Transfection with the Isolated Herpes Simplex Virus Thymidine Kinase Genes. II. Evidence for Amplification of Viral and Adjacent Cellular DNA Sequences

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SUMMARY

We have previously described several series of biochemical transformation experiments in which small defined portions of herpes simplex virus (HSV-1 and HSV-2) DNA encompassing the thymidine kinase (TK) gene were introduced into Ltk- cells by the calcium transfection procedure. The presence of authentic virus TK enzyme in several subcloned cell lines derived from these experiments was confirmed by either the specific incorporation of $^{[125]}$I iodo deoxycytidine into their nuclei or the inhibition of cell growth by the antiviral drug arabinosyl thymine. A panel of 24 independent Ltk+ cell lines receiving either isolated virus DNA fragments or cleaved plasmid DNAs was examined by blot hybridization for both the presence and copy number of virus TK DNA sequences. Most cell lines contained a single virus DNA fragment covalently joined to host (or carrier Ltk-) mouse DNA sequences, but several contained multiple copies of the TK gene. Examination of the structural arrangement of the virus DNA in two early passage multicopy cell lines indicated that the TK gene had integrated into Ltk- cell DNA and then subsequently both viral and flanking cellular sequences were amplified to create up to 20 tandem duplications. In one case, mapping of the adjacent cellular sequences has revealed that the total repeat unit is greater than 23 kilobases (kb) in size. On subsequent passaging, even in HAT medium, the amplified repeat units were not stable and fell to only three to four copies per haploid cell genome. These cell lines should prove useful for additional studies to examine the expression of co-selected non-TK virus sequences and the influence of adjacent cellular DNA sequences on transcription and retransfection of the resident TK gene.

INTRODUCTION

Munyon et al. (1971) first showed that infection with inactivated herpes simplex virus (HSV) can complement the defective thymidine kinase gene in mouse Ltk- cells in selective HAT medium. The virus deoxypyrimidine kinase enzyme (TK) responsible for this effect has been studied in some detail and possesses unique characteristics that differ from those of the cellular enzyme for which it can substitute (Dubbs & Kit, 1964; Aswell et al., 1972; Jamieson et al., 1974). Bacchetti & Graham (1977) and Minson et al. (1978) reported that transfection with isolated sheared HSV virion DNA, rather than u.v.-inactivated virus particles, could also be used to biochemically transform mouse or human tk- cells. A 3.5 kilobase (kb) BamHI fragment from HSV-1 carrying the TK gene was identified by biochemical transfection
procedures by Wigler et al. (1977) and subsequently cloned into pBR322 plasmids by Colbere-Garapin et al. (1979), Enquist et al. (1979), Wilkie et al. (1979) and McKnight & Gavis (1980). Approximately 1800 nucleotides of the coding and 3' and 5' flanking regions of the HSV-1 TK gene have been sequenced recently by McKnight (1980) and also by Wagner et al. (1981). The physical location of the TK gene in the HSV-2 genome has also been mapped by biochemical transfection and genetic procedures (Reyes et al., 1979; Halliburton et al., 1980; McDougall et al., 1980).

The first evidence that biochemically transformed Ltk+ cells stably retain portions of the input u.v.-irradiated HSV genome was obtained by liquid hybridization procedures (Kraiselburd et al., 1975; Davis & Kingsbury, 1976; Sugino et al., 1977). Subsequently, Pellicer et al. (1978) provided convincing evidence from blot hybridization experiments that a single copy of the TK gene was physically integrated into carrier or host DNA sequences in several HSV-1 DNA fragment-transformed cell lines. Others have demonstrated by C0t analysis and blot hybridization experiments that variable amounts of virus DNA sequences from close to 0.3 map units in the HSV genome are consistently retained in a number of the TK+ cell lines established by biochemical transformation of mouse tk− cells with u.v.-irradiated virus or sheared HSV DNA (Minson et al., 1979; Leiden et al., 1980; Bastow et al., 1980).

We have recently defined the limits of the minimally active DNA fragments capable of transferring TK activity to Ltk− cells. These sequences map between coordinates 0.303 and 0.3135 (1.6 kb) in the HSV-1(MP) genome and between 0.3035 and 0.315 (1.65 kb) in HSV-2(333) (Hayward et al., 1981; Reyes et al., 1982). In this report, we have examined and compared the copy number and organization of the resident virus DNA sequences in a number of subcloned cell lines derived from transfection with isolated fragments or cloned plasmid DNAs. Our goal in these studies has been to learn how to control and manipulate the copy number and size of adjacent sequences introduced into cells along with the virus TK gene by linked co-selection procedures. Initial attempts to introduce the morphological transforming BglII N fragment of HSV-2 (Reyes et al., 1979) into Ltk− cells and to establish panels of cell lines containing sequences spanning the entire HSV genome will be described elsewhere (G. Reyes, D. Ciufo & G. Hayward, unpublished results).

