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F. Colbere-Garapin, S. Chousterman, F. Horodniceanu, P. Kourilsky, A. C. Garapin

Proc. Natl. Acad. Sci. USA
Vol. 76, No. 8, pp. 3755–3759, August 1979
Biochemistry

Cloning of the active thymidine kinase gene of herpes simplex virus type 1 in *Escherichia coli* K-12

(biochemical transformation/selective marker/thymidine kinase-deficient mouse L cells/genetic engineering/gene expression)

FLORENCE COLBERE-GARAPIN*, SUZANNE CHOUSTERMAN†, FLORIAN HORODNICEANU*, PHILIPPE KOURILSKY‡, AND AXEL-CLAUDE GARAPIN‡

*Unité de Virologie Médicale, Institut Pasteur, 75724 Paris, Cedex 15, France; †Institut de Recherches Scientifiques sur le Cancer, B.P. 8, 94899 Villejuif, France; and ‡Groupe de Biologie Moléculaire du Gène, Unité de Génie Génétique, Institut Pasteur, 75724 Paris, Cedex 15, France

Communicated by André Lwoff, May 14, 1979

ABSTRACT **A herpes simplex virus DNA fragment that is produced by digestion with *Bam*HI endonuclease and carries the thymidine kinase (TK; ATP:thymidine 5′-phosphotransferase, EC 2.7.1.21) gene has been cloned in *Escherichia coli*. A recombinant plasmid, pFG5, has been analyzed extensively and a detailed restriction map is presented. pFG5 DNA efficiently transforms TK⁻ mouse L cells. The TK coding sequence in the cloned fragment has been localized and a smaller recombinant plasmid, pAG0, also carrying an active TK gene, has been constructed to serve as a more convenient vector for transfer, into TK⁻ cells, of genes previously cloned in *E. coli*.**

Herpes simplex virus (HSV) codes for a thymidine kinase (TK; ATP:thymidine 5′-phosphotransferase, EC 2.7.1.21) (1). An enzyme with a similar activity is found in animal cells, but TK⁻ cell lines have been isolated (1). Their cellular defect can be complemented by the HSV TK gene. TK⁺ cells can easily be selected (1). Wigler *et al.* (2) have isolated a *Bam*HI restriction fragment carrying the HSV TK gene that induces the biochemical transformation of TK⁻ cells into TK⁺ cells. A unique copy of the TK gene is covalently integrated in the DNA of all transformants (3). These results raise the possibility that the HSV TK gene can be used as a selective marker for the cotransfer of other genes into animal cells and, in particular, of genes previously cloned and characterized in *Escherichia coli*. Because it is likely that *E. coli* is not adequate for proper *in vivo* expression of genes from the higher eukaryotes (4), the transfer of such genes into animal cells may be important for performing functional analyses. As a first step in the development of such a transformation system, we have cloned in *E. coli* and characterized extensively the *Bam*HI HSV1 DNA fragment (2) that carries an active TK gene.

MATERIALS AND METHODS

Biohazards. Biohazards associated with the experiments described in this publication have been examined previously by the French National Control Committee, and the experiments were carried out according to the rules established by this committee.

Cells, Virus, and Viral DNA Extraction. Normal mouse L cells and TK⁻ mouse LM cells (clone 1D) were grown in Leibovitz L15 medium containing 5% calf serum (kindly provided by J. C. Guillon and G. Buttin, respectively). TK⁺ cells were selected in the same medium supplemented with thymidine, adenosine, guanosine, glycine, and aminopterin (Sigma) (HAT medium) as described (1). Transformants were maintained in this medium. F3 cells are 1D cells transformed to the TK⁺ phenotype by using sheared HSV1 F strain DNA (kindly provided by R. Crainic). The absence of mycoplasma was confirmed by C. Tram. HSV1 strain F (kindly provided by P. Sheldrick) was propagated in human diploid MRC5 cells. Extracellular virus was clarified and pelleted as described (5). Viral DNA was extracted and separated from cellular DNA by equilibrium density centrifugation in NaI gradients (5, 6).

