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Altering Genotype and Phenotype by DNA-Mediated Gene Transfer

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When cultured mammalian cells are exposed to DNA, a small subpopulation stably integrate exogenous genes into their chromosomes in a form which is recognized by the replicative and transcriptional apparatus of the host cell. This process is known as transformation (*1*). The transforming elements can be maintained within the host genome for hundreds of generations and frequently express products which alter the phenotypes of the recipient cell. Since transformation in most cell populations is a rare event, identification of transformants requires the use of genes coding for either selectable or readily identifiable functions. Thus, DNA from viruses or eukaryotic cells has been used to transfer genes coding for growth transformation (*2*), thymidine kinase (TK) (*3-7*), adenine phosphoribosyltransferase (APRT) (*8*), and hypoxanthine-guanine phosphoribosyltransferase (HGPRT) (*9, 10*) to mutant cells deficient in these functions.

Transformation therefore provides an opportunity to alter the genotype of a cell by the stable introduction of new genetic information and to examine the expression of exogenous DNA sequences in the transformed host. We will discuss the application of transformation to four basic areas of eukaryotic genetics. (i) The integration of transforming elements into the chromosome, as well as their excision from the chromosome, may involve recombinational systems which reflect the capacity of a somatic cell to reorganize its genome. (ii) The ability to introduce specific wild-type and mutant genes into new cellular environments provides a system in which the functional significance of various features of DNA sequence organization can be studied in vivo. (iii) Transformation has facilitated the isolation of cellular genes coding for APRT and TK; for these two genes classical methods of molecular cloning dependent on messenger RNA (mRNA) enrichment are exceedingly difficult (*11, 12*). (iv) Transformation can be used to analyze the molecular nature of mutation and phenotypic variation in somatic cells.

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Viral Thymidine Kinase as a Model System

The development of a successful transformation system for the transfer of eukaryotic genes was initially dependent on the appropriate choice of three basic components: a source of DNA coding for a readily selectable biochemical function, a competent recipient cell deficient in this function, and a selection schema permitting the identification of the rare transformant. In our initial studies, we developed a model system to effect the isolation and transfer of a specific DNA fragment containing the thymidine kinase gene from the herpes simplex virus (HSV-1) genome (*4*). The choice of this system was dictated by several consid-

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erations. First, the viral genome is orders of magnitude less complex than the eukaryotic genome. This greatly enhances the prospect for successful transformation and allows the purification of active restriction fragments by size alone. Second, the 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 (*13, 14*). There exist cell lines deficient in TK with low rates of spontaneous reversion to the Tk^+ phenotype which can be used as recipients (*15*). Third, the *tk* gene is an ideal subject for mutational analysis because either the Tk^+ or the Tk^- phenotype can be selected under appropriate conditions. Fourth, the gene product, thymidine kinase, is a well-characterized viral protein of known function that is readily distinguishable from the cellular enzyme.

Treatment of mouse fibroblasts deficient in TK with HSV-1 DNA cleaved with Bam HI restriction endonuclease results in the appearance of numerous surviving colonies which stably express the Tk^+ phenotype. The enzyme expressed by these cells is immunologically and biochemically identical to the viral kinase and readily distinguishable from host murine TK. Through a series of electrophoretic fractionations in concert with transformation assays, we have isolated and cloned a fragment of 3.6 kilobase pairs (kbp) which contains the *tk* gene and which is competent in the biochemical transformation of mouse Ltk^- cells (*4*).

In this system, we obtain a transformation frequency of one colony per 10^6 cells on addition of 20 picograms of purified *tk* gene. Thus, one transformant is obtained for every 5×10^5 molecules of *tk* gene added to the culture. In the mammalian genome, a single-copy gene is present at about 1 part per million. If we extrapolate from the transformation efficiency observed for transfer of the viral *tk* gene and estimate the size of the haploid mouse genome to be 3.5 pg, we can expect to observe the transfer of specific cellular genes once per 10^6 cells per 20 micrograms of genomic DNA. Our present transformation systems therefore permit the transfer of single-copy cellular genes when total genomic DNA is used as donor. The DNA from Tk^+ cultured cells or tissues from various species of mammals and birds can be used to transfer TK activity to murine Ltk^- cells (*5*). The resultant TK activity in transformants derives from expression of donor DNA.

The generality of the transformation process has been demonstrated by the successful transfer of genes for HGPRT (*9, 10*), APRT (*8*), and a methotrexate-resistant dihydrofolate reductase (*16, 17*) with total cellular DNA used as donor. Perhaps the most efficient method for effecting DNA-mediated gene transfer, developed by Graham and van der Eb (*18*), involves the formation of a calcium phosphate–DNA coprecipitate, which is added directly to cell cultures. The method used to transfer these genes can, in principle, be applied to any gene for which conditional selection criteria are available. In practice, the efficiency of gene transfer can be expected to be a function of the recipient cell, the source of the gene being transferred, and the stringency of the selection criteria. For gene transfer to be readily detectable, it must occur at a frequency higher than the spontaneous rate of mutation of the recipient to the phenotype selected. The frequencies observed for transfer of the *tk* gene from vertebrate cellular DNA to Ltk^- cells range from 1×10^{-6} to 1×10^{-5}. This is also the frequency range observed for spontaneous mutation at many interesting loci in cultured somatic cells. Improvements in transformation efficiency or prior fractionation of donor DNA can be expected to extend the usefulness of this technique.

Summary. Transformation, or DNA-mediated gene transfer, permits the introduction of new genetic information into a cell and frequently results in a change in phenotype. The transforming DNA is ultimately integrated into a recipient cell chromosome. No unique chromosomal locations are apparent; different lines contain the transforming DNA on different chromosomes. Expression of transformed genes frequently results in the synthesis of new polypeptide products which restore appropriate mutant cells to the wild-type phenotype. Thus transformation provides an in vivo assay for the functional role of DNA sequence organization about specific genes. Transforming genes coding for selectable functions, such as adenine phosphoribosyltransferase or thymidine kinase, have now been isolated by utilizing transformation in concert with molecular cloning. Finally, transformation may provide a general approach to the analysis of complex heritable phenotypes by permitting the distinction between phenotypic changes without concomitant changes in DNA and functional genetic rearrangements.

