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Biochemical transformation of thymidine kinase (TK)-deficient mouse cells by herpes simplex virus type 1 DNA fragments purified from hybrid plasmids

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ABSTRACT

The thymidine kinase (TK) gene of HSV-1 has been cloned in Escherichia coli K12 plasmids, pMH1, pMH1A, and pMH4. These plasmids contain a 1,920bp HSV-1 TK DNA sequence, which replaces a 2,067 bp EcoR I to Pvu II sequence of plasmid pBR322 DNA. Superhelical DNAs of plasmids pMH1, pMH1A, and pMH4 as well as plasmid DNAs cleaved by EcoR I, Hinc II, Bgl II, Sma I, and Pvu II transformed TK-deficient LM(TK^-) cells to the TK^+ phenotype. A 1,230bp EcoR I-Sma I fragment purified from pMH1 DNA (and from plasmid pAGO DNA, the parent of pMH1) also transformed LM(TK^-) cells. Serological and disc PAGE studies demonstrated that the TK activity expressed in biochemically transformed cells was HSV-1-specific. The experiments suggest that the HSV-1 TK coding region may be contained within a 1.1kbp DNA sequence extending from about the Hinc II (or Bgl II) cleavage site to the Sma I site. ^{35}S-methionine labeling experiments carried out on cell lines transformed by Hinc II-cleaved pMH1 DNA and by the EcoR I-Sma I fragment showed that the TKs purified from the transformed cells consisted of about 39-40,000 dalton polypeptides.

INTRODUCTION

The acquisition of TK activity by herpes simplex virus type 1 (HSV-1)-infected mouse LM(TK^-) cells was first described by Kit and Dubbs in 1963 (1). Subsequently, evidence has accumulated demonstrating that the virus-induced TK activity is coded by HSV-1 DNA (for review, see Reference 2). Wigler et al. (3) have demonstrated that a BamH I fragment (about 3.4kbp) of HSV-1 DNA can biochemically transform LM(TK^-) cells to the TK^+ phenotype. This BamH I fragment as well as a 2kbp Pvu II subfragment of the BamH I fragment have been cloned in E. coli plasmid pBR322 (4-6). Studies by Colbère-Garapin et al. (4) suggest that the HSV-1 TK-coding region may be contained within a 1.3kbp region extending from the Pvu II to the Sma I cleavage site of the Pvu II fragment (HSV-1 DNA map units from about 31.1-30.2) (7). The orientation of transcription of the TK gene (5'-3') appears to be Pvu II-EcoR I-Hinc II-Sma I-Pvu II (8) (Fig. 1). Recent experiments from our laboratory have confirmed that the 1.3kbp HSV-1 Pvu II-Sma I fragment has transforming activity and have further

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shown that LM(TK$^-$) cells biochemically transformed by this fragment express an HSV-1-associated nuclear antigen (HANA) (9).

The 3.4kbp BamH I fragment which codes for HSV-1 TK contains two EcoR I recognition sites, one of which is also found in the 2kbp Pvu II fragment. Two reports have indicated that digestion of the 3.4kbp BamH I fragment with EcoR I completely abolishes its transforming activity (3,5). In contrast, we observed that a 1.2kbp EcoR I-Sma I fragment purified from E. coli plasmid pAGO (4) (Fig. 1) retains weak transforming activity for LM(TK$^-$) cells and that cells biochemically transformed by this fragment express HSV-1-specific TK and HANA. Smaller recombinant plasmids, pMH1 and pMH4, have now been constructed by deleting a 2,172bp sequence from plasmid pAGO. The present study demonstrates that DNAs from plasmids pMH1 and pMH4 efficiently transform LM(TK$^-$) cells, and suggest that the functional TK-coding sequence may be 3' to the Hinc II cleavage site of the HSV-1 EcoR I-Pvu II fragment (Fig.1).

MATERIAL AND METHODS

Cells:BrdUrd-resistant and TK-deficient mouse LM(TK$^-$) cells (10) were grown in Eagle's minimal essential medium (APMEM) (Auto Pow, Flow Laboratories, Inc., Rockville, Maryland) supplemented with 10% calf serum and 25µg/ml BrdUrd. All LM(TK$^-$) cell lines biochemically transformed to the TK$^+$ phenotype by HSV-1 DNA fragments were grown in APMEM + 10% calf serum or fetal calf serum and HATG (hypoxanthine, 10^{-4}M; aminopterin, 10^{-6}M; thymidine, $4x10^{-5}$M; and glycine, 10^{-5}M). E. coli K12 strain 1106 (803$r_k^-m_k^-$ sup E sup F) containing plasmid pAGO was grown in ML broth with 25µg/ml ampicillin and, in some instances, with 10µg/ml tetracycline (4). E. coli K12 strain RR1 (F$^-$ pro leu thi lacY Str^r $r_k^-m_k^-$) was used as the recipient in plasmid transformation experiments (11). The latter cells were grown in ML broth or in M9-glucose minimal medium (12).

Preparation of plasmid DNA: Plasmid DNA was amplified by the addition of chloramphenicol (200µg/ml) to logarithmic-phase cultures in M9-glucose broth. To label plasmid DNA, ^{3}H-thymidine (4mCi/liter, 52.4Ci/mmole, New England Nuclear, Boston, Massachusetts) and uridine (25µg/ml) were added together with chloramphenicol. Cultures were harvested 17hr later. Bacterial cells were suspended in 1/20th vol of 25% sucrose (w/v) in 50mM Tris-HCl, pH 8 and incubated with 200mg of chick lysozyme per liter bacterial suspension at 4°C for 10 min. For each liter, 3ml of 0.1M EDTA was added for 5 min at 4°C, followed by 20ml of 0.4% Triton X-100 in TE buffer (0.1M Tris-HCl, pH 8 and 0.1M EDTA) for another 30 min. The viscous solution was centrifuged in a Beckman L5-65 centrifuge at 30K rpm for 1hr at 4°C, and the supernatant was collected and treated with pro-

teinase K (100µg/ml clear lysate) for 30 min at 37°C. The supernatant was diluted with TE buffer and deproteinized with phenol. After passing the DNA solution through a 22 gauge needle to shear E. coli DNA, the plasmid DNA was purified by density gradient centrifugation in CsCl-ethidium bromide and by velocity centrifugation for 20hr at 5°C and 24,000rpm in a 5-40% (w/v) sucrose gradient (in 50mM NaCl, 10mM Tris-HCl buffer, pH 7.5, 1mM EDTA) (Spinco centrifuge rotor SW41 Ti). The concentration of DNA was determined by fluorometric analyses. Alternatively, the rapid alkaline extraction procedure of Birnboim and Doly (13) was used for the preparation and screening of plasmid DNAs.

Transformation of bacteria and LM(TK$^-$) cells: E. coli K12 RR1 cells were prepared for transformation by $CaCl_2$ treatment (14). 100µl of DNA (5ng to 1µg) in 10mM Tris-HCl, pH 7.6, 10mM $CaCl_2$ was added to 200µl of $CaCl_2$-treated cells. After 30 min at 4°C, the temperature was raised to 37°C for 5 min, 0.3ml of ML medium was added, and incubation was continued for 45 min at 37°C with gentle shaking. Samples were plated on trypticase soy agar plates supplemented with antibiotics. Transformation efficiencies were about 10^6-10^7 transformed colonies per µg of plasmid pAGO or pMH1 DNAs.

Biochemical transformation experiments of LM(TK$^-$) cells by DNA containing the HSV-1 TK gene were carried out essentially as described by Bacchetti and Graham (15) and Wigler et al. (16). (See legend to Table 1.) TK$^+$ transformants were selected in growth medium supplemented with HATG.

