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# Transfer of Purified Herpes Virus Thymidine Kinase Gene to Cultured Mouse Cells

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## Summary

**Treatment of Ltk$^-$, mouse L cells deficient in thymidine kinase (tk), with Bam I restriction endonuclease cleaved DNA from herpes simplex virus-1 (HSV-1) produced tk$^+$ clones with a frequency of $10^{-6}$/2 $\mu$g of HSV-1 DNA. Untreated cells or cells treated with Eco RI restriction endonuclease fragments produced no tk+ clones under the same conditions. The thymidine kinase activities of four independently derived clones were characterized by biochemical and serological techniques. By these criteria, the tk activities were found to be identical to HSV-1 tk and different from host wild-type tk. The tk$^+$ phenotype was stable over several hundred cell generations, although the rate of reversion to the tk$^-$ phenotype, as judged by cloning efficiency in the presence of bromodeoxyuridine, was high (1–5 × $10^{-3}$). HSV-1 DNA Bam restriction fragments were separated by gel electrophoresis, and virtually all activity, as assayed by transfection, was found to reside in a 3.4 kb fragment. Transformation efficiency with the isolated fragment is 20 fold higher per gene equivalent than with the unfractionated total Bam digest. These results prove the usefulness of transfection assays as a means for the bioassay and isolation of restriction fragments carrying specific genetic information. Cells expressing HSV-1 tk may also provide a useful model system for the detailed analysis of eucaryotic and viral gene regulation.**

## Introduction

The isolation of specific fragments of eucaryotic DNA has permitted an analysis of the structural organization of specific genes and may ultimately provide information on the mechanism regulating the expression of these genes. Cleavage of the genome with restriction endonucleases followed by molecular cloning of these fragments in bacterial plasmids has permitted the isolation and amplification of specific genes (Cohen and Chang, 1974). Identification of eucaryotic genes within recombinant plasmid DNAs, however, requires molecular hybridization with purified RNA or DNA probes capable of reacting specifically with a single gene. Analysis of the biological activity of isolated DNA fragments by transfection provides an alternate means of identifying specific eucaryotic genes. This approach further demonstrates that the DNA contained within a given fragment includes the information required to code for the entire structural gene, in a form recognizable by the transcriptional and translational machinery of the host cell.

This experimental design has been used to identify specific fragments of the SV40 and adenovirus genomes containing the genes for malignant transformation (Graham et al., 1975). Another gene amenable to isolation by restriction endonuclease cleavage in concert with transfection assays is the thymidine kinase (tk) gene of herpes simplex virus (HSV-1). Infection of cells with ultraviolet-irradiated herpes virus results in the introduction and stable expression of multiple viral gene functions (Macnab and Timbury, 1976). HSV-1 thymidine kinase activity has been transferred to tk-deficient mouse L cells (Ltk$^-$) by infection with inactivated virions (Munyon et al., 1971).

We have therefore attempted to isolate a specific DNA fragment containing the thymidine kinase gene from the HSV-1 genome using transfection of this gene function as a bioassay. The choice of this system was dictated by several considerations. First, the viral genome is orders of magnitude less complex than the eucaryotic genome. This greatly enhances the prospects for successful transfection and allows the possibility of purification of active restriction fragments by size alone. Second, the tk$^+$ phenotype can be efficiently selected over a tk$^-$ phenotypic background by utilizing growth conditions in which the salvage pathway enzyme thymidine kinase is necessary for survival. There exist cell lines deficient in tk with low rates of spontaneous reversion to tk$^+$ which can be used as recipients. Third, the tk gene is an ideal subject for mutational analysis because the tk$^+$ or the tk$^-$ phenotype can be selected under various conditions. Fourth, the gene product, thymidine kinase, is a well characterized viral protein of known function.

In this report, we demonstrate the stable transfer of HSV-1 tk activity to mouse L cells (Ltk$^-$) by transfection with HSV-1 DNA cleaved by restriction endonuclease Bam I. The tk gene can be transfected using an electrophoretically pure fragment 3.4 kb in length. The transformed mouse cells with restored tk activity synthesize an enzyme with antigenic and electrophoretic properties identical to that coded for by the herpes virus genome. Furthermore, this gene function is stably expressed

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under selective pressure for several hundred generations.

## Results

### Transfection of tk Activity with Fragments of HSV DNA

The isolation of a specific fragment of the HSV-1 genome containing the thymidine kinase gene requires that we identify a restriction endonuclease capable of digesting HSV DNA, which makes no internal cleavages within the tk gene. Identification of such a DNA fragment in addition requires a cell line that will stably express the tk function upon competent transfection. Ltk$^-$ clone d, a clone of mouse cells resistant to bromo-deoxyuridine (BdUrd) and deficient in cytoplasmic thymidine kinase (Kit et al., 1963) was therefore chosen for transfection experiments. Ltk$^-$ cells are unable to grow in medium containing HAT (hypoxanthine, aminopterin and thymidine), in which survival depends upon the presence of both salvage pathway enzymes thymidine kinase and hypoxanthine–guanosine phosphoribosyl transferase (Littlefield, 1963). The cells have a very low rate of spontaneous reversion to the tk$^+$ phenotype, as judged by ability to form colonies in HAT-containing medium, and were used as host recipients to demonstrate that ultraviolet-inactivated HSV-1 virions could infect and stably confer HSV tk activity (Munyon et al., 1971).

Viral DNA for transfection was extracted from virions grown in Vero cells and purified free of contaminating host sequences by velocity sedimentation or CsCl equilibrium density centrifugation. Purity was monitored by isopycnic centrifugation in CsCl. This DNA was then cleaved with a series of restriction endonucleases. The DNA products of these digestions were separated by electrophoresis on 0.5% agarose slab gels (Figure 1). Although all the enzymes used for cleavage require the recognition of a unique hexanucleotide pair for activity, significant differences in the number of cleavage sites for the different enzymes is apparent. The gel profiles shown in Figure 1 reflect complete digestion by the endonuclease since first, incubation for an additional 3 hr results in no change in the band pattern; second, the addition of a second dose of enzyme at 3 hr followed by a second incubation did not alter the digestion profile; and third, adenovirus 2 DNA was completely digested under our reaction conditions.