Cotransformation with Nonselectable Genes

The transformation systems discussed so far have been restricted to transforming elements coding for selectable biochemical functions. By using the *tk* gene as a vector, it is possible to identify cells cotransformed with virtually any prokaryotic or eukaryotic gene (*19*). The experimental design we developed derives from studies of transformation in bacteria which indicate that only a subpopulation of cells is competent in transformation and those cells incorporate selectable as well as nonselectable DNA sequences (*20, 21*). If this is also true for animal cells, then biochemical transformants will represent a subpopulation of competent cells which are likely to integrate other unlinked genes at higher frequencies than the general population. This cotransformation system should allow the introduction and stable integration of virtually any defined gene into cultured cells. Ligation to either viral vectors or selectable biochemical markers is not required.

Cotransformation experiments were performed with the HSV *tk* gene as the selectable biochemical marker in the presence of various cloned prokaryotic and eukaryotic genes. The Tk^+ transformants were cloned and analyzed by blot hybridization (*22*) for cotransfer of additional DNA sequences. A representative analysis of a Tk^- rat liver cell cotransformed with a variant human growth hormone (hGH) gene (*23*) along with the viral *tk* gene as vector is shown in Fig. 1. The DNA from six cotransformants was analyzed for the presence of growth hormone sequences by blot hybridization. The results of these and additional experiments with several other genes permit the following conclusions: (i) The frequency of cotransformation is high; 80 percent of the Tk^+ transformants contain sequences homologous to the nonselectable gene. (ii) Most cotransformed sequences are in the high-molecular-weight DNA fraction within the cell. (iii) The number of integrated copies varies from one to more than 100 in independent clones. (iv) Some clones integrate virtually intact cotransformed genes, while others contain only small segments of these genes. (v) All the cloned eukaryotic genes we have studied can be introduced into mammalian cells with roughly equal efficiency. The properties

of the cotransformants appear to be independent of the cloned DNA sequence.

The stability of transformed genes in the chromosome, however, may depend on the nature of donor sequences. Cotransformed sequences such as ϕX maintain a stable genotype for many generations under selective pressure. Sequences with the potential for transposition such as retrovirus (*24*) reveal genotypic instability after transformation.

This vector system overcomes the limitations on the nature of donor DNA sequences and permits the introduction of any cloned gene into cultured cells. Detection of gene transfer with selectable markers such as TK, however, restricts transformation to available mutant recipients, which may not provide an appropriate environment for the expression of cotransformed genes. We therefore cotransformed cells with a dominant-acting drug resistance marker, the gene coding for a mutant dihydrofolate reductase (*25*), which renders cells resistant to high concentrations of methotrexate (*17*). The use of this gene or other more convenient drug resistance markers such as the bacterial *gpt* gene (*26*) as a vector in cotransformation systems may permit the transfer of virtually any genetic element into a variety of new cellular environments.

Fate of Transforming DNA

The addition of the *tk* gene as a calcium phosphate precipitate to cultured cells results in Tk^+ transformants which integrate other unlinked DNA sequences at high frequency. Molecular hybridization with highly radioactive DNA homologous to the *tk* vector as well as to cotransformed sequences permits an analysis of the ultimate fate of transforming elements in the DNA and chromosomes of transformed cells. Blot hybridization experiments with a *tk* probe (*27*) have shown that the *tk* gene is present at least once in all transformed clones examined, with many clones containing a single copy per chromosomal complement; the *tk* gene is integrated into high-molecular-weight nuclear DNA; and integration is not site-specific and occurs at different loci in the DNA of all transformants. Similarly, cotransformed genes integrate into high-molecular-weight DNA at different sites in all clones examined.

What are the genetic and physical relations between the selectable marker and cotransformed sequences? Cotransformed cells stably express the selectable biochemical marker for hundreds of generations if cells are maintained under selective pressure. Similarly, cotransformants constructed with ϕX maintain the ϕX DNA in several subclones for numerous generations without significant loss or translocation of information when maintained under selective conditions (*19*). However, in neutral medium the selectable phenotype is lost at frequencies which range from < 0.1 to 30 percent per generation. Perucho *et al.* (*28*) examined the *tk* and ϕX genotype in several independent Tk^- revertants selected in bromodeoxyuridine (BrdU), and in every instance reversion was associated with loss of the *tk* gene. Half of the revertants also lost all cotransformed ϕX sequences; in the remaining lines only a fraction of the original ϕX sequences persisted. This coordinate segregation of *tk* and ϕX DNA suggests genetic linkage of the vector and cotransforming sequences. Direct evidence for physical linkage of these sequences derives from the isolation of recombinant clones containing the *tk* gene immediately adjacent to cotransformed sequences. These data have led to the proposal (*28*) that nonhomologous, unlinked DNA sequences, through a series of multiple ligations, become covalently associated to generate a large concatameric structure. Maintenance of such a transforming element is likely to require the presence of only a single selectable gene. Although the recombination systems responsible for the construction of concatameric transforming elements are unknown, it is likely that large stretches of sequence homology are not essential in this process, since ϕX can be efficiently integrated adjacent to nonhomologous cotransforming DNA.

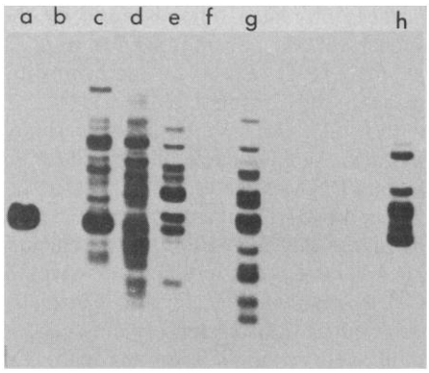

Fig. 1. Human growth hormone genes in cotransformed BRL cells. The BRL Tk^- cells were cotransformed with 1 μg of pTK, 1 μg of phGH (a 2.6-kbp Eco RI fragment containing a variant hGH gene inserted into pBR322) (*22*), and 20 μg of Ltk^- DNA as carrier. Surviving colonies in HAT medium were picked and grown into mass culture. High-molecular-weight DNA from several colonies was digested with Eco RI and electrophoresed on a 0.8 percent agarose gel. The DNA was denatured in situ, transferred to nitrocellulose, and hybridized with ^{32}P-labeled 2.6-kbp Eco RI fragment containing the variant hGH gene. Lanes represent (a) 250 pg of phGH digested with Eco RI, (b) 20 μg of BRL Tk^- DNA digested with Eco RI, and (c to h) 20 μg each of DNA from six independently isolated Tk^+ transformants digested with Eco RI.