METHODS

Cell cultures. LMtk− cells and the thymidine kinase-converted LH-7 line [biochemically transformed with the 3.5 kb BamHI P fragment from HSV-1(F) DNA] were obtained originally from Dr S. Silverstein (Columbia College of Physicians and Surgeons, New York, U.S.A.). The thymidine kinase-negative Swiss/3T3 4(E) cells were received from Dr H. Green (Massachusetts Institute of Technology, U.S.A.). Cell cultures were routinely grown in either Dulbecco's or Eagle's minimal essential medium (EMEM) supplemented with 10% foetal calf serum. Media for Ltk− cells contained in addition 30 μg/ml bromodeoxyuridine. Some TK-transformed cell lines were grown continuously in HAT-G medium (EMEM containing 15 μg/ml hypoxanthine, 1 μg/ml aminopterin, 5 μg/ml thymidine and 15 μg/ml glycine) for up to 30 passages.

TK plasmids. The pHSV106 plasmid contains the 3.5 kb BamHI O fragment from HSV-1(MP) (McKnight & Gavis, 1980). Plasmids pGR1 and pGR3 contain the 5.6 kb SalI G fragment from HSV-2(333) inserted in opposite orientations into the SalI site in the tetracycline resistance gene of pBR322 (Reyes et al., 1982). Plasmid DNA was extracted by a modification of the cleared lysate method of Clewell & Helinski (1969) and purified by banding to equilibrium in CsCl–ethidium bromide density gradients.
**Ara-T and iododeoxycytidine assays.** Growth inhibition by arabinosyl thymine (Ara-T) at $2 \times 10^{-6}$ M (1 $\mu$g/ml) was used as a specific assay for the presence of virus TK enzyme in transfected cell lines (Aswell *et al.*, 1972). The Ara-T was obtained from Dr G. Gentry, Department of Microbiology, University of Mississippi, U.S.A. Cultures were seeded at $5 \times 10^3$ cells per 2.5 cm$^2$ well in plastic Linbro plates and grown at 37 °C in the presence or absence of Ara-T. At 24-h intervals triplicate cultures were trypsinized and counted in a haemocytometer. Alternatively, the presence of virus-specific TK was demonstrated autoradiographically by the selective incorporation of iododeoxycytidine into the cell nuclei. Monolayer cultures were grown on 8-well LabTek slides for 12 h in the presence of $10 \mu$Ci/ml $^{[125]}$Iiododeoxycytidine and 50 $\mu$g/ml tetrahydrouridine (Summers & Summers, 1977). The cells were washed, fixed in methanol, and exposed under a film of Kodak NTB-2 emulsion for 24 h before developing the autoradiographs.

**Blot hybridization.** Cellular DNA preparations from roller bottle cultures of the transfected LH1 or LH2 cell lines (see results) were extracted from isolated nuclei prepared by Dounce homogenization. The nuclei were lysed with 0.5 % Sarcosyl, 0.1 % SDS and then incubated for 4 h with 0.5 mg/ml Pronase. The DNA was extracted by multiple phenol and chloroform/isoamyl alcohol extractions followed by ethanol precipitation, incubation with ribonuclease at 20 $\mu$g/ml, a further phenol extraction and dialysis against 0-01 M-tris, 0-001 M-EDTA pH 8.4. Samples of cleaved cellular DNA (15 $\mu$g/slot) were electrophoresed in 1 or 1.4 % agarose slab gels, photographed after ethidium bromide staining, and then denatured and transferred to nitrocellulose filters as described by Southern (1975). The probes consisted of 0.5 to 1 $\mu$g samples of isolated virus DNA fragments or plasmid DNAs labelled *in vitro* with $^{[32P]}$TTP and $^{[32P]}$dCTP by nick translation (Rigby *et al.*, 1977) to specific activities of at least $5 \times 10^7$ ct/min/$\mu$g. The hybridization buffer consisted of 0.8 M-NaCl, 0.05 M-tris, 0.001 M-EDTA, 0.2 % SDS pH 7.4 plus 20 % formamide and 1 × Denhardt's mixture (0.02 % Ficoll, 0.02 % polyvinylpyrrolidone and 0.02 % bovine serum albumin). The filters were preincubated in the same buffer plus 50 $\mu$g/ml sheared and denatured calf thymus DNA. Hybridization was carried out for 48 to 72 h with $5 \times 10^7$ ct/min in 8 ml vol. in sealed plastic bags at 58 °C. The filters were washed exhaustively after hybridization in 2 × SSC, fresh hybridization buffer at 58 °C, 2 × SSC, and finally 0-01 × SSC at room temperature. The autoradiographs were usually exposed for several days with enhancing screens.

