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# Transformation of Mammalian Cells with Genes from Procaryotes and Eucaryotes

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## Summary

**We have stably transformed mammalian cells with precisely defined procaryotic and eucaryotic genes for which no selective criteria exist. The addition of a purified viral thymidine kinase (tk) gene to mouse cells lacking this enzyme results in the appearance of stable transformants which can be selected by their ability to grow in HAT. These biochemical transformants may represent a subpopulation of competent cells which are likely to integrate other unlinked genes at frequencies higher than the general population. Co-transformation experiments were therefore performed with the viral tk gene and bacteriophage ΦX174, plasmid pBR322 or the cloned chromosomal rabbit β-globin gene sequences. $Tk^+$ transformants were cloned and analyzed for co-transfer of additional DNA sequences by blot hybridization. In this manner, we have identified mouse cell lines which contain multiple copies of ΦX, pBR322 and the rabbit β-globin gene sequences. The ΦX co-transformants were studied in greatest detail. The frequency of co-transformation is high: 15 of 16 $tk^+$ transformants contain the ΦX sequences. Selective pressure was required to identify co-transformants. From one to more than fifty ΦX sequences are integrated into high molecular weight nuclear DNA isolated from independent clones. Analysis of subclones demonstrates that the ΦX genotype is stable through many generations in culture. This co-transformation 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.**

## Introduction

Specific genes can be stably introduced into cultured cells by DNA-mediated gene transfer. The rare transformant is usually detected by biochemical selection. In this manner, we have isolated cells transformed with a variety of cellular and viral genes coding for selectable biochemical markers (Wigler et al., 1977, 1978, 1979). The isolation of cells transformed with genes which do not code for selectable markers, however, is problematic since current transformation procedures are highly inefficient. This paper demonstrates the feasibility of co-transforming cells with two physically unlinked genes. Co-transformed cells can be identified and isolated when one of these genes codes for a selectable marker. We have used a viral thymidine kinase gene as a selectable marker to isolate mouse cell lines which contain the tk gene along with either bacteriophage ΦX174, plasmid pBR322 or the cloned rabbit β-globin gene sequences stably integrated into cellular DNA. The introduction of cloned eucaryotic genes into animal cells may provide a means for studying the functional consequences of DNA sequence organization.

## Results

### Experimental Design

The addition of the purified thymidine kinase (tk) gene from herpes simplex virus to mutant mouse cells lacking tk results in the appearance of stable transformants expressing the viral gene which can be selected by their ability to row in HAT (Maitland and McDougall, 1977; Wigler et al., 1977). To obtain co-transformants, cultures are exposed to the tk gene in the presence of a vast excess of a well defined DNA sequence for which hybridization probes are available. $Tk^+$ transformants are isolated and scored for the co-transfer of additional DNA sequences by molecular hybridization.

### Co-transformation of Mouse Cells with ΦX174 DNA

We initially used ΦX DNA in co-transformation experiments with the tk gene as the selectable marker. ΦX replicative form DNA was cleaved with Pst I, which recognizes a single site in the circular genome (Figure 1) (Sanger et al., 1977). 500 pg of the purified tk gene were mixed with 1–10 μg of Pst-cleaved ΦX replicative form DNA. This DNA was then added to mouse $Ltk^-$ cells using the transformation conditions previously described (Wigler et al., 1979). After 2 weeks in selective medium (HAT), $tk^+$ transformants were observed at a frequency of one colony per $10^6$ cells per 20 pg of purified gene. Clones were picked and grown into mass culture.

We then asked whether $tk^+$ transformants also contained ΦX DNA sequences. High molecular weight DNA from the transformants was cleaved with the restriction endonuclease Eco RI, which recognizes no sites in the ΦX genome. The DNA was fractionated by agarose gel electrophoresis and transferred to nitrocellulose filters, and these filters were then annealed with nick-translated $^{32}P$-ΦX DNA (blot hybridization) (Southern, 1975; Botchan, Topp and Sambrook, 1976; Pellicer et al., 1978). These annealing experi-

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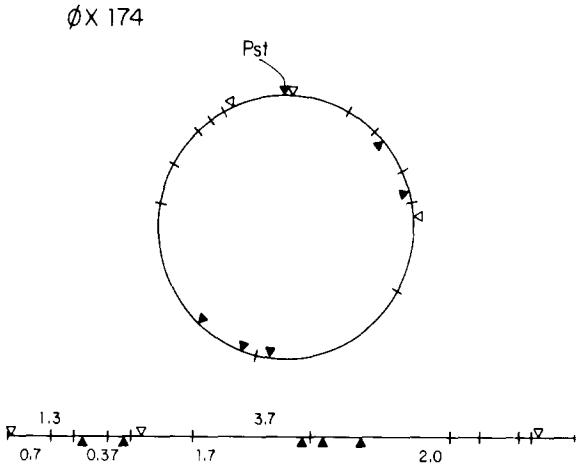


Figure 1. Cleavage Map of the ΦX174 Genome

Cleavage sites for the restriction endonucleases Pst I, Hpa I (▽), Hpa II (▼) and Hae III (I) are shown for circular RFI and Pst I-linearized ΦX174 DNA (Sanger et al., 1977). The numbers above the line refer to the sizes of the internal Hpa I fragments in kbp, while those below the line refer to the sizes of the Hpa II fragments.