Does this transforming element persist within the recipient cell as an autonomously replicating extrachromosomal unit, or is it stably integrated into a host chromosome? Perhaps the most direct approach to this problem involves in situ hybridization to detect the presence of donor DNA sequences in host metaphase chromosomes. The Buffalo rat liver (BRL) cells cotransformed with an hGH gene (Fig. 1) provide ideal candidates for this sort of analysis. First, cell lines have been constructed that contain 3 to 100 growth hormone genes, which overcomes the limitations of sensitivity frequently experienced with unique genes. Second, the BRL line is euploid, which allows precise localization of the transforming element within the chromosomal complement.

The 7-kbp hGH plasmid (phGH) used in the construction of the hGH transformants was nick-translated with ^{125}I-labeled deoxycytidine 5′-triphosphate (dCTP) to a specific activity of 8×10^8 counts per minute per microgram and was used as probe in annealing in situ to metaphase spreads of four transformants (*29*). Illustrative results with line BG-1, which contains about 100 copies of hGH sequences (see Fig. 1), are shown in Fig. 2. Examination of more than 50 spreads consistently revealed an intense cluster of grains restricted to the short arm of chromosome 11. No annealing was observed with the other homolog or with any chromosomes of the parental BRL Tk^- line. Analysis of three additional lines with as few as three copies of hGH sequences revealed discrete hybridization to a single chromosomal location.

This preliminary study indicates that in all four lines a large portion (if not all) of the transformed hGH sequences is maintained within a chromosome on the recipient cell. In each line, annealing is restricted to a single chromosomal site; only one of the homologs contains a transforming element. The findings are in accord with conclusions based on microcell hybrid experiments with a single transformed line (*30*). The presence of a single major site is consistent with the formation of a long concatenate consisting of most (if not all) donor DNA, which is ultimately inserted into the chromosome. We cannot exclude minor sites of insertion in other chromosomes

which do not generate a detectable annealing signal. Finally, within a given cell line, the site of insertion is invariant. Different lines, however, contain the transforming element on different chromosomes; the site of insertion is not restricted to a unique chromosome. It is therefore unlikely that either *tk* or hGH sequences are directing the site of insertion through homologous recombination with rat growth hormone genes.

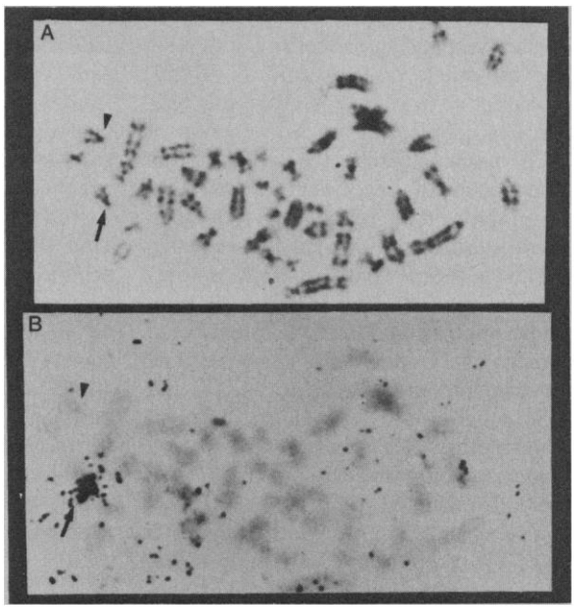

Fig. 2. Localization of hGH sequences to the chromosomes of cotransformed BRL cells. Metaphase chromosomes from a BRL cotransformant containing many copies of an hGH gene (see lane d in Fig. 1) were prepared and hybridized in situ as described by Henderson *et al.* (*56*). Plasmid phGH was nick-translated with ^{125}I-labeled dCTP to a specific activity of 8×10^8 cpm/μg; the concentration of ^{125}I-labeled growth hormone plasmid used as hybridization probe was 0.3 μg/ml. Slides were exposed for 3 days and photographed. (A) Banded metaphase spread before annealing, (B) identical spread after annealing with phGH. The arrows identify the two homologs of chromosome 11.

Expression of Transformed Genes

The introduction of cloned genes into animal cells offers an opportunity to study the expression of heterologous genes in a transformed host and therefore provides a system in which the functional significance of various features of DNA sequence organization can be examined in vivo. Successful transfer of biochemical markers requires transcription and appropriate RNA processing to generate a protein capable of altering the phenotype of the recipient cell. In early studies, we demonstrated that the DNA from various species of mammals and birds transfers either TK or APRT activity to mutant mouse cells with equivalent efficiency (*15*). These observations suggest that a murine cell is capable of transcribing and translating these gene sequences into a functional protein regardless of the vertebrate species of origin.

A number of laboratories have analyzed the expression of cotransformed genes which afford no apparent survival advantage to the recipient cell. In one study (*31*), Aprt$^-$ Tk$^-$ L cells were cotransformed with wild-type genomic DNA along with the cloned HSV *tk* gene. Selection was imposed for the Aprt$^+$ phenotype, and Aprt$^+$ transformants containing an intact *tk* gene were identified by blot hybridization. All cotransformants which contained the *tk* gene also expressed functional TK enzyme. In a similar study, a bacterial plasmid containing the early region of simian virus 40 (SV40) was cotransformed into L cells again, using the *tk* gene as vector (*32*). Half of the cotransformants analyzed synthesized T antigen, although expression within a given transformant was heterogeneous. Expression of these viral genes therefore occurs with high frequency in cotransformants irrespective of selective growth conditions. It should be noted that the mouse L cell recipient in these studies normally supports expression of these viral functions on viral infection.

In a second series of cotransformation experiments in mouse fibroblasts, cloned genes were introduced whose expression is usually restricted to specific differentiated cell types. We examined the expression of the rabbit β-globin gene in six independent transformants containing 1 to 20 copies of the cloned chromosomal globin gene (*33*). Rabbit globin transcripts were detected in two of these transformants at steady-state concentrations of five and two copies per cell. RNA blotting techniques indicated that in one transformed line the rabbit globin sequences were present in the cytoplasm as a polyadenylated, 9*S* species. Further analysis indicated that the two intervening sequences present in the original globin transcript were correctly processed. Surprisingly, 45 nucleotides present at the 5′ terminus of mature rabbit mRNA were absent from the β-globin RNA sequence detected in the cytoplasm of this transformed fibroblast. Appropriate processing of the rabbit β-globin gene was also observed in Tk$^+$ mouse cell transformants in which the globin and TK plasmids were ligated before transformation (*34*). Similar results were obtained when viral vectors were used to introduce the rabbit globin gene into monkey cells (*35, 36*). Taken together, these results suggest that nonerythroid cells from heterologous species contain the enzymes necessary to correctly process intervening sequences of a rabbit gene whose expression is usually restricted to erythroid cells.