**RPC-5 chromatography.** Fractionation of 300 $\mu$g Ltk$^+$ cellular DNA was performed on a 50 × 1 cm column of RPC-5 essentially as described by Tilghman *et al.* (1977) with the modification of column equilibration with 1.1 M-sodium acetate, 50 mM-tris, 1 mM-EDTA pH 7.5 and elution with a 800 ml linear gradient of 1.3 to 1.5 M-sodium acetate, 50 mM-tris, 1 mM-EDTA pH 7.5. The flow rate was approx. 0.75 ml/min under a pressure of 170 to 200 lb/in$^2$. Individual 6 ml fractions were precipitated with 2 vol. ethanol and subjected to electrophoresis in 1.4 % agarose gel slabs.

**RESULTS**

**TK$^+$ cell lines**

The cell lines used in this work were obtained from experiments in which defined fragments of HSV-1 or HSV-2 DNA were transfected into Swiss/tk$^-$ or Ltk$^-$ cells by the calcium phosphate precipitation procedure (Reyes *et al.*, 1979; Reyes *et al.*, 1982). Selected positive colonies developing in HAT medium were each subcloned at least twice and used to establish a panel of TK$^+$ mouse cell lines which had received either isolated virus DNA fragments or cleaved TK plasmid DNA. The origins of 36 such lines likely to contain varying portions of additional virus DNA sequences directly adjacent to the TK genes are listed in Tables 1 and 2. Where multiple cell lines transformed by the same fragment are listed they
### Table 1. TK⁺ cell lines established by transfection with virus DNA fragments

<table>
<thead>
<tr>
<th>Designation and origin</th>
<th>Source, size (kb) and map location of input DNA fragment</th>
<th>Detection of integrated TK sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Number of sites</td>
</tr>
<tr>
<td>HSV-1 virus fragments</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LH-7(S)</td>
<td>Ltk⁻ (F)</td>
<td>BamHI P 3.5</td>
</tr>
<tr>
<td>LH1-X1</td>
<td>Ltk⁻ (MP)</td>
<td>Xbal D 23.0</td>
</tr>
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</tr>
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<td>Ltk⁻ (MP)</td>
<td>Xbal D 23.0</td>
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<td>Xhol E 8.2</td>
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<td>LH1-B1</td>
<td>Ltk⁻ (MP)</td>
<td>BamHI O 3.5</td>
</tr>
<tr>
<td>LH1-B2 p3</td>
<td>Ltk⁻ (MP)</td>
<td>BamHI O 3.5</td>
</tr>
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<td>BamHI O 3.5</td>
</tr>
<tr>
<td>LH1-B3</td>
<td>Ltk⁻ (MP)</td>
<td>BamHI O 3.5</td>
</tr>
<tr>
<td>HSV-2 virus fragments</td>
<td></td>
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<tr>
<td>LH2-H1</td>
<td>Ltk⁻ (333)</td>
<td>HindIII H 17.5</td>
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<td>Ltk⁻ (333)</td>
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<td>Ltk⁻ (333)</td>
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<td>Ltk⁻ (333)</td>
<td>BstII H 6.1</td>
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<td>Ltk⁻ (333)</td>
<td>BstII H 6.1</td>
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<tr>
<td>SH2-Bg1</td>
<td>S-4Etk⁻ (333)</td>
<td>BglII G 17.0</td>
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<tr>
<td>SH2-Bg2</td>
<td>S-4Etk⁻ (333)</td>
<td>BglII G 17.0</td>
</tr>
</tbody>
</table>

* Approximations based on a combination of the number of BamHI fragments hybridizing with the pHSV106 or pGR1 TK probes and the intensity of the hybridization relative to the LH-7 DNA.

† ND, Not done.

### Table 2. TK⁺ cell lines established by transfection with plasmid DNA

<table>
<thead>
<tr>
<th>Designation and origin</th>
<th>Source, size (kb) and map location of input DNA fragment</th>
<th>Detection of integrated TK DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Number of sites</td>
</tr>
<tr>
<td>HSV-1 plasmid DNA</td>
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<td></td>
</tr>
<tr>
<td>LH1pB1</td>
<td>Ltk⁻ pHSV106</td>
<td>BamHI 3.5</td>
</tr>
<tr>
<td>LH1pB2</td>
<td>Ltk⁻ pHSV106</td>
<td>BamHI 3.5</td>
</tr>
<tr>
<td>LH1pB/Bs1</td>
<td>Ltk⁻ pHSV106</td>
<td>B/BstEII 2.5</td>
</tr>
<tr>
<td>LH1pB/Bs2</td>
<td>Ltk⁻ pHSV106</td>
<td>B/BstEII 2.5</td>
</tr>
<tr>
<td>LH1pB/Pv1</td>
<td>Ltk⁻ pHSV106</td>
<td>B/PvuII 1.9</td>
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<tr>
<td>LH1pB/Pv2</td>
<td>Ltk⁻ pHSV106</td>
<td>B/PvuII 1.9</td>
</tr>
</tbody>
</table>