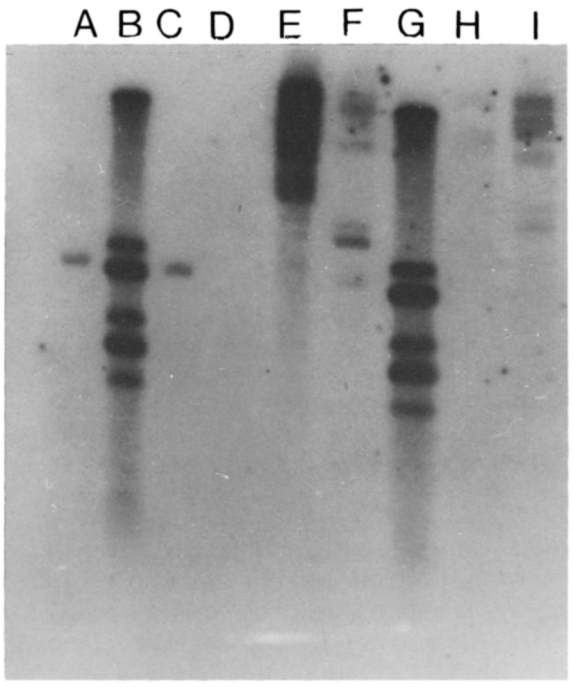


Figure 2. Identification of ΦX Sequences in Cells Transformed with ΦX174 DNA and the HSV tk Gene

Ltk$^-$ aprt$^-$ cells were transformed with ΦX174 DNA and the HSV tk gene using salmon sperm DNA as carrier. Tk$^+$ transformants were selected by growth in HAT, cloned and grown into mass culture in HAT. High molecular weight DNA was extracted from seven independently isolated clones; 15 μg of DNA from each were digested with Eco RI, electrophoresed through 1% agarose gels, denatured in situ and transferred to nitrocellulose filters which were then annealed with $^{32}$P–ΦX174 DNA (5 × 10$^6$ cpm/μg) to identify co-transformants. Lanes B and G are Eco RI digests of $^{32}$P-adenovirus 2 DNA; the six bands are 20.3, 4.2, 3.6, 2.6, 2.2 and 1.8 kbp. Lanes A, C, D, E, F, H and I are the seven independently isolated clones ΦX1–7, respectively. Only clone ΦX3 (lane D) lacks detectable ΦX sequences.

ments (Figure 2) demonstrate that six of the seven transformants had acquired bacteriophage sequences. Since the ΦX genome is not cut by the enzyme Eco RI, the number of bands observed reflects the minimum number of eucaryotic DNA fragments containing information homologous to ΦX. The clones contain variable amounts of ΦX sequences. Clones ΦX1 and ΦX2 (Figure 2, lanes A and C) reveal a single annealing fragment which is smaller than the ΦX genome. In these clones, therefore, only a portion of the transforming sequences persists. In lane D, we observe a tk$^+$ transformant (clone ΦX3) with no detectable ΦX sequences. Clones ΦX4, 5, 6 and 7 (lanes E, F, H and I) reveal numerous high molecular weight bands which are too closely spaced to count, indicating that these clones contain multiple ΦX-specific fragments. These experiments demonstrate co-transformation of cultured mammalian cells with the viral tk gene and ΦX DNA.

### Selection Is Necessary to Identify ΦX Transformants

We next asked whether transformation with ΦX DNA was restricted to the population of tk$^+$ cells or whether a significant proportion of the original culture now contained ΦX sequences. Cultures were exposed to a mixture of the tk gene and ΦX DNA in a molar ratio of 1:2000 or 1:20,000. Half of the cultures were plated under selective conditions, while the other half were plated in neutral media at low density to facilitate cloning. Both selected (tk$^+$) and unselected (tk$^-$) colonies were picked, grown into mass culture and scored for the presence of ΦX sequences. In this series of experiments, eight of the nine tk$^+$ selected colonies contained phage information (Figure 3). As in the previous experiments, the clones contained varying amounts of ΦX DNA. In contrast, none of fifteen clones picked at random from neutral medium contained any ΦX information (data not shown). Thus the addition of a selectable marker facilitates the identification of those cells which contain ΦX DNA.

### ΦX Sequences Are Integrated into Cellular DNA

Cleavage of DNA from ΦX transformants with Eco RI (Figure 2) generates a series of fragments which contain ΦX DNA sequences. These fragments may reflect multiple integration events. Alternatively, these fragments could result from tandem arrays of complete or partial ΦX sequences which are not integrated into cellular DNA. To distinguish between these possibilities, transformed cell DNA was cut with Bam HI or Eco RI, neither of which cleaves the ΦX genome. If the ΦX DNA sequences were not integrated, neither of these enzymes would cleave the ΦX fragments. Identical patterns would be generated from undigested DNA

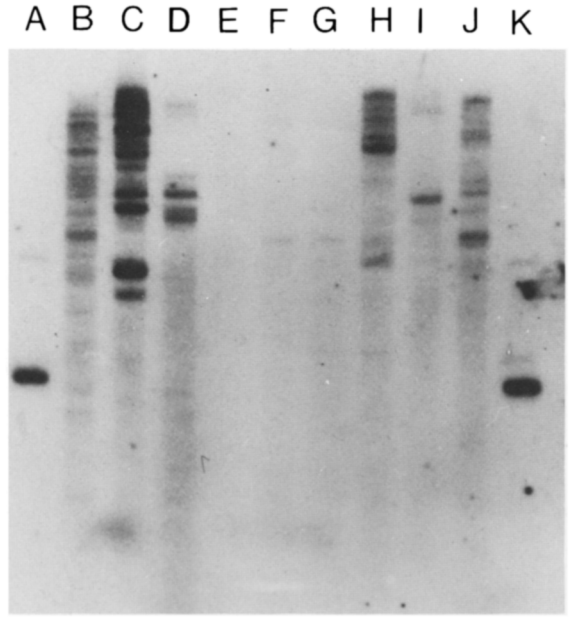


Figure 3. ΦX Sequences in tk$^+$ Transformants

Cells were co-transformed as described in the legend to Figure 2, and half of the cultures were fed HAT, while the other half were replated under cloning conditions in DME. Nine colonies were selected in HAT and assayed for ΦX sequences as described (see Figure 2). Lanes A and K each contain 30 pg (2 gene equivalents) of Pst I-linearized ΦX174 DNA. Lanes B–J contain Eco RI-digested DNA from nine independently isolated tk$^+$ transformants. Only one clone (lane E) does not contain ΦX sequences. None of fifteen clones isolated without selection contained ΦX sequences (blot not shown).