Analyses of cytosol TK activities of biochemically transformed cells: Cytosol extracts were prepared as described previously (17). The extracts contained glycerol (10% v/v), epsilon aminocaproic acid (0.05M) to inhibit proteases, and thymidine, ATP, and Mg^{++} to stabilize TK activity. The TK activity was assayed with reaction mixtures containing ^{3}H-thymidine, ATP, and Mg^{++} (17). Disc PAGE analyses were performed at 4°C in 5% polyacrylamide gels at pH 8.6. ATP, thymidine, and Mg^{++} were added to the gel polymerization mixture and to the upper buffer solutions to protect the TK activities during disc PAGE (17). Serological studies on the inhibition of HSV-1 TK activity by rabbit IgG No.46 were carried out as described previously (18). Detection of herpesvirus-associated nuclear antigen (HANA) in biochemically transformed cells by IF analyses with rabbit IgG No. 46 has also been described (9).

Cellular and viral DNAs: Cellular DNA was extracted from biochemically transformed cells with p-aminosalicylate and phenol and purified as described previously (19). HSV-1 DNA and SV40 DNA were isolated from infected monkey kidney cell cultures by the methods of Pignatti et al. (20) and Hirt (21), respectively.

Restriction nuclease cleavage and gel electrophoresis: Restriction endonucleases were purchased from New England Biolabs, Bethesda Research Laboratories, and Boehringer. Cleavage reactions of plasmid and cellular DNAs were carried out under the conditions recommended by the suppliers. Restriction endocuclease fragments were separated by agarose gel electrophoresis, stained with 0.5µg/ml ethidium bromide, visualized over a long-wave UV-illuminator, and photographed.
Labeling of DNA and molecular hybridization: *E. coli* plasmid pAGO DNA was labeled *in vitro* with α-^{32}P-dCTP (350Ci/mmole, Amersham) and α-^{32}P-dTTP (350Ci/mmole, Amersham) by "nick translation" (22) and used as a probe in molecular hybridization experiments. Restriction nuclease fragments of cellular DNA, which had been separated by electrophoresis on agarose slab gels, were alkali-denatured, neutralized, and transferred to nitrocellulose sheets by the method of Southern (23), dried at 60°C overnight, and baked at 80°C in a vacuum for 3hr. The nitrocellulose sheets were placed in plastic bags containing Denhardt's solution (24) plus 50µg/ml denatured salmon sperm DNA and 10µg/ml poly(A), and incubated at 60°C overnight. The solution was replaced with a hybridization solution [0.6M NaCl, 0.2M Tris HCl, pH 8, 0.02M EDTA, 0.1% SDS, 50µg/ml denatured salmon DNA, 10µg/ml poly(A), and 50% formamide], and the nitrocellulose sheets were incubated at 37°C for 1hr. Heat-denatured, nick-translated DNA probe was added (about 10^7cpm/sheet) to the hybridization solution and incubation was continued at 37°C for 24hr. Nitrocellulose sheets were transferred to trays in a shaking water bath at 37°C and washed once with hybridization solution without poly(A), five times with the hybridization solution lacking both poly(A) and salmon sperm DNA, once with 0.3xSSC, and once with 0.1xSSC. The sheets were air-dried and exposed to X-ray film with a Cronex lighting-plus intensifying screen at -70°C.

RESULTS

Biochemical transformation by DNA fragments isolated from plasmid pAGO. Biochemical transformation of LM(TK$^-$) cells using the 2kbp Pvu II-Pvu II fragment and the 1.3 kbp Pvu II-Sma I fragment isolated from plasmid pAGO has been described (4,9). Although transformation by the 2kbp Pvu II-Pvu II fragment was relatively efficient, that by the 1.3kbp Pvu II-Sma I fragment was not. In our experiments, only eight transformed colonies were obtained on 31 dishes, using 13-100ng Pvu II-Sma I DNA per dish (9). Transformation was also attempted with a 1,230bp EcoR I-Sma fragment isolated from pAGO DNA (Fig. 1), with the expectation that the results would be negative. However, colonies of transformants were consistently obtained, even though the transformation frequency was very low (Table 1, Exp. 1). Cell lines transformed by the Pvu II-Pvu II, the Pvu II-Sma I, and the EcoR I-

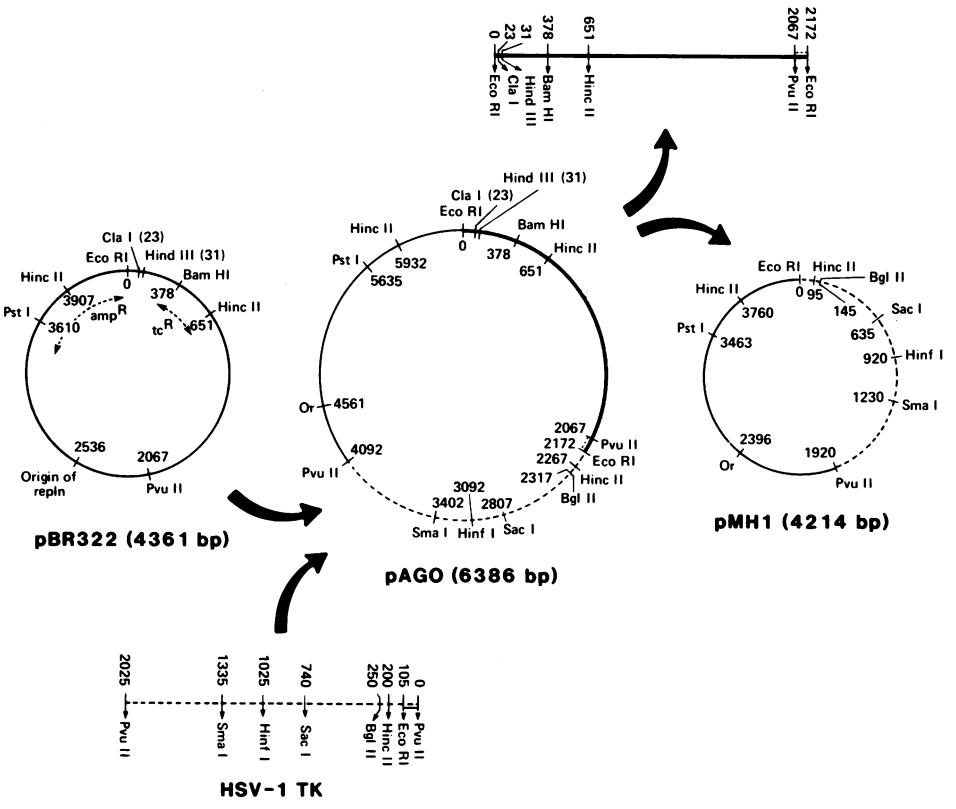


Fig. 1. Derivation of plasmid pAGO by insertion of HSV-1 TK gene (2,025bp Pvu II-Pvu II fragment) at the unique Pvu II site of plasmid pBR322 and derivation of plasmid pMH1 from EcoR I-cleaved pAGO by elimination of a 2,172bp fragment. Restriction maps of the HSV-1 TK gene and of the plasmids are shown. pBR322 and pAGO are ampicillin-resistant (amp^R) and tetracycline-resistant (tc^R), while pMH1 is amp^R but tc^S. The unique pBR322 EcoR I site was chosen as 0 in the maps of pAGO and pMH1. Restriction sites are indicated with their approximate distance from position 0, measured in base pairs and clockwise.

Sma I DNA fragments were named, respectively, LM(TK^-)/TF pAGO PP, LM(TK^-)/TF pAGO PS, and LM(TK^-)/TF pAGO ES.