In initial experiments, Bam I- and Eco RI-digested HSV DNA (Figure 1, slots E and G) were used in transfection assays. Cells were plated at a density of $6 \times 10^5$/100 mm petri dish in growth medium. Culture medium was removed 24 hr later, and cell monolayers were overlaid with restriction-cleaved

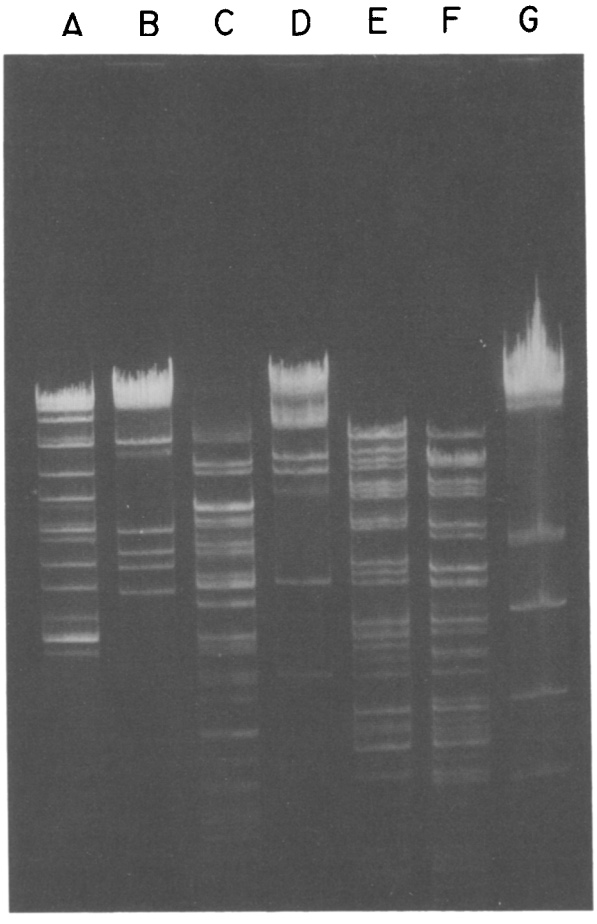


Figure 1. Digestion of HSV-1 DNA with DNA Restriction Endonucleases

1.0 $\mu$g of HSV-1 DNA was incubated with 3 U of various restriction enzymes for 3 hr at 37°C. The resultant DNA fragments were analyzed by electrophoresis on a 17 cm 0.5% agarose slab gel. Gels were stained with ethidium bromide and photographed under short-wave ultraviolet illumination. (A) Hpa I; (B) Bgl II; (C) Sal I; (D) Hind III; (E) Bam I; (F) Bam I + Eco RI; (G) Eco RI.

HSV-1 DNA co-precipitated with calcium phosphate (Graham and van der Eb, 1973). Salmon sperm DNA was used as carrier to yield a total DNA concentration of 20 $\mu$g/ml. After a 30 min exposure to DNA, cells were fed growth medium. 24 hr later, cultures were refed growth medium containing HAT and subsequently fed HAT medium every 2–3 days. After 2 weeks, surviving colonies were counted. Table 1 summarizes the results from four experiments. In all four experiments, untreated cultures, or cultures treated with either Salmon sperm DNA alone or with Eco R1-digested HSV-1, exhibited no surviving colonies in HAT. We estimate from these and other experiments that the reversion rate of these cells to the tk$^+$ phenotype is $<10^{-8}$. By contrast, cultures treated with Bam 1-digested HSV-1 DNA consistently displayed surviving colonies at a frequency of approximately 1 colony per $10^6$ cells per 2 $\mu$g HSV-1 DNA.

Table 1. Summary of Transfection Experiments with Unfractionated Restriction Endonuclease Digests of HSV-1 DNA

| | μg HSV-1 DNA per Dish[a] | Experiment 1 | | Experiment 2 | | Experiment 3 | | Experiment 4 | |
|---|---|---|---|---|---|---|---|---|---|
| | | Σ/Σ[b] | Specific Activity[c] | Σ/Σ | Specific Activity | Σ/Σ | Specific Activity | Σ/Σ | Specific Activity |
| Eco RI-Digested HSV-1 DNA | 4.00 | 0/2 | 0.00 | 0/2 | 0.00 | | | | |
| | 2.00 | 0/2 | | 0/2 | | | | 0/5 | 0.00 |
| | 1.00 | 0/2 | | 0/2 | | | | | |
| | 0.50 | 0/2 | | 0/2 | | | | | |
| | 0.25 | 0/2 | | 0/2 | | | | | |
| Bam I-Digested HSV-1 DNA | 4.00 | | | 4/2 | 0.50 | 7/2 | 0.90 | | |
| | 2.00 | | | 1/2 | 0.25 | 6/2 | 1.50 | | |
| | 1.00 | | | 0/2 | 0.00 | 2/2 | 1.00 | 3/5 | 0.60 |
| | 0.50 | | | 0/2 | 0.00 | 0/2 | 0.00 | | |
| | 0.25 | | | 1/2 | 2.00 | 3/4 | 3.00 | | |
| Bam I-, Eco RI-Digested HSV-1 DNA | 2.00 | | | | | | | 0/5 | 0.00 |
| Salmon Sperm DNA | | 0/5 | | 0/5 | | 0/5 | | 0/5 | |
| Untreated[a] | | 0/5 | | 0/5 | | | | | |

[a] All dishes received 10 μg DNA in 0.5 ml using salmon sperm DNA as carrier, as described in Experimental Procedures, except "untreated" cultures, which did not receive even salmon sperm DNA.
[b] Σ/Σ = total number of colonies per number of replicate dishes.
[c] Specific activity = HAT-resistant colonies per $10^6$ cells exposed per μg of HSV DNA.

These data suggest that Bam I cleavage of HSV-1 DNA generates at least one DNA fragment containing information for the entire tk structural gene. Eco RI fragments display no activity in transfection assay, presumably because cleavage occurs within the gene. To verify this, the infectivity of HSV DNA, which was digested with both Bam I and Eco RI, was assayed by transfection. This preparation of doubly cleaved fragments was not capable of generating HAT-resistant colonies.

## Transformed Cells Express HSV tk Activity

### *Electrophoretic Mobility of tk in Transformed Cells*

Proof that transformation of the mouse Ltk$^-$ phenotype results from the introduction and expression of viral DNA fragments requires us to demonstrate the viral origin of the tk expressed in the transfected clones. To this end, colonies were picked from different culture dishes, grown into mass cultures and further analyzed. Four clones were chosen, and their tk activity was characterized by electrophoretic mobility, immunologic neutralization and substrate specificity.