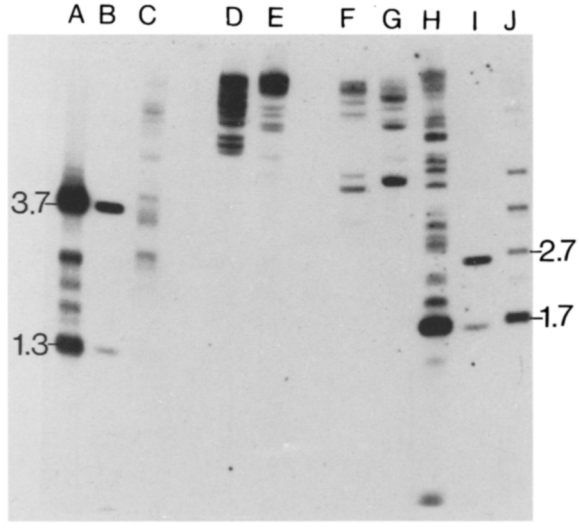


Figure 4. Extent of Sequence Representation in ΦX Co-transformants

High molecular weight DNA from co-transformant clones ΦX4 and ΦX5 was digested with either Eco RI, Bam HI, Hpa I or Hpa II and analyzed for the presence of ΦX sequences as described in the legend to Figure 2. (Lanes B and I) 50 pg (4 gene equivalents) of ΦX RFI DNA digested with Hpa I and Hpa II, respectively. (Lanes A, D, E and H) 15 μg of clone ΦX4 DNA digested with Hpa I, Eco RI, Bam HI and Hpa II, respectively, and analyzed for ΦX sequences by blot hybridization. (Lanes C, F, G and J) 15 μg of clone ΦX5 DNA digested with Hpa I, Eco RI, Bam HI or Hpa II, respectively.

and from DNA cleaved with either of these enzymes. If the sequences are integrated, then Bam HI and Eco RI should recognize different sites in the flanking cellular DNA and generate unique restriction patterns. DNA from clones ΦX4 and ΦX5 was cleaved with Bam HI or Eco RI and analyzed by Southern hybridization (Figure 4: clone 4, lanes D and E; clone 5, lanes F and G). In each instance, the annealing pattern with Eco RI fragments differed from that observed with the Bam HI fragments. Furthermore, the profile obtained with undigested DNA reveals annealing only in very high molecular weight regions with no discrete fragments observed (data not shown). Similar observations were made on clone ΦX1 (data not shown). Thus most of the ΦX sequences in these three clones are integrated into cellular DNA.

### Intracellular Localization of the ΦX Sequences

The location of ΦX sequences in transformed cells was determined by subcellular fractionation. Nuclear and cytoplasmic fractions were prepared, and the ΦX DNA sequence content of each was assayed by blot hybridization. The data (not shown) indicate that 95% of the ΦX sequences are located in the nucleus. High and low molecular weight nuclear DNA was prepared by Hirt fractionation (Hirt, 1967). Hybridization with DNA from these two fractions indicates that more than 95% of the ΦX information co-purifies with the high molecular weight DNA fraction. The small amount of hybridization observed in the supernatant fraction reveals a profile identical to that of the high molecular weight DNA, suggesting contamination of this fraction with high molecular weight DNA.

### Extent of Sequence Representation of the ΦX Genome

The annealing profiles of DNA from transformed clones digested with enzymes that do not cleave the ΦX genome provide evidence that integration of ΦX sequences has occurred and allow us to estimate the number of ΦX sequences integrated. Annealing profiles of DNA from transformed clones digested with enzymes which cleave within the ΦX genome allow us to determine what proportion of the genome is present and how these sequences are arranged following integration. Cleavage of ΦX with the enzyme Hpa I generates three fragments for each integration event (see Figure 1): two "internal" fragments of 3.7 and 1.3 kb which together comprise 90% of the ΦX genome, and one "bridge" fragment of 0.5 kb which spans the Pst I cleavage site. The annealing profile observed when clone ΦX4 is digested with Hpa I is shown in Figure 4, lane A. Two intense bands are oberved at 3.7 and 1.3 kb. A less intense series of

bands of higher molecular weight is also observed, some of which probably represent ΦX sequences adjacent to cellular DNA. These results indicate that at least 90% of the ΦX genome is present in these cells. It is worth noting that the internal 1.3 kb Hpa I fragment is bounded by an Hpa I site only 30 bp from the Pst I cleavage site. Comparison of the intensities of the internal bands with known quantities of Hpa I-cleaved ΦX DNA suggests that this clone contains approximately 100 copies of the ΦX genome (Figure 4, lanes A and B). The annealing patten of clone 5 DNA cleaved with Hpa I is more complex (Figure 4, lane C). If internal fragments are present, they are markedly reduced in intensity; instead, multiple bands of varying molecular weight are observed. The 0.5 kb Hpa I fragment which bridges the Pst I cleavage site is not observed for either clone ΦX 4 or clone ΦX5 (data not shown).