The appearance of an aberrant yet unique 5′ terminus suggests the possibility of incorrect initiation of transcription in this mouse cell line. A similar analysis of cotransformants constructed by ligation of the *tk* and globin gene reveals transcripts with at least two discrete 5′ termini: RNA with 5′ termini indistinguishable from that of mature rabbit mRNA, and RNA with 5′ termini also lacking about 45 nucleotides (*34, 37*). Introduction of chick ovalbumin gene to mouse L cells results in the synthesis of immunoprecipitable ovalbumin protein (*38*). In a separate study, analysis of the RNA indicates that while all seven intervening sequences may be precisely removed from this avian gene by murine enzymes, the transcript is 650 nucleotides longer at the 5′ terminus than mature ovalbumin (*39*).

These studies suggest that if the 5′ terminus of mature mRNA also reflects the site of initiation of a primary transcript, murine fibroblasts do not consistently support correct initiation of cotransformed heterologous genes whose expression is usually restricted to other differentiated cell types. These problems may be addressed by the introduction of these genes into more appropriate environments. To this end, we have developed a cotransformation system which now permits the introduction of cloned globin genes into murine erythroleukemia cells.

Transient Expression of Transformed Genes

Only a small subpopulation of cells within a culture take up and stably express donor DNA. Preliminary experiments in our laboratory, in collaboration with H. Ploegh and J. Strominger, suggest that shortly after the addition of DNA a significantly greater proportion of the cells transiently express newly transformed functions. A mutant line of

human lymphoblastoid cells, Daudi, fails to express detectable quantities of β_2-microglobulin. In wild-type cells, this protein associates with the heavy chain of HLA histocompatibility antigens and is essential for the placement of the HLA-β_2 complex on the cell surface. Although Daudi synthesizes the appropriate heavy chains, it does not express HLA as a surface antigen (*40*). To assay for the transient expression of β_2-microglobulin, Daudi cells were exposed to wild-type human DNA and labeled 0, 24, 48, and 72 hours after transformation with ^{35}S-labeled methionine. Labeled extracts were immunoprecipitated with a monoclonal antibody directed against the HLA-β_2 complex, and the precipitates were analyzed on sodium dodecyl sulfate (SDS) polyacrylamide gradient gels. No synthesis of β_2-microglobulin can be detected in control extracts of Daudi. Immunoprecipitable protein comigrating with β_2-microglobulin begins to appear 12 hours after transformation, reaches a maximum concentration at 48 hours, and is substantially reduced by 72 hours.

These experiments do not permit an accurate determination of the proportion of Daudi cells expressing β_2-microglobulin after transformation. Assuming that, on average, each cell that synthesizes β_2 does so in amounts closely approximating those observed in wild-type cells, we calculate from the total amount of β_2 synthesized that about 0.5 percent of the cells express this protein. Thus, this population of mutant human lymphoid cells transiently expresses a unique gene when exposed to genomic DNA from wild-type cells. It is likely that the unstable expression of β_2-microglobulin results from abortive transformation events. Abortive transformation, observed frequently on transfection with viral DNA (*41*), may reflect a transient and unstable condition in which transforming elements are present within the cell in an unintegrated form, perhaps dissociated from chromosomal elements which could afford stability. These experiments further indicate that detection of transformants can be effected by the introduction of selectable markers or of genes coding for readily identifiable products.

Isolation of Transforming Genes

Isolation of several mammalian genes has been accomplished by a now classical experimental design, with complementary DNA (cDNA) cloning and subsequent screening of recombinant phage or plasmid libraries (*42–44*). For the most part, genes isolated in this manner are represented in relative abundance in mRNA populations. The mRNA's coding for numerous enzymatic functions, however, need be present at only five to ten copies per cell to maintain the required steady-state enzymatic activity (*45*). Isolation of cDNA clones for these mRNA's requires 10^4- to 10^5-fold enrichment and is likely to be exceedingly difficult. Alternative experimental approaches have been designed which should permit the isolation of any gene which can be stably introduced into a recipient cell (*11, 12, 46*).

Two related schemes successfully used to isolate genes coding for TK and APRT are shown in Fig. 3. We will consider the isolation of the hamster *aprt* gene as an example. Cleavage of hamster genomic DNA with Hind III leaves the *aprt* gene functionally intact, since Hind III-digested DNA efficiently transfers hamster APRT activity to Aprt$^-$ mouse L cells. Hind III-cleaved hamster DNA was ligated to a molar excess of Hind III-cleaved plasmid pBR322 DNA to generate a collection of hybrid molecules, so that the *aprt* gene is linked to plasmid sequences. This DNA is used to construct primary Aprt$^+$ transformants which have integrated multiple plasmid sequences because of cotransformation of pBR322, which is abundant in donor DNA. The DNA from primary transformants is transferred to recipient Aprt$^-$ cells to eliminate extraneous plasmid and construct secondary Aprt$^+$ transformants containing the hamster *aprt* gene linked to a single plasmid sequence. At this point, one of two divergent experimental pathways for gene isolation may be chosen. If the plasmid replicative origin and ampicillin resistance gene remain intact in the DNA of secondary transformants, this DNA can now be treated with an endonuclease which does not cleave either pBR322 or the *aprt* gene. The resultant fragments are then circularized with T4 DNA ligase. Transformation of *Escherichia coli* with these circular molecules and subsequent growth in ampicillin selects for an *aprt*-pBR322 recombinant. This approach has been successfully employed to isolate the chicken *tk* gene (*11*).

We have adopted an alternative scheme which does not rely on the maintenance of intact plasmid function through passage in animal cells (*12*). The DNA from secondary transformants is subjected to partial cleavage with Eco RI. Fractions containing 20 kbp are collected and used to construct a library of 10^6 independent recombinant phage in the λ vector Charon 4A. This library of recombinant phage is screened with highly radioactive pBR322 DNA as a hybridization probe.