Electrophoretic profiles of the cytosol fractions derived from Ltk$^-$, L1210 (a mouse leukemic line), Vero and Ltk$^-$HSV$^+$ (Ltk$^-$ 12 hr post-infection with HSV-1 at 10 pfu/cell) are presented in Figure 2. As expected, Ltk$^-$ showed a small peak of activity of mitochondrial tk at an $R_f$ value of 0.9. In contrast, Ltk$^-$HSV$^+$, the infected cell homogenate, has an additional peak migrating with an $R_f$ of 0.45. This value is in agreement with previous studies of the HSV-1-induced tk (Cheng and Ostrander, 1976). The L1210 cell homogenate showed the normal pattern of mouse cytoplasmic and mitochondrial tk with activity migrating at an $R_f$ of 0.2 and 0.9, and with no detectable activity with an $R_f$ of 0.45. Vero cells, in which the HSV-1 was grown, similarly revealed no tk activity at an $R_f$ of 0.45. The second peak of activity at $R_f$ = 0.55 in the electrophoretic pattern of Vero could be due to the mitochondrial tk and has an electrophoretic mobility similar to the human mitochondrial tk (Lee and Cheng, 1976).

Studies were performed on the electrophoretic mobility of four herpes-transformed cell lines: LH1A2-1, LH2-1, LH5-1 derived from cultures exposed to 4, 2 and 0.25 μg DNA (Table 1, experiment 2), and LH5C2-2 derived from cultures exposed to 0.5 μg DNA (experiment 3). These lines were maintained in continuous culture in medium containing HAT for approximately 30 cell doublings. The main tk activity was consistently found at the position $R_f$ = 0.45, in agreement with that of Ltk$^-$HSV$^+$ (Figure 3). Although mouse mitochondrial tk is present at $R_f$ = 0.9, no mouse cytoplasmic tk is found in these lines.

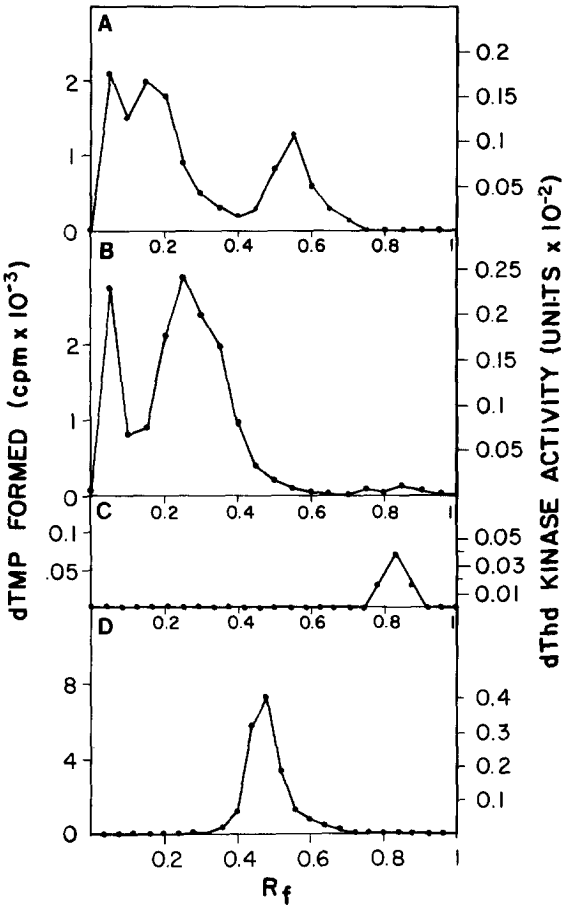


Figure 2. Electrophoretic Pattern of Thymidine Kinase Activities from Cytoplasmic Fractions of Various Cell Lines

30,000 × g supernatants of homogenates were applied to 5% polyacrylamide disc gels. Gels were sliced, and each slice was assayed for thymidine kinase activity as described previously (Lee and Cheng, 1976). Specific activities of samples are as indicated in Table 2. Electrophoretic mobilities ($R_f$ values) were calculated with reference to the electrophoretic mobility of bromphenol blue. (A) Vero; (B) L1210; (C) Ltk⁻; (D) Ltk⁻HSV⁺.

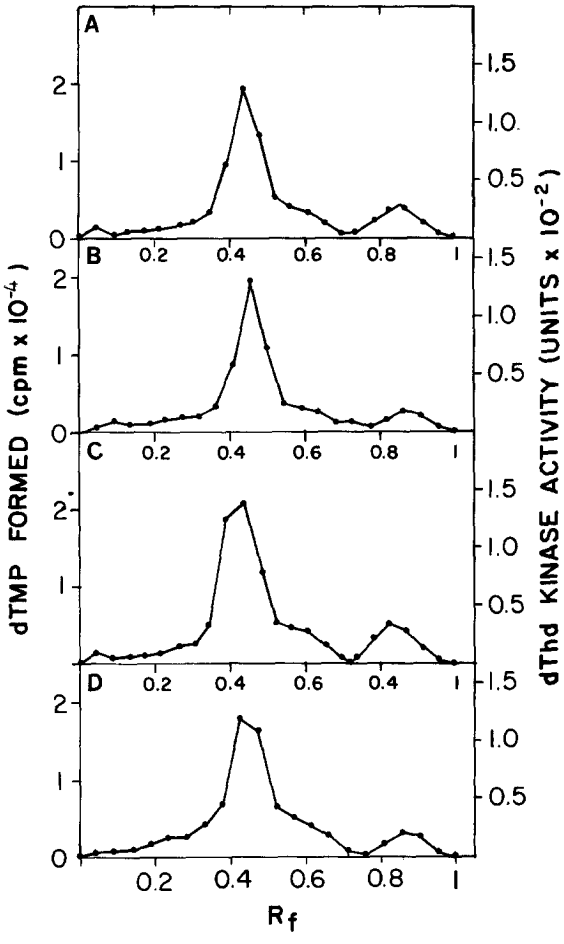


Figure 3. Electrophoretic Pattern of Thymidine Kinase Activities from Cytoplasmic Fractions of Four HSV-1 DNA-Transformed Cell Lines

The 30,000 × g supernatants of four transfected lines of mouse Ltk⁻ cells were assayed for thymidine kinase activity following gel electrophoresis as described in Figure 2. Specific activities of the samples are indicated in Table 2. (A) LH5C2-2; (B) LH5-1; (C) LH2-1; (D) LH1A2-1.