A similar analysis of clone ΦX4 and ΦX5 DNA was performed with the enzyme Hpa II. This enzyme cleaves the ΦX genome five times, thus generating four "internal" fragments of 1.7, 0.5, 0.5 and 0.2 kb, and a 2.6 kb "bridge" fragment which spans the Pst I cleavage site (Figure 1). The annealing patterns for Hpa II-cleaved DNA from ΦX clones 4 and 5 are shown in Figure 4 (clone ΦX4, lane H; clone ΦX5, lane J). In each clone an intense 1.7 kb band is observed, consistent with the retention of at least two internal Hpa II sites. The 0.5 kb internal fragments can also be observed, but they are not shown on this gel. Many additional fragments, mostly of higher molecular weight, are also present in each clone. These presumably reflect the multiple integration sites of ΦX DNA in the cellular genome. The 2.6 kb fragment bridging the Pst I cleavage site, however, is absent from clone ΦX4 (Figure 4, lane H). Reduced amounts of annealing fragments which co-migrate with the 2.6 kb Hpa II bridge fragment are observed in clone ΦX 5 (Figure 4, lane J). Similar observations were made in experiments with the enzyme Hae III. The annealing pattern of Hae III-digested DNA from these clones is shown in Figure 5 (clone ΦX4, lane B; clone ΦX5, lane C). In accord with our previous data, the 0.87 kb Hae III bridge fragment spanning the Pst site is absent or present in reduced amount in transformed cell DNA. Thus in general "internal" fragments of ΦX are found in these transformants, while "bridge" fragments which span the Pst I cleavage site are reduced or absent (see Discussion).

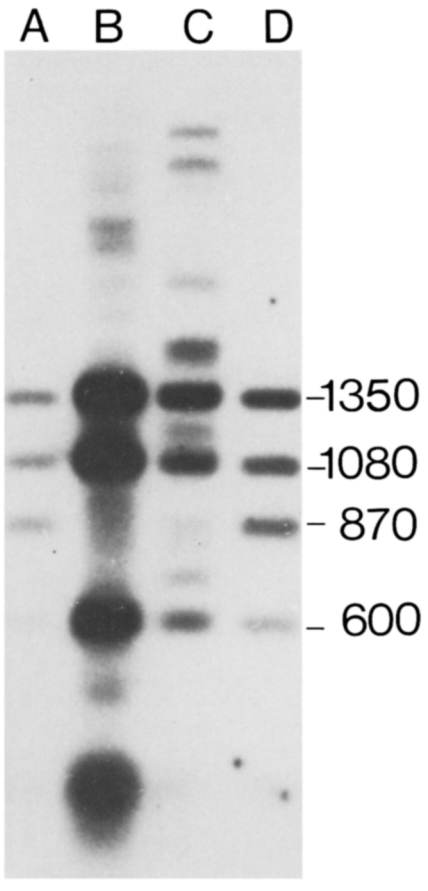


Figure 5. Annealing Pattern of DNA from Hae III-Digested ΦX Co-transformants

High molecular weight DNA from clones ΦX4 and ΦX5 was digested with Hae III, and the annealing profile was compared with that of Hae III-digested ΦXRFI DNA. Lanes A and D contain 30 and 50 pg (2 and 4 gene equivalents, respectively) of Hae III-digested ΦXRFI DNA. Lanes B and C contain 15 μg of Hae III-digested DNA from clones ΦX4 and ΦX5, respectively. The sizes of the prominent ΦX Hae III fragments in lanes A and D are 1350, 1080, 870 and 600 base pairs.

**Stability of the Transformed Genotype**

Our previous observations on the transfer of selectable biochemical markers indicate that the transformed phenotype remains stable for hundreds of generations if cells are maintained under selective pressure. If maintained in neutral medium, the transformed phenotype is lost at frequencies which range from <0.1 to as high as 30% per generation (Wigler et al., 1977, 1979). The use of transformation to study the expression of foreign genes depends upon the stability of the transformed genotype. This is an important consideration with genes for which no selctive criteria are available. We assume that the presence of ΦX DNA in our transformants confers no selective advantage on the recipient cell. We therefore examined the stability of the ΦX genotype in the descendants of two clones after numerous generations in culture. Clones ΦX4 and ΦX5, both containing multiple copies of ΦX DNA, were subcloned and six independent subclones from each original clone were picked and grown into mass culture. DNA from each of these subclones was then digested with either Eco RI or Hpa I, and the annealing profiles of ΦX-containing fragments were compared with those of the original parental clone. The annealing pattern observed for four of the six ΦX4 subclones is virtually identical to that of the parent (Figure 6A). In

