exhibit regulated

expression of only certain types of genes. Thus, e.g., while foreign globin genes can be expressed constitutively at low levels in these cells, there is no evidence that their expression is regulated.[58,59] However, other genes which are normally regulated in fibroblasts can be regulated after gene transfer. Cellular thymidine kinase activity is normally induced at the beginning of S phase. Schlosser et al.[174] have shown that the cellular tk gene from several mammalian sources, when transferred to Ltk$^-$ cells, exhibits a similar induction. In contrast, HSV tk which is not regulated in virus-transformed cells is not induced in S phase in transfectants containing a cloned HSV tk gene. Interferons can be induced in fibroblasts by virus infection or treatment with double-stranded RNAs. Likewise, cloned interferon genes can be similarly induced in these cells after DNA-mediated gene transfer.[150,174-178] Other genes which are normally inducible in fibroblasts and which can be induced after gene transfer include cadmium-regulated metallothionein[178,179] and a *Drosophila* heat shock gene.[180,181]

In addition to these examples, there are now several reports of regulated expression in fibroblasts after DNA-mediated transfer of genes that are normally not expressed in these cells. L cells contain receptors for glucocorticoid hormones and it has been found that genes that can be induced by these hormones in other tissues also can be regulated in transfected L cells. Kurtz[151] showed that the rat $\alpha_{2\mu}$-globulin gene introduced into Ltk$^-$ cells can be induced by dexamethasone treatment and Robins et al.[182] reported similar results for a human growth hormone gene. Dexamethasone also can induce expression of mouse mammary tumor virus (MMTV) genes in DNA-transfected cells.[183,184] Lee et al.[102] linked a mouse dihydrofolate reductace (DHFR) cDNA fragment to a portion of the MTV genome containing a long terminal repeat. When transferred into DHFR$^-$ cells the expression of mouse DHFR was found to be inducible by dexamathosone, thus localizing hormone responsiveness to a region near the promoter of viral transcription.

Finally, there is now a single report of regulated expression following gene transfer into cells that can be induced to undergo differentiation in in vitro culture. Chao et al.[104] have recently reported the inducible expression of globin genes introduced in mouse erythroleukemia cells. Both a fused mouse-human hybrid β-globin gene and an intact human β-globin gene were found to be inducible by dimethylsulfoxide, an inducer of MEL cell differentiation. Induction was specific for globin genes, since a cotransferred hamster APRT gene was not inducible.

The results of these various studies suggest that it will soon be possible to define the regions within and around certain genes that are required for their regulation. Indeed progress for a particular gene may only be limited by the availability of a suitable cell culture system in which regulation can be studied. It should be noted, however, that several aspects of the studies carried out so far suggest that this approach is likely to yield only partial answers to the problem of gene regulation. The studies of tissue-specific gene expression in L cells clearly show that regulation of transfected genes can occur in the absence of expression by the endogenous counterpart. Some transfectant cell clones exhibit regulated expression, while other clones that express the transfected gene constitutively are not responsive. Often the level of inducibility in transfectants is not as high as in the normal tissue and the level of expression per transfected gene copy also is less than normal. These results suggest that aspects beyond primary DNA sequence, such as chromatin structure, developmental history, and distal DNA sequences, are important in gene regulation. Other strategies will need to be devised to reveal the role of these mechanisms.

One approach to studying factors other than primary sequence that are involved in gene regulation is the use of methylated DNA in gene transfer experiments. Many studies have indicated a correlation between hypomethylation of cellular DNA and gene activity. However, it has been difficult to determine whether methylation is a cause of or a consequence of gene inactivation. Recent work in which in vitro methylated genes were transferred to

cultured cells indicates that the DNA remains methylated for many cell generations.[185] The methylation of tk gene was found to inhibit its efficiency of transfer to tk^- cells.[186] This inhibition is not due to an effect on transfer per se, because Stein et al.[185] found that the expression of an APRT gene that had been cotransferred by selection with a tk gene was markedly inhibited by in vitro methylation. In these studies many sites within and around the genes were methylated. It should now be possible to test by DNA transfer the activity of genes methylated at specific sites.

