Thymidine Kinase Deletion Mutants of Herpes Simplex Virus Type 1

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SUMMARY

Deletions in the cloned thymidine kinase (TK) gene of herpes simplex virus type 1 (HSV-1), strain 17 syn+, were produced by two methods. Removal of a 506 base pair fragment from between the unique SsrI and BglII restriction endonuclease sites of pTK1 (HSV-1 BamHI p cloned in pAT153) and subsequent transformation of Escherichia coli resulted in the isolation of 50 deleted plasmids. Sequential digestion of pTK1 with BglII and nuclease BAL 31 followed by ligation and recleavage with BglII resulted in the isolation of 31 deleted plasmids. Three clones, pTK2, pTK3 and pTK4, obtained following BglII and SsrI treatment of pTK1 were recombined with wild-type (wt) HSV-1 (17) syn+ DNA in baby hamster kidney (BHK) cells to produce TK- deletion mutants HSV-1 (17) TK 1301, HSV-1 (17) TK 1302 and HSV-1 (17) TK 1303 respectively. 5-Bromo-2'-deoxyuridine, 5-bromo-2'-deoxycytidine and 9-(2-hydroxyethoxymethyl)guanine were used to reduce the background of TK+ virus in heterogeneous recombinant stocks analysed for the presence of TK- recombinants. All recombinant clones isolated produced a small syncytial plaque morphology in BHK cells. The mutants HSV-1 (17) TK 1301 and HSV-1 (17) TK 1302 were TK-, failed to produce polypeptides of molecular weights 43000 and 19000 found in wt-infected cells and demonstrated one-step growth curves different from wt virus and the TK- mutant HSV-1 (17) dPyk-7. Superinfection studies with HSV-1 (17) TK 1301, HSV-1 (17) TK 1302, HSV-1 (MDK) and HSV-1 (17) dPyk-7 indicated that all TK- mutants except dPyk-7 produce a trans-acting gene product which can switch on the transforming HSV-1 TK gene.

INTRODUCTION

The thymidine kinase (TK) gene of herpes simplex virus type 1 (HSV-1) is eminently suitable for detailed genetic analysis. TK- mutant selection is possible by the use of the base analogues 5-bromo-2'-deoxyuridine (B UdR) (Kit & Dubbs, 1963), 5-bromo-2'-deoxycytidine (BCdR) (Brown & Jamieson, 1978) and 9-(2-hydroxyethoxymethyl)guanine (ACG) (Field et al., 1980). TK+ virus can be selected using HAT medium (Littlefield, 1964). Many TK- mutants fail to produce a polypeptide of mol. wt. 43000 (Summers et al., 1975; Preston, 1977), which is believed to be the TK gene product. The HSV-1 and HSV-2 TK genes have been located and are co-linear between 0.28 and 0.32 genome map units (Wigler et al., 1977; Stow et al., 1978; Halliburton et al., 1980; McDougall et al., 1980). The direction of transcription of the HSV-1 gene has been determined (Smiley et al., 1980). Recently, that portion of the HSV-1 genome coding for the TK gene has been cloned (Wilkie et al., 1979b; Colbere-Garapin et al., 1979; Enquist et al., 1979) and sequenced (McKnight, 1980; Wagner et al., 1981; Preston & McGeoch, 1981). The cloned gene retains biological activity (McKnight & Gavis, 1980; Cordingley & Preston, 1981;
Garapin et al., 1981) and is becoming widely used in genetic manipulation of the HSV genome (Mocarski et al., 1980; Post et al., 1981). TK− deletion mutants are a recent addition to the mutant stocks available for the analysis of HSV gene function (Smiley, 1980; Wilkie et al., 1980; Post et al., 1981).

In this paper we extend our previous report on the production of TK− deletion mutants of HSV-1 (Wilkie et al., 1980). TK− deletion mutants have been selected using three base analogues and all have a small syncytial (SSyn) plaque morphology in BHK cells whereas the wt parent produces Syn+ plaques in which the infected cells individually round up. TK− deletion mutants grow poorly in BHK cells, fail to produce polypeptides of mol. wt. 43000 and 19000 found in wt-infected cells and can provide a product in trans to switch on the HSV-1 TK gene in HSV-1 TK− transformed LMTK− cells. This switch-on was not found following superinfection with the TK− mutant dPyk−7.