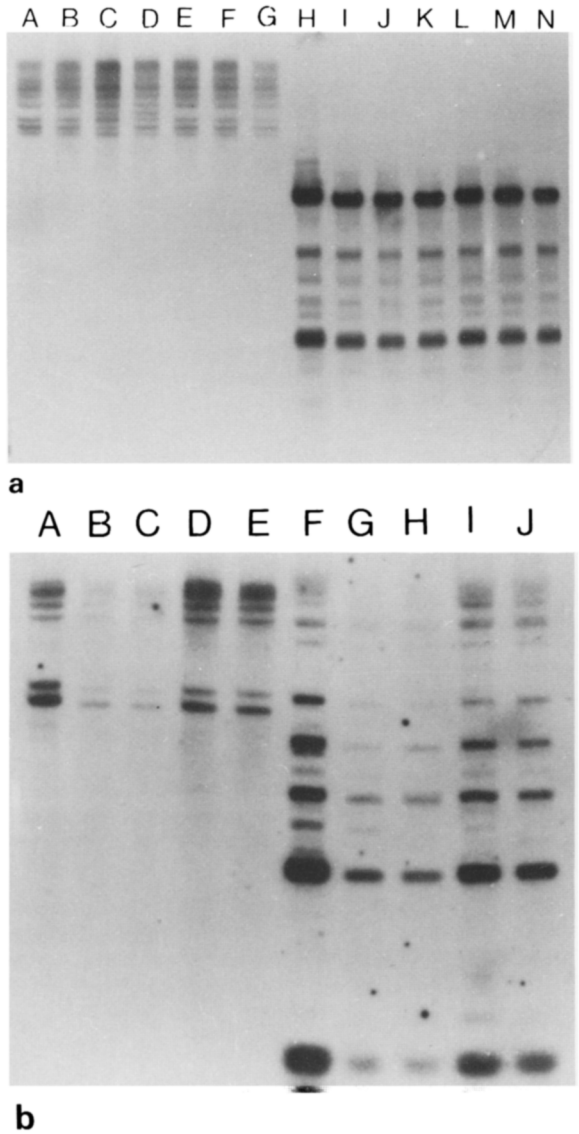


Figure 6. ΦX Sequences in Subclones of Co-transformants

(a) Annealing profiles of DNA from parental clone ΦX4 digested with Eco RI (lane A) and Hpa I (lane H) are compared with DNA from six independent subclones digested with either Eco RI (lanes B–G) or Hpa I (lanes I–N).

(b) High molecular weight DNA from four subclones of clone ΦX5 was isolated, cleaved with either Eco RI or Hpa II and compared with parental clone ΦX5 DNA. Lanes A and F contain clone ΦX5 DNA digested with Eco RI and Hpa II, respectively. DNA from four independently isolated subclones digested with either Eco RI (lanes B–E) or Hpa II (lanes G–J) was analyzed by blot hybridization.

two subclones, an additional Eco RI fragment appeared which is of identical molecular weight in both. This may have resulted from genotypic heterogeneity in the parental clone prior to subcloning. The patterns obtained for the subclones of ΦX5 are again virtually identical to the parental annealing profile (Figure 6B). These data indicate that ΦX DNA is maintained within the ten subclones examined for numerous generations without significant loss or translocation of information.

**Integration of pBR322 DNA into Mouse Cells**

We have extended our observations on co-transformation to the EK2-approved bacterial vector, plasmid pBR322. pBR322 linearized with Bam HI was mixed with the purified viral tk gene in a molar ratio of 1000:1. $Tk^+$ transformants were selected and scored for the presence of pBR322 sequences. The Bgl I restriction map of Bam HI linearized pBR322 DNA is shown in Figure 7. Cleavage of this DNA with Bgl I generates two internal fragments of 2.4 and 0.3 kb. The sequence content of the pBR322 transformants was determined by digestion of transformed cell DNA with Bgl I followed by annealing with $^{32}P$-labeled plasmid DNA. Four of five clones screened contained pBR sequences. Two of these clones contained the 2.4 kb internal fragment (Figure 8). The 0.3 kb fragment would not be detected on these gels. From the intensity of the 2.4 kb band in comparison with controls, we conclude that multiple copies of this fragment are present in these transformants. Other bands are observed which presumably represent the segments of pBR322 attached to cellular DNA.

**Transformation of Mouse Cells with the Rabbit β-Globin Gene**

Transformation with purified eucaryotic genes may provide a means for studying the expression of cloned genes in a heterologous host. We have therefore performed co-transformation experiments with the rabbit β major globin gene which was isolated from a cloned library of rabbit chromosomal DNA (Maniatis et al., 1978). One β-globin clone designated RβG-1 (Lacy et al., 1978) consists of a 15 kb rabbit DNA fragment carried on the bacteriophage λ cloning vector Charon 4a. Intact DNA from this clone (RβG-1) was mixed with the viral tk DNA at a molar ratio of 100:1, and $tk^+$ transformants were isolated and examined for the presence of rabbit globin sequences. A restriction map of RβG-1 is shown in Figure 9. Cleavage of RβG-1 with the enzyme Kpn I generates a 4.7 kb fragment which contains the entire rabbit β-globin gene. This fragment was purified by gel electrophoresis and nick-translated to generate a probe for subsequent annealing experiments. The β-globin genes of mouse and rabbit are partially homologous, although we do not observe annealing of the rabbit β-globin probe with Kpn-cleaved mouse DNA under our experimental conditions (Figure 10, lanes C, D and G). In contrast, cleavage of rabbit liver DNA with Kpn I generates the expected 4.7 kb globin band (Figure 10, lane B). Cleavage of transformed cell DNA with the enzyme Kpn I generates a 4.7 kb fragment containing globin-specific information in six of the eight $tk^+$ transformants examined (Figure 10). In two of the clones (Figure 10, lanes E and H), additional rabbit globin bands are observed which probably re-

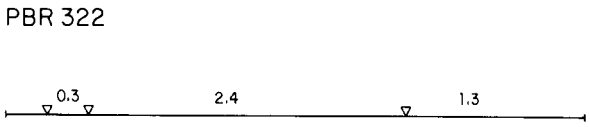


Figure 7. Cleavage Map of pBR322

The Bgl I restriction endonuclease map for Bam HI-linearized pBR322 DNA is shown. The fragment sizes are in kbp, as determined by G. Sutcliffe (personal communication).

sult from the loss of at least one of the Kpn sites during transformation. The number of rabbit globin genes integrated in these transformants is variable. In comparison with control lanes (Figure 10, lanes A and L), some clones contain a single copy of the gene (lanes I, J and K), while others contain multiple copies of this heterologous gene (lanes E, F and H). These results demonstrate that cloned eucaryotic genes can be introduced into cultured mammalian cells by co-transformation.