Molecular hybridization experiments. Molecular hybridization experiments were carried out to verify that the DNAs of cells transformed by pAGO DNA fragments contained integrated HSV-1 DNA sequences. The cellular DNAs were cleaved with restriction endonucleases which would be expected to cleave only cellular DNA sequences flanking the integrated HSV-1 DNA (e.g., Kpn I, BamH I), with restriction endonucleases that cleave the Pvu II-Pvu II fragment of pAGO DNA at one site (e.g., Bgl II, Sac I, Hinf I), or with combinations of restriction endonucleases

TABLE 1

Transformation of LM(TK$^-$) cells by DNA from E. coli plasmids containing HSV-1 TK gene [a]

Expt.	Source of DNA	µg DNA/dish	Number of dishes	Average number of transformed colonies/dish	Name of picked colonies [LM(TK$^-$)/TF]:
1	EcoR I-Sma I fragment purified from pAGO	0.013	20[b]	0.25	pAGO ES1, ES2, ES3
		0.025	24[c]	0.25	pAGO ES4, ES5
2	pMH1 (superhelical)	0.05	4	23	pMH1-1, -2, -3
	pMH1 cut by Pvu II	0.05	4	24	-
		0.005	4[d]	0.75	pMH1 P1, P2, P3
	pMH1 cut by EcoR I	0.05	4	43	-
		0.005	4	7.3	pMH1 E3
3	pMH1A (superhelical)	0.1	3	80	-
		0.01	4[e]	1.0	pMH1A-1, -2, -3
	pMH1A cut by Pvu II	0.1	3	121	-
		0.01	2	73	pMH1A P1, P2, P3
	pMH1A cut by EcoR I	0.1	3	120	-
		0.01	2	63	pMH1A E1, E2, E3
	pAGO (superhelical)	0.1	3	≫200	
		0.01	3	117	
	pAGO cut by Pvu II	0.1	2	174	
		0.01	3	124	
		0.001	2	34	
	pAGO cut by EcoR I	0.1	3	128	
		0.01	3	63	
	pMH4 cut by Pvu II	0.1	3	70	-
		0.01	3	6.3	pMH4 P1, P2, P3
4	pMH1 (superhelical)	0.05	3	225	
	pMH1 cut by Pvu II	0.05	3	201	
	pMH1 cut by Sma I	0.1	4	9.3	pMH1 S1, S2, S3
	pMH1 cut by Hinc II	0.1	3	172	pMH1 Hc1, Hc2, Hc3
	pMH1 cut by Bgl II	0.1	4	2.5	pMH1 Bg1, Bg2, Bg3
5	pMH1 (superhelical)	0.005	6	41	-
	EcoR I-Sma I fragment purified from pMH1	0.1	18[f]	0.28	pMH1 ES1, ES2, ES3

(a) Falcon tissue culture dishes (60mm) were inoculated with 0.5×10^6 LM(TK$^-$) cells in 5ml APMEM supplemented with 10% fetal calf serum. After the cells were incubated at 37°C for 24hr, 0.5ml calcium phosphate-DNA suspension was added for 30 min at room temperature. Then, 7.5ml of APMEM + 10% fetal calf serum was added and incubation was continued for 17hr at 37°C. The medium was replaced with fresh medium and, 24hr later, the medium was replaced with HATG selection medium. Incubation was continued at 37°C in HATG medium for 2 to 4 weeks with media changes every 2 to 3 days. Colonies were isolated from different dishes. The remaining colonies in the dishes were fixed with ethanol, stained with Giemsa, and counted.

(b) Five dishes contained one colony each and 15 contained no colonies.

(c) Six dishes contained one colony each and 18 contained no colonies.

(d) Three dishes contained one colony each and one contained no colonies.

(e) Two dishes contained one colony each, one contained two, and one contained no colonies.

(f) Five dishes contained one colony each and 13 contained no colonies.

(Fig. 1). Cellular DNA fragments were separated by agarose gel electrophoresis, denatured, transferred to nitrocellulose filters by Southern's blotting technique (23), and hybridized with ^{32}P-labeled, nick-translated pAGO DNA. The DNA fragments which hybridized to the probe were detected by autoradiography and are shown in Fig 2 and Table 2. A provisional map of integrated viral DNA and flanking cellular DNA sequences for LM(TK$^-$)/TF pAGO PS7 cells is depicted in Fig. 3.

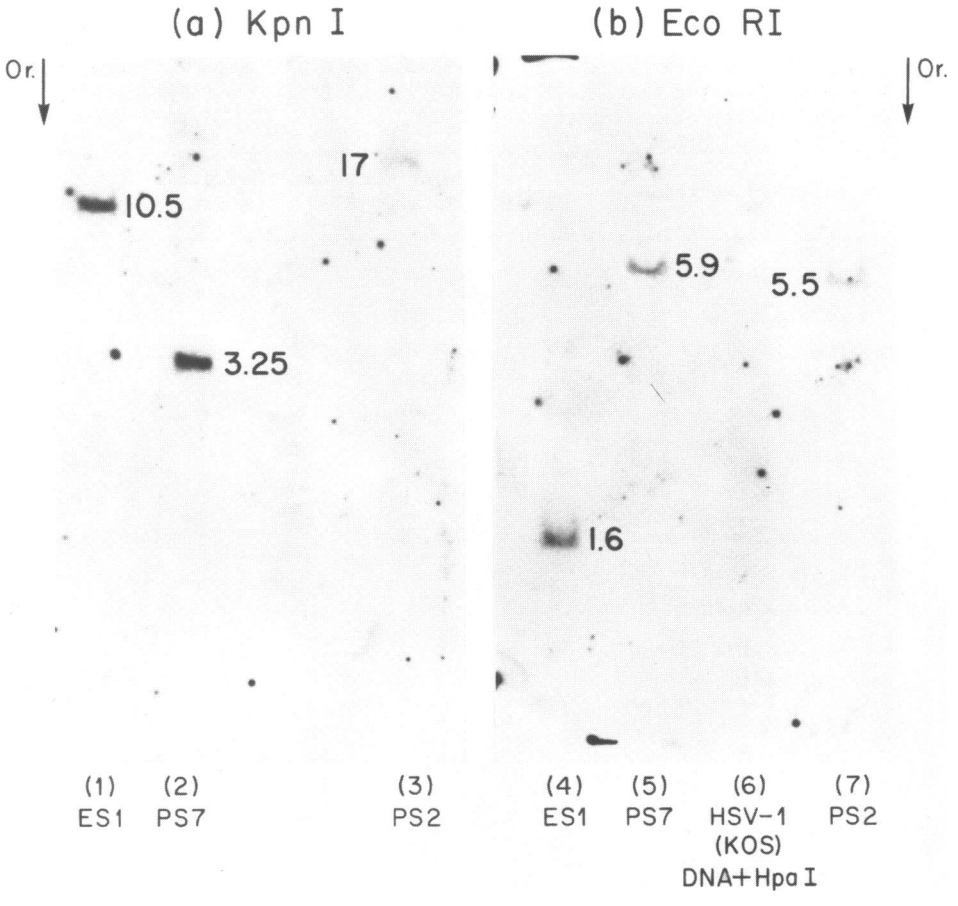


Fig. 2. Detection of restriction nuclease fragments containing HSV-1 TK gene from cells biochemically transformed by purified pAGO DNA fragments. The 1,335bp Pvu II-Sma I and the 1,230bp EcoR I-Sma I fragments (see Fig. 1) were isolated and purified after agarose gel electrophoresis of restriction nuclease-treated plasmid DNAs (25) and used to transform LM(TK^-) cells. Cellular DNA was isolated from clones of cells biochemically transformed by the Pvu II-Sma I fragment [LM(TK^-)/TF pAGO PS2 and LM(TK^-)/TF pAGO PS7] and the EcoR I-Sma I fragment [LM(TK^-)/TF pAGO ES1], and cleaved with restriction endonuclease Kpn I (Fig. 2a) or EcoR I (Fig. 2b); the DNA fragments were separated by electrophoresis, denatured with alkali, transferred to nitrocellulose filters by Southern's blotting technique (23), and hybridized to a nick-translated plasmid pAGO DNA probe. Filters were exposed to X-ray film. The molecular weights ($x10^{-6}$ daltons) of the radioactive bands are shown. For the experiment in Lane 6, HSV-1 DNA was cleaved with Hpa I nuclease. A very light radioactive band with a molecular weight of about $5.8x10^6$ daltons was detected, confirming that the probe hybridized to HSV-1 Hpa I fragment I, which contains the TK gene. (This band is very faint because only about 0.08µg of Hpa I-cut HSV-1 DNA was electrophoresed).

TABLE 2

Hybridization of nick-translated ^{32}P-labeled pAGO DNA probe to fragments produced by cleaving LM(TK$^-$)/TF pAGO PS7 and LM(TK$^-$)/TF pMH1 ES1 cells with restriction endonucleases.