### *Antigenic Identity of tk in Transformed Cells*

Antisera raised against purified HSV tk effectively neutralizes the enzymatic activity of viral, but not cellular, tk (Klemperer et al., 1967). We would therefore predict that the tk activity of our transfected clones should be completely neutralized by these antisera. The experiments shown in Table 2 indicate that the tk activity of four transformed lines, LH2-1, LH5-1, LH5C2-2 and LH1A2-1, can be neutralized by antisera to purified HSV-1 tk. The tk activities of Vero, Ltk⁻ and A9, a mouse L cell with wild-type tk⁺, are not neutralized by the same antisera. These data demonstrate that the tk activity present in the transformed cell lines is antigenically related to purified HSV-1 tk. The residual activity remaining after neutralization of the transformed cell extracts may represent mouse mitochondrial tk activity. It is also apparent from these data that the four transformed lines have at least 20 times the tk activity of the parental Ltk⁻ cell.

### *Substrate Specificity of tk in Transformed Cells*

As a final criterion of identification, the substrate specificity of the tk activity found in the four transformed cell lines was analyzed. Two known inhibitors of herpes-specific tk were used, 5-ethyl deoxyuridine (5-ethyl dUrd) and 5-allyl dUrd (Cheng et al., 1976b). They inhibited phosphorylation of thymidine by 80 and 60%, respectively, in all four cell lines. These same drugs inhibited the tk activity in Ltk⁻HSV⁺ cells, but had no effect on extracts from Vero or A9 cells (Table 3).

### Stability of the tk⁺ Phenotype

Fourteen colonies were picked from experiments 2 and 3 (Table 1) and grown in HAT medium. Of these fourteen colonies, two displayed multiple abortive

Table 2. Specific Neutralization of HSV-1 Thymidine Kinase

| Source of tk | Activity with Preimmune Serum | | Activity with Antiserum | |
|---|---|---|---|---|
| | Units per ml | Units per mg | Units per mg | % Residual Activity |
| Vero | 1.0 | 0.25 | 0.37 | 150 |
| A9 | 1.67 | 1.50 | 1.50 | 100 |
| Ltk$^-$ | 0.06 | 0.007 | 0.007 | 100 |
| Ltk$^-$HSV$^+$ | 20.0 | 2.80 | 0.13 | 5 |
| LH1A2-1 | 1.1 | 0.19 | 0.017 | 9 |
| LH2-1 | 1.15 | 0.15 | 0.019 | 12 |
| LH5-1 | 0.9 | 0.14 | 0.015 | 10 |
| LH5C2-2 | 1.0 | 0.15 | 0.015 | 10 |

30,000 × g supernatants of homogenates (S-30) from various cell lines were mixed with preimmune sera or antisera to purified HSV-1 tk, and tk activity was assayed as described in Experimental Procedures. Activity is expressed both as units per ml of S-30 and units per mg protein within the S-30 fraction.

colonies (that is, formed small colonies which never grew larger than fifty cells) when replated, but twelve could be grown for at least 25 cell doublings under continuous selective pressure. Of these twelve, four were chosen for further study and have now been carried in HAT medium for over 6 months. Similarly, eleven colonies were picked from later experiments (see below, Table 6). One colony formed abortive colonies on replating, and ten lines have now been maintained for several months of continuous passage in HAT medium. We conclude that the acquisition of the HAT resistance phenotype and, presumably, the tk$^+$ phenotype is stable under selective conditions.

To assess the stability of the tk phenotype under other conditions, transformed cells, after approximately 50 doublings in continuous culture in HAT medium, were plated at low density into one of three media, and their cloning efficiency in these media was examined. The media were unsupplemented growth medium, growth medium supplemented with HAT, which selects for the tk$^+$ phenotype, or growth medium supplemented with BdUrd, which selects for the tk$^-$ phenotype. The data presented in Table 4 are summarized as follows; the cloning efficiency of the tk$^+$-transformed cells was the same in either (HAT) or nonselective media, and resembled that of the parental Ltk$^-$ line in nonselective media (30%). The cloning efficiency of these lines in the presence of BdUrd was reduced by two orders of magnitude to between 0.1 and 0.45%. It is of interest that this level of cloning efficiency is some 100 fold higher than that of A9, a mouse L cell derivative, or other tk$^+$ mouse cells. In this respect, these cells are similar to those tk$^+$ cells produced by infection of tk$^-$ cells with ultraviolet-inactivated virus (Davidson, Adelstein and Oxman, 1973).

Table 3. Effect of Pyrimidine Analogs on Thymidine Kinase Activity Derived from Various Sources

| Source of tk | % Activity in the Presence of Analogs | |
|---|---|---|
| | 5-Ethyl dUrd | 5-Allyl dUrd |
| Vero | 105 | 85 |
| A9 | 100 | 100 |
| Ltk$^-$ | 86 | 36 |
| Ltk$^-$HSV$^+$ | 20 | 39 |
| LH1A2-1 | 20 | 42 |
| LH2-1 | 28 | 48 |
| LH5-1 | 26 | 39 |
| LH5C2-2 | 24 | 44 |

Assay of the effect of analogs (100 $\mu$M) was carried out as described in Experimental Procedures. % activity is calculated with respect to the activity in the absence of an analog.

Table 4. Cloning Efficiency of Various Cell Lines in a Variety of Selective and Nonselective Media

| Cell Line | MEM[a] | HAT[b] | BdUrd[c] |
|---|---|---|---|
| A9 | $3.6 \times 10^{-1}$ | ND | $<1.0 \times 10^{-5}$ |
| Ltk$^-$ | $3.0 \times 10^{-1}$ | $<10^{-7}$ | $2.7 \times 10^{-1}$ |
| LH2-1 | $2.9 \times 10^{-1}$ | $2.2 \times 10^{-1}$ | $1.0 \times 10^{-3}$ |
| LH5-1 | $2.0 \times 10^{-1}$ | $2.3 \times 10^{-1}$ | $2.0 \times 10^{-3}$ |
| LH5C2-2 | $2.9 \times 10^{-1}$ | $3.8 \times 10^{-1}$ | $4.5 \times 10^{-3}$ |

[a] Nonselective medium (MEM, 5% fetal calf serum).
[b] tk$^+$ selective medium (MEM, 5% fetal calf serum, 15 $\mu$g/ml hypoxanthine, 1 $\mu$g/ml aminopterin, 5 $\mu$g/ml thymidine, 15 $\mu$g/ml glycine).
[c] tk$^-$ selective medium (MEM, 5% fetal calf serum, 30 $\mu$g/ml BdUrd).
Cells were plated in replicate, and colonies were stained and counted after 2 weeks as described in Experimental Procedures.