Enzymes. All restriction endonucleases were obtained from New England BioLabs. Reaction conditions were as recommended by New England BioLabs. Grade 1 alkaline phosphatase from calf intestine was obtained from Boehringer. DNA (200 ng) was diluted in 100 μl of the reaction mixture containing 0.1 M glycine, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$ (pH adjusted to 8.8 with KOH), and 10 μl of enzyme and incubated for 1 hr at 37°C. DNA was then diluted with an equal volume of 20 mM Tris·HCl, pH 7.4/1 mM EDTA and dialyzed against the same buffer. Phage T4 DNA ligase was obtained from Bethesda Research Laboratories (Rockville, MD). Ligation was carried out as usual (7).

Agarose Gel Electrophoresis and Isolation of Restriction Endonuclease Fragments. DNA restriction fragments were separated by electrophoresis in 20-cm-long gels of 0.4–1.4% agarose medium (Sigma EEO grade I). Electrophoresis was for 10 hr at 10–25 mA in 89 mM Tris/89 mM boric acid/2.5 mM EDTA, pH 8.3.

Restriction fragments were recovered from agarose gel slices either by electrophoresis or by freezing and thawing (8, 9) and further purified as follows: to 1 vol of DNA solution, 1 vol of 2.5 M potassium phosphate buffer (pH 7.6) and 1 vol of 2-methoxyethanol (10) were added. After gentle mixing, the two phases were allowed to separate for 30 min at 4°C and were centrifuged for 30 min at 6000 × *g* at 4°C. The upper phase was then extensively dialyzed against 0.3 M NaCl/1 mM EDTA.

Bacterial Transformation and Construction of Recombinant Plasmids. *E. coli* 1106 (803 $r_k^-m_k^-$ *supE supF*) (11) was used for transformation (12).

The *Bam*HI TK fragment of HSV1 F strain DNA was purified from an 8.3-kilobase (kb) *Hpa* I fragment (13). Equal amounts (500 ng) of the TK fragment and *Bam*HI-cleaved pBR322 (14) were ligated. A transformation efficiency of 165,000 colonies per μg of ligated DNA was obtained. Colonies (1500) were tested for ampicillin resistance and tetracycline sensitivity. We obtained 48 such colonies. Four were analyzed in some detail and were shown to harbor the HSV DNA fragment; one of them (pFG5) was selected for further study. The 2-kb *Pvu* II fragment of HSV1 DNA arising from pFG5 was purified in an agarose gel (100 ng) and recombined with 200 ng of pBR322 cleaved with *Pvu* II and treated with alkaline phosphatase. Colonies (30) were obtained on selective plates; 4 out of 5 were recombinants.

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Abbreviations: HSV, herpes simplex virus; TK, thymidine kinase; HAT, hypoxanthine/aminopterin/thymidine; kb, kilobase.

Eukaryotic Cell Transformation. Restriction endonuclease treated DNAs were heated for 3 min at 65°C prior to transfection in order to eliminate possible end-to-end reassociation. Confluent TK^- L cells in 25-cm^2 Corning flasks were transfected as described (15) with modifications (16). Cultures were switched to HAT selective medium 24 hr later. By 3 weeks, primary colonies were counted microscopically. Transformed cell lines were established by passing colonies in HAT medium.