METHODS

Cells, viruses and plasmids. Baby hamster kidney cells (BHK-21 C13; Macpherson & Stoker, 1962), PyY/CAR/BuDr cells (Hay et al., 1971) and LMTK− cells (Kit et al., 1963) were grown in Eagle's medium supplemented with 10% tryptose–phosphate broth and 10% calf serum (ETC10). Cell monolayers were grown in 50 mm diam. plastic Petri dishes (2 × 10⁶ to 4 × 10⁶ cells) or Linbro trays (6 × 10⁶ cells per well). HSV-1 (Glasgow strain 17) syn+ was grown in BHK cells in Eagle's medium containing 10% calf serum (EC10) after infection at low multiplicities. Titrations of virus were performed on BHK cells under Eagle's medium containing 5% human serum (EHu5). pAT153 (a gift from D. J. Sherratt) was propagated in Escherichia coli K12 strain HB101 (Boyer & Roulland-Dussoix, 1969), a gift from R. Thompson. Large cultures of E. coli containing pAT153 or recombinant plasmids were grown in L-broth (LB) containing 100 μg/ml ampicillin (LBA) and amplified with 200 μg/ml chloramphenicol (CAP). When large numbers of 50 ml cultures were being analysed for deleted recombinant plasmids no CAP was added and the cultures were grown to high density overnight. The mutants HSV-1 (17) TK 1301 and HSV-1 (17) TK 1302 are respectively HSV-1 (17) d1 and HSV-1 (17) d2 of Wilkie et al. (1980).

The BamHI p fragment of P3 (Wilkie et al., 1979b) was purified and ligated into the BamHI site of pAT153 and recombinant plasmid was isolated as described by Wilkie et al. (1979b).

Isolation and labelling of DNA. HSV-1 DNA was extracted as described by Wilkie (1973) from BHK cells infected at 31 °C at a multiplicity of infection (m.o.i.) of 10−2. Plasmid DNA was prepared by the method of Clewell & Helinski (1969). The cell lysates were then sequentially extracted with phenol and chloroform/isoamyl alcohol (24:1) and the DNA was precipitated with isopropanol at room temperature. Purified DNA was stored at −20 °C in 0.1 × SSC (1 × SSC is 150 mM-sodium chloride, 15 mM-trisodium citrate pH 7.4).

HSV-1 and plasmid DNA were labelled in vitro by nick translation (Rigby et al., 1977). Wild-type HSV-1 and recombinant virus were labelled in vitro with [12P]orthophosphate in 50 mm Petri dishes (Wilkie, 1973) or Linbro trays (Lonsdale, 1979).

Restriction endonuclease digestion. All enzymes were obtained from Bethesda Research Laboratories (BRL) or New England Biolabs. All digestions except those with EcoRI were carried out in 50 μl volumes in 6 mm-Tris–HCl pH 7.5, 6 mm-MgCl2, 6 mm-2-mercaptoethanol, 20 mm-KCl and 0.1% (w/v) bovine serum albumin. For EcoRI digestions the KCl concentration was increased to 50 mm.

DNA restriction enzyme profiles were obtained by electrophoresis through 0.4 to 4% agarose and 0.1 to 1% acrylamide gels as described previously (Maniatis et al., 1975; Wilkie, 1973).

Production of deleted pTK1 by SstI : BglII digestion. pTK1 was digested with SstI and BglII to remove the intervening 506 base pair DNA sequence which lies within the TK coding region (Fig. 1). Complete digestion was determined by agarose gel electrophoresis. The digestion of pTK1 with SstI and BglII typically reduced the transforming efficiency from 1.5 × 10⁵ colonies per μg DNA to < 1.0 × 10⁵ colonies per μg DNA. The reaction was terminated by heating at 65 °C for 10 min and 1 μg amounts were used to transform E. coli to ampicillin resistance by the method of Cohen et al. (1972). Individual transformed clones were tested for deletions by picking each into 200 μl LBA in microtest plates for storage at room temperature and also into 50 ml cultures which were grown to high density overnight. DNA was extracted as described above and aliquots of plasmid DNA were compared with parental markers by electrophoresis through 1% agarose gels. DNA was visualized by ethidium bromide staining and u.v. irradiation. Clones bearing deleted plasmids were repurified and stored in 50% glycerol:1% Bactopeptone at −20 °C.

Production of deletions in pTK1 by nuclease BAL 31 digestion. pTK1 DNA was linearized by digestion with BglII and the DNA was purified by phenol extraction and ethanol precipitation. One unit of nuclease BAL 31 (BRL) was added to 30 μg linearized pTK1 in 12 mm-CaCl2, 12 mm-MgCl2, 200 mm-NaCl, 20 mm-Tris–HCl pH 8.1, 1
mM-EDTA at 30 °C. After 1 min, the reaction was terminated by the addition of EDTA to 50 mM. The DNA was phenol-extracted and ethanol-precipitated. The resuspended DNA was ligated with 0-5 units T4 DNA ligase in a volume of 20 μl containing 20 mM-Tris-HCl pH 7.5, 4 mM-MgCl2, 0-5 mM-ATP, 10 mM-dithiothreitol, overnight at 22 °C. The reaction was terminated by heating at 65 °C for 10 min. The ligated DNA was recleaved with BgII and 1 μg amounts were used to transform E. coli to ampicillin resistance. Plasmids were analysed as described above.