### Transformation Competence Is Not Stably Inherited

Our data suggest the existence of a subpopulation of transformation-competent cells within the total cell population. If competence is a stably inherited trait, then cells selected for transformation should be better recipients in subsequent gene transfer experiments than their parental cells. Two results indicate that as in procaryotes, competence is not stably heritable. In the first series of experiments, a double mutant, $Ltk^-$ $aprt^-$ (deficient in both tk and aprt), was transformed to either the $tk^+$ $aprt^-$ or the $tk^-$ $aprt^+$ phenotype using cellular DNA as donor (Wigler et al., 1978, 1979). These clones were then transformed to the $tk^+$ $aprt^+$ phenotype. The frequency of the second transformation was not significantly higher than the first. In another series of experiments, clones ΦX4 and ΦX5 were used as recipients for the transfer of a mutant folate reductase gene which renders recipient cells resistant to methotrexate (mtx). The cell line A29 $Mtx^{RIII}$ contains a mutation in the structural gene for dihydrofolate reductase, reducing the affinity of this enzyme for methotrexate (Flintoff, Davidson and Siminovitch, 1976). Genomic DNA from this line was used to transform clones ΦX4 and ΦX5 and $Ltk^-$ cells. The frequency of transformation to mtx resistance for the ΦX clones was identical to that observed with the parental $Ltk^-$ cells. We conclude that competence is not a stably heritable trait and may therefore be a transient property of cells.

### Discussion

In these studies, we have stably transformed mammalian cells with precisely defined procaryotic and eucaryotic genes for which no selective criteria exist. Our chosen experimental design derives from studies of transformation in bacteria which indicate that a small but selectable subpopulation of cells is competent in transformation (Thomas, 1955; Hotchkiss, 1959; Tomasz and Hotchkiss, 1964; Spizizen, Reilly and Evans, 1966). 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 frequencies higher than the general population. Thus, to identify transformants containing genes which provide no selectable trait, cultures were co-transformed with a physically unlinked gene which provided a selectable marker. This co-transformation 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.

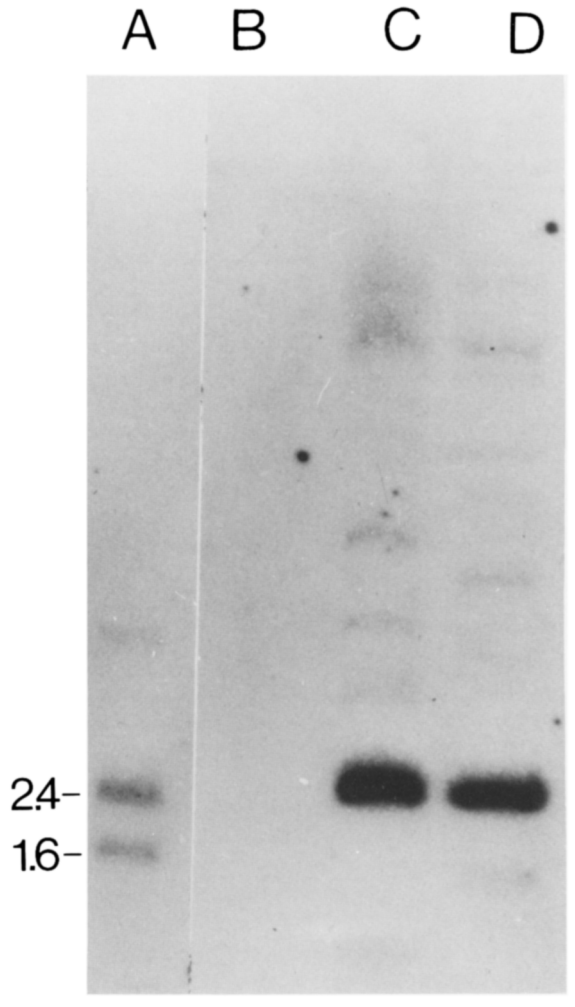


Figure 8. Physical Map of pBR322 Sequences in Co-transformants

Cells were exposed to pBR322 DNA and the viral tk gene and selected in HAT. High molecular weight DNA from three independent clones was digested with Bgl I and electrophoresed on a 1% agarose gel. The DNA was denatured in situ and transferred to nitrocellulose filters which were annealed with $^{32}P$-pBR322 DNA. (Lane A) 5 pg of pBR322 DNA digested with Bgl I; (lanes B–D) 15 μg of DNA from three independent $tk^+$ transformants.

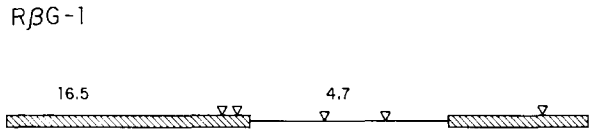


Figure 9. Physical Map of Rabbit β-Globin Phage Clone RβG-1
Cleavage sites for the restriction endonuclease Kpn I within RβG-1 are shown (Lacy et al., 1979). The numbers refer to the sizes of the fragments in kbp.

Co-transformation experiments were performed using the HSV tk gene as the selectable biochemical marker. The addition of this purified tk gene to mouse cells lacking thymidine kinase results in the appearance of stable transformants which can be selected by their ability to grow in HAT. $Tk^+$ transformants were cloned and analyzed by blot hybridization for co-transfer of additional DNA sequences. In this manner, we have constructed mouse cell lines which contain multiple copies of ΦX, pBR322 and rabbit β-globin gene sequences.