## Identification and Isolation of tk Active Bam Fragment

The observation that the DNA products of Bam I cleavage of HSV DNA can stably transfect tk activity suggests the use of this assay to identify the specific DNA fragment containing the thymidine kinase gene. The experimental design we have chosen involves the electrophoretic separation of specific groups of DNA and ultimately of individual DNA fragments. The fragment in which the tk gene resides is then readily identified by transfection with these fractionated populations of DNA. To this end, a Bam I digest of HSV DNA (Figure 1) was fractionated by electrophoresis on a 45 cm, 1% agarose slab gel. These DNA fragments were divided into five size classes and extracted from the agarose slab. DNA was purified free of agarose by hydroxylapatite and Sephadex G-50 chromatography, and was again analyzed by agarose gel electrophoresis (Figure 4).

The isolated size classes seen in Figure 4 were

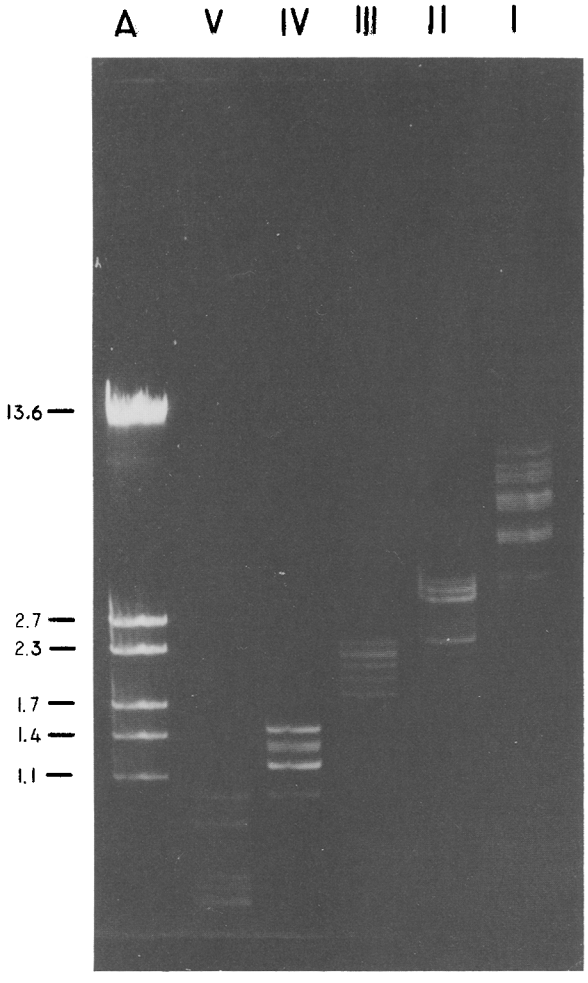


Figure 4. Fractionation of Bam 1-Cleaved HSV-1 DNA

HSV-1 DNA was digested with Bam I endonuclease, and the resultant fragments were separated on a 45 cm 1% agarose slab gel. This preparative gel was sliced, and the DNA corresponding to five discrete size classes was extracted from the gel and electrophoresed on a 0.5% agarose slab. The five size classes of DNA are shown in slots I–V. Slot A contains a preparation of Eco RI-digested Ad2 DNA as a size marker (Pettersson et al., 1973).

then used in transfection experiments following the same protocol as was used for unfractionated total digest. The results of this experiment are summarized in Table 5. Transfection activity is restricted to size class III. The small amount of activity seen in size class II probably results from the contamination of that class with size class III, as can be seen in Figure 4. Of particular interest is the observation that the specific activity of size class III is about 10 colonies per $10^6$ cells per $\mu$g genome equivalent, which is about 20 times the specific activity of the unfractionated genome. These data indicate that the tk gene is located on one of five well resolved fragments ranging in molecular weight from 2.5–3.7 kb. This size class was further fractionated into its five discrete fragments (Figure 5). From the molar yield of these fragments after Bam I digestion of

Table 5. Transfection with Size Classes of Bam I-Cleaved HSV-1 DNA

| Size Class | $\mu$g Equivalents of HSV-1 DNA per Dish | $\Sigma/\Sigma^a$ | Specific Activity[b] |
|---|---|---|---|
| I | 4.0 | 0/5 | 0.0 |
| II | 4.0 | 3/3 | 0.25 |
| III | 4.0 | 93/3 | 7.75 |
| IV | 4.0 | 0/5 | 0.0 |
| V | 4.0 | 0/5 | 0.0 |

[a] See Table 1.

[b] Specific activity calculated as before, but based on $\mu$g equivalents of HSV-1 DNA.

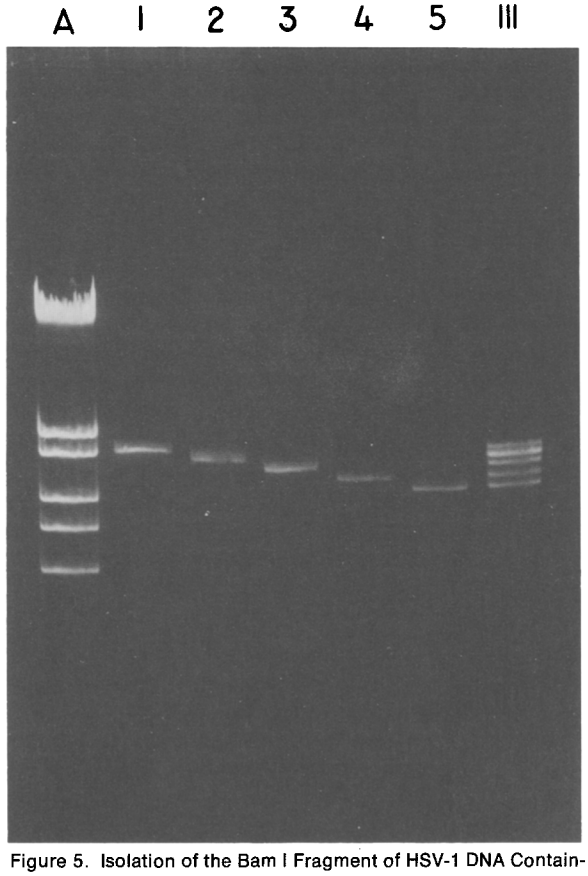


Figure 5. Isolation of the Bam I Fragment of HSV-1 DNA Containing the tk Gene

The DNA bands present in size class III (Figure 4) of a Bam I digest of HSV-1 DNA were fractionated into five fragments on 45 cm agarose slab gels. The isolated fragments were analyzed by electrophoresis on a 1% agarose slab gel. Slot A contains Eco RI-cleaved Ad2 DNA as molecular weight markers. Slots 1–5 contain the isolated fragments of size class III (see Figure 4). Slot III contains the unfractionated DNA of size class III.