D. Modulation of Gene Expression

Davidson and colleagues[70,187,188] first described an interesting phenomenon attributable to modulation of gene activity. They found that mouse Ltk^- cells that had acquired tk activity by infection with UV-inactivated HSV could lose that activity without losing the HSV tk gene. Phenotypic tk^- cells that had been selected for resistance to BUdR were found to be capable of growing in HAT medium at a low frequency (10^{-6}) and these cells were shown to have regained HSV tk activity. This type of modulation of HSV tk activity was also observed for a transfected HSV tk gene by Pellicer et al.,[46] who also found that certain BUdR-resistant clones could revert to HAT resistance at much higher frequencies (10^{-1} to 10^{-2}). Ostrander et al.[47] and Christie and Scangos[189] have shown that the low frequency changes are correlated with alterations in the methylation patterns around the tk gene. Davies et al.[48] have reported that the high frequency events are associated with changes in the local chromatin structure as detected by sensitivity to digestion with DNAse. Recently, Roginski et al.[141] found that the high frequency modulation of tk activity extends to other DNA sequences that have been transferred with tk in ligated cotransfections. They introduced two human globin genes (α and γ) along with HSV tk on a single plasmid vector into Ltk^- cells. The activity of both human globin genes in producing globin mRNA was found to be modulated in parallel with HSV tk during negative selection in BUdR and restoration of activity in HAT medium. The results suggest that alterations in chromatin structure associated with changes in the activity of transfected genes can extend over a distance of at least 20 kb.

VIII. RESCUE AND RECOVERY OF DNA SEQUENCES

Once a specific DNA sequence is introduced into mammalian cells it may remain unintegrated or may become integrated into the host genome. If it is unintegrated it may replicate autonomously. It is of great use to have methods to recover the introduced DNA sequences from the recipient cell. Such rescue and recovery systems are now available and are playing an important role in such diverse processes as developing shuttle vectors and isolation of a number of mammalian genes. Some of these methods and their utility are described below.

A. Rescue of Genes on Viral Vectors

If a gene is introduced via an autonomously replicating vector, such as SV40, into an appropriate host cell like monkey cells, the introduced sequences can be readily isolated by fractionations of DNA based on size.[54] If these have the appropriate sequences they can be used to transform bacteria and be propagated in bacterial hosts. SV40 vectors have the additional advantage that they can be rescued from other mammalian cells by fusion with monkey cells. Hanahan et al.[153] have introduced a recombinant plasmid containing the SV40 early gene region, pBR322 and HSV TK into mouse cells. The plasmid integrated into high molecular weight DNA. They were able to induce release and replication of this plasmid by fusion with monkey cells. Similarly Brietman et al.[90] have shown that an SV40 vector carrying the bacterial gpt gene can be rescued from hamster cells by fusion with monkey cells. Conrad et al.[190] have shown that a relatively small fragment of SV40 containing the

origin of replication is sufficient for the rescue. The products and genes present in monkey cells which permit this autonomous replication are not known, but if they can be isolated, it may be possible to construct vectors which would replicate autonomously in any cell type, making rescue and recovery a simple operation.

In the case of retroviral vectors, if all of the important signals of the viral genome are kept intact, transfection with a helper virus would enable the vector sequences to be repackaged into virions. The virions can be readily isolated and used for reinfection. However, since these virions contain RNA, it is not possible to introduce them into bacterial hosts.

B. Nonviral Vectors

When nonviral vectors or total DNA from an animal species are used as the donor, the rescue systems described above are not useful. However, several alternative methods to rescue the plasmid and their neighboring sequences are available.

C. Plasmid Rescue

The method to be described was first used by Perucho et al.[128] to isolate the chicken thymidine kinase gene. Earlier methods of gene isolation usually required the purification of the appropriate mRNA, constructing the corresponding cDNA, and using it for screening the appropriate genomic library. The plasmid rescue system depends upon isolating a transfected cell line with the gene of interest. The gene could be a selectable gene or one whose expression can be easily assayed in a large number of different cell lines. Chicken TK is a selectable gene. Perucho et al.[128] have tested the ability of chicken DNA to transfer the TK phenotype, to TK^- mouse cells, after digestion with each of a number of restriction endonucleases. They found that Hind III does not destroy the chicken TK gene activity. Hind III-digested pBR322 DNA was covalently ligated to similarly digested chicken DNA and this ligated mixture was used for TK gene transfer. To eliminate extraneous chicken and plasmid sequences, the DNA from primary transfectants was used as the donor to transfer TK in a second round. The DNA from the secondary transfectant was digested with EcoRI and ligated under cyclization conditions. The resulting circular molecules were used to transform *E. coli* and selected for a drug resistance marker within the plasmid sequence. Since appropriate bacterial plasmid sequences are the only ones which are capable of replicating in bacteria, all circular molecules which do not contain such sequences will be lost, while the others can transform and grow in *E. coli*. The rescued plasmid was tested for its ability to confer TK^+ phenotype to TK^- mouse cells and shown to do so at very high efficiencies.

