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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-irraditated 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

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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

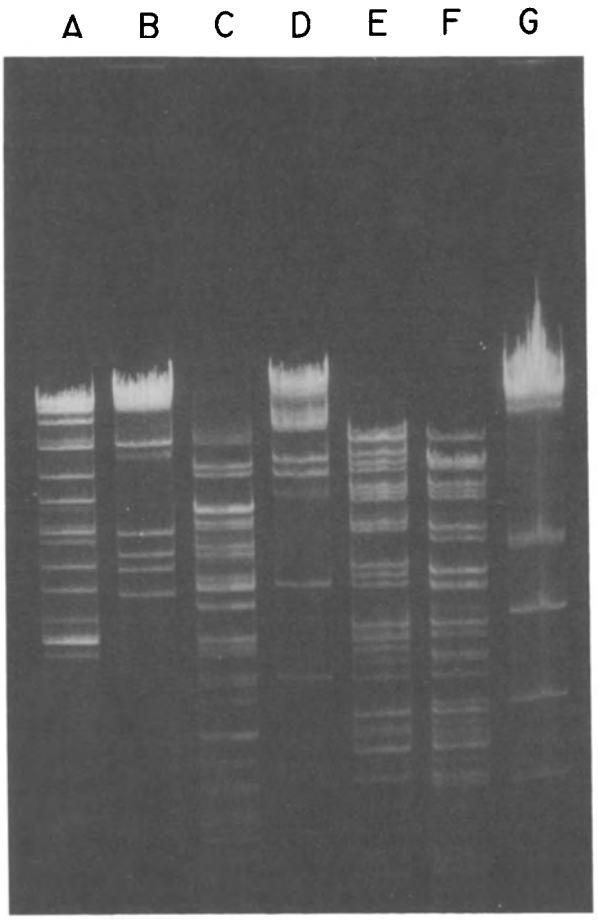


Figure 1. Digestion of HSV-1 DNA with DNA Restriction Endonucleases

1.0 μg 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 μg/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 col-

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 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

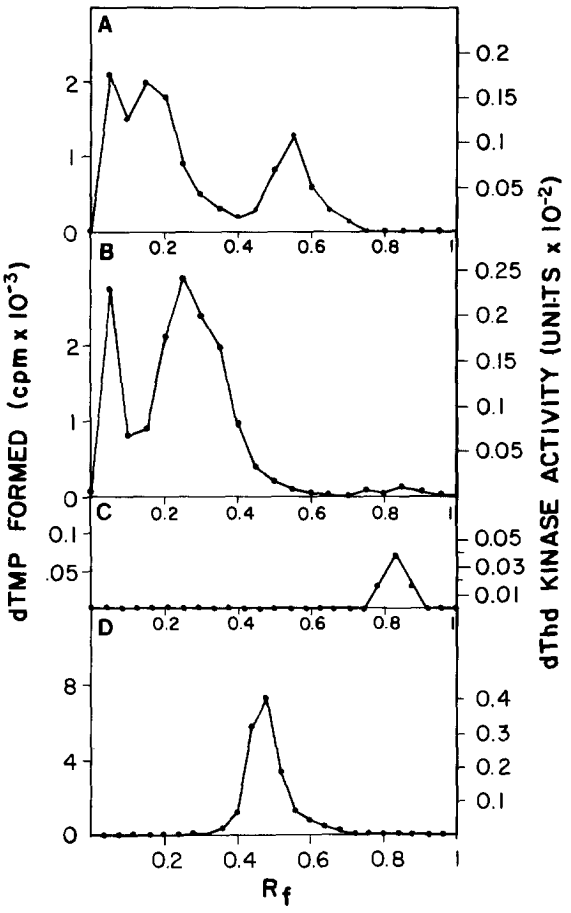


Figure 2. Electrophoretic Pattern of Thymidine Kinase Activities from Cytoplasmic Fractions of Various Cell Lines

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⁺.

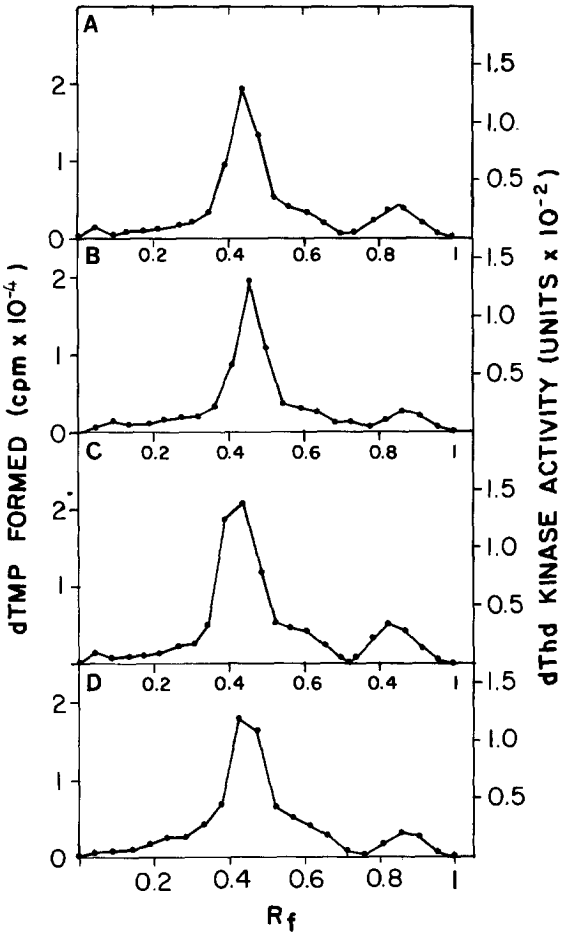


Figure 3. Electrophoretic Pattern of Thymidine Kinase Activities from Cytoplasmic Fractions of Four HSV-1 DNA-Transformed Cell Lines

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

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

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

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 μM) 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 μg/ml hypoxanthine, 1 μg/ml aminopterin, 5 μg/ml thymidine, 15 μg/ml glycine).
[c] tk$^-$ selective medium (MEM, 5% fetal calf serum, 30 μg/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

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