Cell, Vol. 16, 777–785, April 1979, Copyright © 1979 by MIT

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)

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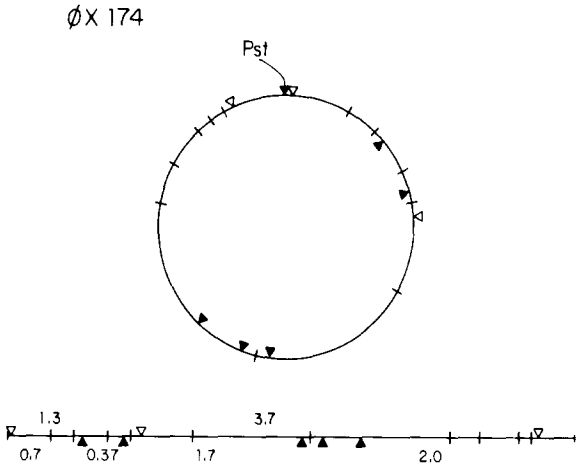

Figure 1. Cleavage Map of the ΦX174 Genome

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.

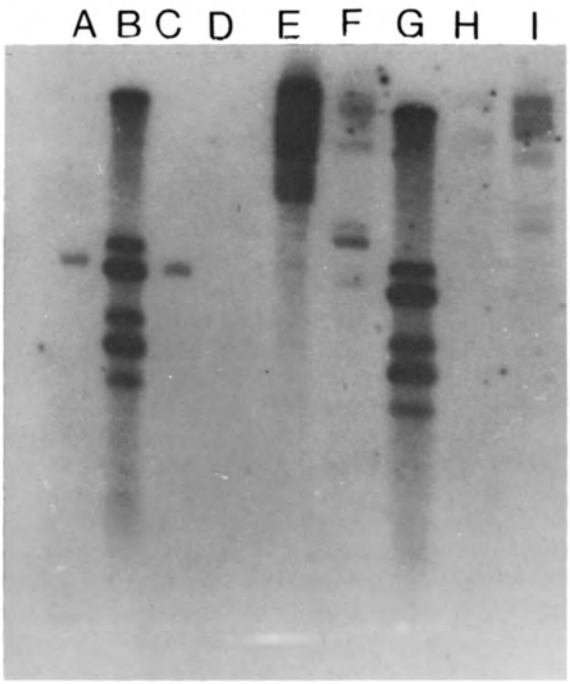

Figure 2. Identification of ΦX Sequences in Cells Transformed with ΦX174 DNA and the HSV tk Gene

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

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

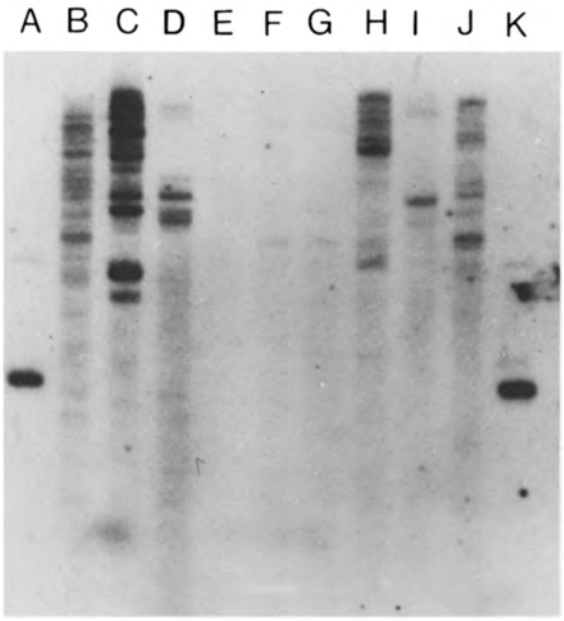

Figure 3. ΦX Sequences in tk$^+$ Transformants

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).