**DNA sequencing.** DNA sequencing of pTK3 and pTK4 was performed according to Maxam & Gilbert (1980).

**Recombination between plasmids and HSV-1 DNA.** pTK1, pTK2, pTK3 and pTK4 were linearized by digestion with *SalI* which cleaves the pAT153 portion of each plasmid once (Fig. 1). The linearized molecules were transfected together with wt HSV-1 (17) syn+ DNA at molar ratios of between 1 and 250 : 1 (plasmid :HSV DNA) by the calcium phosphate precipitation technique (Graham & van der Eb, 1973) and dimethyl sulphoxide boost (Stow & Wilkie, 1976). Monolayers were overlaid with EC10, incubated at 37 °C and cells harvested in the medium when the cytopathic effect (c.p.e.) was 100%. Virus was released into the medium by sonication and titrated on BHK cells under EHu5 supplemented with 25 μg/ml BUDR (Hones & Watson, 1977), 100 μg/ml BCDR (Brown & Jamieson, 1978) or 0-25 ng/ml ACG (D. Dargan & J. H. Subak-Sharpe, personal communication).

**Thymidine kinase assays.** The TK activities of mock-infected and virus-infected cells were estimated according to Wilkie et al. (1979 b). PyY/CAR/BudR cells were infected at an m.o.i. of 10 and harvested 18 h post-infection. One-tenth of the sample was removed for protein estimation by the method of Lowry et al. (1951). In superinfection studies, P9 and LMTK- cells were similarly treated.

**Polypeptide analysis.** Mock-infected and virus-infected BHK cells were labelled with [35S]methionine (Amersham International; > 600 Ci/mmol) in 30 mm Petri dishes according to Marsden et al. (1976). Polypeptides were separated by electrophoresis on 5 to 12-5% gradient polyacrylamide gels containing SDS (SDS-PAGE) according the method of Laemmli (1970) as modified by Marsden et al. (1976). Polypeptide nomenclature is that of Marsden et al. (1978).

**One-step growth curves.** BHK cell monolayers in 30 mm tissue culture dishes were infected at an m.o.i. of 5. After 1 h adsorption, the cells were washed twice with 2 ml EC10, overlaid with 2 ml EC10 and incubated at 37 °C. At appropriate times after infection the cells were harvested in the medium and stored at −70 °C. Virus was released into the medium by sonication, serially diluted and titrated at 31 °C on BHK cells overlaid with EHu5.

**RESULTS**

**Analysis of pTK1**

The orientation of the HSV-1 *BamHI* p fragment in pTK1 was determined by restriction endonuclease digestion and is shown in Fig. 1. No difference in restriction sites was found between the *BamHI* p fragments of P3 and pTK1 following recloning. The TK gene of pTK1 is biologically active and can transform LMTK- cells to a TK+ phenotype (J. Lang & N. M. Wilkie, unpublished results).

**Analysis of deleted plasmids**

Following transformation of *E. coli* with *SstI*- and *BgII*-digested linear pTK1, 50 non-clonally related deleted plasmids were obtained from 173 ampicillin-resistant clones analysed. Fig. 2 shows a comparative gel of these deleted 'SB' clones analysed after *BamHI* digestion of purified DNA. The individual clones have lost different amounts of DNA and clones 153 and 159 have each lost one *BamHI* site. A number of clones were analysed in detail with restriction endonucleases and a summary of the extent of their deletions is shown in Fig. 3. Three plasmids pTK2, pTK3 and pTK4 were chosen as suitable candidates for recombination with wt HSV-1 DNA to make TK- deletion mutants. DNA sequencing of pTK3 and pTK4 has shown that they possess identical deletions, although they were produced in separate transformations. The plasmids pTK3 and pTK4 were produced by *in vivo* recombination within the four base-pair CACA repetition at positions 296 to 299 and 964 to 967 in the TK DNA sequence of Wagner et al. (1981), as shown in Fig. 4.

Following treatment of *BgII*-linearized pTK1 with BAL 31, 31 deleted plasmids were isolated from 50 ampicillin-resistant clones analysed. A summary of these 'BL' clones analysed in detail is given in Fig. 5.

Analysis of deleted pTK1 clones also showed that the orientation of fragments *AluI* a and e of HSV-1 *BamHI* p as presented by Wilkie et al. (1979 b) was incorrect; the orientation is as shown in Fig. 1.
Recombination between deleted plasmids and wt HSV-1 DNA

Preliminary experiments using both high and low molar ratios of linearized plasmid to wt HSV-1 DNA, with selection for TK⁻ virus applied during the recombination period, failed to produce TK⁻ recombinants at a detectable frequency (see Table 1).

