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The Transfer and Stable Integration of the HSV Thymidine Kinase Gene into Mouse Cells

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Summary

Treatment of mutant mouse cells (Ltk$^-$) deficient in thymidine kinase with Bam I restriction endonuclease-cleaved HSV-1 DNA results in the appearance of numerous surviving colonies which stably express the tk$^+$ phenotype. Through a series of electrophoretic fractionations in concert with transfection assays, we isolated a 3.4 kb fragment which contains the thymidine kinase gene and which alone is competent in the biochemical transformation of Ltk$^-$ cells. In this report, we have examined the distribution of *tk* sequences in the DNA of several transformed clones following stable gene transfer. A series of complementary experiments involving reassociation kinetics in solution and annealings with *tk* DNA to restriction-cleaved cellular DNA following electrophoresis and transfer to filters allow us to make the following general conclusions concerning the fate of the *tk* gene in all clones examined: the *tk* gene is present in all cells at a frequency of one copy per chromosomal complement; the *tk* gene is stably integrated in the DNA of all transformants; and integration is not site-specific and occurs at different loci in the DNA of all transformants examined. The existence of a single active *tk* gene in tk$^+$ transformants now facilitates an analysis of the sequence organization of tk$^-$ mutant cells and provides a useful model system for studies on the transfer of cellular genes.

Introduction

The introduction of foreign DNA into cells can result in a stable and heritable change in phenotype. This process is known as transformation. Biochemical transformation involves the stable acquisition of a new trait which can be readily detected by the ability of cells to grow under appropriate selective conditions. In eucaryotes, biochemical transformation has been effected by debilitated virus (Munyon et al., 1971) and metaphase chromosomes (McBride and Ozer, 1973; Willecke and Ruddle, 1975), as well as purified DNA (Bachetti and Graham, 1977; Maitland and McDougall, 1977; Wigler et al., 1977). The ability to transfer purified genes provides an opportunity to study the function and physical state of exogenous genes within the transformed host.

The herpes simplex virus thymidine kinase gene is an ideal genetic element for studies on gene transfer. The tk$^+$ or tk$^-$ phenotype can be efficiently selected utilizing growth conditions in which synthesis of the salvage pathway enzyme, thymidine kinase, either is necessary for survival or results in cell death (Kit et al., 1963; Littlefield, 1964). The stable transformation of tk$^-$ mouse cells (Ltk$^-$) to the tk$^+$ phenotype results from transfection with restriction endonuclease-generated fragments of herpes simplex virus DNA. Through a series of electrophoretic fractionations in concert with transfection assays, we have isolated a unique 3.4 kb fragment of viral DNA which, in the absence of any additional HSV information, is capable of efficiently transfecting *tk* activity to Ltk$^-$ cells (Wigler et al., 1977).

In the present study, we determine the copy number of the transfected gene and demonstrate the stable integration of this gene into the DNA of the recipient host cell in several independently derived clones. The experimental approach we have adopted to examine the physical state of the transfected *tk* gene involves restriction endonuclease treatment of cellular DNA followed by an analysis of the size and number of the DNA fragments which contain information homologous to this gene. This experimental design derives from elegant hybridization techniques originally introduced by Southern (1975), and was previously utilized to examine the integration of SV40 DNA in cloned lines of cells transformed with SV40 virus (Ketner and Kelly, 1976; Botchan, Topp and Sambrook, 1976). This experimental approach in concert with solution hybridization studies on the DNA of transformed cells permits us to conclude that all transformed clones examined contain only a single copy of the *tk* gene per genome; that the *tk* gene is covalently integrated into the DNA of all transformants; and that the site of integration into host DNA is stable within a given clone, but differs in all independently derived clones examined. These results demonstrate the stable integration of HSV DNA into mammalian cells.

Results

Isolation and Mapping of the Thymidine Kinase Gene

Cleavage of HSV-I DNA with the restriction endonuclease Bam I yields about 25 discrete fragments which can be resolved on agarose gels. Transfection of mouse cells (Ltk$^-$) deficient in thymidine

kinase with the 3.4 kb Bam I fragment gives rise to cells able to form colonies in tk^+ selective medium (HAT medium). These cells stably express the viral thymidine kinase for hundreds of generations, from which we conclude that the 3.4 kb fragment contains the structural gene for *tk* (Wigler et al., 1977).

Analysis of the fate of the *tk* gene in transformed cells requires that we isolate this gene free of contaminating HSV-1 DNA. Treatment of the 3.4 kb fragment with a variety of endonucleases, however, consistently generated a series of discrete DNA fragments whose molecular weights summed to 6.8 kb. This indicates that the 3.4 kb fragment consists of two different DNA fragments of identical molecular weight. To isolate the *tk* gene-containing fragment free of the contaminant, we proceeded to identify a larger restriction endonuclease-generated fragment that contained within it the 3.4 kb Bam I fragment. This was effected by isolating an 8.3 kb fragment of HSV DNA generated by Hpa I cleavage which is active in transfection assays. This fragment, when further digested with Bam I, generates the 3.4 kb fragment containing the *tk* gene in pure form (Figure 1).

A restriction endonuclease map of the purified 3.4 kb fragment is shown in Figure 2. The fragment contains a single site of cleavage for the enzyme Hinc II, two sites for Eco RI and three sites for Pst I. In addition, several enzymes were identified, including Hpa I, Hind III and Kpn I, which have no cleavage sites in the 3.4 kb fragment. The availability of a homogeneous fragment, active in transfection assays for which a restriction map is known, now permits an analysis of the fate of this DNA in transformed cells.

tk Gene Frequencies by Solution Hybridization

In initial experiments, we determined the frequency of the *tk* gene in a number of transformed clones by solution hybridization. In these studies, the kinetics of reassociation of a nick-translated *tk* gene were compared with the kinetics observed when the gene was permitted to reassociate in the presence of a vast excess of transformed cell DNA. Accurate estimates of the copy number from experiments involving double-stranded DNA probes require the use of DNA of exceedingly high specific activity. Nick translation of the purified *tk* gene with ^{32}P-deoxynucleoside triphosphates routinely provided DNA with specific activities from 100–300 $\times 10^6$ cpm/μg. This DNA reannealed at saturation to values ranging from 40–60%. To overcome this difficulty, the probe was reassociated to a Cot value of 0.1, and those DNA sequences capable of reannealing were isolated on hydroxyapatite columns. DNA containing the *tk* gene isolated in this way reassociates with second-order kinetics to saturation values of 75–80% (Figure 3). The $Cot_{1/2}$ of this annealing reaction is 2×10^{-3}, while the $Cot_{1/2}$ observed with nick-translated total HSV-1 DNA is 10^{-1}. These values are consistent with molecular weight determinations which indicate that this fragment represents about 1/40 the complexity of the total viral genome.