Restriction Nuclease	Size of fragment hybridized (kbp) LM(TK$^-$)/TF pAGO PS7	LM(TK$^-$)/TF pMH1 ES1
Kpn I	5.0	5.0
BamH I	3.7	3.8
Pvu II	20.0	2.1
Sma I	>25.0	1.9
EcoR I	9.0	2.8
Sac I	1.0	5.3, 1.4
Hinf I	1.4, 0.6	1.5, 1.0
Bgl II	2.9, 1.4	2.1
Kpn I + EcoR I	1.6	
Kpn I + BamH I	2.6	
Kpn I + Pvu II	5.0	
Kpn I + Sma I	5.0	1.9
Kpn I + Sac I	1.0	3.1, 1.4
Kpn I + Hinf I	1.4, 0.6	1.5, 0.9
Kpn I + Bgl II	2.9, 1.4	
BamH I + Sma I	3.5	
Sac I + EcoR I		2.0, 0.8
Sac I + Pvu II		1.3, 0.8
Hinf I + Pvu II		1.0, 0.9
Hinc II		1.7
Hinc II + Hinf I		0.8
Hinc II + Pvu II		1.7
Hinc II + BamH I		1.6
Pvu II + Bgl II		1.8

The following points may be noted: (i) the probe hybridized to only one Kpn I fragment of LM(TK$^-$)/TF pAGO PS2 and PS7 and LM(TK$^-$)pAGO ES1 cells, showing that the viral DNA fragments were integrated at one site in these cells; (ii) the molecular weights of the radioactive Kpn I bands were different in these three transformants, indicating that the integration sites were also distinctive; (iii) the probe hybridized to Pvu II and Sma I fragments of LM(TK$^-$)/TF pAGO PS7 cells, which

were larger than the 1.3kbp transfecting Pvu II-Sma I fragment, suggesting that Pvu II and Sma I recognition sites were not restored at the junctions of viral and cellular DNAs; and (iv) the probe hybridized to only one EcoR I fragment of LM(TK$^-$)/TF pAGO PS2 and PS7 cells. This fragment was presumably produced by cleavage of the Pvu II-Sma I fragment at the EcoR I site of the integrated HSV-1 DNA and by cleavage of the flanking cellular DNA at an EcoR I recognition site (Fig. 1). The 105bp viral DNA sequences extending from the EcoR I to the Pvu II site of the integrated HSV-1 DNA (with the flanking cellular DNA sequences) was apparently too small to be detected by the probe in these experiments. In the case of LM(TK$^-$)/TF pAGO ES1 cells, the size of the EcoR I fragment (1.6×10^6 daltons, 2.4kbp) which hybridized to the probe was larger than the DNA fragment (1.2 kbp) used to transform the cells, also suggesting that the EcoR I and/or the Sma I recognition sites of the transfecting fragment had been altered during the integration of the TK gene into cellular DNA. The experiments confirm that the transfecting HSV-1 DNA fragment was integrated with cellular DNA.

Biochemical transformation by plasmid pMH DNA. The preceding experiments suggested that the functional coding sequence for the HSV-1 TK gene was on the 3' side of the EcoR I recognition site (map position = 2,172bp; Fig. 1) of pAGO DNA. To verify this, it was essential to obtain a plasmid with the EcoR I-Pvu II sequence (map positions = 2,172 to 4,092 of pAGO DNA), but lacking the Pvu II-EcoR I sequence (map positions = 2,067 to 2,172) (Fig. 1). The simplest way to do this is to eliminate the pAGO DNA EcoR I sequence, which extends from map positions 0 to 2,172 and which contains the plasmid gene for tetracycline resistance. Therefore, pAGO DNA was cleaved with EcoR I, the restricted DNA was used to transform *E. coli* K12 RR1, and colonies resistant to ampicillin but sensitive to tetracycline were selected. The plasmid DNAs from ten colonies of amp^{R}tc^S cells were isolated and analyzed by agarose gel electrophoresis. All ten plasmid DNAs contained superhelical DNA molecules of 4.2kbp, as would be expected from the deletion of 2.2 kbp from pAGO DNA (6.4kbp). Two of the plasmid DNAs, designated pMH1 and pMH4, were chosen for further study. Plasmid pMH1 DNA was also used to transform additional *E. coli* K12 RR1 cells, and the plasmid from the resulting transformant was designated pMH1A.

Agarose gel electrophoresis of restriction nuclease-cleaved plasmid DNAs. DNAs isolated from plasmids pMH1, pMH1A, and pMH4 were cleaved with several restriction endonucleases and the cleaved DNAs were analyzed by electrophoresis on agarose gels. As controls, the DNAs of plasmids pBR322 and pAGO, and the DNAs of simian virus 40 (SV40) and phage PM2, were studied. The analyses of pMH4 DNA gave the same results as those for pMH1 and pMH1A DNAs, so that only the latter are shown (Fig. 4).

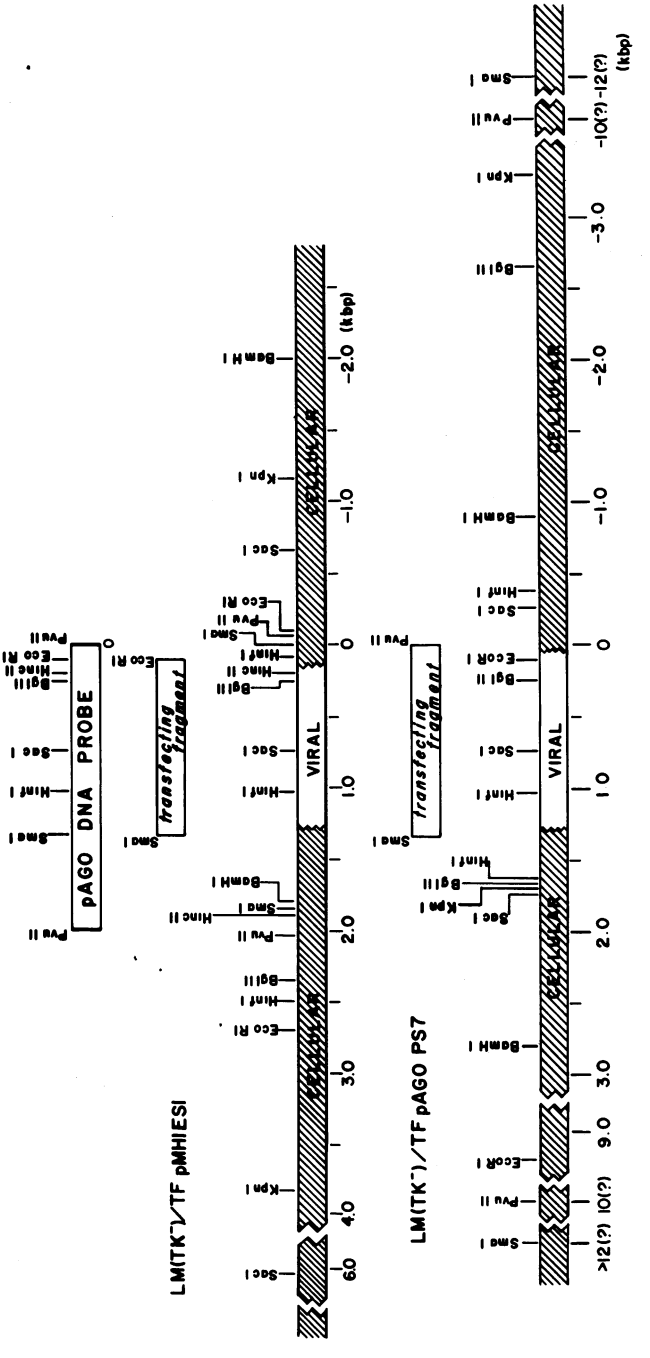


Fig. 3. Restriction map of integrated viral DNA and flanking cellular DNA sequences of biochemically transformed LM(TK$^-$)/TF pMH1 ES1 and LM(TK$^-$)/TF pAGO PS7 cells. (See Table 2).