total HSV DNA (Figure 1), it is probable that each of these fragments represents a homogeneous species of DNA. These individual fragments obtained from two separate preparations of HSV DNA were then assayed for their ability to transfect the tk gene (Table 6). Experiments 1 and 2 both indicate

that significant transfection activity resides only in fragment 2 of size class III. The other purified fragments of class III as well as class II DNA have little or no activity. The structural gene for tk is therefore contained within a single DNA fragment 3.4 kb in length. Again it should be noted that the specific activity of band 2 is about 20 fold higher per genome equivalent than unfractionated total digest, with colonies appearing in cultures treated with as little as 0.05 $\mu$g HSV-1 DNA equivalents or about 1 ng of DNA.

Table 6. Transfection with Fractionated Size Class III of Bam I-Cleaved HSV-1 DNA

| | $\mu$g Equivalents per Dish | Experiment 1 | | Experiment 2 | |
|---|---|---|---|---|---|
| | | $\Sigma/\Sigma$[a] | Specific Activity[b] | $\Sigma/\Sigma$ | Specific Activity |
| Size Class II | 2.0 | | | 0/2 | 0.0 |
| | 0.7 | | | 0/2 | 0.0 |
| Size Class III | | | | | |
| Band 1 | 2.0 | | | 1/2 | 0.25 |
| | 0.7 | | | 0/2 | 0.0 |
| Band 2 | 1.5 | 48/4 | 8.0 | | |
| | 0.5 | | | 19/2 | 19.0 |
| | 0.15 | | | 6/2 | 18.0 |
| | 0.05 | | | 5/3 | 30.0 |
| Band 3 | 1.0 | 0/4 | 0.0 | | |
| | 2.0 | | | 7/2 | 1.75 |
| | 0.7 | | | 3/2 | 2.0 |
| Band 4 | 1.5 | 0/4 | 0.0 | | |
| | 2.0 | | | 0/2 | 0.0 |
| | 0.7 | | | 0/2 | 0.0 |
| Band 5 | 1.5 | 0/4 | 0.0 | | |
| | 2.0 | | | 0/2 | 0.0 |
| | 0.7 | | | 0/2 | 0.0 |

[a] See Table 1.
[b] See Table 5.

## Discussion

The genome of herpes simplex virus consists of about $10^8$ daltons of DNA, sufficient information to direct the synthesis of 50–100 proteins (Kieff, Bachenheimer and Roizman, 1971). The enzyme thymidine kinase consistently appears shortly after infection, and considerable evidence indicates that it is coded for by the viral genome (Honess and Watson, 1974; Summers, Wagner and Summers, 1975). Considering the relatively small size of the viral genome, we would expect that a specific fragment could be obtained which could transfer viral tk activity to $tk^-$ cells. In this report, we describe the isolation of a 3.4 kb fragment of HSV-1 DNA which contains the structural gene for tk and which can stably transfect cells lacking this activity. The ease with which this gene can be identified in transfection experiments suggests that this approach may be applied to the localization of any gene for which selection criteria are available.

Our studies indicate that treatment of $Ltk^-$ cells with Bam I-digested HSV-1 DNA results in the stable transformation of a population of cells now expressing a $tk^+$ phenotype and capable of forming colonies in HAT selection media. Eco RI-treated HSV-1 DNA does not show this activity, presumably because this enzyme cleaves within the tk gene. It is possible, however, that Eco RI does not fragment the tk gene but liberates a series of fragments capable of transfecting lytic functions of HSV. This would result in cell death and obviously obscure the transfer of tk activity. If this were the case, we would expect that digestion of HSV DNA with both Bam I and Eco RI should leave the tk structural gene intact and result in fragmentation of the DNA coding for lytic functions. Preliminary results from our laboratory indicate that this doubly digested DNA is not competent in transfection assays, suggesting that Eco RI indeed cleaves within the tk gene. More definitive data will be obtained by transfection with the Eco RI cleavage productions of the 3.4 kb Bam I fragment.

It is of obvious importance to demonstrate that tk activity transfected by Bam I-cleaved HSV DNA is of viral origin. The spontaneous rate of reversion of the recipient $Ltk^-$ cells is $<10^{-8}$. Nevertheless, it was necessary to characterize carefully the tk activity expressed by the transformed cell clones. The tk activity of these HAT-resistant clones is at least 20 times greater than the activity detected in the $Ltk^-$ parent. The biochemical and antigenic properties of this enzyme were characterized by examining the neutralization of activity by specific antisera raised against HSV-1 tk; the electrophoretic mobility of the enzymatic activity; and the selective inhibition of tk activity by agents specific for the viral enzyme. For all these parameters, the enzyme was indistinguishable from HSV-1 tk and differed from either mouse or monkey cell tk. The conclusion that the tk activity appearing in transfected $Ltk^-$ cells results from the introduction and expression of viral DNA therefore appears firm.

Bam I-treated DNA, competent in transfection assays, can be resolved into about 25 bands on agarose gels. Through a series of electrophoretic fractionations in concert with transfection assays, we have identified and isolated a 3.4 kb fragment of viral DNA containing the tk gene. This fragment is capable of efficiently transfecting tk activity to $Ltk^-$ cells in the absence of any additional HSV information.