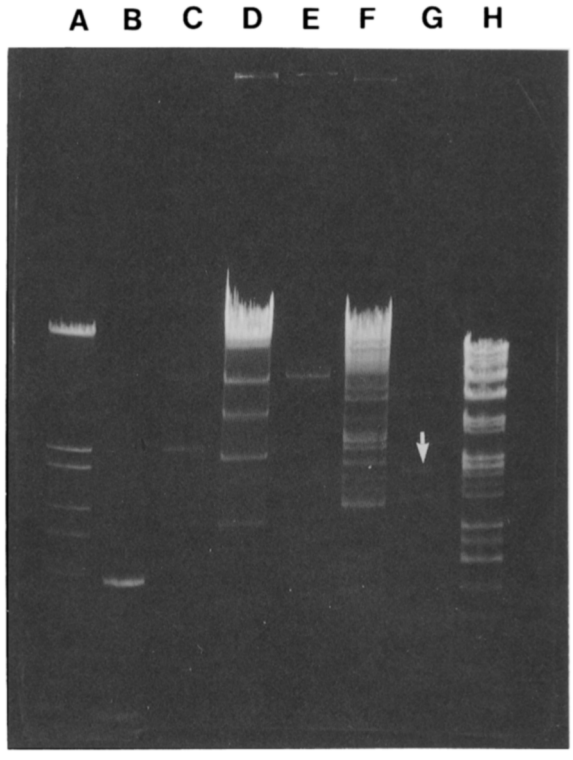

Figure 1. Isolation of the Restriction Fragments of HSV-I DNA Containing the *tk* Gene

HSV-I DNA was cleaved with the enzyme Hpa I, and the DNA fragments obtained were fractionated on 0.5% agarose slab gel electrophoresis. The DNA fragment containing the *tk* gene was identified by transfection assays. Virtually all the transfection activity resided in a fragment 8.3 kb in length. This fragment was purified from the gel and is shown in slot E. The fragments generated upon treatment of this 8.3 kb DNA with Bam I are shown in slot G. The arrow points to the pure 3.4 kb fragment which retains transfection activity and which was used as a probe in subsequent hybridization experiments. Slot C reveals the fragments obtained upon Eco RI cleavage of the purified 8.3 kb fragment. For comparison, digests of total HSV-I DNA with Eco RI (slot D), Kpn I (slot F) and Bam I (slot H) are shown. The markers include an Eco RI digestion of adenovirus 2 DNA (slot A) and the two upper bands of an Hae III digestion of SV40 DNA (slot B).

This ^{32}P-tk DNA was now permitted to reanneal in the presence of excess quantities of cellular DNA from Ltk^-- and tk^+-transformed cell lines. We observe the kinetics of reassociation of the *tk* gene with Ltk^- or transfected clone Ib DNA in Figure 4 (see Table 1). It should be noted that the Cot values in this figure refer to the concentration of cellular DNA rather than probe DNA as in Figure 3. The kinetics of annealing of the *tk* gene in the presence or absence of Ltk^- DNA are virtually identical. We therefore conclude that the viral *tk* gene shares no

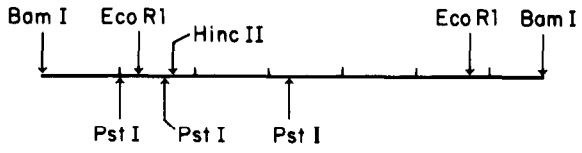

Figure 2. Restriction Map of the 3.4 kb Fragment of HSV DNA Containing the *tk* Gene

HSV-I DNA was cleaved first with Hpa I and then with Bam I to generate a pure 3.4 kb fragment which retains transfection activity. The location of the sites for the enzymes Eco RI, Hinc II and Pst I are shown. The fragment is calibrated in 0.5 kb units.

sequence homology with the normal mouse cell genome. In the presence of transformed cell DNA, however, the annealing of *tk* DNA occurs with a $Cot_{1/2}$ of 1.5×10^3, a value 10 times greater than that observed when the gene is allowed to reassociate with unrelated DNA. Under identical conditions, total single-copy DNA of the mouse anneals with a $Cot_{1/2}$ of 800. In this clone, therefore, the *tk* gene is present at low frequency, perhaps uniquely per cell. As an additional control, ^{3}H-mouse globin cDNA was added to the annealing reaction with ^{32}P-tk DNA (Figure 4). Globin cDNA anneals with clone Ib DNA with a $Cot_{1/2}$ of 400, a value about one fourth that of *tk* DNA. Since the individual globin genes are believed to be present 1-3 times per haploid genome (Leder et al., 1973), these data support our conclusion that the *tk* gene is present as a unique gene.

Analysis of annealing data as described above is complicated by the contribution due to self-annealing of tk probe. Deviation from second-order kinetics, which can result from unequal representation of the probe in transformed cell DNA, can often be obscured. To overcome this difficulty, Sharp, Pettersson and Sambrook (1974) suggested an alternative analysis which we used to determine the *tk* gene content of several transformed clones (Figure 5). In this method of analysis, the reciprocal of the fraction of the probe remaining single-stranded is plotted as a function of time. A linear plot is obtained for an ideal single component, second-order reaction. The gene frequency is determined from the ratio of the slopes observed when the probe is annealed with transformant and normal Ltk$^-$ DNA. Unequal representation of different portions of the *tk* fragment are reflected in a deviation from linearity. The kinetics of annealing of the *tk* DNA with DNA from a variety of different clones (see Table 1) is strikingly similar (Figure 5). For all clones, we calculate that the *tk* gene represents only one part in 10^6 of the mouse genome. No deviation from linearity is observed in Figure 5, suggesting that most of the transfecting *tk* fragment is equally represented in transformed cell DNA.