The results may be summarized as follows: (i) superhelical forms of viral and plasmid DNAs (no restriction nuclease treatment) had a higher electrophoretic mobility, as expected, than nicked or linear DNA forms: (ii) preparations of superhelical DNA sometimes contained small amounts of dimeric molecules as well as nicked-circular DNA (e.g. PM2 DNA); (iii) pBR322 and pAGO DNAs were converted by Cla I to linear DNAs; (iv) superhelical pAGO DNA was converted to unit length linear DNA by Hind III, Sma I, and BamH I (Fig. 4A), to two DNA fragments by EcoR I (2.2 and 4.2kbp) and by Pvu II (2 and 4.4kbp), and to three DNA fragments by Hinc II (1.1, 1.6, and 3.7kbp); (v) the DNAs of plasmids pMH1, pMH1A, and pMH4 were not cut by Hind III or BamH I, but they were converted to unit length linear DNAs by EcoR I, Sma I, and Pvu II; and (vi) pMH1, pMH1A, and pMH4 DNAs were cleaved by Hinc II to two DNA fragments (0.55 and 3.7kbp). The analyses confirm the restriction map of pMH1, pMH1A, and pMH4 DNAs (Fig. 1).

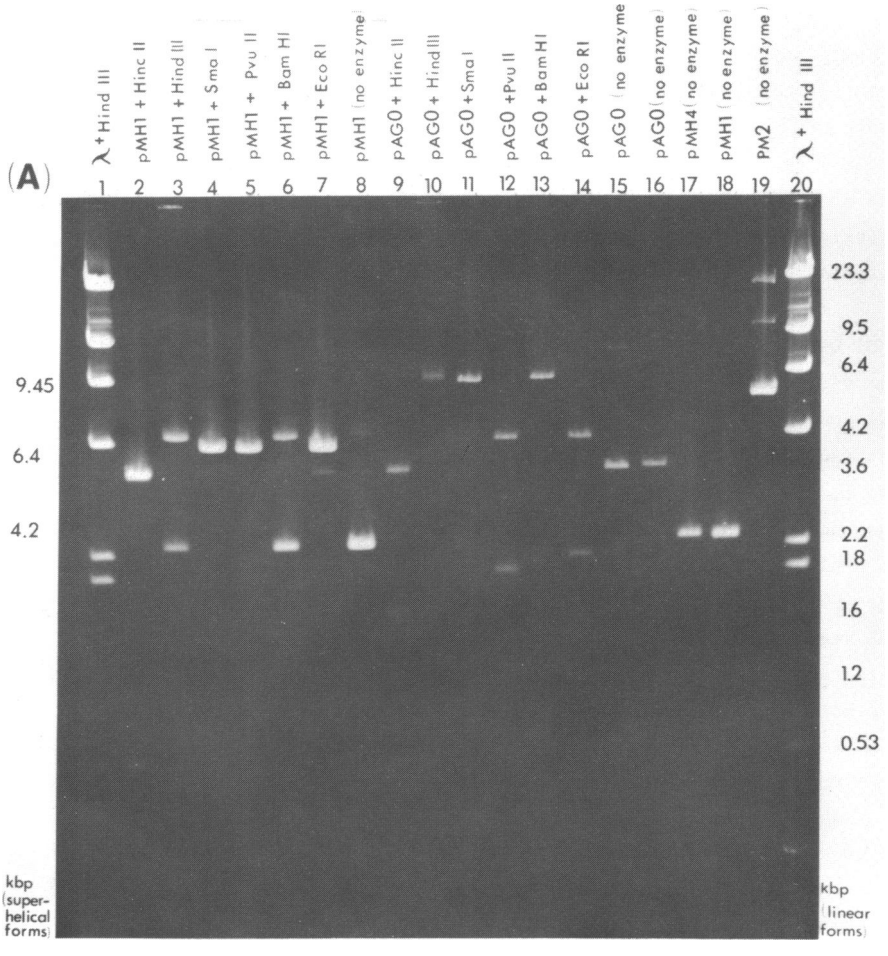
Transformation of LM(TK$^-$) cells by DNAs from pMH plasmids. Superhelical DNAs from plasmids pMH1, pMH1A, and pMH4 and the linear DNAs obtained by cutting the plasmid DNAs with one-cut enzymes (Pvu II, EcoR I) biochemically transformed LM(TK$^-$) cells (Table 1). Biochemical transformation experiments utilizing pAGO DNA are shown for comparison. In all experiments, cleavage of the DNAs by the restriction endonucleases was verified by agarose gel electrophoresis prior to carrying out the biochemical transformation experiments on the LM(TK$^-$) cells.

LM(TK$^-$) cells were also transformed by Hinc II-, Bgl II-, and Sma I-cleaved pMH1 DNAs and by an EcoR I-Sma I DNA fragment (1,230bp) purified from pMH1 DNA. However, the frequency of transformation was low with the Bgl II-cleaved pMH1 DNA and with the EcoR I-Sma I fragment from pMH1 (Table 1, Fig. 1). In contrast, no transformed colonies were obtained when pMH1A DNAs which had been cleaved by Sac I, Hinf I, and Bgl I were used for transformation. The latter restriction endonucleases have cleavage sites 3' to the Bgl II site and 5' to the Sma I site of pMH1A DNA (Fig. 1)(4,6). These results suggest that the coding region of the HSV-1 TK gene is contained within the Hinc II-Sma I (or the Bgl II-Sma I) DNA sequence.

Integrated viral and flanking cellular DNA sequences of LM(TK$^-$)/TF pMH1 ES1 cells The molecular hybridization experiments presented in Table 2 were carried out to analyze the integrated viral and flanking cellular DNA sequences of LM(TK$^-$)/TF pMH1 ES1 cells. A restriction map based on these analyses is shown in Fig. 3. As expected, only one radioactive band was detected when the probe was hybridized to cellular DNA cleaved by Kpn I, BamH I and Pvu II. Also, only one radioactive band was observed after the probe was hybridized to Sma I-, EcoR I-, Bgl II- and Hinc II-cleaved cellular DNA, but two labeled bands were observed when the

probe was hybridized to Sac I- and Hinf I-cleaved cellular DNAs (Table 2). The EcoR I- and Sma I-cleaved LM(TK$^-$)/TF pMH1 ES1 bands which hybridized to the probe were larger than the transfecting EcoR I-Sma I fragment, suggesting that they consisted of both viral and cellular DNA sequences. Cleavage of cellular DNA with Hinc II plus Hinf I resulted in a labeled 0.8kbp band, as expected (Fig. 3). The results indicate that DNA sequences 3' to the Hinc II site and 5' to the Hinf I site of the EcoR I-Sma I fragment were definitely integrated and that the restriction endonuclease cleavage sites in flanking cellular DNAs were different in LM(TK$^-$)/TF pMH1 ES1 from those of LM(TK$^-$)/TF pAGO PS7 cells.

Characteristics of the thymidine kinase from cells transformed by fragments of plasmid DNAs. The biochemically transformed LM(TK$^-$) cells were selected in HATG



