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Hybrid plasmids containing an active thymidine kinase gene of Herpes simplex virus 1

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ABSTRACT

The gene for the thymidine kinase (TK) of Herpes simplex virus type 1 (HSV-1) is located in the KpnI m and BamHI p fragments of the genome (Wigler et al., Cell 11, 223-232 (1977)). These fragments have been inserted into the EcoRI and BamHI sites, respectively, of plasmid pBR322, and propagated in *E.coli*. The TK gene contained in the recombinant plasmids was shown to be biologically active when introduced into TK^- mouse L cells. Detailed restriction site maps of the BamHI p fragment have been constructed and the approximate location of the TK gene has been determined. Mouse cells transformed with cloned HSV-1 tk^+ DNA produced HSV-1-specific thymidine kinase; superinfection with HSV-1 tk^- virus increased the level of TK activity tenfold, suggesting that the BamHI p sequences present in transformed cells respond to virus-encoded regulatory gene product(s).

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) codes for both a thymidine kinase (TK) and a deoxycytidine kinase which appear to be genetically and biochemically identical (1,2). Infection of mouse LTK^- cells which lack cytoplasmic TK activity (3) with UV-irradiated virus results in the appearance of colonies which express virus TK (4,5). The gene coding for HSV-1 TK has been physically mapped using two techniques. In one, isolated restriction fragments of viral DNA were used to transform mouse LTK^- cells (6). In the other, bromodeoxycytidine was used to select TK^- recombinants produced by co-infection of tissue culture cells with intact DNA of HSV-1 tk^+ and isolated restriction fragments of HSV-1 tk^- DNA (7). The TK gene is located within the HSV-1 KpnI m and BamHI

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p fragments (see Fig. 1).

Since TK^+ cells can be selected in medium containing hypoxanthine, aminopterin and thymidine (HAT) (8,9), HSV TK may be used as a selectable marker for the introduction of exogenous genes into cultured cells (10-12). In this communication we describe the isolation of recombinant plasmids grown in E.coli which contain the HSV-1 TK gene and are able to transform mouse LTK^- cells efficiently. The cloned virus fragments have been mapped and the approximate location of the TK gene determined. The level of TK activity in cells transformed with the HSV-1 tk^+ BamHI p fragment increased tenfold or more after superinfection with HSV-1 tk^- virus.

MATERIALS AND METHODS

Cells and viruses.

E.coli strain X1776 (13) (obtained from Roy Curtiss III) was grown in L broth containing 100 µg/ml diaminopimelic acid (DAP), 10 µg/ml nalidixic acid (NAL) and 30 µg/ml thymidine (THY). E.coli strain C600 containing the plasmid pBR322 was grown in L broth containing 100 µg/ml ampicillin and 5 µg/ml vitamin B1. Baby hamster kidney cells (BHK21-C13) and thymidine kinase deficient mouse L (LTK^-) cells were originally obtained from S. Kit and propagated in monolayers as described previously (14,3). HSV-1 Glasgow strain 17 was grown in BHK21-C13 cells after infection at low input multiplicities (15). HSV-1 tk^- (strain MDK) was originally obtained from S.Kit.

Isolation of DNA.

HSV-1 DNA was extracted as described (15), and further purified by isopycnic banding in CsCl followed by rate zonal sedimentation in preformed CsCl gradients (16). Two methods were used for the preparation of plasmid DNA. (A) Bacterial cells were grown to an apparent A_{630} of 0.6; chloramphenicol was added to 200 µg/ml (E.coli C600) or 20 µg/ml (E.coli X1776) and incubation was continued overnight. After harvesting, cells were resuspended in 1/50th vol of 25% sucrose in 50 mM Tris-HCl (pH 8.0) and incubated with 500 µg/ml lysozyme at

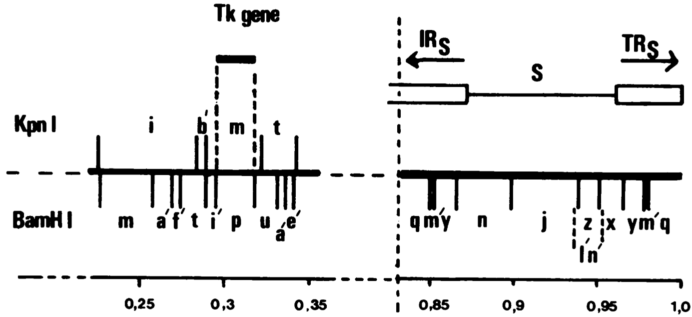

Figure 1. Restriction site maps of parts of the HSV-1 genome. Physical maps for the region comprising the thymidine kinase locus and the short (S) region are depicted. The location of the TK gene is taken from the results of Wigler et al. (6) and Stow et al. (7). The inverted repetitive regions which bound the short region (39) have identical sequences, as judged by restriction endonuclease mapping and electron microscopy of self-annealed single strands. Map units are given as fractional length (HSV-1 DNA, 96 x 10^6 daltons).