Cell Extract Preparation and TK Assays. Cell extracts were prepared as described (1) except that cells were washed three times in phosphate-buffered saline and lysed with 0.5% Nonidet P40 in 0.01 M maleic acid/Tris to pH 6.5/0.15 M KCl/0.02 M $MgCl_2$/0.01 M 2-mercaptoethanol, 200 μM ATP/10 μM thymidine, 200 μM phenylmethylsulfonyl fluoride (Sigma)/100 μM diisopropyl fluorphosphate (Serva, Heidelberg, West Germany). Nuclei were removed by spinning at 5000 × *g*. The supernatants were clarified at 30,000 × *g* for 30 min at 4°C. The standard assay mixture consisted of 0.1 M maleic acid/Tris to pH 6.5/0.01 M ATP/0.02 M $MgCl_2$/10 mM NaF/10 μM [^{3}H]thymidine (500 mCi/mmol, Commissariat à l'Energie Atomique, Saclay, France; 1 Ci = 3.7×10^{10} becquerels). After 3-hr incubation at 37°C, samples were spotted on Whatman DE-81 paper disks, which were washed four times in ethanol and assayed for radioactivity. For neutralization, 3 μl of control serum or purified IgG against HSV1-infected rabbit cells that specifically neutralized HSV TK [a generous gift of D. R. Dubbs (17)] were added to 15 μl of each enzyme sample, which contained 2–6 mg of protein per ml (18). The samples were incubated overnight at 4°C, clarified at 12,000 × *g* for 10 min, and assayed for TK activity.

RESULTS

Construction of pFG5, a Recombinant Plasmid Carrying the HSV1 TK Gene. The *Bam*HI fragment of HSV1 DNA carrying the TK gene was purified as described by Pellicer *et al.* (13) from an 8.3-kb *Hpa* I fragment of the viral genome and recombined with *Bam*HI-cleaved pBR322 (19). The HSV fragments were isolated from agarose gels by a method (described in *Materials and Methods*) that reproducibly provided, with a high yield, materials suitable for restriction endonuclease cleavage, ligation, and electron microscopy. Because insertion at the *Bam*HI site of pBR322 inactivates resistance to tetracycline, bacteria harboring recombinant plasmids are easily scored as ampicillin resistant and tetracycline sensitive (14). One recombinant plasmid, pFG5, which generated a fragment of 3.6-kb upon digestion with *Bam*HI, was analyzed further.

Restriction Map of the *Bam*HI HSV1 DNA Fragment. After cleavage of pFG5 by *Bam*HI, the cloned HSV fragment comigrated in agarose gel with a 3.6-kb fragment obtained after digestion of pBR322 with *Eco*RI and *Pst* I (19). The cloned HSV DNA could be separated from the pBR322 vector by electrophoresis in an agarose gel (Fig. 1) or by centrifugation in a NaI density gradient (not shown). The TK fragment was not cleaved by *Bcl* I, *Hin*dIII, *Hpa* I, *Kpn* I, *Sal* I, or *Xba* I. It was cut once by *Bgl* II, *Hin*cII, and *Sac* I and twice by *Eco*RI and *Pvu* II. There were three *Pst* I cleavage sites, four *Hin*fI sites, and five *Sma* I sites. The *Hin*cII site has an asymmetric location in the HSV1 *Bam*HI cloned DNA fragment such that digestion of pFG5 by *Hin*cII determines the orientation of HSV DNA with respect to the vector. Cleavage of pFG5 by *Eco*RI yielded three fragments of 4.5, 2.4, and 1.05 kb, which were isolated by sedimentation in a sucrose gradient (not shown). The data in Fig. 1 were sufficient to localize the *Sma* I sites closest to the ends of the 3.6-kb *Bam*HI fragment. For the other three *Sma* I sites, the purified 2.4-kb *Eco*RI fragment was used. Digestion with *Sma* I generated four fragments of 1.2, 0.77, 0.21, and 0.19 kb. In partial digests, fragments of 2 and 0.4 kb were observed (indicating that the two larger and the two smaller fragments are contiguous), as well as a 1-kb fragment, showing that the 0.77-kb fragment was adjacent to one of the smaller fragments. One *Hin*fI site was found in the 2.4-kb *Eco*RI fragment and mapped in relation to the *Sac* I and *Pst* I sites. Digestion of the 4.5-kb *Eco*RI fragment by *Hin*fI showed the presence of one site in the HSV DNA close to the *Bam*HI site. This *Hin*fI site was mapped more precisely by digestion of a 0.8-kb *Sal* I/*Eco*RI fragment purified by sucrose gradient sedimentation. The purified 1.05-kb *Eco*RI fragment was digested by *Hin*fI into fragments of 0.44, 0.37, and 0.24 kb, which were ordered by partial digestion. The restriction map was otherwise established by conventional means (Figs. 1 and 2).