Following co-transfection of BHK cells with linearized pTK2, pTK3 or pTK4 and wt HSV-1 DNA at molar ratios of up to 250:1 (plasmid:wt DNA), recombination was allowed to take place without selection for TK⁻ progeny. Infected cell monolayers were subsequently harvested and virus titrated in the presence of BUdR, BCdR or ACG to reduce the level of non-recombinant TK⁺ progeny. The mutants obtained following the examination of wt and TK⁻ recombinant DNA with BglII or BamHI are summarized in Table 1. Fig. 6(a) shows the BamHI restriction profiles for clones analysed after BCdR selection for TK⁻ progeny.

An analysis was also made during the BCdR selection to determine whether use of this drug was necessary to obtain TK⁻ progeny at a detectable frequency. Table 2 shows that of 20 plaque isolates picked in the absence of BCdR only one, clone 1, was as resistant as any of the 17 picked
in the presence of the drug. Clone 1 was not a TK- deletion mutant and, like the majority of plaques picked in the presence of BCdR, is probably a virus containing a mutant, not recombinant, TK gene. Only one of the clones picked in the presence of BCdR, clone 21, had exchanged the deleted BamHI p fragment for the wt fragment. The results therefore suggest that a reduction of non-recombinant TK+ progeny by the use of drug selection is required to detect rare TK- recombinants.

All TK- recombinant progeny isolated also had a small syncytial (SSyn) plaque morphology in BHK cells (Fig. 7b). This term was chosen because a small proportion (<0.1%) of the plaques produced by the wt DNA stock used in this study produced large syncytial plaques (LSyn) in BHK cells (Fig. 7c).

In selecting for TK- virus with ACG, only clones having an apparently SSyn morphology were isolated and DNA restriction profiles examined. Table 3 and Fig. 6(b) show that all six SSyn plaques picked using ACG were deleted in BamHI p. Some HSV-1 TK- deletion mutants are therefore detectable by screening heterogeneous populations for SSyn plaques. Table 1 summarizes the plasmids and TK- recombinant virus described in this study.

**Analysis of recombinant DNA profiles from purified virion DNA**

The restriction analyses performed on the initial deleted isolates were performed on nuclear DNA, which is concatemeric (Jacob et al., 1979). The relative abundance of certain virus bands therefore is not found when the same restrictions are performed on virion DNA.
Fig. 3. Summary of the sizes of deletions in SB clones as determined by restriction enzyme analysis. Vertical dashed lines indicate restriction sites absent; vertical solid lines indicate restriction sites present. Horizontal dashed lines indicate regions of uncertainty; horizontal solid lines indicate sequences definitely present. Sequences absent are left blank.

Fig. 4. DNA sequences involved in recombination to produce the non-clonally related pTK1 derivatives pTK3 and pTK4, which are identical. Positions of nucleotides are as in Wagner et al. (1981), with the T at position 962 changed to C to produce the SstI site found in HSV-1 (17), which is indicated by the asterisks. The CACA repeat in which recombination has occurred is boxed. The upper row of each sequence is 5' to 3', left to right. Regions of homology are underlined.
Virus DNA labelled \textit{in vivo} with $^{32}$P orthophosphate was obtained for TK 1301 and TK 1302 and analysed by digestion with \textit{BamHI}, \textit{PvuII}, \textit{HpaI}, \textit{XhoI}, \textit{EcoRI} and \textit{KpnI}. The profiles are shown in Fig. 8 and restriction maps in Fig. 9. It should be noted that all deletion mutants isolated labelled poorly with $^{32}$P orthophosphate \textit{in vivo}, suggesting that the TK gene is either not as indispensable as previously thought for HSV replication or that some other genome function has been affected by the deletion to produce this effect.

Both TK 1301 and TK 1302 showed altered mobilities in their \textit{BamHI} $p$, \textit{HpaI} $i$, \textit{KpnI} $m$ and \textit{PvuII} $y$ fragments. TK 1302 had also lost \textit{PvuII} $z$, which is adjacent to \textit{PvuII} $y$ in the genome. TK 1301 had lost \textit{XhoI} $f$ and the same fragment is absent in TK 1302 (data not shown). Comparison of these profiles with the maps of pTK2 and pTK3 is consistent with the incorporation of the \textit{BamHI} $p$ of these two plasmids into TK 1301 and TK 1302 respectively.