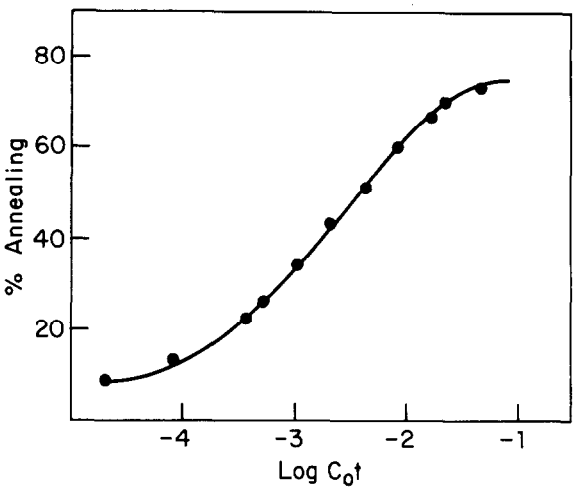

Figure 3. Kinetics of Reassociation of the Purified 3.4 kb Bam I-Generated Fragment Containing the *tk* Gene

The 3.4 kb DNA fragment generated following Hpa I and Bam I cleavage of HSV-I DNA was purified from gels and nick-translated with ^{32}P-deoxynucleoside triphosphates. Following nick translation, snapback DNA was removed, and the DNA capable of reassociation was isolated by hydroxylapatite chromatography (see Experimental Procedures). This purified ^{32}P-*tk* DNA (0.05 ng; spec. act. 200×10^6 cpm/μg) was annealed with 30 ng of the purified 3.4 kb template in a total volume of 100 μl. Annealing reactions were performed at 69°C, and duplex formation was monitored with S1 nuclease.

The Physical State of the *tk* Gene in Transformed Cells

Restriction endonuclease treatment of the eucaryotic genome generates several thousand fragments which result from cleavage at precisely defined loci within the genome. The enormous complexity of eucaryotic DNA does not permit resolution of discrete fragments which contain *tk* gene sequences. It is possible, however, to determine the size, number and arrangement of the *tk* DNA fragments by eluting restriction-cleaved DNA from agarose gels onto nitrocellulose filters (Southern, 1975). Highly radioactive *tk* DNA is annealed with these filters, and the distribution of *tk* sequences within transformed cell DNA is then determined by autoradiography. These experiments permit us to determine the precise number of genes within a given clone; to determine whether integration of the *tk* gene into cellular DNA has occurred; and to compare the site of integration within cellular DNA from different clones.

Kpn I

The 3.4 kb *tk* DNA fragment which we have stably transferred to Ltk$^-$ cells contains no restriction sites cleaved by the enzyme Kpn I. If integration of the 3.4 kb *tk* fragment has occurred, then treatment of transformed cell DNA with Kpn I should generate a discrete fragment larger than 3.4 kb. This frag-

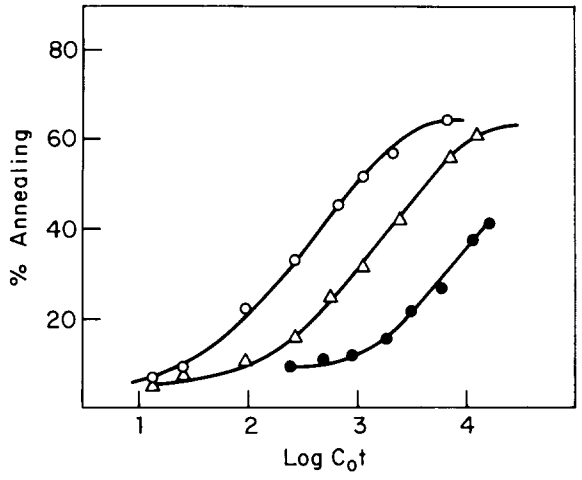

Figure 4. Kinetics of Annealing of Purified *tk* DNA with Transformed Cell and Ltk⁻ Cellular DNA

A ^{32}P-*tk* DNA probe was prepared by nick translation of the 3.4 kb fragment as shown in Figure 3 and described in Experimental Procedures. 0.05 ng of this *tk* DNA (spec. act. 200 × 10^6 cpm/μg) were annealed with 3 mg of sonicated DNA derived from Ltk⁻ cells of tk⁺-transformed clone 1b. As an additional control, 0.5 ng of ^{3}H-globin cDNA (spec. act. 15 × 10^6 cpm/μg) were included in the reaction mixture with clone 1b DNA. Hybridization of *tk* DNA with Ltk⁻ DNA (●—●) or clone 1b DNA (△—△). Hybridization of globin cDNA with Ltk⁻ DNA or clone 1b DNA (○—○).

Table 1. Derivation of Transformed Clones

Clone	Transforming DNA	Dose of DNA (μg Equivalents of HSV-1 DNA per Dish)
LH 2-1	Bam I-treated HSV-1 DNA	4.0
LH5C2-2	Bam I-treated HSV-1 DNA	4.0
LH1b	Bam I-generated 3.4 doublet	1.5
LH2b	Bam I-generated 3.4 kb doublet	1.5
LH3b	Bam I-generated 3.4 kb doublet	1.5
LH4b	Bam I-generated 3.4 kb doublet	1.5
LH7	Bam I-generated 3.4 kb doublet	0.05
HB-I	Hpa-Bam I-generated 3.4 kb fragment	1.0

ment would result from cleavage at sites in cellular DNA flanking both ends of the newly integrated DNA. From the number of different molecular weight fragments containing *tk* information, we can obtain a minimum value for the number of integrated genes.