We screened 6×10^5 plaques to identify a single clone containing plasmid sequences. This phage, λH*aprt*-1, when added to Aprt$^-$ cells, either in the form of intact phage particles or as naked DNA, generates Aprt$^+$ surviving colonies at a

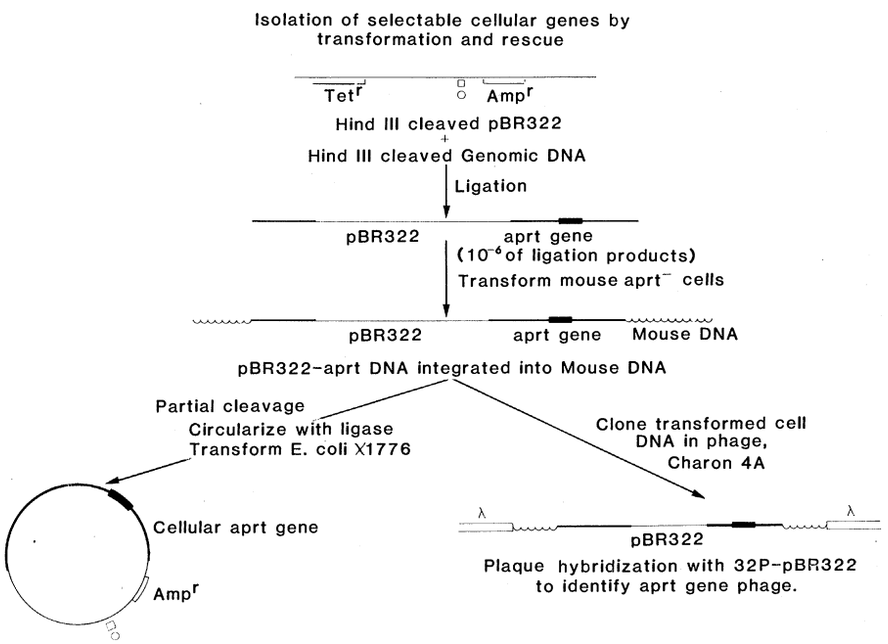

Fig. 3. Scheme for the isolation of transformed genes. Thin lines represent pBR322 DNA; O, the origin of replication; thicker lines, hamster genomic DNA; dark rectangles, hamster *aprt* gene; and wavy lines, mouse genomic DNA.

frequency of one colony per 400 pg per 5×10^5 cells. This efficiency of transformation is five to six orders of magnitude greater than that obtained with total genomic DNA and is equivalent to that observed with the purified viral *tk* gene.

More direct evidence that λH*aprt*-1 carries the hamster *aprt* gene derives from an analysis of the APRT activity in transformed clones. Isoelectric focusing in polyacrylamide gels clearly distinguishes murine and hamster APRT activity. Five transformed clones obtained after transfer with λH*aprt*-1 all express APRT activity with an isoelectric point characteristic of hamster (Fig. 4). Thus, the transformants we obtain do not result from reversion or reactivation of the murine gene, but from transfer of a cloned hamster *aprt* gene.

λH*aprt*-1 contains an insert of 17.5 kbp of eukaryotic DNA including the marker pBR322 sequences. Preliminary mapping data combined with transformation localizes the functional *aprt* gene to an 8-kbp Hind III fragment. Blot hybridizations indicate that this fragment is present in wild-type hamster DNA. Furthermore, transformed clones all contain new fragments homologous to the 8-kbp *aprt* sequence.

This experimental design, involving transformation in concert with molecular cloning, should in theory permit the isolation of any gene coding for selectable or identifiable functions for which DNA-mediated gene transfer can be effected.

Mutation of Transformed Genes

Mutation in higher eukaryotes can be analyzed by the identification or selection of variant proteins. The molecular nature of the mutation can frequently be deduced from the variant protein sequence. Molecular analysis of mutants deficient in a specific polypeptide product, however, requires the isolation of specific genes, which may then be utilized as probes for sequence alterations within the DNA. This approach has been used to characterize a number of globinopathies in man (*47*) and to analyze mutations in viral-infected cells (*48*). The viral *tk* gene is ideal for this sort of analysis since it has been cloned in bacterial plasmids and the Tk^+ and Tk^- phenotypes are readily selectable under appropriate growth conditions.

Analysis of mutation of transformed genes also provides an opportunity to examine mutation of a haploid gene present in an autosome (*49*). Mutation to the recessive phenotype for diploid autosomal markers usually requires two mutational events. If one of these events is "catastrophic," involving chromosome loss or extensive rearrangement, a second event of this magnitude occurring in the homolog may generate lethals which are lost from the population and therefore excluded from analysis. Introduction of a single selectable gene into one homolog of a diploid pair eliminates this experimental bias and permits quantitation and characterization of all types of mutational events.

If maintained under selective pressure, Tk^+ transformants stably express the *tk* gene for hundreds of generations. On removal of selective pressure, Tk^- revertants are obtained which fall into two discrete classes: a stable class that rereverts to the Tk^+ phenotype only rarely (frequency $< 10^{-6}$) and an unstable class that rereverts at unusually high frequencies ($> 10^{-2}$). Thus, sets of mutants can be isolated that switch phenotype from Tk^+ to Tk^- to Tk^+ and so on, with each switch occurring at a frequency greater than 1 percent.

The most prevalent Tk^- mutants observed do not rerevert to the Tk^+ phenotype since they result from deletion of most, if not all, of the transformed *tk* gene. Blot hybridization to one such mutant (K_2B_7, Fig. 6B) with *tk* probes shows no evidence of hybridizing fragments.

We analyzed the TK phenotype and genotype of two transformants, K_1 and K_2, obtained after transformation with a 5-kbp Kpn fragment of HSV, which switch phenotype from Tk^+ to Tk^- to Tk^+ (*50*). These Tk^+ transformants are phenotypically stable under selective pressure, but when plated into counterselective medium (BrdU) they generate Tk^- colonies at a frequency of 10 percent for K_1 and 1 percent for K_2. The revertant cell lines are stable in BrdU, but when they are plated in hypoxanthine-aminopterin-thymidine (HAT), surviving colonies are again observed to continue the switching cycle (Table 1).

The pattern of phenotypic switching may be caused by phenotypic changes transmissible to progeny in the absence of concomitant changes in DNA. Alternatively, switching may result from frequent but reversible changes in DNA. To distinguish between these alternatives, we performed a series of transformation experiments with purified DNA from transformant, revertant, and rerevertant cell lines. If reversion to the Tk^- phenotype occurs without genetic change, DNA from these Tk^- cells should transfer the *tk* gene to Ltk^- recipients as effi-

Table 1. Cloning and transformation frequencies of unstable transformed cell lines.