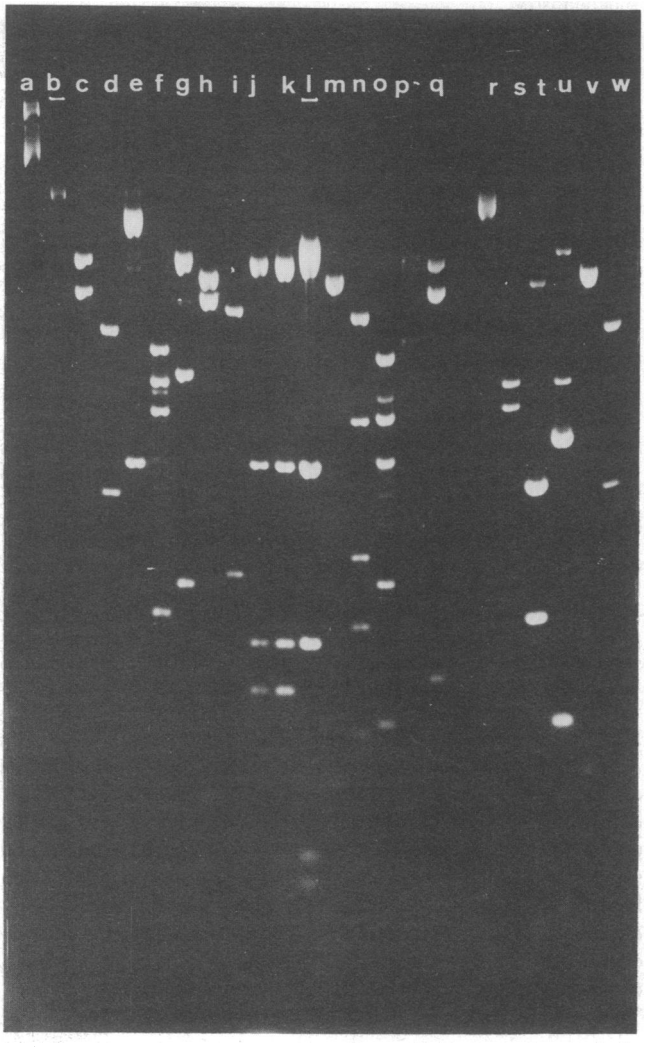

FIG. 1. Digestion of pFG5 DNA with restriction endonucleases. DNA fragments were analyzed by electrophoresis in a 1% agarose slab gel for 12 hr at 25 mA. Uncleaved pFG5 DNA (a); pFG5 DNA cleaved by *Hin*dIII (b), *Bam*HI (c), *Hin*dIII + *Sac* I (e), *Hin*dIII + *Pvu* II (f), *Hin*dIII + *Eco*RI (g), *Hin*dIII + *Bam*HI (h), *Hin*dIII + *Sma* I (j, k), *Sma* I (l), *Bam*HI + *Pst* I (n), *Bam*HI + *Pvu* II (o), *Bam*HI + *Bgl* II (p), *Bgl* II (r). The 2.4-kb DNA fragment obtained after digestion of pFG5 with *Eco*RI was further cleaved with: *Eco*RI + *Pst* I (t) and *Sac* I + *Bgl* II (u). pBR322 DNA fragments of known molecular weight were used as markers (19): pBR322 *Eco*RI + *Ava* I (d, w), *Ava* I + *Bam*HI (i), *Bam*HI + *Eco*RI (m, v), *Eco*RI + *Sal* I (q), *Pvu* II + *Eco*RI (s).