Some variation can also be seen in the migration of the \textit{BamHI} $x$ and \textit{KpnI} $j$ and $k$ fragments. This is due to size heterogeneities in the repetitive regions of the genome and is not a consequence of the deletions in the \textit{BamHI} $p$ fragments (Lonsdale \textit{et al}., 1980).
Table 1. *Summary of recombination experiments between pTK2, pTK3, pTK4 and HSV-1 syn+ TK+ DNA*

<table>
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<tr>
<th>Linearization before transfection</th>
<th>Plasmid: HSV-1 DNA ratio</th>
<th>Initial recombination in presence of BcdR</th>
<th>Selection for TK- virus</th>
<th>Analysed</th>
<th>Deleted</th>
<th>Percentage deleted</th>
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<td>100</td>
<td>4, 6, 9, 11</td>
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*ND, Not done; u, undesignated.
† Possibly clonally related. Clone 65 was designated TK 1302.
 HSV-1 TK deletion mutants

Fig. 6. BamHI digests of [\(^{32}\)P]orthophosphate-labelled DNA isolated from putative pTK4-HSV-1 recombinants. TK~ clones (numbered) were selected by (a) BCdR and (b) ACG. The position of wt HSV-1 BamHI p is indicated; other variations in migration (e.g. of BamHI k) are due to known heterogeneities. Clones 21 (a) and 4, 6, 9, 11, 16 and 19 (b) all had a SSyn plaque morphology and lacked their BamHI p fragment.

Properties of the recombinants HSV-1 (17) TK 1301 and HSV-1 (17) TK 1302: one-step growth curves

The growth of TK 1301 and TK 1302 was compared on BHK cells to that of wt HSV-1 (17) and the TK~ mutant HSV-1 (17) dPyk~. The latter was added as a control to indicate whether the cells were indeed capable of supporting the growth of TK~ virus (Jamieson et al., 1974).

Fig. 10 shows that wt HSV-1 and dPyk~ had very similar profiles with a lag period up to 6 h after infection, followed by an increase in virus progeny which started to decline between 12 and 16 h post-infection. TK 1301, however, had no well-defined lag period and its final titre was at least one \(\log_{10}\) down on HSV-1 (17). TK 1302 had a lag period of 8 h and then produced virus progeny at a rate far lower than cells infected with wt virus or dPyk~. The absence of a lag period with TK 1301 is perhaps due to slow adsorption and uncoating of virus, suggesting a defect in a membrane (or capsid) component. The inability of either deletion mutant to grow as efficiently as a TK~ mutant or wt HSV-1 (17) virus is possibly the result of an effect on a second function, as well as on the virus TK.
Fig. 7. Plaque morphologies, to scale, of BHK cells infected with HSV-1 and grown under EHu5 for 3 days at 37 °C. (a) HSV-1 (17) wt (syn+); (b) HSV-1 (17) TK− deletion mutant (ACG clone 4, SSyn plaque morphology); (c) one of the rare (<0.1%) LSyn plaques from the HSV-1 (17) wt population.

Table 2. Analysis of putative pTK4–HSV-1 recombinants*

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<th>Plaques picked plus BCdR</th>
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<td>17</td>
<td>2.1 × 10^{-2}</td>
</tr>
<tr>
<td>18</td>
<td>3.2 × 10^{-2}</td>
</tr>
<tr>
<td>19</td>
<td>3.7 × 10^{-2}</td>
</tr>
<tr>
<td>20</td>
<td>6.1 × 10^{-2}</td>
</tr>
</tbody>
</table>

* Plaque isolates were obtained following pTK4 co-transfection and subsequent selection with and without 100 μg/ml BCdR. Plaques were picked, purified twice, plate lysates grown and subsequently titrated with and without BCdR. Results are expressed as the ratio of the virus titres obtained in EHu5 with and without BCdR.

† Titres of clones 1 to 20 minus BCdR were 10⁶ to 10⁷ p.f.u./ml.

‡ Titres of clones 21 to 37 minus BCdR were 10⁷ to 10⁸ p.f.u./ml.