The suggestion that these observations could result from contaminating procaryotic cells in our cultures is highly improbable. At least one of the rabbit β-globin mouse transformants expresses polyadenylated rabbit β-globin RNA sequences as a discrete 9S cytoplasmic species (B. Wold et al., manuscript in preparation). The elaborate processing events required to generate 9S globin RNA correctly are unlikely to occur in procaryotes.

The ΦX co-transformants were studied in greatest detail. The frequency of co-transformation is high: 14 of 16 $tk^+$ transformants contain ΦX sequences. The ΦX sequences are integrated into high molecular weight nuclear DNA. The number of integration events varies from one to more than fifty in independent clones. The extent of the bacteriophage genome present within a given transformant is also variable; while some clones have lost up to half the genome, other clones contain over 90% of the ΦX sequences. Analysis of subclones demonstrates that the ΦX genotype is stable through many generations in culture. Similar conclusions are emerging from the characterization of the pBR322 and globin gene co-transformants.

Hybridization analysis of restriction endonuclease-cleaved transformed cell DNA allows us to make some preliminary statements on the nature of the integration intermediate. Only two ΦX clones have been examined in detail. In both clones, the donor DNA was Pst I-linearized ΦX DNA. We have attempted to distinguish between the integration of a linear or circular intermediate. If either precise circularization or the formation of linear concatamers had occurred at the Pst I cleavage site, and if integration occurred at random points along this DNA, we would expect cleavage maps of transformed cell DNA to mirror the circular ΦX map. The bridge fragment, however, is not observed or is present in reduced amounts in digests of

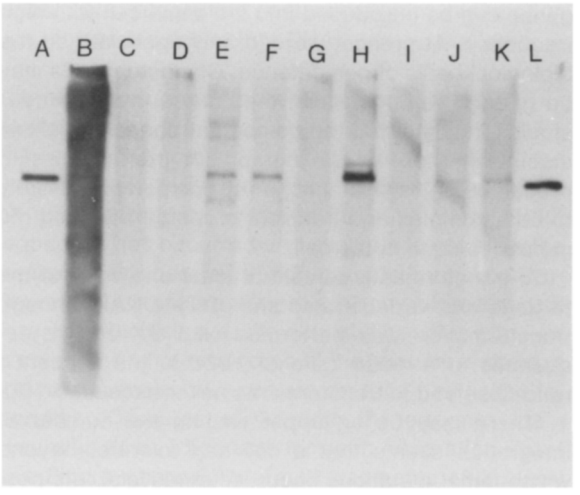


Figure 10. The Rabbit β-Globin Gene Is Present in Mouse DNA
Cells were exposed to RβG-1 DNA and the viral tk gene and selected in HAT. High molecular weight DNA from eight independent clones was digested with Kpn I and electrophoresed on a 1% agarose gel. The DNA was denatured in situ and transferred to nitrocellulose filters, which were then annealed with a $^{32}P$-labeled 4.7 kbp fragment containing the rabbit β-globin gene. (Lanes A and L) 50 pg of the 4.7 kbp Kpn fragment of RβG-1; (lane B) 15 μg of rabbit liver DNA digested with Kpn; (lane C) 15 μg of $Ltk^-$ $aprt^-$ DNA; (lanes D–K) 15 μg of DNA from each of eight independently isolated $tk^+$ transformants.

transformed cell DNA with three different restriction endonucleases. The fragments observed are in accord with a model in which ΦX DNA integrates as a linear molecule. Alternatively, it is possible that intramolecular recombination of ΦX DNA occurs, resulting in circularization with deletions at the Pst termini (Lai and Nathans, 1974). Random integration of this circular molecule would generate a restriction map similar to that observed for clones ΦX4 and ΦX5. Other more complex models of events occurring before, during or after integration can also be considered. Although variable amounts of DNA may be deleted from termini during transformation, most copies of integrated ΦX sequences in clone ΦX4 retain the Hpa I site, which is only 30 bp from the Pst I cleavage site. Whatever the mode of integration, it appears that cells can be stably transformed with long stretches of donor DNA. We have observed transformants containing contiguous stretches of donor DNA 50 kb long (B. Wold et al., unpublished studies).

We have attempted to identify cells transformed with ΦX sequences in the absence of selective pressure. Cultures were exposed to ΦX and tk DNA and cells were cloned under nonselective conditions. ΦX sequences were absent from all fifteen clones picked. In contrast, 14 of 16 clones selected for the $tk^+$ phenotype contained ΦX DNA. The simplest interpretation is that a subpopulation of cells within the culture is competent in the uptake and integration of DNA. In this subpopulation of cells, two physically unlinked

genes can be introduced into the same cell with high frequency. At present we can only speculate on the biological basis of competence. Competent cells may be genetic variants within the culture; however, our studies indicate that the competent phenotype is not stably inherited. If we extrapolate from studies in procaryotes, the phenomenon of competence is likely to be a complex and transient property reflecting the metabolic state of the cell.

Co-transformants contain at least one copy of the tk gene and variable amounts of ΦX DNA. Although transformation was performed with ΦX and tk sequences at a molar ratio of 1000:1, the sequence ratio observed in transformants never exceeded 100:1. There may be an upper limit to the number of integration events that a cell can tolerate, beyond which lethal mutations occur. Alternatively, it is possible that the efficiency of transformation may depend upon the nature of the transforming fragment. The tk gene may therefore represent a more efficient transforming agent than phage DNA.