Biochemical Transformation of TK^- Mouse L Cells with pFG5 DNA. Transformation of TK^- mouse L cells with su-

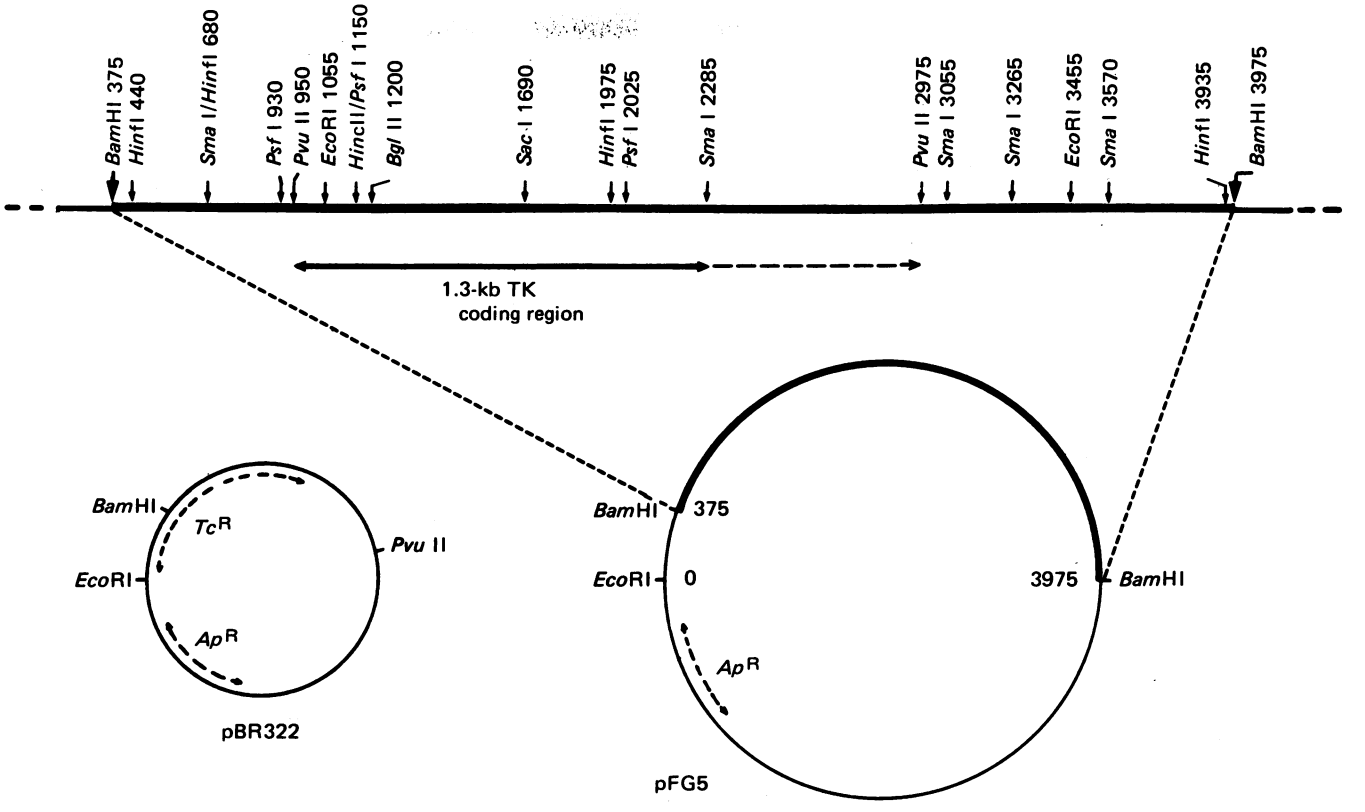

FIG. 2. Restriction map of the HSV1 3.6-kb *Bam*HI DNA fragment inserted at the *Bam*HI site of pBR322 and cloned as pFG5. The unique pBR322 *Eco*RI site was chosen as position 0 in the map of pFG5. Ampicillin resistance (Ap^R) and tetracycline resistance (Tc^R) genes in pBR322, and Ap^R in pFG5, are shown. Restriction sites are indicated with their approximate distance from position 0, measured in base pairs and clockwise. The TK coding region is indicated by the arrow.