| HSV-2 plasmid DNA      |                                                          |                     |                  |                                     |
| LH2pS1                 | Ltk⁻ pGR1                                               | SalI 5.6            | 1                | 1-2                                    | 10.5 |
| LH2pS2                 | Ltk⁻ pGR1                                               | SalI 5.6            | ND†              | ND†                                     |
| LH2pH1                 | Ltk⁻ pGR1                                               | HindIII 8.5*        | ND†              | ND†                                     |
| LH2pH2                 | Ltk⁻ pGR1                                               | HindIII 8.5*        | ND†              | ND†                                     |
| LH2pH/Bg1              | Ltk⁻ pGR1                                               | H/BglII 4.0         | 1                | 3-4                                    | 4.6  |
| LH2pH/Bg2              | Ltk⁻ pGR1                                               | H/BglII 4.0         | 2                | 2                                     | 5.5 10 |
| LH2pH/Hc1              | Ltk⁻ pGR1                                               | H/HincII 3.9        | 1                | 1                                     | >20  |
| LH2pH/Hc2              | Ltk⁻ pGR1                                               | H/HincII 3.9        | ND†              | ND†                                     |
| LH2pH/Xh1              | Ltk⁻ pGR1                                               | H/Xhol 7.9*         | 7                | 15                                     | 4.1-17 |
| LH2pH/Xh2              | Ltk⁻ pGR1                                               | H/Xhol 7.9*         | 2                | 4-5                                    | 12-20 |
| LH2pH/Bs1              | Ltk⁻ pGR1                                               | H/BstEII 6.7*       | ND†              | ND†                                     |

* Includes 3.7 kb of pBR322 sequences from SalI rightwards to HindIII.

† ND, Not done.
Evidence for the virus origin of the thymidine kinase enzyme in transfected cell lines

Although growth in HAT medium ensures that the cell lines we generated must have regained thymidine kinase activity, it was necessary to show that the enzyme produced was indeed of virus origin. We did this in two ways not previously applied to cell lines of this kind: first, by demonstrating the extreme sensitivity of the cells to the antiviral pyrimidine analogue Ara-T (Aswell et al., 1972), and second, by observing the selective incorporation of $[^{125}]$iododeoxycytidine into the nuclei of cells expressing the HSV-specific deoxypyrimidine kinase (Summers & Summers, 1977). Fig. 1 illustrates growth inhibition and cell killing effects of 1 µg/ml Ara-T for the LH1-B2, LH1-X2 and LH2-H2 cell lines in comparison to the negligible effects on Vero and Ltk$^-$ cells. Because Ltk$^+$ cells tend to revert to Ltk$^-$ quite rapidly in non-selective media (Davidson et al., 1973) we maintained all of the transfected cell lines in HAT selective medium throughout this study. Autoradiographic assays for grains over the nuclei of cells grown for 24 h in $[^{125}]$iododeoxycytidine (Fig. 2) confirmed that the LH1-B2, LH1-X2, and LH2-B1 cell lines all stably retained the ability to express virus TK for at least 30 passages (Fig. 2d, f). Neither Ltk$^-$ nor Vero cells gave positive nuclei with $[^{125}]$iododeoxycytidine under these conditions (Fig. 2b, e).

Integrated virus DNA sequences in DNA fragment-transformed cell lines

Twenty-four of our Ltk$^+$ cell lines have been examined for the presence of virus TK DNA sequences by blot hybridization to BamHI-digested cell DNA using in vitro $^{32}$P-labelled BamHI O and SalI G DNA probes prepared either from isolated virus DNA fragments or from cloned plasmid DNAs. Pellicer et al. (1978) have previously shown that the 3-5 kb BamHI fragment from HSV-1 DNA is integrated at one copy/cell in the LH-7 cell line and we used this DNA as a consistent reference for comparing the copy numbers in our sets of
cell DNAs. Most of the LH1 and LH2 cell DNAs from either virus or plasmid sources contained only one or two bands of hybridizing virus sequences whereas the Ltk− parent cells had none (see Tables 1 and 2). The sizes of the hybridizing bands varied considerably among different cell lines including subclones which had received identical input fragments. With only a few exceptions (see below) the intensity of hybridization of each band observed in HSV-1 TK+ cell lines was approximately equal to that obtained with LH-7 DNA. Because of the relatively low cross-homology between the HSV-1 and HSV-2 TK probes (approx. 5 to 10% in these experiments) we were unable to detect HSV-2 TK DNA sequences directly with the heterologous BamHI O fragment probe from HSV-1 DNA. However, when 300 μg EcoRI-cleaved LH2-B1 cell DNA were prefractionated by RPC-5 column chromatography and each sample eluting at a different salt concentration was further resolved by agarose gel electrophoresis, a single band of 6 kb was revealed using the cloned heterologous HSV-1 TK DNA probe (Fig. 3). This hybrid band eluted near the centre of the genomic RPC-5 DNA pattern, providing further evidence for integration of the input HSV-2 Bg/II G fragment at a
Fig. 3. RPC-5 chromatography of LH2-Bg1 DNA. Demonstration of single integration site for an HSV-2 TK DNA fragment and detection with a heterologous probe. The figure shows both the ethidium bromide-stained gels of EcoRI-cleaved cellular DNA (upper panel) and the corresponding autoradiographs of Southern blots after hybridization with a nick-translated probe of 32P-labelled pHHSV106 DNA (lower panel). Only the central group of fractions from the RPC-5 column is shown. The LH2-Bg1 cell line was derived from transfection of Ltk- cells with the Bg/II G fragment from HSV-2(333) DNA (map coordinates 0.203 to 0.305). The input fragment contains a single EcoRI site at 0.313 near the 5' end of the TK gene (0.3045 to 0.313). The pHHSV106 probe represents the BamHI O fragment from HSV-1(MP) DNA (map coordinates 0.292 to 0.316).