Cell line*	Relative cloning efficiency†		Relative Tk transformation efficiency‡
	HAT	BrdU	
K_1	0.98	0.13	1.0
K_1B_6	0.50	0.52	0.21
K_1B_6N	< 0.0001	0.87	0
$K_1B_6H_1$			0.67
$K_1B_6H_2$	0.03	0.47	0.47
K_2	0.99	0.02	1.0
K_2B_6	0.01	0.97	0.05
$K_2B_6H_4$	0.79	0.08	1.7
K_2B_7	< 0.0001	0.87	

*K_1 and K_2 refer to two independent Tk^+ transformants. The Tk^- revertant clones derived from both K_1 and K_2 are designated K_1B_n and K_2B_n; the Tk^+ rerevertants are designated $K_1B_nH_n$ and $K_2B_nH_n$; K_1B_6N is a variant of K_1B_6 which emerged on long-term culture. †We plated 100 to 10,000 cells in triplicate into either neutral medium (hypoxanthine or thymidine) or selective medium (HAT or BrdU). ‡Relative transformation efficiency reflects the frequency of transformation with Tk^- revertant or Tk^+ rerevertant DNA compared with the frequency obtained with original Tk^+ transformants. Results are averages for five to ten dishes, each containing 80 μg of DNA. The Tk transformation efficiency was 0.2 to 0.3 colony per microgram of DNA for K_1 and 0.1 to 0.2 colony per microgram of DNA for K_2.

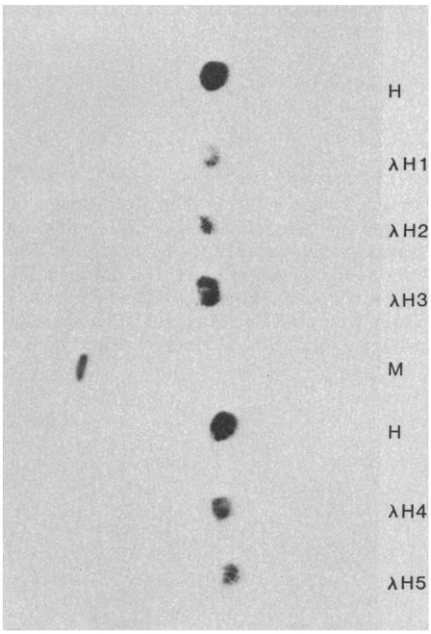

Fig. 4. Identification of APRT activity in transformants. The Ltk^- $Aprt^-$ cells were treated with 10^9 phage particles designated λH*aprt*-1 in the presence of 20 μg of $Aprt^-$ carrier DNA. Colonies surviving in azaserine-adenine were picked and grown into mass culture. Supernatants from high-speed centrifugation of homogenates of wild-type and transformed cells were focused on 4.5 percent acrylamide gels. For development of enzyme activity [2-^{3}H]adenine was used, and the product was blotted onto polyethyleneimine cellulose and localized by fluorography (*8*). Abbreviations: *H*, Chinese hamster ovary (CHO) cell extract; *M*, mouse L cell extract; and λ*H1* to λ*H5*, five transformants obtained on transfer of λH*aprt*-1 to Ltk^- $Aprt^-$ mouse cells.

ciently as DNA from Tk$^+$ cells. On the other hand, if alterations in DNA are associated with the phenotypic switch, transfer would be far less efficient with DNA from the Tk$^-$ revertants. As expected, DNA from the Tk$^+$ transformants or Tk$^+$ rerevertants is an efficient donor of the *tk* gene, generating 10 to 30 Tk$^+$ colonies per 80 μg of DNA per 5 $\times$ 10^5 cells. In contrast, DNA from two independent revertants generated 50- to 100-fold fewer colonies than were observed with DNA from Tk$^+$ lines (Table 1). The potential inhibition of DNA transfer by BrdU substitution in revertant DNA was avoided by growth of revertant lines in neutral medium (Dulbecco's modified Eagles medium) for five generations before isolation.

Two observations suggest that a specific but reversible genetic alteration of the *tk* gene accompanies phenotypic switching. The efficiency of gene transfer with DNA from revertants is roughly correlated with the frequency at which Tk$^-$ revertants plate in HAT medium (Table 1). Furthermore, DNA from all cell lines efficiently transfers the cellular *aprt* gene to recipient Aprt$^-$ cells without regard to the TK phenotype.

We analyzed the organization of the viral *tk* gene sequences in cell sets consisting of transformant, revertant, and rerevertants by restriction endonuclease mapping and blot hybridization. A restriction map of the donor fragment along with maps of the *tk* gene in DNA from transformed cell sets is shown in Fig. 5. Blotting data obtained with the enzyme Bgl II, which cleaves only once within the donor fragment, are presented in Fig. 5. The data clearly indicate that the *tk* gene is integrated at a single site in transformed cell DNA. With one exception, mapping data at more than 30 restriction enzyme sites are invariant from transformant to revertant, and in all instances rerevertants are identical to the revertant from which they were derived (Figs. 5 and 6). Thus, within the limitations of this analysis, four of the five unstable Tk$^-$ revertants do not reveal rearrangements. These studies are far from exhaustive. Transpositions or inversions involving large blocks of DNA beyond the boundaries analyzed, as well as smaller rearrangements, may not have been detected. Identification of such rearrangements will require fine-structure analysis of cloned segments of cellular DNA containing these *tk* genes.

In one mutant, K_1B_5, changes in sequence organization have occurred at both sides of the integrated viral fragment, suggesting that in this revertant the *tk* gene has rearranged from one chromosomal location to another. Cleavage of DNA from K_1B_5 with Bgl II generates two annealing fragments, as expected for an enzyme which cuts once within the *tk* gene, but both fragments differ from those obtained on digestion of the original Tk$^+$ transformant (Figs. 5 and 6).