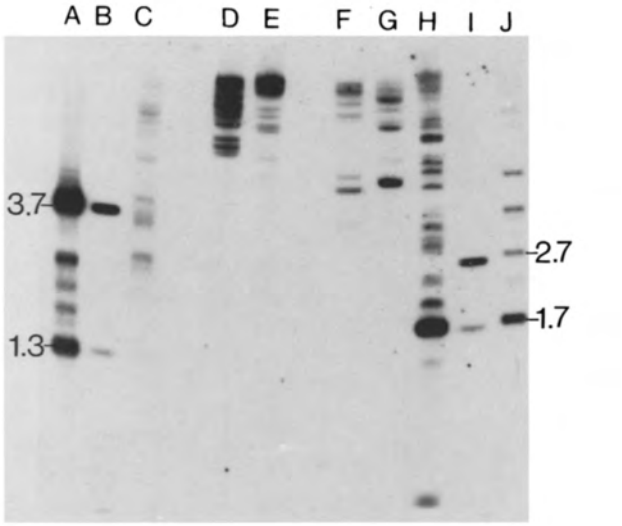

Figure 4. Extent of Sequence Representation in ΦX Co-transformants

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

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

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).

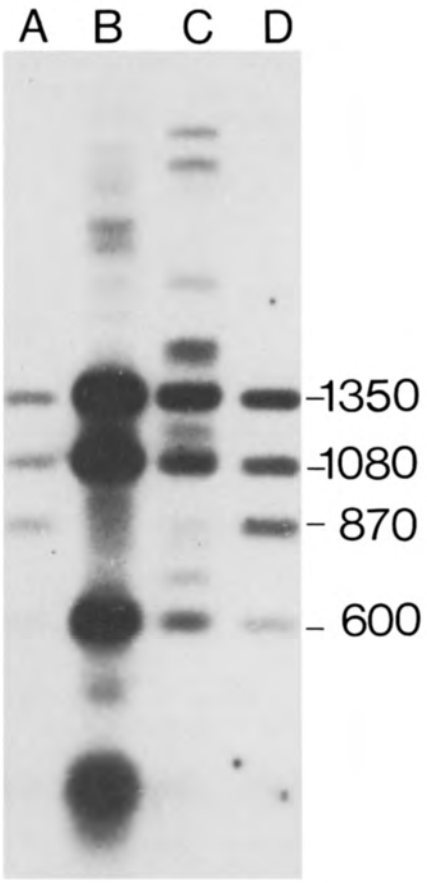

Figure 5. Annealing Pattern of DNA from Hae III-Digested ΦX Co-transformants

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

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

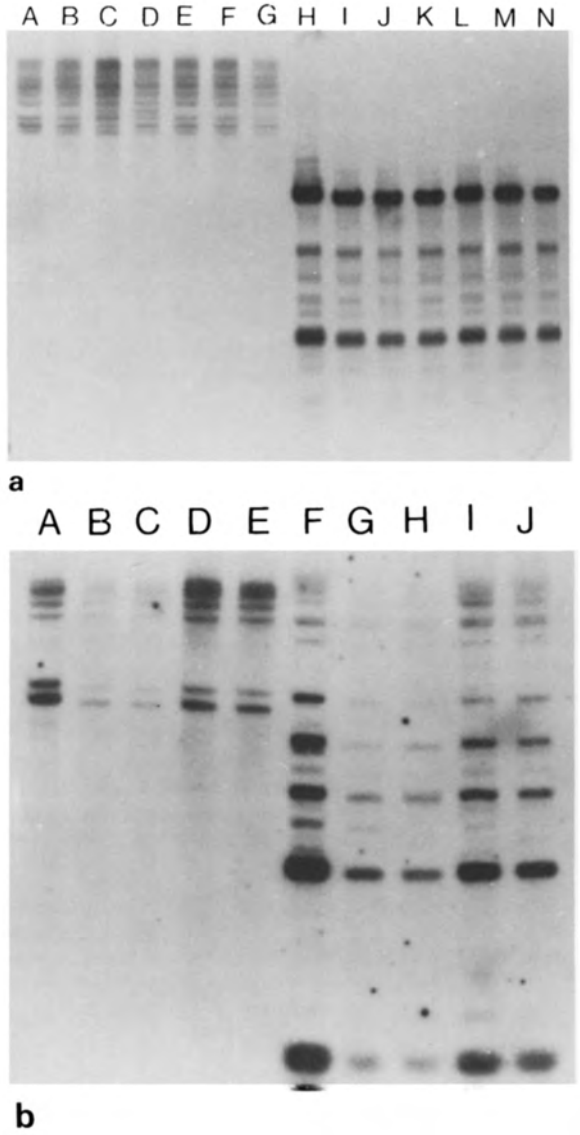

Figure 6. ΦX Sequences in Subclones of Co-transformants

(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 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

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