percoiled pFG5 DNA resulted in the appearance of TK^+ colonies in HAT medium. The increase in the number of TK^+ colonies was approximately linear with input DNA up to amounts of about 1 μg per 25-cm^2 flask (Fig. 3). The efficiency of transformation by pFG5 DNA varied between 20 and 400 colonies per μg of pFG5 DNA. When calculated from Fig. 3, it is of about 300 colonies per μg of pFG5 DNA, or one colony per 10^4 cells with saturating amounts of pFG5 DNA (these figures may be slightly overestimated, due to the appearance of a few secondary colonies). No colony was observed after treatment with salmon sperm DNA or pBR322 DNA. With *Bam*HI-cleaved pFG5 DNA, the dose–response curve was also linear. However, transformation in this case, as well as with pFG5 linearized by digestion with *Hin*dIII, was reproducibly 2 to 5 times more efficient than with uncleaved pFG5 DNA.

TK Activity of Cells Transformed with pFG5 DNA. Rabbit anti-HSV1 IgGs that specifically neutralized the viral TK activity in HSV1-infected cells also neutralized efficiently the TK activity present in cells transformed by either sheared HSV1 DNA or pFG5 DNA (Table 1). As previously noticed by others (20), neutralization was more complete in virus-infected cells than in transformed cells. When cells were lysed by 0.5% Nonidet P40 rather than by sonication, neutralization was improved, which suggests that part of the TK activity may be associated with cell membranes. The proportion of TK activity remaining after incubation with anti-HSV1 antibody never exceeded 10%. Therefore, TK activity in pFG5-transformed cells has the expected characteristics of viral enzyme, and must be encoded by the fragment cloned in pFG5.

Localization of the TK Gene within the Cloned HSV1 DNA Fragment. pFG5 DNA was cleaved by various restriction enzymes, and the digests were assayed for their ability to transform TK^- cells. We observed no TK^+ colonies with pFG5 DNA treated with any of the following enzymes: *Alu* I, *Bgl* II, *Hae* III, *Hha* I, *Msp* I, *Sac* I, and *Taq* I. In contrast, pFG5 cleaved with *Pvu* II reproducibly induced the appearance of TK^+ colonies with an efficiency comparable (albeit slightly lower) to that of *Bam*HI-cleaved pFG5. Cleavage by *Sma* I yielded an intermediate result: a few colonies (about 5–10% of the number obtained with DNA cleaved by *Bam*HI) appeared after longer incubation (3 to 4 instead of 1 or 2 weeks) but subsequently grew at the same rate. Partial digestion is unlikely because digestions were systematically monitored by gel electrophoresis. Reassociation of DNA fragments is not probable, especially because *Sma* I generates completely double-stranded

Table 1. Inactivation of TK by specific rabbit IgG

Source of enzyme	TK specific activity*	% activity remaining after neutralization†
TK^+ LM	18	89
TK^- LM 1D	0.05	—
HSV1 F-infected 1D cells	24	1
HSV1 F DNA-transformed 1D cells (F3)	6.6	6
pFG5 DNA-transformed 1D cells	3	8

Results are averages of duplicate samples.

* TK specific activity in crude cell extracts is expressed as pmol of thymidine phosphorylated in 3 hr at 37°C per μg of protein.

† Neutralization was with rabbit anti-HSV1 IgG at a 1:6 dilution overnight at 4°C. Control values were obtained after incubation with normal rabbit serum. Similar control values were obtained with extraction buffer or rabbit serum raised against spontaneously transformed rabbit cells.