The frequency of transfection from experiments 2 and 3 in Table 1 is clearly too low to allow a

reliable statistical analysis of dose dependence. When transfection is performed with the purified tk fragment, the frequency of successful transfection increases and a clear dose dependence emerges. The precise nature of the dose dependence clearly requires additional experiments spanning broad ranges of gene concentrations. These experiments may provide useful information for understanding the mechanism of transfection, which is currently not understood. Nevertheless, several interesting questions emerge as a result of our data. The efficiency of transfection we observe with the purified fragment of Bam I digests of HSV-1 DNA is about 10 colonies per $\mu$g DNA per $10^6$ cells. Transfer of tk activity occurs with about 2% the efficiency of lytic transfection as assayed by plaque formation using purified intact HSV-1 DNA (personal observations). It is possible that this difference results in part from the fact that lytic transfection requires only the transient expression of viral functions in recipient cells. Detection of the transfer of tk activity requires the stable expression of this enzyme through several cell doublings.

Of further interest is the observation that the isolated Bam I fragment containing the tk gene is about 20 times more effective in transfection assays than the unfractionated Bam I digest of total HSV-1 DNA. This is an unexpected result for which we have no certain explanation. It is possible that transfection with the total DNA digest results in the transfer of either lytic functions or of other viral gene products capable of regulating tk expression. Transfer of these functions need only be transient to obscure the stable expression of tk activity. Expression of these inhibitory functions may therefore be far more frequent than the stable expression of tk activity, resulting in the lower efficiency of tk transfer observed with unfractionated, Bam I-cleaved HSV DNA.

At present, we have no information on the state of the viral tk gene in our transfected cell lines. Our observations indicate that the transfected Ltk$^-$ cells continue to express tk activity for hundreds of generations under selective pressure. The cloning efficiency of these transformed cells, either in the presence or absence of selective pressure, is equivalent to the cloning efficiency of the parental Ltk$^-$ line. The cloning efficiency of the transformed lines in the presence of BdUrd, however, which selects for the tk$^-$ phenotype, was about one hundredth the efficiency observed in HAT medium. This relatively high rate of reversion to the tk$^-$ phenotype may reflect the loss of the viral information from recipient cells or may result from suppression of tk expression or an unusually high mutation rate. It is of interest that high reversion rates were similarly observed in Ltk$^-$ cells infected with ultraviolet inactivated herpes virus (Davidson et al., 1973), and that tk activity in these phenotypic revertants could be reactivated with high efficiency.

A final point concerns the possible control of viral tk gene expression by the regulatory machinery of the host cell. Our data indicate that the 3.4 kb fragment, containing the tk structural gene, can be efficiently transcribed by host RNA polymerase. Transcription of this gene may proceed via initiation at a viral promoter or, if chromosomal integration has occurred, at a host promoter. The size of the viral thymidine kinase, 44,000 daltons (Honess and Watson, 1974; Summers et al., 1975), requires a DNA coding segment 1.3 kb in length. The 3.4 kb fragment is therefore likely to contain the tk structural gene as well as any regulatory elements which may exist adjacent to structural gene information. If such regulatory sequences exist, then this system may permit a direct analysis of the mechanism of control of viral gene expression in host cells and serve as a model for the regulation and expression of eucaryotic genes.

## Experimental Procedures

### Cell Culture

Vero cells were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 10% calf serum. L1210, a mouse leukemic cell line obtained from Dr. A. Bloch, was maintained in suspension culture in RPMI 1640 supplemented with 5% fetal calf serum (FCS). Mouse Ltk$^-$ cells were obtained from Dr. P. Spear and maintained in minimal essential medium (MEM) supplemented with 5% FCS and 30 $\mu$g/ml BdUrd. Mouse A9 cells, obtained from Dr. O. J. Miller, were maintained in MEM with 5% FCS. All lines except L1210 were grown as monolayer cultures at 37°C under an humidified atmosphere of 5% $CO_2$.

### Cloning Efficiency

For cloning efficiency experiments, cells were seeded at 10, $10^2$, $10^3$, $10^4$ or $10^5$/100 mm petri dish, at least in triplicate, directly into selective or nonselective medium. After 10–17 days, cells were fixed and stained. Only large colonies were counted, and cloning efficiency was calculated as number of colonies per plate divided by number of cells initially seeded.

### Transfection and Selection of the tk$^+$ Phenotype

Transfections were performed as described by Graham and van der Eb (1973). HSV-1 DNA was mixed with salmon sperm DNA (Worthington) to a final concentration of 20 $\mu$g/ml in Hepes-buffered saline: 8.0 g/l NaCl, 0.37 g/l KCl, 0.125 g/l $NA_2HPO_4 \cdot 2H_2O$, 1.0 g/l dextrose and 5.0 g/l Hepes (pH 7.10). 2 M $CaCl_2$ were added to a final concentration of 125 mM, and the mixture was allowed to stand at room temperature for 30 min before overlaying cells. Prior to transfection, Ltk$^-$ cells were grown for three cell doublings in the absence of BdUrd. Cells were then seeded at $6 \cdot 10^5$/100 mm petri dish. 24 hr later, the cell number was $10^6$ per dish. Growth media were aspirated, and cells were overlaid with 0.5 ml of the previously prepared DNA/Ca phosphate mixture. After 30 min, an additional 10 ml of MEM + 5% FCS were added. After 12 hr, media were aspirated, and cultures were fed 10 ml of MEM + 5% FCS. After another 12–24 hr, media were again aspirated, and cultures were fed HAT selection media (15 $\mu$g/ml hypoxanthine, 1 $\mu$g/ml aminopterin, 5 $\mu$g/ml thymidine, 15 $\mu$g/ml glycine in MEM + 5% FCS). Cultures were

thereafter fed every 2-3 days with HAT until the experiment was terminated.

After 2 weeks, colonies were counted in stained or unstained plates. Where HAT-resistant colonies had developed, isolated colonies of cells were picked using the sterile cylinder technique (Ham, 1972).

**Assay of Thymidine Kinase Activity**

Cells growing in monolayer cultures were scraped into phosphate-buffered saline. After washing with phosphate-buffered saline, the cell pellet was suspended in 5 vol of extraction buffer: 0.01 M Tris-HCl (pH 7.5), 0.01 M KCl, 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol and 50 $\mu$M thymidine. The cell suspension was frozen and thawed 3 times, and the KCl concentration was adjusted to 0.15 M. After sonication, the homogenate was centrifuged at 30,000 × g for 30 min, and the supernatant was used for tk assays as previously described (Lee and Cheng, 1976). One unit of thymidine kinase is defined as the amount of enzyme which converts 1 nmole of thymidine into thymidine monophosphate (TMP) per minute.

