Virus-specific DNA Sequences Present in Cells which Carry the Herpes Simplex Virus Thymidine Kinase Gene

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SUMMARY

Two independently derived cell lines which carry the herpes simplex type 2 thymidine kinase gene have been examined for the presence of HSV-2-specific DNA sequences. Both cell lines contained 1 to 3 copies per cell of a sequence lying within map co-ordinates 0·2 to 0·4 of the HSV-2 genome. Revertant cells, which contained no detectable thymidine kinase, did not contain this DNA sequence. The failure of EcoRI-restricted HSV-2 DNA to act as a donor of the thymidine kinase gene in transformation experiments suggests that the gene lies close to the EcoRI restriction site within this sequence at a map position of approx. 0·3. The HSV-2 kinase gene is therefore approximately co-linear with the HSV-1 gene.

INTRODUCTION

The thymidine kinase gene of herpes simplex virus can be introduced into the genotype of cells lacking this enzyme by infecting cells either with virus inactivated by u.v. light (Munyon et al. 1971) or with virus DNA sequences which have been sheared or restricted to destroy their infectivity (Bacchetti & Graham, 1977; Maitland & McDougall, 1977; Wigler et al. 1977). These 'transformed' cells contain the virus-specific enzyme and virus-specific DNA sequences (Kraizelburd et al. 1975; Davis & Kingsbury, 1976). Selection for the thymidine kinase gene can result in the acquisition of additional non-selected virus-specific functions which have been identified by the ability of the transformed cells to compensate the defect in virus mutants (Minson et al. 1978, 1979). Selection in 5-bromodeoxyuridine (BrdUrd) results in reversion to a thymidine kinase-negative phenotype and the simultaneous loss of non-selected virus-specific functions. By analogy with revertants of SV40-transformed cells (Steinberg et al. 1978) a frequent mechanism for reversion of HSV-tk+ cells to a tk− phenotype would be deletion of virus-specific sequences from the cell genome. However, it is clear that, at least in some instances, reversion to a kinase-negative phenotype is due to failure to express a resident tk gene, since clones of tk− revertants have been shown to regain a kinase-positive phenotype (Davidson et al. 1973; Kaufman & Davidson, 1975), and a quiescent kinase gene in revertant cells has been activated by infection with a tk− herpes simplex virus mutant (Kit & Dubbs, 1977).

It is apparent that cells carrying the HSV thymidine kinase gene provide a useful model for the study of the inheritance and control of virus genes acquired by mammalian cells. In this paper we describe the identification of the virus-specific DNA sequences carried by two cell lines which have been 'transformed' to a tk+ phenotype with HSV-2 DNA and the examination of three tk− revertant cell lines for the presence of these sequences. Using the data we have asked the following questions: (i) can the transformed cells acquire non-contiguous virus DNA sequences? (ii) Does reversion to a tk− phenotype result from deletion
Fig. 1. Separation of probe sequences. EcoRI-digested HSV-2 DNA was electrophoresed in 0.5% agarose gels and the smallest fragment was retarded in a 2% gel. The order of these fragments in the HSV-2 genome is given as determined by Cortini & Wilkie (1978), and is shown in the 'prototype' arrangement. All map positions given in the text also refer to the prototype isomer. Comparison of the EcoRI digestion products of the DNA of the HSV-2 strain used in this work, and those of the strain used by Cortini & Wilkie (strain HG52) showed that they were indistinguishable by electrophoresis in 0.5% gels.

of virus specific sequences? (iii) Is the DNA content of the transformed lines consistent with the map position of the HSV-2 thymidine kinase gene determined by Maitland & McDougall (1977).

METHODS

Cells and virus. Cell lines D21 and D25 are thymidine kinase-positive cell lines derived by treatment of LMTK- cells with sheared HSV-2 DNA. D21R1, D21R2 and D21R3 are tk- cell lines derived from D21 cells by selection in BrdUrd. The derivation, culture and properties of these cells have been described previously (Minson et al., 1978, 1979). All experiments described here were performed with cells of passage number 10 or lower. Kinase-positive cells were cultured in selective medium and tk- revertants in non-selective medium. HSV-2 strain 25766 was used throughout.

Preparation of hybridization probes. A 200 µg amount of HSV-2 DNA was restricted with EcoRI restriction endonuclease and the fragments separated by electrophoresis in 0.5% agarose tube gels. The smallest fragment was retarded by a 2% gel at the bottom of the tube. All molar and half-molar fragments (Fig. 1) were sliced from the gels. The half-molar fragment H cannot be separated from the molar fragment G, so the quarter-molar fragment E (H + M) was therefore also harvested to provide an independent source of H sequences. Pooled gel slices were melted at 100 °C, sonicated to decrease the nucleotide chain length and the gel allowed to re-set in tubes. The DNA was then eluted from the gels by electrophoresis into dialysis sacks below the tubes. The eluted DNA, in a vol. of 0.2 ml, was precipitated by addition of two vol. ethanol, precipitated once with cetyltrimethylammonium bromide and twice more with ethanol. Final yields varied from 10% for the higher mol. wt. fragments to 25% for the lower mol. wt. fragments. This difference probably results from the more conservative removal of the better separated smaller fragments.