Amplification of virus DNA fragments

Three TK+ lines that received the 29 kb XbaI F fragment and three that received the 3.5 kb BamHI O fragment from HSV-1(MP) DNA were selected for study in greater detail. Fig. 4 shows blot hybridization with a pHHSV106-cloned DNA probe to each of these cell DNAs after cleavage with BamHI or EcoRI. LH1-X3, LH1-B1 and LH1-B3 all gave a single band in the BamHI digests which had an intensity equivalent to that obtained with LH-7 DNA (i.e. single copy). LH1-X1 gave two such bands. Similarly, LH1-B2 and LH1-X2 gave one and two hybridizing bands respectively in the BamHI digests, but each band exhibited up to 20-fold greater intensity than that obtained with equivalent amounts of DNA from the single copy lines. This result has been observed consistently with several DNA preparations from both cell lines, and we conclude that they contain multiple copies of the TK DNA sequences.

The LH1-B1, LH1-B2 and LH1-B3 cell DNAs (all transfected with the 3.5 kb BamHI O fragment) show unambiguous evidence for integration because they have each lost their terminal BamHI sites and the probe now hybridizes to single BamHI species of 17 kb, 6.5 kb and 7.5 kb respectively, which must therefore contain flanking cellular DNA in addition to
Fig. 4. Identification of integrated TK DNA in HSV-1 DNA fragment-transformed mouse cell lines.
The figure shows a gel blot hybridization experiment with a cloned HSV-1 TK probe (pHSV106) and both BamHI and EcoRI digests of total DNA from six subcloned Ltk+ lines that received either the isolated XbaI F or BamHI O fragments from HSV-1(MP) DNA. The cleaved cellular DNA was fractionated by electrophoresis through a 1.4% agarose slab gel, transferred to a nitrocellulose sheet and hybridized with nick-translated 32P-labelled pHSV106 DNA. The resulting autoradiograph is shown in the left-hand panel and an interpretative drawing is given in the right-hand panel. Samples of 15 μg of cleaved cell DNA were loaded into each gel slot as follows: 1, LH-7; 2, LH1-X1; 3, LH1-X2 (passage 3); 4, LH1-X3; 5, LH1-B1; 6, LH1-B2 (passage 3); 7, LH1-B3; 8, Ltk-. Channel 9 received HindIII-cleaved phage lambda DNA for size references (given in kilobase pairs). The positions of the unintegrated linear 3.5 kb BamHI O fragment and of the 2.4 kb internal EcoRI N fragment are indicated. Comparison of the relative band intensity with that obtained using single copy LH-7 DNA (arrowed) reveals the approximate copy number in the other cell lines.

the virus sequences. Although integration of the original 3.5 kb BamHI DNA fragment eliminates the BamHI sites, the EcoRI sites at 0.296 and 0.3125 and internal 2.4 kb EcoRI N fragment remained intact in all three cell lines (Fig. 4), suggesting that no major rearrangements of the virus sequences had occurred. Since the multiple copies of TK DNA in LH1-B2 and LH1-X2 gave rise to only one and two distinct species of BamHI fragments respectively (each larger than the 3.5 kb probe DNA) we conclude that either all of these copies independently integrated into identical cellular (or carrier) sequences or more likely that a single integrated TK fragment was somehow amplified to create a cluster of adjacent tandemly repeated copies.