The usefulness of the co-transformation method will depend to a large extent on its generality. To date, we have limited experience with other cell lines. The use of tk as a selectable marker restricts host cells to $tk^-$ mutants. In unpublished studies, we have demonstrated the co-transfer of plasmid pBR 322DNA into $Ltk^-$ $aprt^-$ cells using $aprt^+$ cellular DNA as donor and aprt as selectable marker. Furthermore, the use of dominant acting mutant genes which can confer drug resistance may extend the host range for co-transformation to virtually any cultured cell.

The stable transfer of ΦX DNA sequences to mammalian cells serves as a model system for the introduction of defined genes for which no selective criteria exist. We have used the tk co-transformation system to transform cells with the bacterial plasmid pBR322 and the cloned rabbit β-globin gene. Experiments which indicate that several of the pBR transformants contain an uninterrupted sequence which includes the replicative origin and the gene coding for ampicillin resistance (β-lactamase), suggest that DNA from pBR transformants may transfer ampicillin resistance to E. coli. Work in progress suggests that the rabbit β-globin gene is transcribed in at least one of the mouse transformants we have examined. Although preliminary, these studies indicate the potential value of co-transformation systems in the analysis of eucaryotic gene expression.

## Experimental Procedures

### Cell Culture

$Ltk^-$ $aprt^-$, a derivative of $Ltk^-$ clone D (Kit et al., 1963), was obtained from R. Hughes and maintained in Dulbecco's modified Eagle's medium (DME) containing 10% calf serum and 50 μg/ml of diaminopurine (DAP). Prior to transformation, cells were washed and grown for three generations in the absence of DAP. A Chinese hamster cell line containing an altered dihydrofolate reductase (rendering it resistant to methotrexate) A29 $Mtx^{RIII}$ (Flintoff et al., 1976) was obtained from L. Siminovitch. These cells were propagated in DME supplemented with 3X nonessential amino acids, 10% calf serum and 1 μg/ml amethopterin.

### Isolation of the HSV tk Gene

Intact herpes simplex virus (HSV) DNA was isolated from CV-1-infected cells as previously described (Pellicer et al., 1978). DNA was digested to completion with Kpn I (New England Biolabs) in a buffer containing 6 mM Tris (pH 7.9), 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 6 mM NaCl and 200 μg/ml bovine serum albumin. The restricted DNA was fractionated by electrophoresis through 0.5% agarose gels (17 × 20 × 0.5 cm) for 24 hr at 70 V, and the 5.1 kb tk-containing fragment was extracted from the gel as described by Maxam and Gilbert (1977).

### Source of DNAs

ΦX174 am3 RFI DNA was purchased from Bethesda Research Laboratories. Plasmid pBR322 DNA was grown in E. coli HB 101 and purified according to the method of Clewell (1972). The cloned rabbit β major globin gene in the λ Charon 4A derivative (RβG-1) was identified and isolated as previously described (Maniatis et al., 1978).

### Co-transformation of Defined DNA Sequences and the HSV tk Gene

$Ltk^-$ $aprt^-$ mouse cells were transformed with either 1–10 μg of ΦX174, 1 μg of pBR322 or 1 μg of RβG-1 DNA in the presence of 1 ng of HSV-1 tk gene and 10–20 μg of salmon sperm carrier DNA, as previously described (Wigler et al., 1979). $Tk^+$ transformants were selected in DME containing hypoxanthine, aminopterin and thymidine (HAT) and 10% calf serum. Isolated colonies were picked using cloning cylinders and grown into mass cultures.

### Transformation

Methotrexate-resistant transformants of $Ltk^-$ $aprt^-$ cells were obtained following transformation with 20 μg of high molecular weight DNA from A29 $Mtx^{RIII}$ cells and selection in DME containing 10% calf serum and 0.2 μg/ml amethopterin.

Transformation and selection for $aprt^+$ transformants were as described by Wigler et al. (1979).

### Isolation of Transformed Cell DNA

Cells were harvested by scraping into PBS and centrifuging at 1000 × g for 10 min. The pellet was resuspended in 40 vol of TNE [10 mM Tris–HCl (pH 8.0), 150 mM NaCl, 10 mM EDTA], and SDS and proteinase K were added to 0.2% and 100 μg/ml, respectively. The lysate was incubated at 37°C for 5–10 hr and then extracted sequentially with buffer-saturated phenol and $CHCl_3$. High molecular weight DNA was isolated by mixing the aqueous phase with 2 vol of cold ethanol and immediately removing the precipitate that formed. The DNA was washed with 70% ethanol and dissolved in 1 mM Tris, 0.1 mM EDTA.

Nuclei and cytoplasm from clones ΦX4 and ΦX5 were prepared as described by Ringold et al. (1977). The nuclear fraction was further fractionated into high and low molecular weight DNA as described by Hirt (1967).

### Filter Hybridization

DNA from transformed cells was digested with various restriction endonucleases using the conditions specified by the supplier (New England Biolabs or Bethesda Research Laboratories). Digestions were performed at an enzyme to DNA ratio of 1.5 U/μg for 2 hr at 37°C. Reactions were terminated by the addition of EDTA, and the product was electrophoresed on horizontal agarose slab gels in 36 mM Tris, 30 mM $NaH_2PO_4$, 1 mM EDTA (pH 7.7). DNA fragments were transferred to nitrocellulose sheets, hybridized and washed as previously described (Weinstock et al., 1978) with two modifications. Two nitrocellulose filters were used during transfer (Jeffreys and Flavell, 1977b). The lower filter was discarded, and following hybridization the filter was washed 4 times for 20 min in 2 × SSC, 25 mM

sodium phosphate, 1.5 mM $Na_4P_2O_7$, 0.05% SDS at 65°C and then successively in 1:1 and 1:5 dilutions of this buffer (Jeffreys and Flavell, 1977a).

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