§ Syncytial morphology.
Table 3. Analysis of putative pTK4–HSV-1 recombinants

<table>
<thead>
<tr>
<th>ACG-selected clone</th>
<th>Titre in EHu5 + BCDR</th>
<th>Efficiency of plating</th>
<th>Infected cell morphology</th>
<th>Deletion*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2  4.4 x 10^8</td>
<td>3.5 x 10^7</td>
<td>7.9 x 10^-2</td>
<td>Syn+/SSyn</td>
</tr>
<tr>
<td></td>
<td>3  2.1 x 10^8</td>
<td>1.5 x 10^6</td>
<td>7.1 x 10^-4</td>
<td>Syn+</td>
</tr>
<tr>
<td></td>
<td>4  9.2 x 10^8</td>
<td>6.0 x 10^5</td>
<td>6.2 x 10^-2</td>
<td>SSyn+</td>
</tr>
<tr>
<td></td>
<td>6  1.05 x 10^7</td>
<td>9.5 x 10^5</td>
<td>8.9 x 10^-2</td>
<td>SSyn+</td>
</tr>
<tr>
<td></td>
<td>8  2.3 x 10^6</td>
<td>9.6 x 10^7</td>
<td>4.1 x 10^-1</td>
<td>LSyn</td>
</tr>
<tr>
<td></td>
<td>9  7.8 x 10^7</td>
<td>4.0 x 10^6</td>
<td>5.0 x 10^-2</td>
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<tr>
<td></td>
<td>10 1.19 x 10^6</td>
<td>4.85 x 10^6</td>
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<td>LSyn ND</td>
</tr>
<tr>
<td></td>
<td>11 3.1 x 10^4</td>
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<td>3.2 x 10^-1</td>
<td>SSyn+</td>
</tr>
<tr>
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<td>6.9 x 10^-2</td>
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<td>4.0 x 10^-1</td>
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<tr>
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<td>4.5 x 10^-5</td>
<td>Syn+ ND</td>
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<tr>
<td></td>
<td>16 3.6 x 10^4</td>
<td>2.5 x 10^4</td>
<td>6.9 x 10^-3</td>
<td>SSyn+</td>
</tr>
<tr>
<td></td>
<td>19 3.9 x 10^5</td>
<td>1.4 x 10^6</td>
<td>3.5 x 10^-1</td>
<td>SSyn+</td>
</tr>
<tr>
<td>HSV-1 (17) wt</td>
<td>4.35 x 10^9</td>
<td>9.0 x 10^6</td>
<td>2.0 x 10^-3</td>
<td>Syn+ ND</td>
</tr>
<tr>
<td>HSV-1 (17) dPyK-7</td>
<td>2.4 x 10^7</td>
<td>4.5 x 10^6</td>
<td>1.8 x 10^-1</td>
<td>Syn+ ND</td>
</tr>
</tbody>
</table>

* Symbols indicate: -, clone has wt BamHI p; +, clone deleted in BamHI p; ND, not done.

Analysis of infected cell polypeptides

Fig. 11 shows the polypeptide profiles of mock-infected, wt-, TK 1301- and TK 1302-infected cells labelled with [35S]methionine from 2 to 24 h post-infection. Lanes 3 and 4 show the absence of the putative TK polypeptide (Vmw 43), after infection with the mutants TK 1301 and TK 1302. A second alteration can be seen on longer exposure of the lower portions of the gel, where a polypeptide of approximate mol. wt. 19000 appears to be absent in the mutant infections.

Thymidine kinase assays

The thymidine kinase activity of the mutants TK 1301, TK 1302 and of wt HSV-1 (17) virus was assayed on TK-, PyY/CAR/BUDR monolayers. Fig. 12 shows the result of an assay performed on cells harvested 18 h post-infection (Wilkie et al., 1979b). Both deletion mutants completely lacked TK activity and reduced the TK activity of the infected cells below that of mock-infected controls. This effect on the host has been reported previously by Dubbs & Kit (1964) and Leiden et al. (1976).

Superinfection of LMTK- cells biochemically transformed with the HSV-1 TK gene

It has been reported that infection of TK- cells which have been biochemically transformed by HSV-1 TK to produce a TK+ cell phenotype can, on superinfection by phenotypically TK- virus, produce increased levels of HSV-1-specific TK (Lin & Munyon, 1974; Leiden et al., 1976; Kit et al., 1978a; Wilkie et al., 1979b). This would suggest that the transforming HSV-1 TK DNA still has control sequences which can respond in trans to a product of another HSV-1 gene. TK- deletion mutants provide an improved molecule with which to superinfect such cells.

Superinfection of P9 cells (Wilkie et al., 1979b) was therefore performed with wt HSV-1, TK 1301, TK 1302 and two other TK- mutants, HSV-1 (MDK) and dPyk-7. The mutant MDK produces TK mRNA (Wilkie et al., 1980) but not a TK polypeptide (Kit et al., 1978b) and can switch on the transforming HSV TK in transformed LMTK- cells (Lin & Munyon, 1974). HSV-1 dPyk-7 produces no TK polypeptide (Preston, 1977). A control in which LMTK- cells were also infected confirmed the previous absence of TK activity in TK- cells infected by the TK- mutants (data not shown).

Fig. 13 shows the TK activity assayed in P9 cells harvested at 18 h post-infection was markedly increased following infection with TK 1301 and TK 1302. The TK activity following wt infection was also increased, whereas no increase was found following dPyk-7 or MDK superinfection. In two subsequent experiments in which cells were harvested for TK assays 6,
Fig. 8. Restriction enzyme digests of $[^{32}P]$orthophosphate-labelled virion DNA. The lanes are coded 'P' for parental (wt) HSV-1 DNA, '1' for TK 1301 DNA, and '2' for TK 1302 DNA. Positions of parental and recombinant fragments of interest are marked by the squares and circles respectively.
Fig. 9. Restriction maps for HSV-1. (a) Partial restriction maps for the region between 0.2 and 0.45 map units showing the fragments affected by recombination between deleted plasmids pTK2, pTK3 and pTK4 and wt HSV-1 (17). The partial maps are based on those published by Wilkie (1976), Preston et al. (1978) and Wilkie et al. (1979a) and on the previously unpublished XhoI and PvuII maps shown in (b), upper and lower respectively. Fragments affected in all deletion mutants are indicated by thick black lines in the partial maps, those affected only in pTK2–HSV-1 recombinants by cross-hatching.