0^oC for 30 min. EDTA was added to 0.05 M and after 30 min at 0^oC NaCl was added to 1.0 M and sodium dodecylsulfate to 1%. The lysate was stored for at least 3 h at 0^oC and centrifuged at 20'000 rpm in a Beckman A1.30 rotor. The supernatant fluid was diluted with an equal volume of water and extracted twice with phenol. After dialysis against 1x SSC, supercoiled plasmid DNA was purified by isopycnic banding in CsCl containing 120 µg/ml ethidium bromide. The dye was removed by extraction with CsCl-saturated isopropanol, the DNA precipitated with ethanol and dissolved in 10 mM Tris-HCl (pH 7.4), 1 mM EDTA. (B) Cultures of plasmid-containing E.coli X1776 were grown in 200 ml of medium containing per liter: 10 g Bacto tryptone, 1 g yeast extract (Difco), 1 g glucose, 8 g NaCl and 0.3 g $CaCl_2$ in 2-liter flasks, to an apparent A_{650} of 1 (the estimation was visual, to avoid manipulation of the culture). Then 800 ml of medium containing 170 mg/L chloramphenicol (Siegfried, Zofingen, Switzerland) were added and incubation was continued overnight at 37^oC with shaking (70 rpm). Bacteria were killed with chloroform (17) and collected by centrifuga-

tion. Plasmids were isolated by a combination of published methods. In short, cells were lysed by the successive addition of lysozyme, EDTA and sodium dodecylsulfate. After addition of NaCl the lysate was kept at $0^{o}C$ for at least 7 h (18). After centrifugation the supernatant was treated with phenol, chloroform and RNase A. The DNA was precipitated by the addition of polyethylene glycol (19), redissolved, and linear molecules were denatured at pH 12.5 (20). After neutralization and phenol extraction, form I DNA was precipitated with ethanol, redissolved, and centrifuged through a sucrose density gradient.

Enzymes.

Restriction endonucleases EcoRI, BamHI, HindII and AluI were purchased from Boehringer and New England Biolabs. BglII and KpnI were purified by chromatography on Ultragel (LKB), DE-52 (Whatman) and Biorex (Biorad) or purchased from New England Biolabs. SstI and SmaI were the kind gifts of P. Rigby and J. Arrand. T4 DNA ligase was purchased from New England Biolabs. Terminal deoxynucleotidyl transferase (21) and λ 5'-exonuclease (22) were prepared as described.

Labeling of DNA, restriction endonuclease cleavage and gel electrophoresis.

Intact HSV-1 or plasmid DNA was labeled in vitro with the four α-^{32}P-deoxyribonucleoside triphosphates (300 Ci/mmole, Amersham) by "nick translation" (23). The termini of BamHI--cleaved DNA fragments were labeled directly with T4 DNA polymerase: 0.2 μg of DNA were incubated for 10 min at $37^{o}C$ in 20 μl of a solution containing 0.025 units of T4 DNA polymerase (Bethesda Research Laboratories), 75 pmoles each of α-^{32}P-dGTP, α-^{32}P-dATP and α-^{32}P-dTTP (specific activity 300 Ci/mmole each), 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 1 mM dithiothreitol and 50 μg/ml bovine serum albumen. Only the three nucleotides were used since the overhanging 5' end of BamHI-cleaved DNA is GATC. Restriction analysis showed that only termini-containing fragments were labeled.

Unlabeled and radioactive DNA samples were cleaved with restriction endonucleases, the fragments separated by agarose

gel electrophoresis, and purified on hydroxyapatite as previously described (16). Polyacrylamide gel electrophoresis was at 3.4 V/cm at room temperature on gel slabs formed from solutions containing 5% or 15% w/v of 29:1 w/w acrylamide: N,N'-methylene-bisacrylamide. The gel and tank buffer was 89 mM Tris base (Trizma), 89 mM boric acid, 2.5 mM EDTA. Gels were dried and autoradiographed at -70°C using preflashed Kodak X-Omat H film with an intensifier screen.

Construction of recombinant plasmid DNA molecules.

Hybrid plasmids containing HSV-1 DNA were prepared using two methods.