The LH1-X2 line contains two separate families of multicopy tandem repeats: one retains both BamHI sites and the other appears to have lost the BamHI site at 0.292 during the integration event. LH1-X1 DNA exhibits a very similar pattern to that of LH1-X2 but both integrated fragments are single copy. The LH1-X series cells were also tested for the presence of the adjacent EcoRI F DNA sequences (not shown), which represent a 16 kb portion of the input XbaI F fragment mapping from 0.313 to 0.417. This analysis is complicated because certain virus sequences within this region (even after cloning) cross-hybridize specifically to a single BamHI band of 20 kb and a slightly larger EcoRI band in Ltk− cells (Reyes et al., 1979). However, the LH1-X1 and LH1-X2 cell DNAs, but not the LH1-B series, gave several additional bands which hybridized with the EcoRI F probe. The most intense EcoRI species in LH1-X2 is only 7 kb in size, implying that only a portion of the adjacent linked EcoRI F sequence is present at the most prominent TK integration site (see Fig. 6). An experiment in which cleaved cellular DNA from the LH1-B2 and LH1-X2 lines after 21 passages was compared directly with DNA from the same subclones at third passage (Fig. 5, lanes 5 to 8) revealed that the amount of amplified TK DNA within each tandem repeat
Integrated HSV TK DNA

Fig. 5. Variations in copy number of the TK DNA at different passages of the LH1-B2 and LH1-X2 cell lines as detected by blot hybridization. The experiment was similar to that described in Fig. 4 except that cell DNAs from different passages were used. The left-hand panel contains a u.v.-light photograph of the ethidium bromide-stained cellular DNA pattern after electrophoresis of BamHI-cleaved cellular DNA through a 1.4% agarose gel. Sample channel 1, 15 μg Ltk− cell DNA; 2 and 3, control reconstructions with 15 μg calf thymus DNA plus the pHSV107 plasmid in amounts equivalent to 30 and 3 copies/diploid cell respectively (i.e. 1 ng and 0.1 ng); 4, single copy LH-7 DNA; 5, LH1-X2 DNA (passage 3); 6, LH1-X2 DNA (passage 21); 7, LH1-B2 DNA (passage 3); 8, LH1-B2 DNA (passage 21).

cluster was reduced considerably after several months of continuous culture. Reconstruction controls included in the experiment suggest that in both cases the copy number dropped fivefold, from approx. 20 down to 4 copies/cell.

Our interpretations of the approximate number of integration sites, total TK DNA copy number and size of the major BamHI fragment(s) containing the virus TK sequences are listed in Table 1 for fragment-transformed cell lines and in Table 2 for plasmid-transformed cell lines. These results indicate that: (i) in probably every case most of the virus DNA is integrated into the cellular or carrier Ltk− genome; (ii) the sites of integration differ in each independent cell line but do not alter during passaging; (iii) single copy integration is the most common outcome, with multiple integration events or tandem duplications being relatively rare. Fig. 6 summarizes our current understanding of the gross structure of the integrated virus DNA sequences for the six LH1-X and LH1-B cell lines.

Size of the tandem repeat unit in LH1-B2 DNA

We believe that the amplified repeat unit in the LH1-B2 cell genome consists of a single segment of at least 3 kb from the original input 3.5 kb BamHI fragment of virus TK DNA plus at least 20 kb of Ltk− cell DNA. Some of the experimental evidence for this interpretation is presented in Fig. 7 and is summarized as a physical map of the integrated TK DNA plus associated cellular sequences in Fig. 8. The internal EcoRI, KpnI, BglII and BstEII sites retain their original positions relative to one another in the integrated DNA but both virus BamHI sites are missing. At third passage all 20 copies of the TK sequence in every cell genome lie within reiterated fragments of 6.5 kb after cleavage with BamHI or of 7.4 kb after cleavage with HindIII. Enzymes that cleave once within the original 3.5 kb BamHI fragment generate two multicycopy species of 6.7 kb and 7.8 kb in the case of KpnI
Fig. 6. Organization of integrated virus DNA sequences in six Ltk+ cell lines derived by transfection
with the isolated XbaI F or BamHI O fragments from HSV-1(MP) DNA. Open bars indicate the input
fragments, with hatched bars representing the TK genes. Solid bars depict integrated virus sequences
and the wavy lines show Ltk− cellular or carrier DNA sequences. Broken lines denote uncertainties in
map boundaries. LH1-X1 and LH1-X2 contain two copies of the TK DNA either at two separate
locations or adjacent to one another within a single integration site. The LH1-X2 and LH1-B2 cell lines
appear to contain multiple tandem duplications of the integrated TK genes and flanking mouse DNA
sequences.

(not shown) and 5.8 kb and 17 kb in the case of BstEII. This indicates that cellular DNA
sequences well beyond the BamHI and HindIII sites are also reiterated. The most logical
interpretation of these results is that a cellular DNA segment containing the virus TK gene
has been amplified in the form of a tandem repeat unit of at least 23 kb in size (and possibly
much larger). The structure of this repeat unit is apparently identical at both the third and
21st passages despite the at least fivefold reduction in average copy number/cell at the later
passage level.