12, 18 and 24 h after infection, we observed a switch-on of the virus TK in P9 cells with MDK at 12 h post-infection but have not been able to demonstrate any increase in TK activity following superinfection with dPyk−7 (data not shown).

DISCUSSION

Production of deletions in pTK1 by digestion with SstI and BglII is an extremely efficient method of producing extended deletions but has an obvious drawback in that there is no control over the size of the deletion. The mechanism within the host bacterium which allows recombination in a recA− background to recircularize the linear pTK1-derived DNA is at present unknown. The plasmids pTK3 and pTK4 did, however, recombine within a four base pair "CACA" repeat, adjacent to G + C-rich areas, 668 bp apart and the sequences had 12 out of 14 bp in common (Fig. 4). Such non-tandem direct repeats have been found to be preferred sites for recombination in both prokaryotes and eukaryotes (Edlund & Normark, 1981; Kataoka et al., 1981; Weisberg & Adhya, 1977).

The deletions produced after treatment with nuclease BAL 31 are much smaller than those
Fig. 10. One-step growth curves of wt HSV-1 (17) (○), dPyk-7 (□), TK 1301 (■) and TK 1302 (●).

Fig. 11. [35S]methionine-labelled polypeptide profiles for mock-infected (lanes 1 and 5), wt HSV-1- (lane 2), TK 1301- (lane 3) and TK 1302- (lane 4) infected BHK cells from 2 to 24 h post-infection. The lower half of the figure shows a longer exposure of the same gel. The positions of Vmw 43 and the 19K polypeptide are shown.

using BglII:SstI digestion but it cannot be determined whether the deleted plasmid clones obtained were produced by ligation of blunt-ended molecules or by intramolecular recombination within the host. The clone BL9, which retains the EcoRI site 5' to the TK mRNA (this site is lost in all SstI:BglII-produced deletions), is at present being recombined into wt HSV-1 DNA. This EcoRI site may be within a sequence modulating transcription (Dierks et al., 1981). Digestion of HSV-1 BamHI p with EcoRI has been reported to abolish the TK-transforming activity of the fragment (Wigler et al., 1977) although in more recent studies a low level of transforming activity has been reported following EcoRI digestion (Colbere-Garapin et al., 1979). Deletion mutants retaining this site are therefore of obvious value in determining the role of sequences upstream of the TK mRNA-coding region.
**HSV-1 TK deletion mutants**

Fig. 12. Thymidine kinase assays performed on extracts of mock-infected (○), HSV-1 wt- (■), TK 1301- or TK 1302- (▲) infected PyY/CAR/BUdR cells at 18 h after infection. Cells were infected at an m.o.i. of 10. The results from TK 1301 and TK 1302 have not been distinguished from each other.

Fig. 13. Thymidine kinase assays performed at 18 h post-infection on P9 cells mock-infected (■) or superinfected with wt HSV-1 (○), TK 1301 (●), TK 1302 (□), MDK (△) and dPyK-7 (▲).

Selections for TK− virus with BCdR, BUdR, ACG and Ara-T have previously been reported (Brown & Jamieson, 1978; Honess & Watson, 1977; Kit & Dubbs, 1963; Post et al., 1981; Smiley, 1980; Wilkie et al., 1980). All of these compounds have proved suitable for reducing background levels of TK+ virus. In this study, although we have selected for TK− virus with three different analogues BCdR, BUdR and ACG, we consider that only BCdR provides a definitive test for TK− virus in TK+ cells. BUdR may have a mutagenic effect and ACG can select for DNA polymerase mutants (Field et al., 1980). ACG, however, was the drug which most efficiently reduced the titre of heterogeneous stocks from which TK− deletion mutants were selected. The efficiencies of plating of such stocks were $10^{-3}$, compared to $10^{-2}$ for stocks titrated plus or minus BUdR (25 μg/ml) or BCdR (100 μg/ml). Because selection with ACG was biased by the isolation of plaques with a more syncytial phenotype, we cannot make a direct comparison between the frequency of isolation of TK− deletion mutants with this drug and the use of BCdR or BUdR. The isolation frequency of TK− recombinants which do not have such a change in morphology cannot therefore be estimated, but on the basis of the BCdR and BUdR selections it would appear to vary considerably, between 25% and 4%. Obviously, an important but uncontrollable factor is the efficiency of recombination, which in basic genetic studies has been shown to vary widely between experiments performed on different days in different batches of cells (Brown et al., 1973).