1. Direct ligation of BamHI cohesive ends.

A mixture of 7 μg/ml pBR322 DNA and 20 μg/ml HSV-1 DNA was incubated with BamHI at 37°C in TMM buffer (6 mM Tris-HCl (pH 7.4), 6 mM $MgCl_2$, 6 mM β-mercaptoethanol and 1 mg/ml bovine serum albumen (Sigma, St. Louis)). The reaction was terminated by incubation at 70°C. T4 DNA ligase was added to a final concentration of 0.33 U/ml along with 1/10 volume of 300 mM Tris-HCl (pH 8.0), 40 mM $MgCl_2$, 12 mM EDTA, 2 mM ATP, 100 mM dithiothreitol, 50 μg/ml bovine serum albumen. After 2h at 17°C the DNA was used directly to transform bacteria.

2. Poly(dT) and poly(dA) tailing.

pBR322 was digested with EcoRI, treated with λ 5' endonuclease to remove an average of 6 nucleotides from the 5' end, and then terminal transferase used to add poly(dT) tails as described previously (24).

Cleavage with restriction endonuclease KpnI results in molecules with exposed 3' single strand termini (25), which can be "tailed" directly. HSV-1 DNA was digested to completion with KpnI in TMM buffer and incubated at 70°C to terminate the reaction. The DNA was diluted to 30 μg/ml in a solution containing (final concentration) 100 mM sodium cacodylate (pH 7.2), 10 mM NaH_2PO_4, 1 mM dATP, 5 mM $MgCl_2$, 50 μg/ml bovine serum albumen and 360 units/ml terminal transferase. After 45 min at 37°C the dA-tailed HSV-1 DNA KpnI fragments were mixed with the dT-tailed plasmid DNA

and diluted to 1 μg/ml each in 100 mM NaCl, 50 mM Tris-HCl (pH 7.4), 5 mM EDTA. The mixture was kept for 10 min at 65°C and then for consecutive one hour-periods at 46°C, 37°C and 23°C.

Transformation of E.coli X1776.

A single colony of E.coli X1776 was picked into 100 ml of L broth supplemented with DAP, NAL and THY, and the culture incubated at 37°C until it reached an apparent A_{630} of 0.6. The cells were harvested by centrifugation for 5 min at 0°C and 4000 rpm in an SS34 Sorvall rotor. All subsequent manipulations were at 0°C. After washing once in 20 ml 10 mM NaCl the cells were resuspended in 20 ml 100 mM $CaCl_2$, left for 1 h at 0°C, harvested by centrifugation as above, but at 3000 rpm, resuspended in 2 ml chilled 100 mM $CaCl_2$, and left overnight at 0°C. 100 μl of the cell suspension were added to 100 μl 10 mM $CaCl_2$, 10 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.5) containing up to 100 ng of pBR322 DNA joined to HSV-1 DNA fragments. After 30 min at 0°C, the temperature was raised to 23°C for 15 min, one ml of L-broth containing DAP, NAL and THY was added and incubation continued for 10 min at 0°C, and then for 90 min at 37°C with gentle shaking. Samples were plated on L-broth agar plates supplemented with DAP, NAL, THY and 50 μg/ml ampicillin. Transformation efficiencies were up to 10^7 transformed colonies per μg of ligated pBR322 DNA. "Tailed" pBR322 DNA alone yielded only 15 colonies per μg; after annealing with "tailed" HSV-1 KpnI fragments, 2-3 x 10^4 colonies per μg were obtained.

Isolation of colonies containing the HSV-1 TK gene.

About 300 ampicillin-resistant colonies obtained after transformation with hybrids containing KpnI fragments were picked, plated onto reference agar plates and transferred to replicate gridded nitrocellulose membranes. The filters were processed as described (26). The cells were lysed in situ and hybridized to ^{32}P-labeled total HSV-1 DNA or purified ^{32}P-labeled HSV-1 KpnI m DNA which contains the gene coding for HSV-1 TK (Fig. 1). About 80% of the colonies showed positive

hybridization with total HSV-1 DNA and nine out of the 300 colonies hybridised strongly to the KpnI m fragment; the plasmids in these colonies were designated M1 to M9. Clones of M2, M3, M4 and M5 were grown in liquid culture for the preparation of plasmid DNA.

E.coli X1776 transformed with BamHI-cleaved pBR322 joined to BamHI fragments of HSV-1 DNA were plated at a density of several hundred colonies per dish. Colonies were "lifted" on to nitrocellulose membranes, lysed and hybridised to total ^{32}P-HSV-1 DNA. 5-10% of the ampicillin-resistant colonies contained HSV-1 DNA inserts. Positive colonies were picked into L-broth in microtiter plates, replica-plated onto nitrocellulose membranes, lysed and hybridized to ^{32}P-HSV-1 KpnI m DNA. Two clones which hybridised strongly with the probe (designated P1 and P3) were grown for preparation of plasmid DNA.