DISCUSSION

There have been several other studies relating to the size and state of virus TK DNA
sequences retained in DNA fragment-transfected cell lines. Pellicer et al. (1978) demonstrated
for the first time unambiguous evidence that most of the virus DNA sequences were
integrated at one copy/cell in LH-7 and similar lines derived from transfection with the
isolated BamHI P fragment from HSV-1(F). Minson et al. (1979) showed by Cot analyses
that lines transfected with sheared total HSV-2 DNA retained sequences from 0.2 to 0.4 map
units including adjacent non-TK virus sequences. The same group subsequently documented
losses of some or all of these sequences in phenotypically reverted cell lines (Bastow et al.,
1980). More recently, Kit et al. (1980) have also detected virus DNA sequences in lines
transfected with HSV-1 TK plasmids.

The panels of cell lines established here represent the first set of biochemically transformed
cells derived by transfection with well-defined HSV DNA fragments of various sizes. Our blot
Fig. 7. Mapping of the integrated TK sequences in cell line LH1-B2. The figure shows autoradiographs (left-hand panels) and interpretative diagrams (right-hand panels) from blot hybridization experiments using nick-translated $^{32}$P-labelled pHSV106 DNA as a probe after electrophoresis of cleaved cellular DNA samples through 1.4% agarose gels. (a) Channels 1 to 4, LH1-B2 DNA (passage 3); 5 to 12, LH1-B2 DNA (passage 21); 13, Ltk-; 14, LH-7; 15 to 17, reconstructions (R) of 15 μg calf thymus DNA plus 1.5 ng pHSV106 DNA (40 copies/cell). The DNA was cleaved with the following enzymes: 1 and 5, BamHI; 2 and 6, BstEII; 3 and 7, SalI; 4 and 8, EcoRI; 9, HindIII; 10, HindIII/BamHI; 11, HindIII/BstEII; 12, HindIII/BglII; 13 and 14, BamHI; 15, BstEII (the lower band represents uncleaved supercoiled plasmid DNA); 16, BamHI; 17, EcoRI. (b) Channels 1 to 11, LH1-B2 DNA (passage 21); 12, Ltk-; 13, LH-7; 14 to 17, reconstructions (R) of 15 μg calf thymus DNA plus 1.5 ng pHSV106 DNA (40 copies/cell). The DNA was cleaved with the following enzymes: 1, BamHI; 2, BamHI/BglII; 3, BglII; 4, BamHI/EcoRI; 5, EcoRI; 6, BamHI/BstEII; 7, BstEII; 8, EcoRI/BstEII; 9, BglII/BstEII; 10, HindIII; 11, XhoI; 12 to 14, BamHI; 15, BamHI/BglII; 16, BamHI/BstEII; 17, EcoRI. Arrows denote the 15 kb single copy reference band in LH-7 DNA. The HindIII fragments from phage lambda DNA were used as size markers (kb).

hybridization studies provide evidence for integration of HSV-1 and HSV-2 TK DNA sequences in numerous cell lines derived from transfection with both isolated virus DNA fragments and cloned TK plasmid DNAs. In general, only one or two copies are retained and they appear to have been integrated at different sites in each case. Of 24 cell lines examined,
Fig. 8. Physical map of the integrated HSV-1 TK gene and adjacent cellular DNA sequences in the multicopy cell line LH1-B2. Solid horizontal lines indicate the sizes (in kilobase pairs) and relative map positions of multicopy cellular DNA fragments which hybridize to the pHSV106 probe. Broken lines indicate either tentative assignments or bands that were expected but apparently hybridized below the levels of detection in experiments such as those shown in Fig. 7. The sizes of two 3' flanking fragments with slightly anomalous mobilities are given in parentheses. Restriction enzyme cleavage sites are abbreviated as follows: BamHI (B), BglII (Bg), BstEII (Bs), EcoRI (E), HindIII (H) and KpnI (K). Note that the total size of the tandem repeat unit given here is a minimum value only.

22 revealed only one or at most two distinct virus integration sites. However, several lines were exceptional in having multiple copies of the TK DNA sequences (up to 15 or 20 per cell genome). In two cases (both receiving plasmid DNA) the multiple copies were displayed in as many as four to seven different hybridizing bands. Unfortunately, since it is apparent that losses of input sequences accompany integration and stabilization events, the usefulness of these cell lines for studying gene expression from viral or cellular sequences adjacent to the TK gene is somewhat limited until detailed maps of integrated sequences are established in each case.