A change in plaque morphology due to selecting for TK− mutants is surprising and has not previously been reported. Because TK 1301 and TK 1302 were grown in TK+ cells, and BUdR was used to reduce the levels of TK+ non-recombinant progeny, it is possible that there is a second mutation due to incorporation of this base analogue. However, it would be unlikely that both deletion mutants have the same second mutation producing a change in plaque morphology and deletion mutants subsequently selected using BCdR and ACG have a similar syncytial plaque morphology.

The molecular basis for syncytium formation has been suggested to involve two gene
products, a fusion factor and a regulator which in Syn+ infections acts to suppress syncytium formation (Keller, 1976; Manservigi et al., 1977). It has become obvious, however, that many loci spread over the L region of the genome can affect plaque morphology and the number of genes involved is at least six (Manservigi et al., 1977; Read et al., 1980; Ruyechan et al., 1979; Yamamoto & Kabuta, 1977). The host cell is also involved and may provide one or more factors determining whether the virus infection produces Syn or Syn+ plaques (Bzik & Person, 1981; Lee & Spear, 1980).

Of the loci previously implicated in plaque morphology, those nearest to the mutated BamHI p fragment of mutants TK 1301 and TK 1302 are glycoprotein gB, which maps 0·05 units to the right between 0·37 and 0·42 map units (Marsden et al., 1978; Morse et al., 1978), and a second locus at 0·31 to 0·33 map units (Spear & Roizman, 1980). It is not possible, however, to correlate the latter locus, which was described for HSV-1 HFEM, with the sequences within BamHI p (0·29 to 0·315 map units) of HSV-1 strain 17. We cannot as yet prove that deletion of material from within the BamHI p fragment is the cause of the change in plaque morphology. A number of other possibilities exist, such as a mutation at some sequence outside the TK-coding region or the incorporation of pAT153 DNA sequences adjacent to the BamHI p fragment in recombinants. We are at present producing TK+ recombinants using TK 1302 and pTK1 to investigate these possibilities. It is probable, however, that there is a gene, at least partly within HSV-1 BamHI p, which has an effect on plaque morphology.

Analysis of the polypeptides produced in wt-, TK 1301- and TK 1302-infected BHK cells (Fig. 11) shows the absence of the Vmw 43 and a second polypeptide of approximate mol. wt. 19000 in the two mutants. No shortened TK polypeptides have been identified in cells infected with TK 1301 and TK 1302 and none would be expected from an analysis of the published TK gene sequences (McKnight, 1980; Wagner et al., 1981; Preston & McGeoch, 1981). Most, if not all, of the DNA sequences believed to be important for RNA polymerase binding and the initiation of TK mRNA transcription and translation have been removed in both deletion mutants. The genome sequences coding for the polypeptide of mol. wt. 19000 are at present unknown, but an mRNA crossing the BglII site of HSV-1 BamHI p on the DNA strand opposite that coding for the Vmw 43 has been detected at late times after infection (Wilkie et al., 1980). It remains to be determined whether the 19000 mol. wt. polypeptide is a translation product of this mRNA and whether this polypeptide defect is connected with the change in plaque morphology of the deletion mutants.

The deletion mutants as expected produce no TK activity yet retain a gene function which can act in trans to switch on the transforming HSV-1 TK in LMTK−-transformed cells. It has been shown that an immediate–early (IE) polypeptide, the Vmw IE 175, has a vital role in activating the TK gene. A double mutant, deleted within the TK gene and temperature-sensitive in the IE gene function, would be useful for analysing IE gene function. The lack of switch-on by dPyk−7 in superinfection studies is surprising and raises the possibility that the lesion in this mutant is not at the TK locus, but in a separate controlling function. Alternatively, this lack of TK induction in superinfection experiments could be related to the m.o.i. used in this study. Leiden et al. (1976) reported a maximum induction on superinfection at an m.o.i. of 2 with no induction detectable at an m.o.i. of 10. It may well be that different mutants have different potentials for induction depending on their rates of growth and other virus–cell interactions and that the m.o.i. required for optimum induction may vary. It is also possible that the high level of HSV TK activity found in the uninfected P9 cells (data not shown) is masking any small degree of induction by the dPyk−7 infection; cells with a lower level of TK activity may be of value in this respect.

The one-step growth of ts mutants is often delayed at permissive temperature compared to wt virus, but follows a similar sigmoid pattern (Brown et al., 1973; Halliburton & Timbury, 1976; Mechic, 1974). The deletion mutants TK 1301 and TK 1302, however, have a flattened growth curve in conditions where the TK− mutant dPyk−7 grows as well as wt virus. This again suggests that a second gene function as well as the TK gene is affected in the TK− deletion mutants.

We wish to thank Mrs Cathie Adair for carrying out the initial cloning of pTK1. This and subsequent propagation of plasmids was carried out in 1979 to 1980 under Category II EK-2 containment conditions at this
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HSV-1 TK deletion mutants


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