Transformation of LTK$^-$ cells with HSV-1 tk$^+$ DNA.

DNA containing the HSV-1 TK gene was introduced into LTK$^-$ cells by the calcium phosphate precipitation technique of Graham and Van der Eb (27) as described by Wigler et al. (28), using 10 μg of calf thymus DNA as carrier. TK$^+$ transformants were selected in Dulbecco's modified Eagle's medium supplemented with glutamine, non-essential amino acids and 10% foetal calf serum, containing 100 μM hypoxanthine, 0.8 μM aminopterine, 15 μM thymidine and 3 μM glycine (HAT medium (8,9)).

RESULTS

Mapping of HSV-1 sequences present in recombinant plasmids M2, M3, M4, M5, P1 and P3.

Hybrid plasmids containing BamHI or KpnI fragments of HSV-1 were labeled by nick-translation and hybridized to a Southern blot of BamHI-cleaved total HSV-1 DNA (Fig. 2). Plasmids M2, M3, M4 and M5, containing KpnI fragments, hybridised only with the p, uv and i' bands. Fig. 1 shows that only the BamHI i', p and u segments are contained in the KpnI m fragment, and we conclude that all four plasmids contain the HSV-1 Kpn m fragment. P1 DNA hybridized to both BamHI p, x, and n DNA (Fig. 2). Cleavage of plasmid P1 with BamHI gave intact pBR322 DNA

Figure 2. Hybridization of ^{32}P-labeled recombinant plasmid DNAs to restriction fragments of HSV-1 DNA. HSV-1 DNA was cleaved with BamHI, the fragments were separated by agarose gel electrophoresis and transferred to a nitrocellulose membrane (40). The probes were prepared by nick-translating (a) HSV-1 DNA, and (b) P1, (c) M2, (d) M3, (e) M4, and (f) M5 hybrid plasmid DNAs with the four α-^{32}P-labeled deoxynucleoside triphosphates (specific activity, 300 Ci/-mmole each). Strips from the nitrocellulose sheet were hybridized with approximately 100'000 cpm of each plasmid DNA at 80°C for 18 h in 5 ml 3 x SSC, 1x Denhardt's solution (41). The strips were washed at 50°C in 3x SSC for 5 h and autoradiographed.

and two other fragments with electrophoretic mobilities identical to HSV-1 BamHI p and x but not n (not shown). Fig. 1 shows that the BamHI n and x share sequences from repetitive regions IR_S and TR_S. It was concluded that P1 contains both BamHI p and BamHI x, and that fragment n hybridizes because of sequences shared with fragment x. In the same way it was demonstrated that plasmid P3 contains both HSV-1 BamHI fragments p and i'. Since these are adjacent on the physical map (Fig. 1), P3 may have been formed by the insertion of an uncleaved i'-p fragment.

The BamHI fragment p was isolated from P3 DNA, and restriction endonuclease maps for SstI, BglII, HindII, AluI, EcoRI, PvuII and SmaI were constructed using the conventional double digestion technique (cf. Fig. 4). Location of the SmaI and AluI restriction sites was also deduced by the method of Smith and Birnstiel (29). Terminally labeled BamHI p DNA was digested with SstI, which cleaves it at a single site, and the resulting two fragments were separated. Fig. 3 shows the appearance of