The results of this experiment with Kpn I-treated DNA from two transformed clones, 7 and 2b, are shown in Figure 6. High molecular weight cellular DNA was digested with Kpn I and fractionated by electrophoresis on 1% agarose gels. The DNA was denatured in situ and eluted onto nitrocellulose filters which were then annealed with a highly radioactive *tk* probe prepared by nick translation of *tk* DNA. Slot D contains 50 pg of the 3.4 Bam I fragment. As predicted, a single band containing the *tk* gene was observed with a molecular weight of 3.4 kb. Slots B and C contain 50 μg of Kpn I-treated DNA from two different clones. In each case, only a single band was observed with a molecular weight significantly larger than that of transfecting fragment. In clone 2b, the size of the *tk*-containing fragment was 19 kb, while in clone 7 it was about 30 kb. We conclude from these data that the *tk* gene does not exist as a 3.4 kb DNA linear or circular molecule, but is probably integrated into the DNA of the transfected cells. The site of integration in the host DNA must differ in the two clones examined. Furthermore, the presence of only a single band of *tk* DNA suggests that only one copy of the *tk* gene exists at a unique location in the chromosomes of each clone.

Hinc II

A second class of restriction endonucleases useful in delineating the number and location of the integrated *tk* genes are those enzymes which recognize and cleave at a single site within the *tk* DNA. One such enzyme is Hinc II, which cleaves the 3.4 kb *tk* fragment at a single site, generating two fragments 2.4 and 0.9 kb in length. From the previous data with Kpn-digested DNA, we would predict that cleavage of transformed cell DNA with Hinc II should only generate two fragments containing *tk* sequences if the fragment is present only once per chromosome complement. These two fragments should result from a unique cleavage within the gene and cleavages at Hinc II sites in adjacent cellular DNA on both sides of the integrated fragment. From the number of bands observed, we can obtain an accurate measure of the number of copies of the *tk* gene within each clone.

The results of this experiment, in which the *tk* sequences are localized in a Hinc II digest of cellular DNA, are shown in Figure 7. Slot C contains 50 pg of 3.4 kb Bam fragment treated with Hinc II. We predict two bands at 2.5 and 0.9 kb, which are clearly observed in the autoradiograph. An additional band which results from incomplete digestion is observed at 3.4 kb. We observe the profile obtained with clone 2b DNA in slot B. Only two bands, 3.2 and 1.5 kb in length, are present. The 3.2 kb fragment is likely to contain the 2.5 kb fragment covalently attached to a 0.7 kb segment of cellular DNA. Similarly, the 1.5 kb fragment will contain the 0.9 kb fragment joined to a short 0.6 kb segment of host DNA. A similar situation is observed for the *tk* gene sequences in clone 7 (Figure 7, slot A). Only two *tk* fragments are generated with

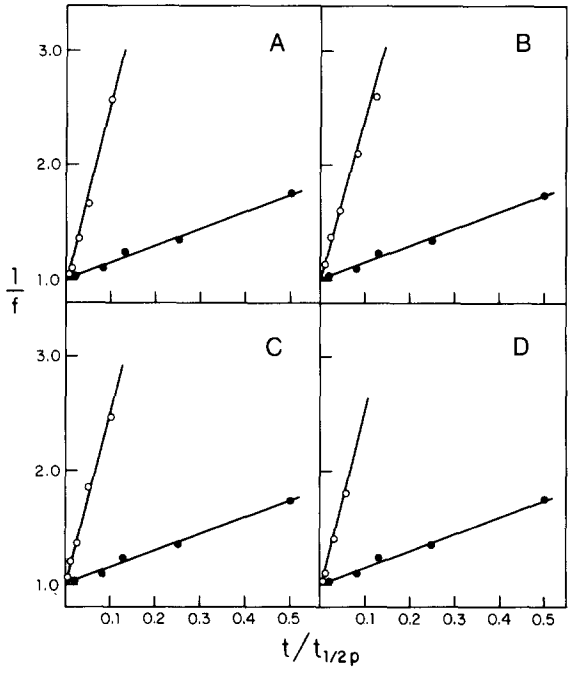

Figure 5. Renaturation of ^{32}P-labeled *tk* DNA in the Presence of tk$^+$-Transformed Clone DNA

Annealing reactions contained 0.05 ng of ^{32}P-*tk* DNA (spec. act. 200 × 10^6 cpm/μg) and 3 mg of DNA derived from transformed cell clones LH1b (A), LH3b (B), LH4b (C) and LH7 (D). For comparison, *tk* DNA was also annealed with an equal quantity of Ltk$^-$ cellular DNA. The data are plotted according to Sharp et al. (1974) where 1/f represents the fraction of probe DNA which is single-stranded at time t, and $t_{1/2}$ p is the time required for half of the probe DNA to renature in the presence of untransformed cell DNA. Hybridization of *tk*$^+$ DNA with transformed cell DNA (○—○) or with Ltk$^-$ DNA (●—●).

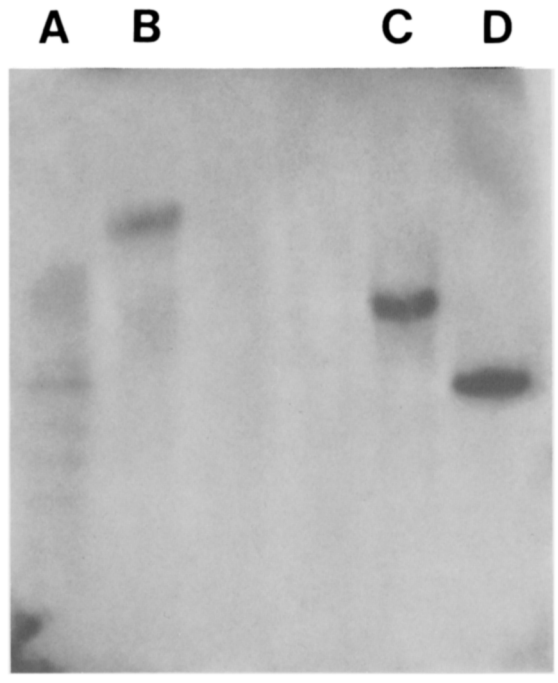

Figure 6. Identification of the *tk*-Specific Sequences in DNA Fragments Generated by Kpn I Cleavage of Transformed Cell DNA