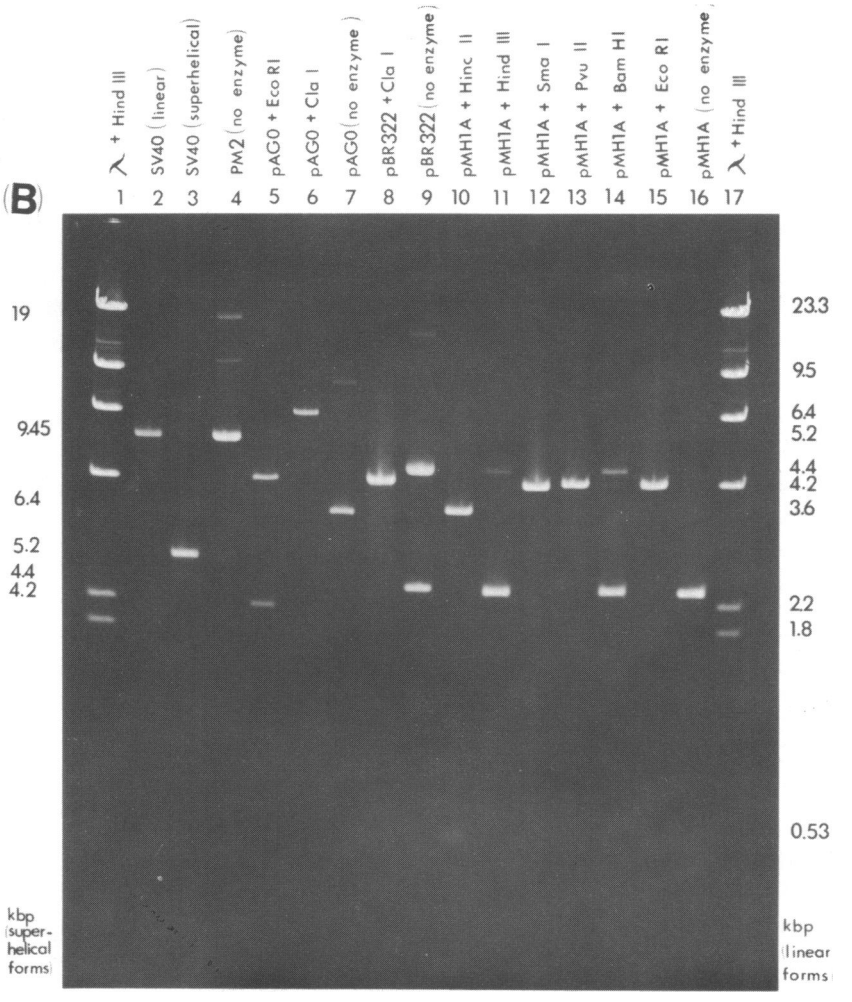


Fig. 4. (A & B). Analyses of superhelical and restriction endonuclease-digested pAG0, pMH1 and pMH1A DNAs by electrophoresis on 1% agarose slab gels (20x20x0.4cm) (Sea Kem HGT, Marine Colloids Division, FMC Corporation, Rockland, Maine) for about 18hr at 4°C (60 volts, about 20mA) in 0.03M NaH_2PO_4, 0.001M EDTA, 0.04M Tris, pH 8.1. The DNA fragments were stained with 0.5µg/ml ethidium bromide, visualized over a long-wave UV-illuminator, and photographed. Lambda DNA Hind III fragments were utilized as molecular weight markers for linear DNA molecules. The sizes in kbp of the seven lambda DNA-Hind III fragments are shown at the right of the figures. As molecular weight markers for superhelical DNAs, phage PM2 DNA (9.45kbp) (Boehringer), pBR322 DNA (4.4kbp)(Bethesda Research Laboratories), and SV40 DNA (5.2kbp) were used. Numbers at the left of the figures refer to the sizes in kbp of superhelical DNA molecules. The figures show that the size of pAG0 DNA is about 6.4kbp and that pMH1, pMH1A and pMH4 DNAs are about 4.2kbp in length.

medium. To verify that the cell lines were not merely aminopterin-resistant cells and that they expressed the type-specific HSV-1 TK and not mouse TK, cytosol extracts were prepared from several transformants, and serological experiments with anti-HSV-1 IgG No. 46 and disc PAGE analyses were carried out. The anti-HSV-1 IgG No. 46 inhibits HSV-1 TK activity, but not mouse (LM), human (HeLa S3), or monkey (CV-1) cytosol TK. The IgG No. 46 also reacts with an HSV-1-associated nuclear antigen (HANA) present in human and mouse cells transformed by UV-irradiated HSV-1 and by the Pvu II-Pvu II fragment of pAGO DNA (4,9,18). The HANA detected in cells biochemically transformed by the Pvu II-Pvu II fragment of pAGO DNA probably contains the HSV-1 TK polypeptide. The present serological experiments demonstrated that anti-HSV-1 IgG No. 46, at a dilution of 1:7, inhibited the cytosol TK activities of LM(TK^-)/TF pAGO ES1, LM(TK^-)/TF pMH1-1, LM(TK^-)/TF pMH1 E3, LM(TK^-)/TF pMH1 ES1, LM(TK^-)/TF pMH1 Hc1 and Hc2, and LM(TK^-)/TF pMH1 Bg1, Bg2 andBg3 by 66-92%. This antiserum inhibited the cytosol TK activity of LM(TK^-) cells productively infected with HSV-1(KOS) by 97%, but did not inhibit the cytosol TK activity of LM(TK^-) cells biochemically transformed by sheared fragments of HeLa S3 DNA [LM(TK^-)/TF HeLa-1]. The HSV-1-associated nuclear antigen was detected in the five cell lines transformed by the EcoR I-Sma I fragment purified from pAGO DNA, in the three cell lines transformed by the EcoR I-Sma I fragment purified from pMH1 DNA, in cell lines transformed by superhelical pMH1 and pMH1A DNAs, in cell lines transformed by Pvu II-cleaved pMH1, pMH1A, and pMH4 DNAs and by EcoR I-cleaved pMH1 and pMH1A DNAs, and in cell lines transformed by Hinc II- and Bgl II-cleaved pMH1 DNAs.

Disc PAGE analyses on cytosol extracts from HSV-1-infected cells and from several transformants are shown in Fig. 5. The cytosol TK activity of HSV-1-infected cells has a relative electrophoretic mobility (Rm) of about 0.6 (Fig. 5B). This Rm value is distinctly different from the Rm values of the cytosol TKs of human (Rm = 0.25) or mouse (Rm = 0.35) cells (2,17). Figure 5A shows that the Rm value of the cytosol TK of LM(TK^-) cells transformed by sheared HeLa S3 DNA was about 0.24, as expected. The Rm values for the cytosol TKs of cells transformed by the EcoR I-Sma I fragment purified from pAGO DNA [LM(TK^-)/TF pAGO ES1] and by EcoR I-cut pMH1 DNA [LM(TK^-)/TF pMH1 E3] were 0.62 and 0.55, respectively, within the range observed for the HSV-1 TK of lytically infected cells (Fig. 5C, 5D). However, minor TK activities migrating slower and faster than the principal TK activity were also detected, indicating electrophoretic heterogeneity of TK molecular forms. Likewise, electrophoretic heterogeneity of TK molecular forms was found for the cytosol TK activities of LM(TK^-)/TF pMH1-1, LM(TK^-)/TF pMH1 ES1, LM(TK^-)/TF pMH1 Hc1, and LM(TK^-)/TF pMH1 Bg2