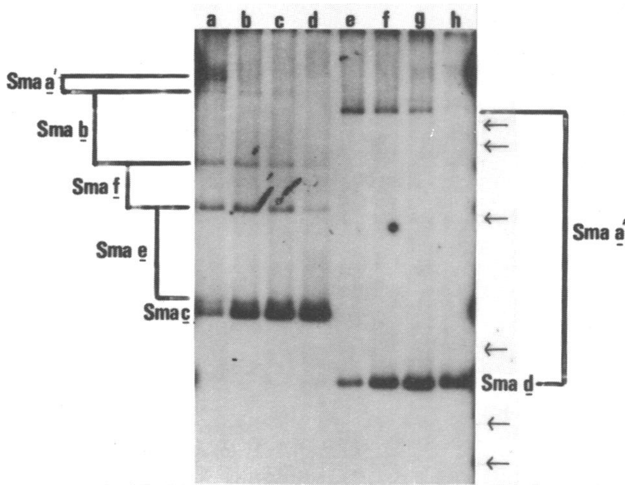

Figure 3. SmaI restriction site analysis of the BamHI p fragment of HSV-1 DNA by partial cleavage of end-labeled fragments. BamHI fragment p was isolated from hybrid plasmid P3 and terminally labeled as described under Materials and Methods. The DNA was cleaved with SstI and the 2300 and 1290 bp fragments separated by electrophoresis through a 0.7% agarose gel. Each fragment was digested with varying amounts of SmaI, the products were separated by electrophoresis through a 5% polyacrylamide gel, and radioactive fragments detected by autoradiography. Tracks a to d show the 2300 bp fragment and tracks e to h the 1290 bp fragment, each cleaved with increasing amounts of SmaI. HaeIII fragments of ØX174 RFI DNA were used as molecular weight markers in parallel tracks. The arrows (from top to bottom) indicate fragments of 1078, 872, 606, 310, 234 and 194 bp, respectively. The size increments of the terminally labeled partial products match the size of the SmaI fragments determined in separate experiments (1800, 800, 370, 270, 230 and 190 bps respectively (N. Wilkie, unpublished results)). Sma a' and Sma a" refer to the fragments produced by SstI cleavage of the SamI a fragment of BamHI p.

end-labeled partial products with decreasing digestion with SmaI. The increments in molecular weight agreed with the molecular weights of limit SmaI fragments (cf. Fig. 3). A similar procedure was used for the mapping of the AluI sites.

The appearance of two partial product bands very close to the position of SmaI c was consistently observed, and is probably due to two small fragments, which are tentatively designated g and h on the map of Fig. 4.

BamHI cleavage of hybrids M2 and M4, which contain the KpnI m sequence, yielded 4 fragments. One of these had the same EcoRI, SstI, PvuII, HindII and BglII cleavage points as deduced for the BamHI p fragment recovered from plasmid P3; another one was identified as BamHI fragment i'. Additional digestions with PstI, and PstI plus EcoRI, served to construct the PstI map of plasmid M2. A composite restriction map for plasmid M2 is shown in Fig. 4.

The orientation of the insert in M3, M4 and M5, as determined by double digestion with BglII and HindIII, is opposite to that of M2 (data not shown).

Transformation of LTK^- cells with hybrid plasmids.

Transformation of LTK^- cells with BamHI or SalI-cleaved M2, M4 and P1 DNA resulted in colonies which grew in the presence of HAT (Table 1). A transformation efficiency of 95-160 colonies per µg DNA was obtained and no colonies were observed with calf thymus DNA alone. Transformation with BamHI fragment p (isolated from plasmid P3) also resulted in the appearance of HAT-resistant colonies, with an efficiency of about 230 colonies per µg (Fig. 5). This value corresponds to about 500 colonies per pmole of DNA and is close to the value of 600-750 colonies per pmole obtained for SalI-cleaved M2 and M4 DNA.

Cleavage of BamHI fragment p with SstI, BglII, HindII or EcoRI completely abolished its capacity to induce the appearance of HAT-resistant colonies (Fig. 5). Wigler et al. (6) have previously reported that EcoRI abolishes the transforming ability of a BamHI fragment of HSV-1 DNA. BamHI fragment p has two EcoRI sites (Fig. 4), however, the clustering of the single sites for SstI, BglII, HindII and EcoRI at 2.2-2.9 kbp suggests that the TK gene is located in this region.

TK activity in cells transformed with cloned BamHI fragment p and superinfected with HSV-1 tk^- virus.

The TK activity of cell extracts was determined by measuring the conversion of ^{3}H-dThd to ^{3}H-thymidine phosphates in the presence of 200 µM TTP, which inhibits cellular TK 95% (2). As shown in Fig. 6, the enzyme activity measured in transformed