High molecular weight transformed cell DNA was digested with Kpn I, and the fragments were electrophoresed on 1% agarose gel. The DNA was denatured in situ and transferred to nitrocellulose filters which were then annealed with ^{32}P-*tk* DNA as described in Experimental Procedures. Kpn I-digested DNA from clone 7 (slot B) and clone 2b (slot C) is shown. As a control, 50 pg of the purified 3.4 kb Bam-generated *tk* fragment were run in slot D. The markers shown in slot A include ^{32}P-adenovirus 2 DNA cleaved with the enzyme Eco RI.

lengths of 4.5 and 1.6 kb. The observation that the *tk* sequences of each clone localize to only two fragments whose molecular weights differ in each clone strongly supports the conclusion that the *tk* gene is present at a single locus in each clone and that this locus differs in the two clones examined. The sum of the molecular weights of the two fragments exceeds 3.4 kb for both clones, again suggesting that integration into cellular DNA has occurred.

Eco RI

A third class of restriction enzymes that may provide additional information on the organization of the integrated *tk* gene are those endonucleases that recognize and cleave at multiple sites within *tk* DNA. Eco RI cleaves the 3.4 kb *tk* DNA at two sites, generating three fragments 2.2, 0.68 and 0.52 kb in length. Cleavage of the *tk* gene in its integrated form with this enzyme should also reveal three *tk*-containing fragments. If the *tk* gene has not undergone extensive rearrangement or nucleolytic cleavage prior to or upon integration, we would predict *tk* sequences to be localized in a 2.2 kb fragment which results from the two internal cleavages. Two additional fragments are expected with molecular weights >0.68 and 0.52 kb which result from one cleavage within the gene and a second site in the flanking host DNA.

The location of the *tk* sequences in Eco RI-digested, transformed cell DNA is shown in Figure 8. The control is slot A includes 2 ng of total HSV-1 DNA digested with both Eco RI and Bam I. As expected, the most intense band corresponds to a fragment 2.2 kb in length and reflects the internal fragment generated upon Eco RI cleavage. Additional lower molecular weight bands are present at a position corresponding to the 0.52 and 0.68 kb fragments. Slot B contains Eco RI-treated DNA from clone 2b, and reveals three distinct bands 0.98, 2.2 and 3.6 kb in length. Similarly, only three bands are observed with Eco RI-digested DNA from clone 7—0.92, 2.2 and 4 kb in length. The 2.2 kb fragment, present in the control and in both transformant DNAs, is of significantly greater intensity than the remaining bands. Although we have not yet obtained accurate quantitation in these sorts of

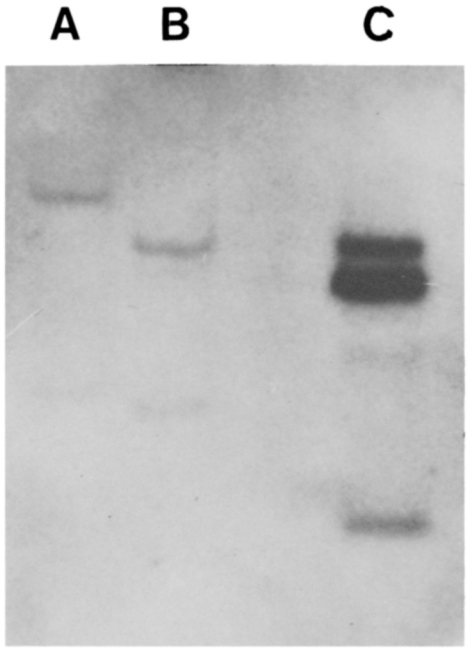

Figure 7. Identification of *tk*-Specific Sequences in Transformed Cell DNA Cleaved with Hinc II

High molecular weight DNA obtained from clone 7 (slot A) and clone 2b (slot B) was cleaved with the enzyme Hinc II and electrophoresed on 1% agarose gels. The DNA was transferred to nitrocellulose filters for annealing with ^{32}P-*tk* DNA (see Experimental Procedures). As a control, 50 pg of the purified 3.4 kb Bam fragment were digested with Hinc II and run in slot C. The additional minor bands shown in this slot result from incomplete digestion.

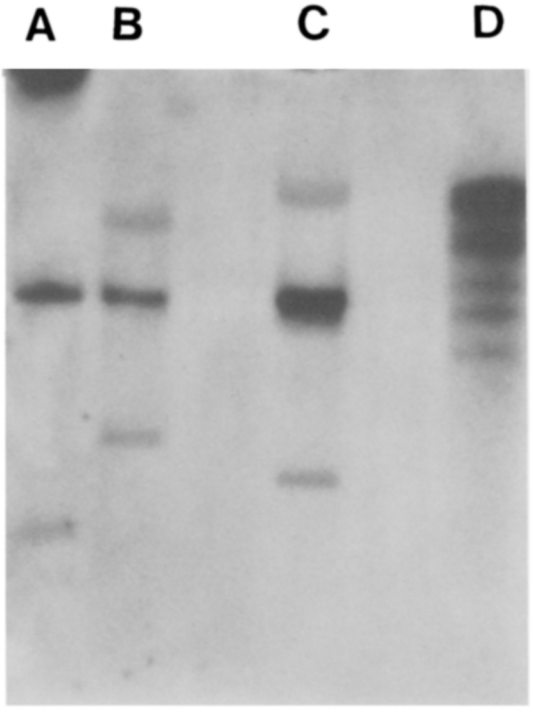

Figure 8. Identification of *tk*-Specific Sequences in DNA Fragments Generated by Cleavage of Transformed Clone DNA with Eco RI

High molecular weight DNA obtained from clone 2b (slot B) and clone 7 (slot C) was digested with the enzyme Eco RI and electrophoresed on 1.5% agarose gels. The DNA was transferred to nitrocellulose filters and annealed with ^{32}P-*tk* DNA (see Experimental Procedures). As a control, 2 ng of HSV-I DNA digested with Eco RI and Bam I were run in slot A. Slot D contains ^{32}P-adenovirus cleaved with Eco RI as markers.

annealing reactions, we can tentatively assume that the 2.2 kb results from two internal cleavages with the *tk* DNA, since this fragment contains 2–3 times as much homologous DNA as the two flanking fragments liberated from the ends of the 3.4 kb *tk* DNA.