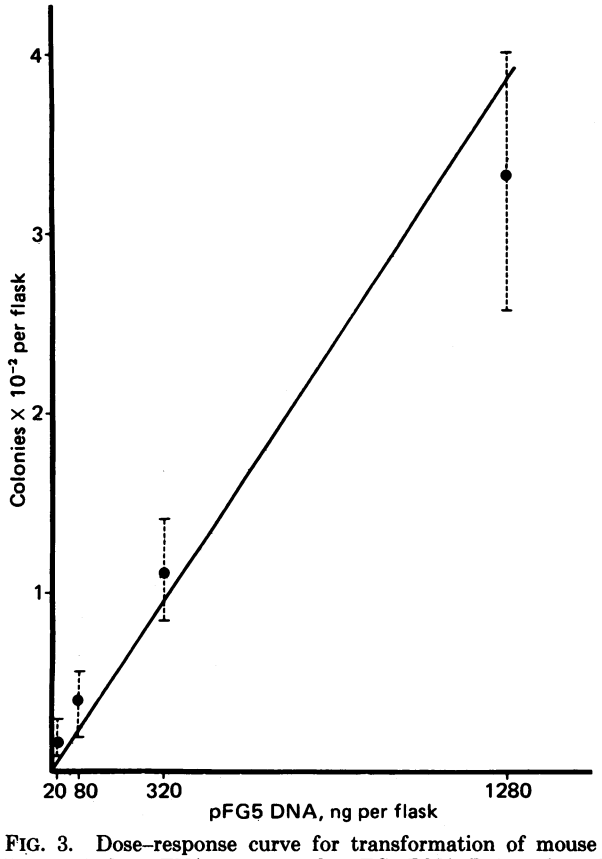

FIG. 3. Dose–response curve for transformation of mouse L cells from TK^- to TK^+ phenotype by pFG5 DNA. Points show the average of four determinations in the limits of observed values.

ends. It appears possible, therefore, that the TK gene is encoded by one of the *Sma* I fragments.

Comparison with the restriction map in Fig. 2 suggests that the active TK gene is contained in the 2-kb *Pvu* II HSV DNA fragment, perhaps in the 1.3-kb region extending from the *Pvu* II to the *Sma* I site located at positions 950 and 2285 in the map of Fig. 2. This region would be large enough to code for the 43,000-dalton TK protein (21).

To demonstrate that the 2-kb *Pvu* II fragment does contain the active gene, we inserted it in the *Pvu* II site of pBR322. One recombinant plasmid, pAG0, was cloned and characterized. pAG0 induced the formation of TK^+ colonies in HAT selective medium with the same efficiency as pFG5.

DISCUSSION

We have constructed a recombinant plasmid, pFG5, in which the HSV1 *Bam*HI fragment coding for the viral TK has been integrated in the *Bam*HI site of the *E. coli* plasmid pBR322. [In a recent report Enquist *et al.* (22) mention the isolation of a similarly constructed plasmid.] We present a detailed restriction map of the cloned fragment, which fits the less extensive mapping data previously obtained with a similar HSV1 *Bam*HI fragment not cloned in *E. coli* (13). pFG5 DNA effectively transforms TK^- mouse L cells into cells with a TK^+ phenotype. In contrast, control experiments with pBR322 DNA were entirely negative. More than 90% of the TK activity present in cells biochemically transformed by pFG5 could be neutralized by IgGs that react with the viral, but not the cellular, TK. All together, these results demonstrate that, in pFG5, we have cloned an active viral TK gene.

Biochemical transformation of TK^- cells by sheared HSV1 DNA or pFG5 has similar characteristics. In both cases, the dose–response curve is linear (Fig. 3 and ref. 23), so that one TK gene is presumably enough to induce biochemical transformation. In addition, the efficiencies are comparable (1–10 TK^+ colonies per μg of sheared HSV1 DNA versus a few hundred colonies per μg of *Bam*HI-cleaved pFG5 DNA—*i.e.*, about 2–20 TK^+ colonies per 10^{10} gene equivalents). In this instance the activity of a gene in an animal cell can be compared to that of the same gene cloned in *E. coli*. The results suggest that DNA cloned in *E. coli* has not undergone changes (such as restriction-modification) that interfere with its expression in mouse cells.