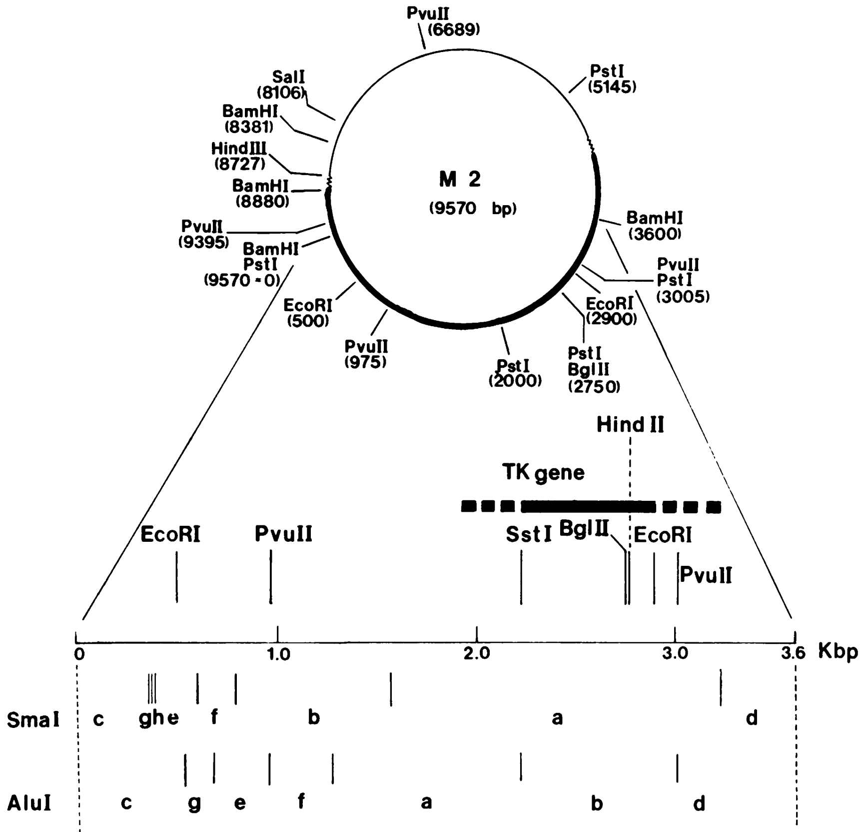

Figure 4. Composite restriction site map for the HSV-1 TK DNA-containing hybrid plasmid pBR322 (Eco)/HSV1tk-M2. The map of the hybrid plasmid was obtained from total cleavages with the restriction endonucleases indicated and double cleavage with EcoRI and PstI, as well as mapping according to Smith and Birnstiel (29) from the BglII site. The detailed map in the lower part of the figure is based on analyses of HSV-1 BamHI p DNA excised from hybrid plasmid P3. The fragment was cleaved with SstI, HindII, PvuII and EcoRI singly and in all double combinations. Double digests were also carried out with SmaI on the one hand and BglII, HindII, SstI and EcoRI on the other. In addition, terminally labeled BamHI p DNA was cleaved with SstI and mapping according to Smith and Birnstiel (29) was carried out with SmaI (cf. Fig. 3) and with AluI. The location of the TK gene indicated is derived from the observation that cleavage of the BamHI p fragment with SstI, BglII, HindII or EcoRI inactivated the transforming activity of the DNA (cf. Fig. 5).

TABLE 1 Transformation of LTK^- cells by HSV-1 TK gene-containing hybrid plasmids.

TK hybrid plasmid	μg DNA/dish	Colonies/number dishes* Expt. 1[(a)]	Expt. 2[(b)]	Transformants per μg hybrid DNA per dish
P1	0.02	6/1		
	0.07	5/1		
	0.25		81/2	160
	0.30	35/1		
M2	0.02	2/1		
	0.07	2/1		
	0.25		83/2	95
	0.30	4/1		
M4	0.02	2/1		
	0.07	10/1		
	0.25		78/2	122
	0.30	17/1		
Mock transfected		0/2		
Untreated		0/1	0/6	-

*8 x 10^5 LTK^- cells/dish, cultured in HAT medium, were transformed as described (28).
(a) Plasmids digested with BamHI.
(b) Plasmids digested with SalI.
Average of all experiments.

LTK^- cells (lines p3 and p9) is 60 to 80 times higher than in their non-transformed counterparts.

HSV-1 tk^- virus has been observed to enhance TK activity 2 to 5 fold in cultured cells containing HSV DNA sequences (30, 31,32). We therefore examined the effect of superinfecting HSV-1 tk^+ plasmid-transformed cell lines with MDK virus, a tk^- strain of HSV-1.

Transformed cell lines p3 and p11 (generated by transformation of LTK^- cells with cloned BamHI p DNA) as well as LTK^- cells were infected with HSV-1 tk^- strain MDK. The cells were harvested 15 h after infection, cell extracts prepared and TK activities determined. Cell extracts from MDK-infected LTK^- cells showed no increase in TK activity over mock-infected cells (Table 2, experiment 2). However, an approximately ten-fold increase in TK activity was observed in extracts from MDK-infected p3 and p11 cells.