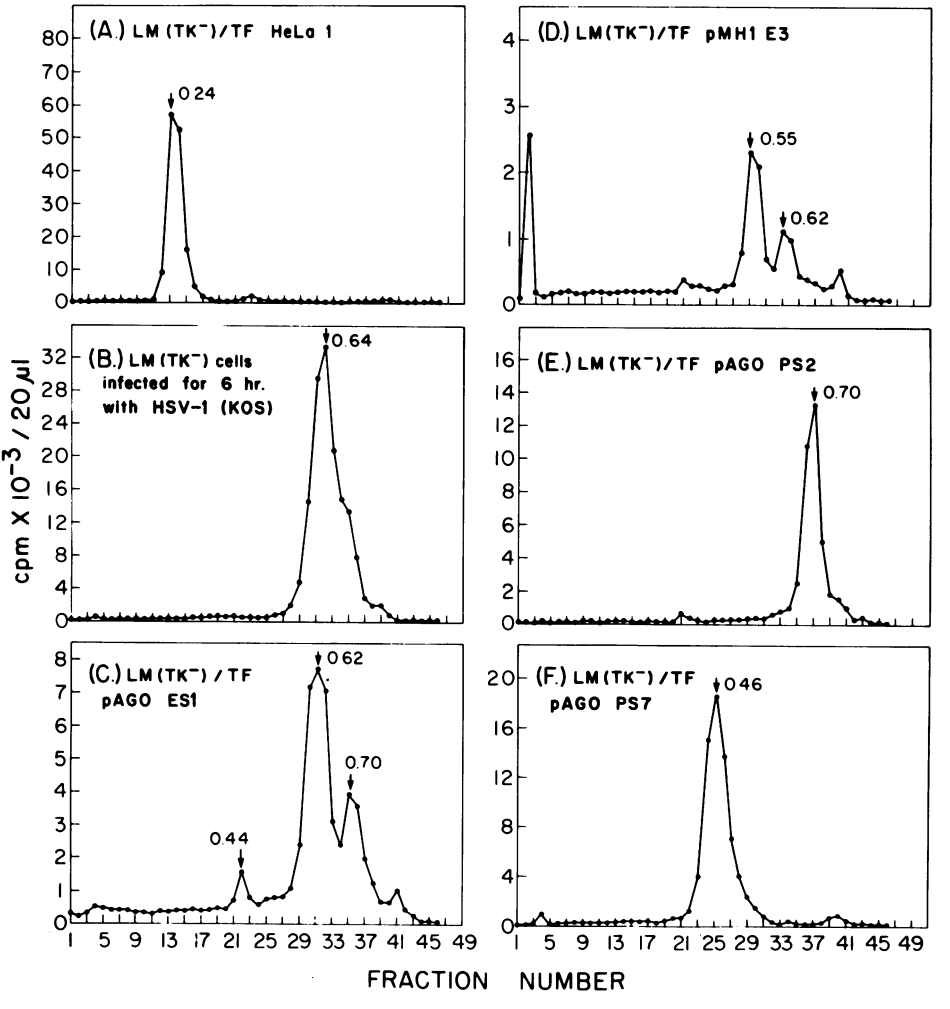


Fig. 5. Disc PAGE analyses (17) of cytosol TK from LM(TK$^-$) cells infected for 6hr with HSV-1(KOS) (B) and of cytosol extracts from cells biochemically transformed by fragments of plasmid DNAs containing the HSV-1 TK gene (C-F), or by sheared HeLa S3 cell DNA (A). Numbers above the peaks signify electrophoretic mobilities relative to the tracking dye.

cells (data not shown). It is interesting that the cytosol TK activity of cell lines transformed by the Pvu II-Sma I fragment purified from pAGO DNA [LM(TK$^-$)/TF pAGO PS2 and PS7] exhibited Rm values that were either less than or greater than the Rm of TK from HSV-1-infected cells (Fig. 5E, 5F). The serological and disc PAGE analyses demonstrate that cell lines transformed by HSV-1 restriction nuclease fragments express HSV-1-specific TK activities, but that somewhat alter-

ed translation products may be obtained in some cases.

Analyses of ^{35}S-methionine-labeled cell lines. Previous experiments have shown that the HSV-1 TK synthesized in productively infected cells has a molecular weight of about 80,000 daltons and consists of two subunits of about 40,000 daltons (2,8,26). To investigate the size of the TK polypeptides synthesized in cells biochemically transformed by fragments of plasmid DNAs, LM(TK$^-$)/TF pMH1 ES1 and LM(TK$^-$)/TF pMH1 Hc2 cells were labeled with ^{35}S-methionine and the TK activities were purified by $(NH_4)_2SO_4$ precipitation, preparative disc PAGE, a second $(NH_4)_2SO_4$ precipitation step, and by glycerol gradient centrifugation (26,27). Fractions containing the purified TK activity were treated with anti-HSV-1 IgG No. 46 or with normal rabbit IgG and immunoprecipitated with Staph. aureus protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, New Jersey). The labeled polypeptides were eluted from the protein A-Sepharose complexes by boiling with Tris buffer-mercaptoethanol-sodium dodecyl sulfate (SDS) solution, and the eluates were anlyzed by SDS-polyacrylamide slab gel electrophoresis and autoradiography (see legend to Fig. 5 for details). These experiments demonstrated that the principal TK activities of LM(TK$^-$)/TF pMH1 ES1 and LM(TK$^-$)/TF pMH1 Hc2 cells consisted of polypeptides of about 39,000 to 40,000 daltons (Fig. 6).

DISCUSSION

Hybrid plasmids containing an active HSV-1 TK gene have been cloned in E. coli K12 (4,5). In the present study, hybrid plasmids which contain a 1,920bp HSV-1 TK DNA sequence (EcoR I-Pvu II) are described. These plasmids, designated pMH1, pMH1A, and pMH4, biochemically transform LM(TK$^-$) cells when they are presented to the cells as superhelical closed-circular DNAs, and also after they have been cleaved with restriction endonucleases EcoR I, Hinc II, Bgl II, Sma I, and Pvu II, which have only one recognition site in the HSV-1 TK sequence of the plasmids (Fig. 1, Table 1). A 1,230bp nucleotide sequence (EcoR I-Sma I) purified from pMH1 and pAGO (from which pMH1 was derived) also transformed LM(TK$^-$) cells to the TK$^+$ phenotype.

Biochemical transformation of LM(TK$^-$) cells entails the integration of HSV-1 TK DNA into cellular DNA (3-5,9,16)(Fig. 2). Although the detailed molecular mechanisms leading to integration are not known, it appears that the termini of the restricted fragments are modified as a consequence of the integration events. Molecular hybridization experiments utilizing a pAGO DNA probe and cellular DNAs cleaved by a series of restriction endonucleases have been carried out on LM(TK$^-$)/TF pAGO PS7 and LM(TK$^-$)/TF pMH1 ES1 cells to analyze integrated viral and flank-

ing cellular DNA sequences. These studies indicate that the original Pvu II and Sma I, and the original EcoR I and Sma I cleavage sites, respectively, were absent from the viral DNAs integrated in LM(TK$^-$)/TF pAGO PS7 and LM(TK$^-$)/TF pMH1 ES1 cells. The DNA fragments actually integrated may be somewhat smaller than the DNA fragments presented to the cells (Fig. 3).

With these considerations in mind, the frequencies of transformation by DNAs treated with different restriction endonucleases become significant. The data of Table 1 show that the transformation frequencies were relatively high when plasmid pMH DNAs cut by EcoR I, Hinc II, and Pvu II were used for transformation. Partial digestion is unlikely because digestions were systematically monitored by gel electrophoresis. Reassociation of DNA fragments appears unlikely because of the high transformation frequency. In contrast, the transformation frequency was low when plasmid pMH DNAs cut by Bgl II and Sma I or when purified EcoR I-Sma I fragments were used for transformation. The preceding results are consistent with the hypothesis that the functional coding region for HSV-1 TK is contained within the DNA sequence extending from Hinc II(or Bgl II)to Sma I in the orientation 5'-3' (4,8).

In support of this interpretation, we have recently cloned a new hybrid plasmid (pMH110) derived from plasmid pAGO, but lacking the pAGO DNA sequence from the BamH I to the Bgl II cleavage sites (Fig. 1). LM(TK$^-$) cells were readily transformed to TK$^+$ by superhelical pMH110 DNAs and by EcoR I- and Hinc II-cleaved pMH110 DNAs (unpublished experiments).

In this and previous studies (3,4,9), it was shown by serological analyses with rabbit anti-HSV-1 IgG No. 46, that the TK activity expressed in the biochemically transformed cells was HSV-1-specific. Disc PAGE analyses also indicated that the TK activities of biochemically transformed cells resembled the HSV-1 TK induced in cytolytic infection and not mammalian cytosol TK. However, the TK activities of biochemically transformed cells often exhibited electrophoretic heterogeneity. Furthermore, the TK activities of cells biochemically transformed by purified Pvu II-Sma I fragments were either altered in size or in charge, as compared with the HSV-1 TK of cytolytically infected cells (Fig. 5). These observations together with the findings that LM(TK$^-$) cells can be biochemically transformed by Hinc II- and Bgl II- cleaved plasmid DNAs and by a purified 1.2kbp EcoR I-Sma I fragment, which has barely enough genetic information to code for the 40,000 dalton HSV-1 TK polypeptide, raised the possibility that fused polypeptides, which are translated from viral and flanking cellular DNA sequences, or truncated polypeptides, could have been made in some biochemically transformed cells (29).