Examination of two unusual multicopy cell lines that had received isolated HSV-1 virus DNA fragments, provided evidence that sometimes the input virus DNA sequence first integrates within a segment of cellular or carrier DNA which is subsequently amplified to create a multiple tandemly duplicated structure with a repeating unit greater than 23 kb in size. Amplification of this kind has been detected previously in several other systems, for example, as a response to selective conditions for resistance to methotrexate (Alt et al., 1978), and after chromosome-mediated cell TK gene transfer (Scangos et al., 1979). Perhaps similar
selective advantages for cells expressing relatively large amounts of virus thymidine kinase may have occurred transiently in our experiments. The highest copy numbers were observed at third passage in the LH1-B2 and LH1-X2 cell lines, but after subsequent passaging the copy number decreased from approx. 20 to 4 in both cases (although the structure of the repeat units themselves was unchanged). This result suggests that the conditions favouring amplification occurred very early after transfection, that regular HAT medium does not normally provide selective conditions favouring amplified copy numbers, and that the amplified copies are probably unstable or disadvantageous to the cell. We cannot rigorously exclude the possibility that these multiple copies represent episomal forms rather than tandem repeats integrated into cellular chromosomes, but note that each repeat unit must still be at least 23 kb in size. Furthermore, the virus sequences migrated with high molecular weight cell DNA (30 to 200 kb in size) after cleavage with enzymes such as SalI and XhoI that have no sites within the input 3.5 kb BamHI fragment, whereas relatively small episomal structures would have migrated much faster.

Studies on the biochemical transformation of Ltk- cells by the HSV TK gene raise several important questions about the nature of the control of TK gene expression and the sites of integration. The TK gene is a member of the second or 'delayed-early' class of virus genes and as such is not normally transcribed from the input infecting genomes until after the 'immediate-early' class of proteins is synthesized. In particular, Preston (1979) and Watson & Clements (1980) have used a temperature-sensitive mutant (tsK) to show that expression of delayed-early and late virus genes, including TK, are dependent on continued expression of the nuclear 175K protein product of immediate-early gene IE IV. How then is the TK gene expressed in transformed cells in the absence of the normal virus transcriptional regulatory factors? Can all HSV early genes bypass these cascade regulatory controls or is the TK gene a special case designed to operate under a separate regulatory control pathway during the establishment and maintenance of (or reactivation from) the latent state in neurons? It should be noted that, firstly, the isolated vaccinia virus TK gene cannot substitute for the Ltk- cellular gene in HAT selection assays, and secondly, the HSV TK gene, whilst dispensible in cell culture systems, is essential for efficient establishment of latent infections in animals (Field & Darby, 1981). These considerations draw attention to the nature of the cellular or carrier DNA sequences surrounding the integrated TK gene and especially the observations by Minson et al. (1978) and Wigler et al. (1978) that retransfection of Ltk- cells in HAT medium with genomic DNA from the biochemically transformed cell lines occurs at an efficiency several orders of magnitude higher than that with the original isolated or cloned virus DNA fragments (i.e. 4 × 10^4 colonies/μg equivalent of TK gene sequences). It appears clear from the ability to induce or enhance TK enzyme levels up to 10-fold after superinfection with tk- HSV-1 virus (Leiden et al., 1976; Kit & Dubbs, 1977; Wilkie et al., 1979) that the TK gene can operate under the control of its own virus promoter in transformed cells. However, it seems quite plausible that cellular factors may substitute for the normal virus regulatory proteins and that the integrated TK genes require some adjacent cellular cis-acting sequences to function effectively.

The cell lines described in this paper, and especially the mapping of adjacent cellular sequences in the LH1-B2 cell line, provide an initial framework for further research to study these questions in some detail. We now know, for example, that the predominant virus mRNA from the l strand in our LH1-B1 cell line possesses exactly the same 5' terminus (50 bp upstream from the BglII site) as the TK-coding mRNA from HSV-1-infected Vero cells (Hayward et al., 1981; McKnight et al., 1981), supporting the notion that the normal virus TK promoter is operating in the transformed cells. As many as four additional 'promoter-like' regions exist within the 1800 bp portion of pHSV106 DNA that has been sequenced (McKnight, 1980; Hayward et al., 1981). We do not as yet know whether these potential
promoters operate in infected or transformed cells nor to what class of virus genes they might belong; however, an examination of their functional state in transformed cell lines will become a matter of considerable importance if the cloned TK genes are to be used extensively as vehicles for linked co-selection of foreign genes into eukaryotic cells.

One of the most important aspects of the thymidine kinase transfection system is the opportunity it provides to incorporate other genes (non-selectable) into the cell by either linked or unlinked co-selection (Wigler et al., 1979; Mantei et al., 1979; Lai et al., 1980). The possibility of eventually being able to control the TK gene amplification process described here in a linked co-selection system seems to offer additional attractions. Consequently, with this aim in mind, we have constructed a set of TK co-selection plasmids derived from pHSV106 and pGR1 which provide considerable versatility in available cleavage sites for inserting foreign sequences. The structure of these plasmid vehicles and their use in establishing panels of cell lines receiving most of the BglII fragments from the HSV-1 and HSV-2 genomes by linked co-selection will be described elsewhere (G. Reyes, D. Ciufo & G. Hayward, unpublished results).

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