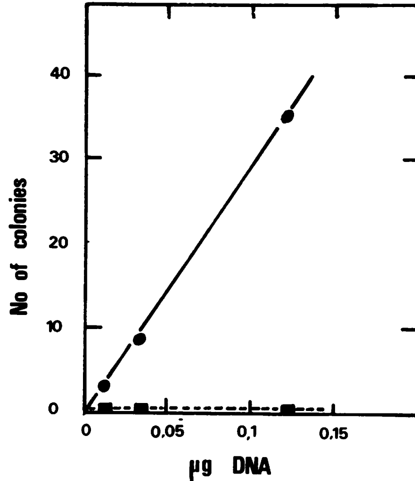

Figure 5. Transformation of LTK⁻ cells with cloned BamHI p DNA. BamHI fragments p from plasmid P3 was isolated by electrophoresis on 0.7% agarose and purified by chromatography on hydroxylapatite as described (16). Monolayers of LTK⁻ cells in 50 mm dishes were infected with the amounts of BamHI p DNA indicated, using calf thymus DNA as carrier, and cultured in HAT medium, as described in Materials and Methods. The number of HAT-resistant colonies present at 21 days after infection are plotted against the amount of DNA added to each plate. Each point represents the average number of colonies from three dishes of cells. ■ , BamHI p DNA digested for 3 h with EcoRI, HindII, BglII or SstI in TMM buffer. ● , intact BamHI p DNA incubated under the same conditions without restriction endonuclease.

DISCUSSION

We have characterized six recombinant plasmids which contain the gene locus for HSV-1 TK. Four (M2 to M5) contain the HSV-1 KpnI m fragment inserted into the EcoRI site of pBR322 by poly(dA)-poly(dT) tailing; both orientations are represented. The other two plasmids (P1 and P3) contain the HSV-1 BamHI p fragment inserted into the BamHI site of pBR322, and each contains an additional, but different, small BamHI fragment of HSV-1 DNA.

SalI-cleaved M2 and M4 DNAs transformed mouse LTK$^-$ cells to an LTK$^+$ phenotype with an efficiency of about 530 colonies

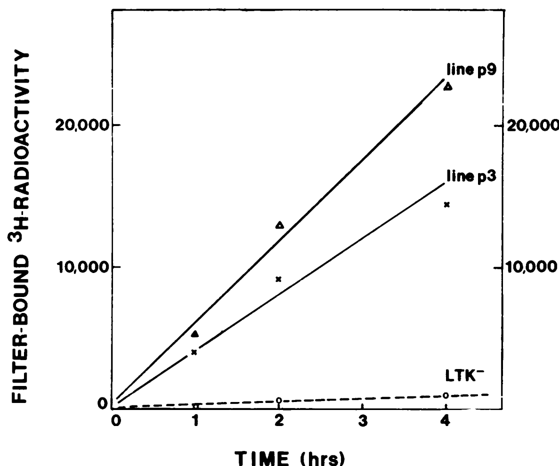

Figure 6. TK activity in LTK⁻ cells and cells transformed with cloned BamHI p DNA. Cell monolayers in 35 mm dishes were harvested, washed in phosphate-buffered saline, suspended in 500 μl 50 mM Tris-HCl (pH 7.5), 5 mM β-mercaptoethanol, 5 μM dThd, and disrupted by sonication in a bath sonicator. Aliquots of each extract were incubated at 37°C in 100 μl containing (final concentration): 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM ATP, 4.4 μM dThd, 5 μCi ^{3}H-dThd (Amersham, 21 Ci/mmole) and 0.2 mM TTP which inhibits L cell TK activities (2). 25 μl aliquots were removed at 1, 2 and 4 h to determine the conversion of dThd to TMP and higher phosphates by binding to DE81 discs (Whatman) as described (2). The results are expressed as ^{3}H cpm bound per disc, and each point represents the average of 3 determinations from separate dishes. Cell densities were determined from separate dishes and the number of cell equivalents present in each 100 μl reaction calculated: transformed cell line P9 (▲), 3 x 10^4; transformed cell line P3 (x), 2.7 x 10^4; LTK⁻ cells (o), 1.1 x 10^5.

per pmole plasmid. BamHI-cleaved P1 DNA (about 10'000 bp) had a transformation efficiency of about 1000 colonies per pmole DNA, whereas purified BamHI fragment p from P3 gave about 500 colonies per pmole. These values can be considered equivalent as they fall within the normal range of variation of transformation experiments. They are somewhat less than the values (2000-3000 colonies per pmole DNA) we calculated from the data of Wigler et al. (6) for purified BamHI fragment p. Such differences may however be due to differences in the technique and

TABLE 2 Thymidine kinase activity in cells infected with HSV-1 tk^- (MDK) virus.