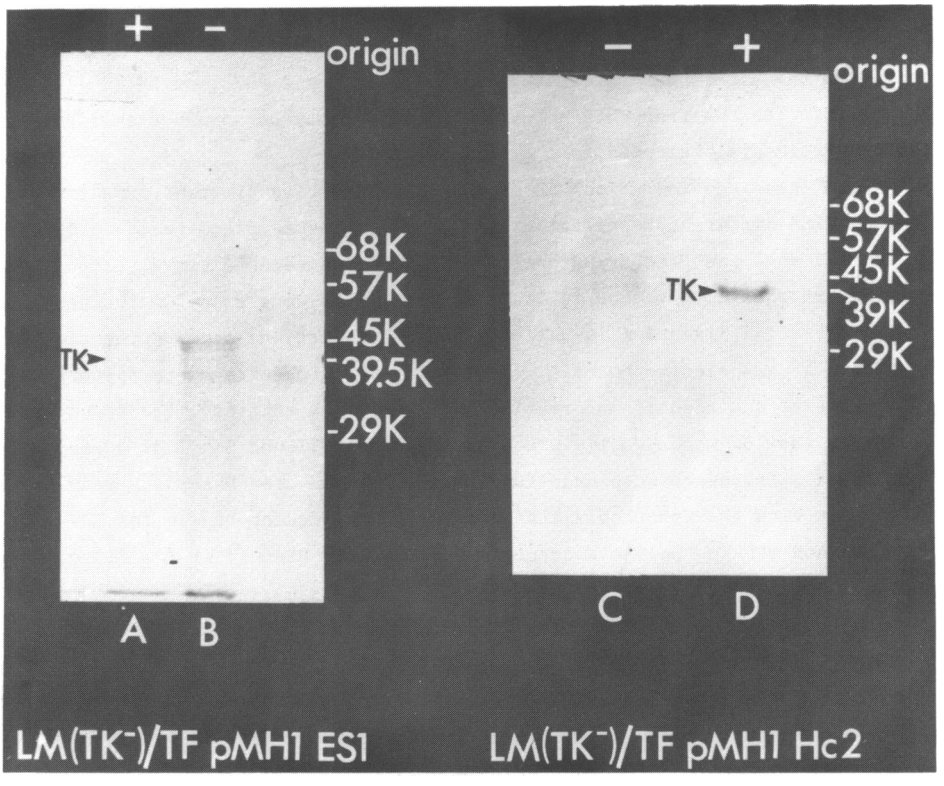


Fig. 6. Autoradiograms of 10% polyacrylamide (SDS) slab gels showing ^{35}S-methionine-labeled HSV-1 TK polypeptides from biochemically transformed cells. (+) indicates precipitation with anti-HSV-1 IgG No. 46 and (-) indicates precipitation with normal rabbit IgG. Monolayer cultures (about 5 X 10^8 cells) of LM(TK$^-$)/TF pMH1 ES1 and LM(TK$^-$)/TF pMH1 Hc2 cells were labeled for 4hr with ^{35}S-methionine (about 160µCi/ml) in 3ml of methionine-deficient medium (modified McCoy's containing 2% dialyzed calf serum and HATG) and cytosol extracts were prepared (17,26,27). Extracts were treated at 4°C with 0.06ml of 10% polymin P per ml of extract and the precipitated nucleic acids were removed by centrifugation. Ammonium sulfate (243mg/ml) was added to each supernatant fraction, the suspension was stirred for 30 min at 4°C, the precipitate was collected by centrifugation, and redissolved in modified enzyme buffer [0.15M KCl, 0.003M 2-mercaptoethanol (ME), 0.01M Tris-HCl, pH 8, 2.5mM ATP, 1.25mM Mg^{2+}, 0.2mM thymidine, 0.05M epsilon aminocaproic acid (EACA), and 5% glycerol]. The HSV-1 TK activity was then purified by preparative polyacrylamide gel electrophoresis at 4°C, as described previously (26,27), except that a 1.9% stacking gel was used together with the 5% separating gel and fractions were collected in tubes containing 0.5mg normal rabbit IgG. Fractions containing the HSV-1 TK activity were pooled, the HSV-1 TK activity was reprecipitated with ammonium sulfate, redissolved in modified enzyme buffer, and further purified by centrifugation in 10-30%(v/v) glycerol gradients (26). An immunoprecipitation procedure based on the protocol of Luborsky and Chandrasekaran (28) was then employed. Fractions containing HSV-1 TK activity were pooled, half of the material

(0.95ml) was incubated at 4°C for 1hr with 75µl of anti-HSV-1 IgG No. 46, and the other half with normal rabbit IgG. The IgG preparations had previously been absorbed with LM(TK$^-$) and HeLa(BU25) cells. Next, 0.4ml of washed, swollen Staph. aureus protein A-Sepharose CL-4B, at a concentration of 50%(v/v) in 0.15M KCl,3mM ME,0.05M Tris-HCl pH 8.0, 2.5mM EDTA, 10% glycerol, 0.05% Nonidet P40, and 0.02% of sodium azide was added, and the reaction mixture was incubated with vigorous shaking for 3hr at 4°C. After the protein A-Sepharose complexes had been washed six times with 0.5M LiCl, 0.1% ME, 0.1M Tris, pH 9.0, and once with modified enzyme buffer, they were resuspended in 75µl of gel electrophoresis sample buffer (62.5mM Tris, pH 7.0, 3% SDS, 5% ME, and 10% glycerol) and placed in boiling water for 3 min. Supernatant solutions were then analyzed by electrophoresis in SDS-polyacrylamide (10%) vertical slab gels and autoradiography (26,27). Molecular weights of radioactive polypeptide bands were estimated by comparing their electrophoretic mobilities with those of actin (45K) and the protein standards previously described (27).

To investigate the size of the TK polypeptides made in biochemically transformed cells, ^{35}S-methionine-labeling experiments were carried out (Fig. 6). These experiments demonstrated that the principal TK activities purified from LM(TK$^-$)/TF pMH1 ES1 and LM(TK$^-$)/TF pMH1 Hc2 cells consisted of polypeptides of about 39,000 to 40,000 daltons, which is about the same, within experimental error, as the size of the HSV-1 TK polypeptides made in cytolytically infected cells (26). These results further support the hypothesis that the functional region of the HSV-1 TK polypeptide is contained within the Hinc II to Sma I DNA sequence, but do not rule out the possibility that the amino- and/or carboxy-terminal fragments of the TK polypeptides made in biochemically transformed cells may be different from those made in HSV-1-infected cells. Peptide mapping experiments (30, 31) could disclose whether or not this is so.

In this connection, the nucleotide sequence analyses on the HSV-1 TK gene recently completed by M.J. Wagner, J.A. Sharp, and W.C. Summers (personal communication) are relevant. Their data indicate that: (i) the DNA sequence complementary to the 5' end of the message is located about 50 base pairs upstream from the Bgl II cleavage site; (ii) the most probable start codon is located about 55 base pairs 3' to the Bgl II site; and (iii) the stop codon and signals for polyadenylation are present 3' to the Sma I cleavage site.
From the latter findings, the prediction can be made that the TK polypeptide synthesized in cells transformed by the purified EcoR I-Sma I fragment has a normal amino terminal peptide sequence but an altered carboxy-terminal peptide sequence.

Finally, the experiments showing that the size of the functional coding region of HSV-1 TK (1.1kbp) approximates the minimum coding length for the HSV-1 TK polypeptide (40,000 daltons) raises the possibility that the coding region of the HSV-1 TK gene has either small intervening sequences which do not dis-

rupt the translational reading frame or no intervening sequences at all. It is of interest that the E. coli TK consists of two 42,000 dalton subunits (32) and that the maximum size of the E. coli TK coding region is 1.5kbp (33). Also, HSV-1-specific TK activity has recently been demonstrated in TK^- E. coli K12 cells which had been transformed by plasmid pAGO (Fig. 1) (34). Intervening sequences in eukaryotic genes are an important barrier to the spontaneous expression of eukaryotic genes in prokaryotes. The expression of HSV-1 TK in E. coli, therefore, supports the hypothesis that intervening sequences may be absent from the coding region of the HSV-1 TK gene.

ACKNOWLEDGEMENTS

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