Several restriction enzymes destroy the transforming activity of the cloned HSV1 DNA, while others do not. This allowed the localization of the TK coding sequence in a 2-kb *Pvu* II fragment, and, perhaps, in the 1.3-kb *Pvu* II/*Sma* I fragment shown in Fig. 2, which is theoretically large enough to encode the 43,000-dalton protein (21). Because we have not as yet subcloned this fragment, we cannot be certain that it contains an active TK coding sequence. The 2-kb *Pvu* II fragment, in contrast, has been recloned in pBR322, and the recombinant plasmid, pAG0, transforms about as efficiently as pFG5. pAG0 is smaller than pFG5, carries both ampicillin and tetracycline resistance genes, and has several unique restriction sites (*Bam*HI, *Hin*dIII, *Sal* I) located in the latter (such that integration of DNA at these sites will cause partial or total loss of resistance to tetracycline). pAG0, therefore, is a more convenient vector than pFG5 for cloning additional genetic material in *E. coli* prior to transfer into animal cells.

The fate of TK DNA in biochemically transformed cells has not been investigated yet. In view of previous results (13), it is possible that the DNA becomes integrated into the host chromosomes. Because supercoiled pFG5 or pAG0 induces biochemical transformation efficiently, it seems possible that plasmid sequences linked to TK become integrated as well. The finding that linear pFG5 DNA transforms 2–5 times better than supercoiled DNA is unexplained.

The availability of the cloned HSV1 TK gene can serve several purposes. The regulation of TK expression during the life cycle of herpes viruses can be more easily studied. The mechanism by which the TK gene possibly becomes integrated into the host chromosomes in biochemical transformation is amenable to molecular analysis. Finally, the HSV1 TK gene may be used for the transfer of genes into animal cells in one of the following ways: (*i*) The cloned *Bam*HI or *Pvu* II fragments may be purified in large amounts from pFG5 or pAG0, ligated with other genetic material, and used for biochemical transformation. (*ii*) Additional genetic material may be directly cloned in *E. coli* by using pFG5, or, more conveniently, pAG0 as vector, and introduced into TK^- cells. (*iii*) The HSV TK gene may also be used in the construction of vectors able to replicate autonomously in animal cells in an episomal state, to allow an easy selection of cells that have received DNA and, perhaps, help to stabilize the episomal state. Prior to their transfer into animal cells by any of these means, genes could be manipulated *in vitro* in such a way as to modify genetic elements expected to be involved in their expression and regulation.

While this work was in progress, the biochemical transformation of TK^- cells by HSV1 DNA fragments cloned in *E. coli* was reported by Weissman *et al.* (24), as well as the use of the TK gene for gene transfer into animal cells.

Note Added in Proof. The 2.4-kb *Eco*RI HSV1 DNA fragment obtained from pFG5 was inserted in the *Eco*RI site of pBR322 and cloned in *E. coli*. One recombinant, pAG1, transformed TK^- cells with a low efficiency.

We thank Dr. S. Michelson for helpful discussion. We are grateful to Dr. B. Blondel for protein measurements, Miss O. Le Bail for pBR322 preparation, and Mrs. J. Lavolé for expert cell culture. We thank Mrs. B. Cami and A. Cyprès for help in the preparation of the manuscript. This work was supported by Institut National de la Santé et de la Recherche Médicale (INSERM) Grant 009 to F.H. and by grants to P.K. from the Centre National de la Recherche Scientifique (Equipe de Recherche Associée 201, plus an exceptional grant, and ATP no. 3558), the INSERM (grants 76.4.319 and 76.4.465), the Délégation Générale à la Recherche Scientifique et Technique, and the Fondation de la Recherche Médicale Française.

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