Cell line	Infected with	TK activity pmoles/hour/10^5 cells
LTK^-	mock-infected	(6; 6)
	MDK-infected	(6; 6.5)
P3	mock-infected	(5; 6)
	MDK-infected	58; 68
P11	mock-infected	7.4; -
	MDK-infected	84; 105

Cell lines P3 and P11 were obtained by transforming LTK^- cells with cloned BamHI p DNA. Confluent monolayers of cells in 50 mm dishes were mock-infected, or infected at a calculated multiplicity of infection of 5 with HSV-1 tk^- virus (MDK). After 18 h the cells were scraped off, washed in phosphate buffered saline, resuspended in 50 mM Tris-HCl (pH 7.5), 5 mM β-mercaptoethanol, 5 μM dThd, and disrupted by sonication in a bath sonicator. Aliquots of each extract equivalent to 10^5 cells were incubated at 37°C in 100 μl of 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM ATP, 10 μM dThd, containing 2.5 μCi ^3H-dThd (Amersham, 20 mCi/mmole). At various times aliquots were removed to measure the conversion of dThd to TMP, essentially as described (2). The zero time value corresponding to about 5 pmoles was subtracted from each point. A linear reaction rate was obtained up to 60 min incubation. The results are expressed as pmoles dThd converted per hour per 10^5 cells. Under the same conditions the activity in LTK^- cells infected with wt HSV-1 (Glasgow strain 17) was 200-300 pmoles per hour per 10^5 cells. The numbers in parentheses are not significantly over the zero-time controls. Since the assay used for these experiments is far less sensitive than that of Fig. 3, the TK activity of the mock-infected cell lines is not evident.

the susceptibility of cells to transformation. We conclude that cloning in bacteria has not substantially affected the biological properties of the TK gene.

Digestion of purified, cloned BamHI p DNA with EcoRI, BglII, HindII or SstI completely abolished its transforming activity. Since sites for these enzymes were clustered in a 0.7 kbp region to the right of the physical map (Fig. 4), we conclude that the coding sequences for TK are located there. The molecular weight of the presumptive TK polypeptide is 43'000 daltons (33), which requires a coding region of at least 1.3 kbp. Colbere-Garapin *et al.* (34) have also cloned the HSV-1 Bam p fragment

and have located the transforming activity in the PvuII fragment comprising the cleavage sites mentioned above.

It is not yet known whether the tk sequences in transformed cells are present as chromosomal or extrachromosomal elements. Pellicer et al. (35) have reported that in their experiments the HSV-1 tk-containing BamHI fragment was present at a copy number of about one, and that the TK activity was lost with a frequency of about 1-5 x 10^{-3} when the cells were grown without selective pressure, i.e. in the absence of HAT (and in the presence of BdUrd).

The TK activity in LTK^- cells transformed with HSV-1 tk plasmids is less than 5% of that of cells infected with HSV-1 virus (N. Wilkie, unpublished results). Although this difference may partly be due to a dosage effect arising as a consequence of viral DNA replication in the infected cell, it is clear that the expression of immediate-early HSV gene(s) located in the repetitive sequences bounding the short region of the virus genome is required for turning on the TK gene of HSV-1 (36-39). The TK activity in cells transformed with a TK gene-containing DNA fragment may represent the basal expression level of the gene; alternatively, the gene may be integrated in a DNA segment which facilitates its transcription. In any event infection of the transformed cells with HSV-1 tk^- virus increased the enzyme activity 10-fold, suggesting that a viral product may activate the resident HSV-1 tk gene.

The HAT selection for a TK^+ phenotype is one of the most powerful selection systems available for eukaryotic cells; therefore TK-gene containing plasmids provide a convenient vehicle for the introduction of exogenous genes into animal cells. Two approaches have already been described. Weissmann and his colleagues (11,12) linked M2, M4 and P1 DNA to plasmid DNA containing the genomic sequences for rabbit β-globin and used the concatemeric DNA to transform LTK^- cells to LTK^+. About 90% of the transformed colonies contained globin sequences, which in 75% of the cell lines were transcribed into RNA. Using a different approach, Wigler et al. (10) reported that exogenous DNA sequences can be stably introduced into mouse L cells by co-transformation without linkage to the TK gene. LTK^+ trans-

formants may therefore originate from a sub-population of cells which are particularly competent for the uptake of exogenous DNA.

It may be possible to construct a circular "mini-chromosome" containing the TK gene and an origin of replication operational in eukaryotic cells, which has suitable restriction endonuclease targets for the insertion of exogenous DNA sequences. Such a cloning vehicle would offer considerable advantages for the analysis of eukaryotic genetic material. Whichever system is used, the availability of the cloned HSV TK gene facilitates experiments designed to study the transfer and expression of selected eukaryotic genes.

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