Though the plasmid rescue is an elegant method for rescue and recovery, it has several drawbacks. The size of the gene of interest may be too large to find an appropriate enzyme which fails to cut it or its size may preclude it from acceptance by the plasmid. It may also be difficult to keep the bacterial replication origin and the drug resistance markers intact through the multiple rounds of transfection. Lowy et al.[18] have used an alternative approach to isolate the hamster aprt gene. The initial strategy in introducing the plasmid-ligated gene into appropriate recipient cells is very similar to that described above. However, the DNA from the secondary transfectant was used to construct a genomic library in bacteriophage vector. This library was screened with labeled plasmid sequences. Phage from the positive plaques was used directly to transfer the aprt gene and was shown to be highly efficient. The phage was thus shown to harbor the hamster aprt gene.

An alternative strategy to that used by Lowy et al.[18] was used by Perucho et al. to isolate a human transforming gene sequence. The principle in these systems is to ligate a known gene or DNA sequence at a site in the immediate vicinity of the gene to be isolated and cotransfer the two sequences into an appropriate recipient cell. After removing extraneous sequences by repeated transfections, the selectable gene is rescued and the desired nonse-

lectable gene will be rescued along with it. In the specific experiments, DNA from the human bladder carcinoma cell line T24 was digested with Bam HI (which was previously shown not to destroy the activity of the oncogene in T24) and was ligated to a bacterial Sup F gene having Bam HI ends. The Sup F gene permits expression of bacterial genes which have the appropriate chain termination mutation. The ligated mixture was used to transform NIH 3T3 cels. Following an additional round of transfection, the transformant cell DNA was used to construct a genomic library and a bacterium with a suppressible gene mutant was infected. The only plaques that formed were those that carry the Sup F gene. The transforming gene was isolated by virtue of its location next to the Sup F gene.

When certain interspecies transfers are involved it is not necessary to ligate the gene of interest to an identifiable marker. For example, when human genes are transferred into rodent cells, the human DNA can be distinguished from the rodent cell DNA because it carries uniformly distributed repeated sequence elements (e.g., Alu family of repeats[192]). Shih and Weinberg[193] and Pulciani et al.[194] have transferred the human bladder carcinoma oncogene into mouse NIH 3T3 cells. After two rounds of transfer, a genomic library of the recipient cell carrying the human oncogene was constructed and screened with human repeated sequence probes. Positive clones were tested for oncogene activity. This permited the isolation of the human bladder oncogene.

Based on the general principles described above, it is possible to develop a variety of alternative methods to rescue genes introduced into mammalian cells. This may involve use of cosmid systems for cloning,[195] utilize other tags (e.g., amplifiable sequences like mouse dhfr[105,106]), or use recombination in bacteria as a rescue system (πVX system[196]). The systems of choice will depend upon the nature and size of the gene and the nature of the donor and host cell systems.

IX. PERSPECTIVES

We have presented various aspects of DNA-mediated gene transfer and how this methodology is being used to further our understanding of gene structure and function. The future for DNA transfer seems to be bright. Some of the problems that are receiving current attention and additional potential of the system are described briefly below.

A. Gene Therapy

The facts that foreign genes can be reliably introduced into mammalian cells and that these genes are usually expressed increase the possibility of using this method for gene replacement therapy. It is possible to envision at least three possible modes of such gene therapy. Several of the genetic disorders in humans are the result of single base pair changes. In some instances the exact base change and the resulting alteration in the amino acid sequence of the protein that the altered gene codes for are known (e.g., sickle cell anemia). It is possible to correct this deficiency by introducing a gene for tRNA so engineered that it would be able to incorporate the correct amino acid though recognizing the mutant codon. The general feasibility of such an approach is demonstrated by Laski et al.[154] who have shown that a *X. laevis* tRNA is functional in mammalian cells. As genes for different mammalian tRNAs are isolated, it would be possible to use site-directed in vitro mutagenesis methods (e.g., Shortle et al.[197]); the tRNA genes can be appropriately altered to suit individual experimental needs.