The organization of *tk* sequences in K_1B_5 (Fig. 5) could result from two deletions, each extending into the donor *tk* fragment, or from a translocation of the *tk* gene to a new chromosomal location. It remains possible, however, that K_1B_5 does not result from a rearrangement but merely represents a rare variant present in the original clone of K_1 cells which

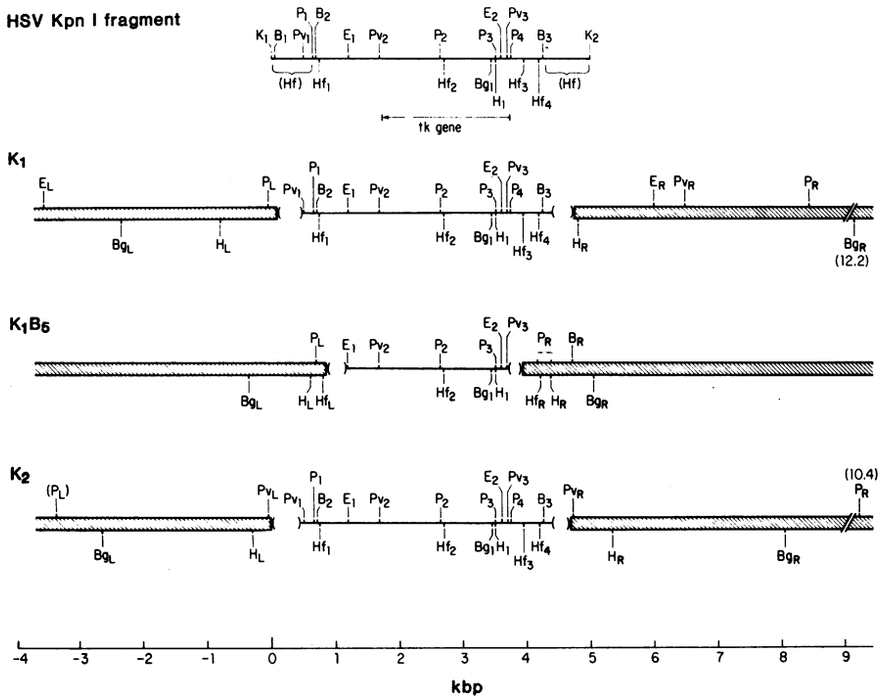

Fig. 5. Restriction endonuclease maps of the HSV *tk* gene region in the 5.0-kbp Kpn I fragment from HSV, the transformant K_1, the revertant K_1B_5, and the transformant K_2. Viral sequences are represented by thin solid lines and nonviral flanking DNA sequences by hatching. The region of the breakpoint between these sequences is bounded by parentheses. The functional *tk* gene (arrow) resides between Pvu II sites 2 and 3 (see text). Restriction sites whose position is uncertain are shown in parentheses. Numbers in parentheses beside restriction sites are map distances in kilobase pairs. Abbreviations: *B*, Bam HI; *Bg*, Bgl II; *E*, Eco RI; *H*, Hinc II; *Hf*, Hinf I; *K*, Kpn I; *P*, Pst I; and *Pv*, Pvu II.

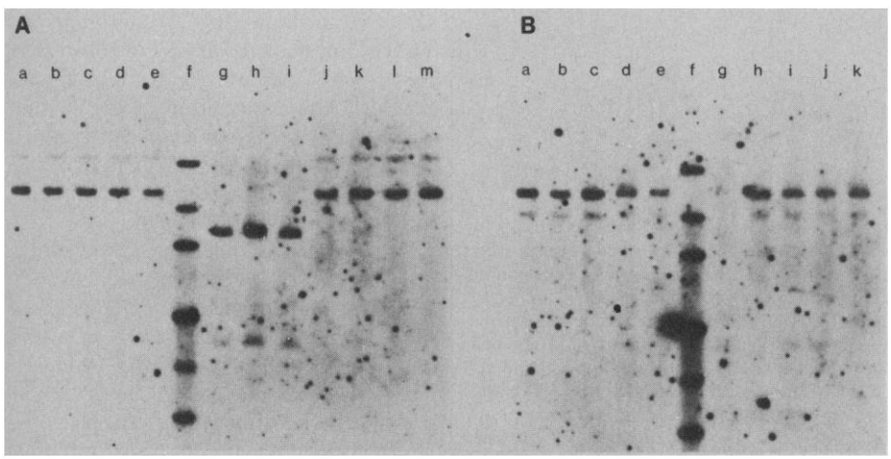

Fig. 6. Bgl II profiles of the *tk* fragment in sets of transformant, revertant, and rerevertant cell lines. (A) K_1 family: lanes a to e, K_1, K_1B_4, $K_1B_4H_1$, $K_1B_4H_2$, $K_1B_4H_3$; lanes g to i, K_1B_5, $K_1B_5H_2$, $K_1B_5H_3$; lanes j to m, K_1B_6N, K_1B_6, $K_1B_6H_1$, $K_1B_6H_2$; lane f, markers (8.0, 5.0, 3.6, 2.0, 1.3, and 0.88 kbp). (B) K_2 family: lanes a to e, K_2, K_2B_6, $K_2B_6H_4$, $K_2B_6H_5$, $K_2B_6H_6$; lane g, K_2B_7; lanes h to k, K_2B_8, $K_2B_8H_4$, $K_2B_8H_5$, $K_2B_8H_6$; lane f, markers as in (A). The lower band in lane e is readily visible in the original autoradiograph. See legend of Table 1 for explanation of cell lines.

was subcloned on counterselection in BrdU. If a rearrangement did occur to generate K_1B_5 its functional consequences remain unclear, since Tk^+ rerevertants of this clone do not reveal further rearrangements. It is possible that numerous alternative pathways exist that facilitate phenotypic switching. In this instance, a positional change may result in the Tk^- phenotype, and a second unrelated event may now generate a Tk^+ state without positional change.

We also examined the pattern of DNA methylation about the *tk* gene sequences in cell sets. Recent studies suggest a general pattern of methylation, correlated with gene activity, such that transcription is associated with undermethylation of precise sites within defined genes (*51–53*). We used restriction enzymes whose recognition sites include the methylatable sequence pCpG to examine multiple sites in and around the *tk* gene. With the one exception discussed below, the *tk* gene in Tk^+ transformants and Tk^- mutants remains unmodified. Therefore, a wave of hypermethylation is not associated with genetic inactivity of the *tk* gene.

We identified one interesting Tk^- variant which emerged in long-term culture, exhibiting extensive methylation of the *tk* gene. The Tk^- mutant, K_1B_6, characterized within a month of its isolation, reverts to the Tk^+ phenotype at high frequency and is not methylated within the *tk* gene. During long-term culture in BrdU, a variant emerged which does not rerevert to the Tk^+ phenotype at measurable frequencies and is modified at many pCpG sites within the *tk* gene. In this clone, extensive methylation is associated with a nonreverting Tk^- phenotype.