If this assumption is correct, the two lighter bands each result from a single internal cleavage and cleavage at a site in adjacent cellular DNA. These data provide further support for the conclusion that within a given clone, the *tk* gene integrates only once per genome and the site of integration is unique to the individual clone. The observation that the 2.2 kb internal fragment persists in transformed cell DNA indicates that extensive rearrangement or cleavage within this large segment of the transfecting fragment has not occurred either prior to or during the integration event.

Analysis of Integration of the *tk* Gene in Additional Clones

We next asked whether the observations on the physical state of the *tk* gene in clones 2b and 7 are also true for other independently derived clones obtained following transfection with fractionated and unfractionated Bam I-cleaved HSV DNA. Clones 2b and 7 both resulted from transfection with the isolated 3.4 kb doublet generated by Bam I digestion. Clone 2b, however, was selected following transfection with 30 times the dose of DNA used in the selection of clone 7 (see Table 1). We have analyzed additional clones resulting from transfection with unfractionated Bam I-treated HSV-1 DNA (LH2-1, LH5C2-2), the 3.4 kb Bam I-generated doublet (LH1b, LH3b, LH4b) and the homogeneous 3.4 kb fragment purified following Hpa I-Bam I digestion (HB-1).

The localization of *tk*-specific sequences in Hinc II-cleaved cellular DNA from four additional clones (LH2-1, LH5C2-2, LH1b and LH3b) is shown in Figure 9. As discussed earlier, Hinc II cleavage of cellular DNA containing a single integrated copy of the *tk* gene should generate two annealing fragments. In each of the four clones examined, only two fragments can be seen that contain sequences homologous to *tk* DNA. Thus each transformed clone contains only a single copy of the *tk* gene. The sizes of the *tk*-specific fragments following

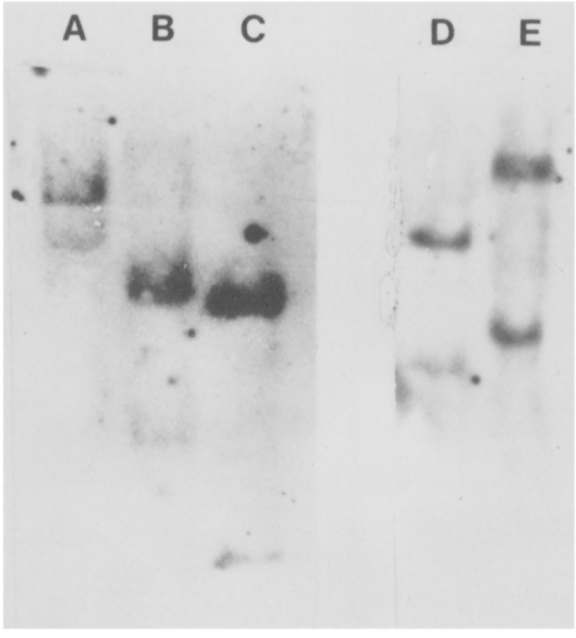

Figure 9. Identification of *tk*-Specific Sequences in DNA Fragments Generated by Hinc II Cleavage of a Variety of Transformed Clone DNAs

High molecular weight DNA isolated from tk^+-transformed cell clones LH5C2-2 (slot A), LH2-1 (slot B), LH3b (slot D) and LH1b (slot E) were digested with Hinc II and electrophoresed on 1% agarose slab gels, transferred to filters and annealed with ^{32}P-*tk* DNA probes. Slot C contains 100 pg of a purified 3.4 kb fragment cleaved with Hinc II to generate a band at 2.5 and 0.9 kb.

Hinc II cleavage differ in all six clones examined, and within each clone the sum of the molecular weights of the fragments exceeds the molecular weight of the transfecting DNA. Thus the *tk* gene is integrated within cellular DNA in all clones examined, and the site of integration differs for each clone. These conclusions are independent of the degree of purity or the dose of the transfecting DNA.

Discussion

Treatment of mutant mouse cells (Ltk^-) deficient in thymidine kinase with Bam I restriction endonuclease-cleaved HSV-1 DNA results in the appearance of numerous surviving colonies which stably express the tk^+ phenotype. The enzyme expressed by these cells is serologically and biochemically identical to the viral kinase and is readily distinguishable from host *tk* (Wigler et al., 1977). Through a serie of electrophoretic fractionations in concert with transfection assays, we isolated a 3.4 kb fragment which contains the thymidine kinase gene and which alone is competent in the biochemical transformation of Ltk^- cells. In this report, we have examined the distribution of *tk* sequences in the DNA of several transformed clones following stable gene transfer. A series of complementary experiments involving reassociation kinetics in solution and annealings with *tk* DNA to restriction-cleaved cellular DNA following electrophoresis and transfer to filters allow us to make the following general conclusions concerning the fate of the *tk* gene in all clones examined: the *tk* gene is present in all cells at a frequency of one copy per chromosomal complement; the *tk* gene is stably integrated in the DNA of all transformants; and integration is not site-specific and occurs at different loci in the DNA of all transformants examined.

In initial experiments, we nick-translated the homogeneous *tk* fragment to high specific activity for use as a probe in solution annealings with a vast excess of transformed cell DNA. The kinetics of annealing of *tk* DNA with the DNA of several different clones are strikingly similar and indicate that *tk* sequences comprise only one millionth of the cell DNA (Figures 4 and 5), a value consistent with the integration of only a single copy of the *tk* gene per cell. Since the mouse globin genes are present in the genome with an average reiteration of three, we performed an additional control in which ^{32}P-*tk* DNA and ^{3}H-globin cDNA were annealed with transformed cell DNA. The globin sequence anneals 4 times faster than *tk* DNA, a finding consistent with our estimate of copy number from acceleration rates (Figure 5). In concert with solution reactions, we exploited the elegant technology of Southern (1975) to determine the number and location of the *tk* sequences which are liberated upon restriction endonuclease treatment of transformed cell DNA. We examined two independently derived clones with three different enzymes—Kpn I, Hinc II and Eco RI, which recognize 0, 1 and 2 sites, respectively, within the 3.4 kb fragment containing the *tk* gene. We have examined four additional clones with Hinc II alone. In all cases, the number of *tk* fragments generated by restriction cleavage conforms to the predictions for the integration of maximally one *tk* gene per chromosomal complement.