An alternative to this approach is true gene replacement therapy. The ability to conduct any gene replacement therapy involves introduction of genes into primary cells in vivo or in vitro followed by their reintroduction into the host. Cline et al.[198] and Mercola et al.[199] have reported experimental results in which they provide evidence for introduction and expression of mouse dhfr and HSV TK genes into mouse bone marrow cells. Confirmation

of these results would provide a basis for attempting additional gene transfer experiments into primary cells derived from animals for reintroduction.

Several investigators have examined the possibility of introducing genes into mouse embryos. Microinjection has been used as the choice method to accomplish this goal. A number of different DNA sequences have been successfully introduced into embryos and the mice that resulted from these embryos carried these sequences.[7-13,200-202] These exogenous sequences were found to be integrated and were detectable in a number of somatic tissues as well as the germ line. The presence of this DNA in the germ line was dramatically demonstrated by showing that they can be passed on from one generation to the next.[9,10]

The expression of exogenous genes in the transgenic mice has been tested. Wagner and colleagues[12] have presented evidence and claimed that an exogenously introduced globin gene is expressed in the transgenic mice. Wagner and Mintz[13] also presented similar evidence. Similar experiments in another laboratory did not result in successful expression of an exogenously introduced globin gene. Palmiter and colleagues have introduced a herpes thymidine kinase gene ligated to a metallothionein promoter and rat growth hormone gene ligated to the same promoter, and in both cases they found expression.[201,202] The question about tissue-specific expression of the genes is not yet answered. It may be possible that such tissue-specific expression of exogenous genes is necessary to provide a realistic basis for embryonic gene therapy. It is also possible that the proper developmental regulation of the exogenous genes is necessary to achieve true gene replacement and to avoid problems that may be associated with ectopic gene expression. Methods to achieve homologous recombination in mammalian cells are underway and information obtained from these experiments would help us understand mechanisms of somatic recombination and homologous recombination; in addition they would help pave the way for embryo manipulation. Some of the scientific and ethical problems associated with these developments are discussed by Williamson.[203]

B. Understanding Mechanisms of Development and Differentiation

If the foreign genes introduced into embryos are regulated in a developmentally specific manner, they may serve as excellent markers for studying cell development and differentiation. Of the several embryo microinjection experiments that are reported, only a few provide evidence for expression of the foreign genes. Additional experiments to determine if the foreign genes are developmentally regulated should yield valuable information.

Because exogenous DNA integrates at random within the recipient cell genome, it is to be expected that it acts as an inducer of mutation. Jaenisch et al.[204] recently reported that a retrovirus sequence introduced into a mouse embryo resulted in a developmental mutation. Once the mutant phenotype is identified, the availability of the pure exogenous DNA probe would help in isolation of the gene whose function must be considered essential for normal development. Strategies of this nature can be partially used to isolate genes which have no special features except their phenotypes which can be readily detected.

C. Mutagenesis

Gene transfer systems are already facilitating the study of mutagenesis in cultured cells. In vitro modifications of DNA on expression of genes are already described. It is now possible to conduct in vitro site-directed mutagenesis, and the effects of such mutations can be tested readily by transfection.

Perhaps a more important application of gene transfer is to introduce a gene or a plasmid whose expression can be selected in mammalian as well as bacterial cells, into mammalian cells and mutagenize such cells with an agent.

The gene or plasmid can be isolated and examined for the nature of the change which has made it a mutant. Such a strategy would permit deduction of the mode of action of a

large number of agents. Shuttle vectors should be considered most useful for these experiments. However, recent experimental results from Razzaque et al.[205] and Calos et al.[206] show that the shuttle vectors are subject to high frequency of mutations and DNA rearrangements even after short-term culture of recipient cells. Though these observations provide new avenues of research, it may be necessary to reevaluate the use of shuttle vectors for mutagenesis studies.

D. Protein Manufacture

The development of recombinant DNA techniques and the concomitant isolation of medically or industrially significant genes and bacterial expression vectors have made possible the production of mammalian proteins in large quantities using nonmammalian cell hosts. However, it is possible that certain proteins will be functional only if they are properly modified and such posttranslational modifications may be possible only in mammalian cells. The gene transfer systems and the modular vectors we have described earlier should make it possible to produce and isolate large quantities of appropriately modified proteins for industrial or therapeutic use.

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