**Electrophoresis of Thymidine Kinase Activity**

Polyacrylamide gel electrophoresis analyses were performed in 5% acrylamide gels as previously described (Lee and Cheng, 1976). Thymidine (50 $\mu$M), mercaptoethanol (2mM) and $MgCl_2$ (2 mM) were added to the gel polymerization mixture to stabilize the enzyme activity. At the end of the run, the gels were cut into 2.5 mm slices and assayed for activity as previously described (Lee and Cheng, 1976).

**Preparation of Antisera to HSV-1-Induced Thymidine Kinase and the Enzyme Neutralization Experiments**

HSV-1-induced thymidine kinase was purified by means of affinity column chromatography (Cheng and Ostrander, 1976). The purified enzyme was injected into New Zealand white rabbits, and the gammaglobulin fraction was partially purified as described (Cheng, Chadha and Hughes, 1976a). The antiserum or preimmune serum was mixed with an equal volume of an enzyme preparation, and ATP-Mg was added to 6.7 mM. The enzyme-antibody mixture was incubated for 30 min at room temperature. The mixture was centrifuged at 2000 × g for 10 min, and the supernatant was assayed for tk activities.

**Effect of Pyrimidine Analogs on the Thymidine Kinase Activity of Extracts from Transformed and Normal Cells**

100 $\mu$M of 5-ethyl dUrd or 5-allyl dUrd were included in the assay mixture that contains 50 $\mu$M $^{14}$C-thymidine and 2 mM Mg-ATP, and the inhibition of the conversion of $^{14}$C-thymidine to $^{14}$C-TMP was measured (Cheng et al., 1976b). The pyrimidine analogs were a gift from Dr. M. Bobek.

**Growth of Virus and Isolation of Intact Viral DNA**

The F strain of HSV-1 was obtained from Dr. Bernard Roizman, and after plaque purification, grown up into stocks by passage at low moi. Virus stocks routinely had titers of 2-5 × $10^9$ pfu/ml.

Vero cells grown in glass roller bottles were infected at an moi of 5 for 2 hr. At this time, the infecting fluid was removed, and cells were overlaid with 50 ml of DME with 2% calf serum. At 24 hr post-infection, the cells were shaken off the glass and harvested by centrifugation. Cells were resuspended in RSB [10 mM Tris-HCl, 10 mM NaCl, 1.5 mM $MgCl_2$ (pH 7.5)] and lysed by the addition of NP-40 to 1%. Nuclei were removed by low speed centrifugation and lysed by the addition of deoxycholate to 1%. After sonication, large debris was removed by centrifugation at 10,000 × g for 10 min, and virus from the cytoplasmic and nuclear fractions was pelleted by centrifugation for 60 min at 15,000 rpm in an SW27 rotor. The virus pellets were resuspended in virus buffer (VB) [0.1 M NaCl, 0.01 M Tris-HCl (pH 7.5)] and centrifuged through 10-50% w/v sucrose gradients made in VB for 1 hr at 23,000 rpm in an SW27 rotor. The visible band of virus was removed, and after dilution in VB, pelleted by centrifugation at 25,000 rpm for 2 hr in an SW27 rotor. The pelleted virus was resuspended in 10 mM Tris, 1 mM EDTA, and DNA was released by addition of SDS to 0.5% followed by treatment with pronase (heat-inactivated for 10 min at 80°C) at 200 $\mu$g/ml. The released DNA was centrifuged through neutral sucrose gradients (10-30% w/v) prepared in 1 M NaCl, 50 mM Tris, 10 mM EDTA (pH 7.5) containing 0.15% Sarcosyl for 12 hr at 20,000 rpm in an SW27.1 rotor. The gradients were scanned at 254 nM using an ISCO density gradient fractionator. The peak corresponding to 56S DNA (Kieff, Bachenheimer and Roizman, 1971) was collected and concentrated by ethanol precipitation. Viral DNA was monitored for contamination with host sequences by isopycnic centrifugation in CsCl and by agarose gel electrophoresis.

**Restriction Endonuclease Digestion**

Restriction endonucleases Bam I, Eco RI, Sal I, Hind III and Hpa I were obtained from New England Biolabs. Bgl II was a gift from Dr. R. Roberts. Reaction mixtures contained in 100λ: 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$ and 3 units of restriction enzyme per $\mu$g of HSV-1 DNA. Reactions were for 3 hr at 37°C and were terminated by the addition of 10λ of 0.2 M EDTA. DNA was extracted with phenol/chloroform and concentrated by ethanol precipitation.

**Gel Electrophoresis of DNA Fragments**

DNA restriction fragments were separated by electrophoresis in 0.5% or 1.0% agarose (SeaKem) slab gels. The cooled 17 cm gel was run for 2.0 hr at 200 V in a buffer system of 0.04 M Tris-HCl, 0.004 M sodium acetate, 0.001 M $Na_2EDTA$ (pH 7.9). Gels were stained in ethidium bromide (1 $\mu$g/ml $H_2O$) and photographed under 254 nm ultraviolet light. For preparative runs, DNA was electrophoresed at 350 V for 13 hr in 45 cm 1.0% agarose slab gels.

**Isolation of DNA Restriction Fragments**

After electrophoresis, slab gels were stained with ethidium bromide and visualized with a short-wave ultraviolet light. Gel slices containing fragments of interest were made and dissolved in 5 vol of 5 M $NaClO_4$, 0.1 M Tris-HCl (pH 8.1). The DNA/agarose solution was loaded onto an hydroxyapatite column at 60°C and washed extensively with 0.14 M phosphate (pH 6.8). The DNA was eluted in a small volume with 0.5 M phosphate and desalted on a G-50 sephadex column preequilibrated with 0.01 M Tris-HCl (pH 7.9), 0.15 M NaCl, 0.001 M EDTA. DNA extracted from a staphylococcal nuclease limit digest of calf thymus nuclei (Axel, 1975) was added as carrier to 10 $\mu$g/ml, and the DNA was concentrated by ethanol precipitation. To calculate yields throughout the procedure, Bam I-digested, $^{32}$P-labeled HSV-1 DNA was added as tracer.

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## Note Added in Proof

The 3.4 kb band we have isolated consists of a partial molar fragment along with a molar fragment containing the tk gene. We have subsequently purified the tk-containing fragment by isolation of a pure 8.3 kb fragment following HPA I digestion which can transfer the tk activity. Cleavage of this fragment with Bam I generates the 3.4 kb fragment containing the tk gene in pure form.