Analysis of the molecular weights of the fragments which contain *tk* sequences following restriction endonuclease digestion of large molecular weight host DNA indicates that in all clones studied, the *tk* gene is covalently integrated into cellular DNA. In every case, the lengths of the DNA fragments generated are larger than we would predict if the *tk* DNA were to exist either as an unintegrated linear or circular form. No two clones share identical sites of integration in cellular DNA. This has been confirmed with three different enzymes for clones 2b and 7, and with a single enzyme for four additional clones. Within any given

clone, however, the site of integration must be stable.

We have found that viral *tk* DNA from six independently derived transformed clones is stably integrated with large molecular weight host DNA. This result does not imply that integration is always the fate of transfected genetic elements in the transformation process in general, or even that it is always the fate of transfected *tk* DNA in particular. At present, we have no information on the potential stability of extrachromosomal genetic elements in mammalian cells. To survive independently as a functional entity, such an extrachromosomal element would at the least require its own promoter for RNA transcription and an origin for DNA replication. It is not known whether the 3.4 kb Bam fragment containing the structural HSV *tk* gene meets either of these requirements.

The evidence is compelling that integration has occurred. We have not, however, demonstrated chromosomal integration; we do not know whether rearrangement of the viral gene has occurred or whether host sequences have been inserted within the viral gene; and we have not defined the sites on the viral gene where insertion into host DNA occurs. These studies are in progress, and will permit us to examine mechanisms of integration and the possibility of genomic rearrangement.

We have presented data indicating that there is probably only one *tk* gene per transformed cell. This is not a surprising observation considering the relatively low efficiency of the transfection process. The maximal efficiency of transfection we observe is about one transformant per ng *tk* gene per 10^6 cells. This reflects a single successful transformation event for only one of 2.7×10^8 molecules of *tk* DNA added. Unless phenomena such as specific cell competence or cooperativity in the integration process are operative, the probability of two independent integration events occurring in the same cell is vanishingly low.

The existence of a single active *tk* gene will facilitate the subsequent study of both its configuration in chromatin and its sequence organization. In preliminary studies, we isolated tk^- revertants which retain the *tk* gene and reexpress this function under appropriate selection conditions. The structure and sequence organization of the *tk* gene in both revertants and parental transformants can therefore be studied. Furthermore, the HSV *tk* gene, present as a single copy in the transformed cell, provides a useful model for studies on the transfer of cellular genes. Work in progress has demonstrated, to our surprise, that the *tk* gene present in transformants can again be transferred to recipient Ltk^- cells using restriction-cleaved, transformed cell DNA (M. Wigler, A. Pellicer, S. Silverstein and R. Axel, manuscript in preparation). The efficiency of this transfer is many times higher than the transfer of the *tk* gene when purified from viral DNA.

One final note concerns the frequency of stable gene transfer in nature and the more general question of genomic fluidity. Our data indicate that cultured cells have the ability to accept exogenous fragments of DNA, stably integrate this DNA into the genome, and ultimately express this information in the form of a functional protein which corrects a genetic deficiency of the cell. At present, we have no indication as to the frequency with which stable gene transfer occurs in nature, nor do we know the frequency of genomic rearrangement. The availability of a purified gene which can be stably transferred to cells, and for which both positive and negative selective conditions exist, provides a unique opportunity to address these problems.

Experimental Procedures

Cells and Viruses

CV-1 cells, a line of African green monkey kidney cells, were grown in roller bottle cultures in Dulbecco's modified Eagle's medium (DME) supplemented with 10% calf serum. Mouse Ltk^- cells (Kit et al., 1963) were obtained from Dr. P. Spear and maintained in DME supplemented with 10% calf serum and 30 μg/ml BUdR. Ltk^+ (HSV) cells lines LH1b, LH2b, LH3b, LH4b, LH7, LHHB-1, LH2-1 and LH5C2-2 were derived by transfecting Ltk^- cells with fragments of HSV-1 DNA (see Table 1) as previously described (Wigler et al., 1977), and were maintained in DME supplemented with 10% calf serum, 15 μg/ml hypoxanthine, 1 μg/ml aminopterin and 5 μg/ml thymidine (HAT medium).

The F strain of HSV-1 was grown and titrated in Vero cells as previously described (Nishioka and Silverstein, 1977).

Isolation of HSV DNA

Confluent roller bottle cultures of CV-1 cells were infected at 5 pfu per cell with HSV-1 in 10 ml of DME containing 1% calf serum. At 2 hr post-infection, 40 ml of DME containing 5 μCi/ml of ^{3}H-TdR and 1% calf serum were added to each bottle, and the infection was permitted to proceed for 36 hr. At this time, the cell culture fluid was collected and centrifuged at 8000 × g for 20 min in the GSA rotor of a Sorvall RC-5 centrifuge to remove cells and debris. Virus was then pelleted from the clarified media by centrifugation for 90 min at 20,000 × g. The pellet was resuspended in 0.1 M NaCl, 0.01 M Tris-HCl (pH 7.5), and digested with 40 μg/ml of RNAase A at 37°C for 30 min. Proteinase K was then added to 250 μg/ml, and the incubation was continued for an additional 15 min. The suspension was brought to 0.5% SDS to lyse the virions and release viral DNA. After an additional 1 hr of incubation, the DNA was purified by extraction with phenol followed by two extractions with chloroform-2% isoamyl alcohol. The DNA was then precipitated with 2 vol of ice-cold ethanol. HSV DNA prepared in this manner has a specific activity of $3–4 \times 10^8$ cpm/μg and is free of cellular DNA as judged by analytical ultracentrifugation in a Beckman model E centrifuge.