Conclusions

Addition of DNA to cultured mammalian cells results in the transformation of a small subpopulation of cells which stably express the newly introduced genetic information. The transforming element consists not of single gene sequences but of an array of several donor sequences ligated to form a large concatameric unit. Physically unlinked donor genes are genetically and physically linked in the transformed cell (*28, 29*). The fate of the transforming element ultimately involves integration into a host chromosome. No unique chromosomal locations are apparent; different lines contain the transforming DNA on different chromosomes. It is likely that two discrete recombinational events are associated with stable transformation. The first is end-to-end ligation of nonhomologous duplexes (as in plasmid to genomic DNA) to generate a concatenate. The second, perhaps related event facilitates integration of the transforming DNA into the chromosome. In yeast the site of insertion of transforming sequences is most frequently directed by homology between donor DNA and the host genome (*54*). In mammalian cells, recombination may occur between nonhomologous sequences. The mammalian genome is about 200 times larger that that of yeast, which serves to dilute potential sites of homology and may thereby increase the ratio of nonhomologous to homologous recombinational events. Alternatively, it is possible that repetitive sequences within the transforming element direct its insertion at one of many homologous loci within the mammalian chromosome.

The potential usefulness of transformation in the study of eukaryotic gene expression will depend on its generality. We demonstrated that cells transformed with selectable genes also integrate other physically unlinked genes at high frequency. The demonstration of physical and genetic linkage of cotransformed sequences in the chromosome of the recipient offers a logical explanation for the phenomenon (*28*). The use of cloned drug resistance markers (*26*) in cotransformation experiments can be expected to expand the repertoire of recipient cells and ultimately permit the introduction of any cloned gene into virtually any cultured cell.

Transformation provides an in vivo assay for the functional role of specific DNA sequences. The transfer of genes coding for several enzymatic functions results in synthesis of functional polypeptides. Transformation of fibroblasts with cloned genes whose expression is usually restricted to other differentiated tissues generates RNA transcripts which are correctly processed but frequently reveal aberrant 5′ termini. These results suggest two interesting and perhaps related questions: Why are these tissue-specific genes transcribed in the fibroblast, a cell in which the endogenous counterpart genes are inactive, and why is initiation so frequently inappropriate? Aberrant transcription of cotransformed genes may result from gene dosage effects or position effects, among several other alternatives. The introduction of tissue-specific genes into more appropriate environments may provide information to distinguish between the alternatives.

We have described two related experimental approaches involving transformation in concert with molecular cloning that permit the isolation of genes which can be stably introduced into cultured cells. To date, this technique has led to the isolation of cellular *tk* and *aprt* genes, but extension of the approach to other selectable or immunologically identifiable genes may lead to the isolation of a host of cellular genes not readily obtainable by any other available technologies. Analysis of mRNA populations suggests the presence of a discrete set of genes whose transcripts are only rarely represented in mRNA; a second set of genes generates transcripts abundantly represented in mRNA. Structural comparisons of those gene sets may afford an explanation for their striking functional differences.

Finally, we have begun to examine the molecular events responsible for mutation and phenotypic variation in transformed cells. The frequency of reversion of *tk* transformants to the Tk^- phenotype is far greater than that observed for other cellular haploid genes (*55*). Furthermore, reversion most frequently results from deletion of the *tk* gene. It is not clear whether these properties are distinctive of transformed genes or reflect frequent catastrophic events acting on autosomal hemizygous genes. One class of Tk^- mutants maintain the *tk* gene and switch phenotypes from Tk^+ to Tk^- to Tk^+ and so on, in a manner reminiscent of phenotypic changes associated with reversible genetic rearrangements in prokaryotes and lower eukaryotes. Although our data suggest that the Tk switching results from heritable but unstable alterations in DNA, mapping data to date do not reveal consistent changes in sequence organization about the *tk* gene in transformants and revertants. Transformation may provide a general approach to the analysis of complex heritable phenotypes by permitting the distinction between self-reinforcing phenotypic changes without concomitant changes in DNA and functional genetic rearrangements.

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Expression of a Bacterial Gene in Mammalian Cells

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The most powerful tools now available for studying the molecular anatomy of eukaryote genes and chromosomes, particularly those of higher vertebrates, are restriction enzymes, simple physical methods for separating and visualizing DNA molecules, molecular cloning, and rapid DNA sequencing. In just a few years, the application of these techniques has produced a qualitative change in our views of gene structure and organization in mammalian and other eukaryote organisms. Such newly coined terms as gene libraries, split genes, pseudogenes, and transposons and picturesque references to "shotgunning," or even "walking and jumping along chromosomes," reflect this revolution and enrich the lexicon of modern genetics. Solving the nucleotide sequence of the chromosomal locus encoding the entire human β-globin-like gene cluster, a region encompassing about 65 kilobase pairs (kbp) (*1*), would have been considered visionary 10 years ago, but now that prospect looms on the horizon as a feasible undertaking.

Nevertheless, in spite of this impressive progress it is worth considering whether knowing the molecular anatomical details of genes can, by itself, explain the subtleties of gene expression and regulation during growth and development. Put another way, can we deduce the mechanisms of transcription initiation, splicing, and polyadenylation from the nucleotide sequences of isolated genes? Can we expect to learn from the nucleotide sequence of the human γ- and β-globin genes why the former is expressed only during fetal life and the latter only in adulthood? Most likely not; at least not without an assay for the biological activity of the genes in question.

About 10 years ago we began to consider how the biological activity of isolated genes could be assayed. That interest coincided with another preoccupation concerned with devising a virus-mediated transducing system for cultured mammalian cells. The overlap of these two interests culminated in a general approach for introducing isolated genes and their modified derivatives into the genomes of cultured mammalian cells (*2*). Our immediate goal was, and still is, to characterize the physical state, expression, and regulation of the new genes in their transduced hosts.

Because bacteriophages had proved to be so versatile for transducing genes into bacterial cells (*3*), simian virus 40 (SV40), a mammalian virus, was adopted as the vector to mediate the gene transfer. SV40 was chosen because its minichromosome propagates vegetatively or becomes stably integrated into selected host cell genomes. SV40 was also attractive because its genes and their corresponding functions had been identified and experiments were under way to map the genes to specific regions of the virus's DNA. Subsequently, the entire 5243-base pair (bp) sequence of the circular viral DNA was solved (*4, 5*), and

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