Isolation of Transformed Cell DNA

Transformed cell lines were grown into mass culture, and the cells were harvested and washed twice in isotonic phosphate-buffered saline. The packed cells were swollen on ice in hypotonic buffer [10 mM Tris (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$] and lysed by dounce homogenization. Nuclei were pelleted by centrifugation at 4000 × g. The pellet was homogenized (and resuspended)

in the above buffer containing 0.2% Triton X-100. After pelleting, the nuclei were resuspended in 10 mM Tris-HCl (pH 7.9), 0.4 M NaCl, 2 mM EDTA, and lysed by addition of SDS to 0.5%. Proteinase K was added to 200 μg/ml, and the mixture was incubated at 37°C for 3 hr. The viscous extract was extracted with phenol and then phenol-chloroform-isoamyl alcohol 50:48:2. The organic phases were reextracted; the aqueous phases were then pooled, extracted with chloroform-2% isoamyl alcohol; and nucleic acid was precipitated by the addition of 2 vol of ethanol. High molecular weight DNA, removed by spooling, was dissolved in 10 mM Tris-HCl (pH 7.9), 2 mM EDTA, and digested for 1 hr at 37°C with 40 μg/ml RNAase A and 10 μg/ml of RNAase T_1. The DNA was reextracted as described above and precipitated with ethanol. If the DNA was to be used for solution hybridization, it was sonicated at low wattage for 10 min in 0.1 M NaOH on ice to obtain 0.3 kb fragments. The sonicated DNA was then incubated at 37°C in 0.4 M NaOH for 8 hr to remove any trace of RNA. The solution was then neutralized, phenol-extracted, precipitated with ethanol and resuspended in 1 mM Tris (pH 7.9), 0.2 M EDTA at 30–50 mg/ml.

Isolation of Restriction Endonuclease Generated Fragments and Preparation of the *tk* Probe

HSV-1 DNA was digested with either Eco RI, Hpa I or Kpn I as previously described (Wigler et al., 1977). The fragments were resolved by electrophoresis through 0.7% agarose gels (40 × 20 × 0.3 cm) for 40 hr at 80 V. Fragments containing the *tk* gene were identified by transfection of Ltk⁻ cells (Wigler et al., 1977). They were 3.4, 5.2 and 8.3 kb for Bam I, Kpn I and Hpa I, respectively. The 5.2 and 8.3 kb fragments were extracted from the gels by solubilizing with 5 M $NaClO_4$, and the DNA was bound to hydroxyapatite at 60°C and eluted with 0.4 M phosphate buffer. The eluate was chromatographed over G-50 to remove phosphate and precipitated with ethanol. These fragments were subsequently digested with Bam, and the unique 3.4 kb fragment was isolated by electrophoresis in 1% agarose gels and extracted as described above.

The purified fragment containing the *tk* gene was labeled to high specific activity by nick translation as described by Maniatis, Jeffrey and Kleid (1975). The reaction mixture contained 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 50 μg/ml BSA, 1 ng/ml DNAase I, 4.0 μM ^{32}P-deoxyribonucleoside triphosphates (200–300 Ci/mmole), 1–2 μg/ml of DNA fragment and 1 unit of DNA polymerase per 0.1 μg of DNA. The reaction was incubated at 15°C for 1 hr, and the product was deproteinized by phenol extraction and separated from unincorporated nucleotides by column chromatography on Sephadex G-50. The final product had a specific activity of 2–4 × 10^8 cpm/μg.

Solution Annealing Reaction

Annealing reactions between ^{32}P viral DNA and cellular DNA were performed in 1 mM Tris (pH 7.9), 0.1 mM EDTA, 0.4 M NaCl at 69°C under paraffin oil. Reactions contained 20–30 mg/ml cellular DNA and 50 pg of ^{32}P viral DNA probe. Aliquots (10 μl) were removed and diluted in 1 ml of 30 mM NaOAc (pH 4.5), 0.1 M NaCl, 0.12 mM $ZnSO_4$. One half the sample was precipitated with 10% TCA, while the other half was assayed for duplex formation by digestion with 1000 units of S_1 nuclease (Miles) for 1 hr at 37°C.

Preparation of ^{32}P-*tk* DNA for Solution Hybridization

Following nick translation of the 3.4 kb fragment to high specific activity, the denatured product usually displayed 10–15% resistance to S_1 nuclease digestion and revealed only 50–60% duplex formation upon self-annealing. To overcome these problems, the probe was denatured and allowed to reassociate to a Cot of 10^{-4}. the foldback DNA was removed by chromatography on hydroxyapatite. The DNA remaining single-stranded at a Cot of 10^{-4} was isolated and allowed to reassociate to a Cot of 10^{-1}. Duplex DNA capable of annealing was isolated by digestion with S_1 nuclease (1000 units per ml) in 30 mM NaOAc (pH 4.5), 0.1 M NaCl, 0.12 mM $ZnSO_4$ for 1 hr at 37°C. 80% of the probe prepared in this way was capable of reassociation at saturation.

Filter Hybridization

The DNA fragments from agarose slab gels (21 × 18 × 0.6 cm) were transferred to nitrocellulose filter sheets essentially as described by Ketner and Kelly (1976). The filter was washed in 6 × SSC and then dried for at least 8 h under vacuum at 80°C. To prevent nonspecific binding of DNA during hybridization, the filter was soaked in 6 × SSC containing 0.02% each of polyvinylpyrrolidone (Sigma, PVP-360), bovine serum albumin and Ficoll (Pharmacia) for 6–10 hr at 65°C (Denhardt, 1966), and subsequently in the same buffer plus 10 μg/ml denaturated E. coli DNA for an additional 3 hr. After drying, the filters were saturated with 2 X SSC–10 mM EDTA containing denatured ^{32}P-*tk* DNA (10–30 ng/ml). Annealing was carried out at 65°C for 20–30 hr. The filters were then soaked in three changes of 2 X SSC, 25 mM sodium phosphate, 1.5 mM sodium pyrophosphate, 0.1% SDS (pH 7.0) at 65°C for a total of 3 hr. Following air-drying, the filters were developed at −70°C against Kodak XR2 X-ray film with intensifying screens (Dupont, lightning plus).

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