The purified DNA fragments were isotope labelled with 125I by an adaptation of the method of Commerford (1971). Denatured DNA (1 to 3 µg) was iodinated with 1.5 mCi 125I in 0.1 M-sodium acetate pH 4.5, 10-2 M-TICl3 in a total vol. of 40 µl. Sufficient KI was added to achieve a final iodide concentration of 2 × 10-5 M. After 20 min at 60 °C the reaction
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Vessel was transferred to ice and the pH raised by the addition of 0.2 ml 0.2 M-ammonium acetate, pH 9.0. 2-Mercaptoethanol (2 µl) was added to reduce excess TiCl₃ and the mixture heated to 50 °C for 10 min to remove unstable iodinated derivatives (Commerford, 1971). The mixture was transferred to ice, brought to pH 7.5 by the addition of 50 µl 1 M-tris-Cl⁻-pH 7.5 and made 0.2 M with respect to NaCl. Denatured salmon sperm DNA (100 µg) was added and the nucleic acid precipitated by addition of two vol. ethanol. The precipitate was collected by centrifugation and the DNA purified by hydroxyapatite chromatography. The eluate was dialysed against 0.05 M-tris-Cl⁻, 0.2 M-NaCl, 10 mM-EDTA, pH 7.5, and stored at −20 °C as an ethanol precipitate. Labelling of all fragments yielded between 0.5 x 10⁸ and 2 x 10⁹ d/min <sup>125</sup>I. The recovery of DNA cannot be measured because of the presence of carrier, but the minimum sp. act. of the fragments was 3 x 10⁷ to 10⁹ d/min/µg, these being the lower limits of activity if the recovery of DNA was 100 %. The only exception was the quarter-molar fragment E (H + M) which labelled very poorly and was recovered with a minimum sp. act. of 4 x 10⁶ d/min/µg.

Fragment I migrates close to fragments G + H (see Fig. 1). To establish the extent of cross-contamination of these probes they were hybridized to a Southern blot (Southern, 1975) of <i>BamH</i> restricted type 2 DNA. The resulting autoradiograph showed that G + H sequences were not detectable in probe I, but that probe G + H consisted of about 15 % I sequences.

Hybridization reactions. Cell DNA, extracted by the method of Sharp <i>et al.</i> (1974), was mixed with <sup>125</sup>I-labelled probe DNA, sonicated to a nucleotide chain length of 300, denatured by heating to 100 °C and annealed at 70 °C in 1 M-NaCl and at a final DNA concentration of 2.5 mg/ml. The experiments were designed such that the cell DNA was in tenfold molar excess over probe DNA (i.e. 10 haploid cell genome copies per probe copy). These conditions were calculated assuming a probe sp. act. of 5 x 10⁶ d/min/µg and assuming the weight of the haploid mouse genome as 5 x 10⁻⁶ µg. Fragment E (of lower sp. act.) was required at higher concentration, and in hybridization reactions using this probe the cell DNA was used in threefold molar excess. Each hybridization experiment included a negative control, in which probe DNA was annealed in the presence of 2.5 mg/ml LMTK<sup>-</sup>cell DNA and a positive control in which probe DNA was annealed in the presence of 2.5 mg/ml LMTK<sup>-</sup> DNA to which had been added 0.075 µg/ml HSV-2 DNA (approx. equivalent to one virus DNA copy per haploid genome copy).

After various annealing reaction times samples were removed and digested with S¹ nuclease in 0.03 M-sodium acetate, pH 4.5, 2 mM-ZnSO₄, 0.3 M-NaCl and at a final DNA concentration of 100 µg/ml. Digestion was for 45 min at 37 °C. The remaining double-stranded <sup>125</sup>I-labelled DNA was measured after TCA precipitation. Samples containing only single-stranded (ss) DNA were about 4% resistant to this S¹ nuclease digestion. Results are expressed as the reciprocal of the fraction of DNA remaining single-stranded (ss) against reaction time, and the fraction remaining ss was corrected using the empirical factor derived by Smith <i>et al.</i> (1975) to allow for the poor reactivity of the ss tails which result from hybridization of randomly sheared sequences.

RESULTS

DNA content of transformed cells

Two transformed cell lines were chosen for study because they were known to express HSV-specific functions in addition to the selected thymidine kinase gene. Cell line D₂₁ compensates the defect in a type 2 mutant, <i>ts-208</i> (isolated by Dr D. Purifoy) and a type 1 mutant <i>ts-N₁₀₂</i>. These two mutants efficiently complement each other and are therefore unlikely to be allelic. Cell line D₂₅ compensates the defect in <i>ts-208</i>, but not the defect in <i>ts-N₁₀₂</i> (Minson <i>et al.</i> 1978, 1979).
Hybridization of the probe DNA sequences in the presence of DNA from cell lines D2₁ and D2₅ is shown in Fig. 2 and 3. A number of points should be made about this data.

1. With the exception of probe fragment E, the probes anneal very little in the absence of additional HSV-2 sequences. Comparison of the positive and negative control hybridizations confirms that the cell DNA is in at least tenfold molar excess over probe DNA.

2. Since all sequences present in the probe DNA must be represented in the HSV-2 sequences added to the positive control hybridizations, these reactions should be linear. Most of the reactions are linear, but some deviate significantly.

3. The negative control hybridizations show little annealing (except those involving fragment E) and the rate of annealing of the positive controls is therefore dependent solely on the concentration of added HSV-2 DNA. Since this was the same for all positive controls, it follows that the positive controls should all exhibit the same annealing rate. In fact, these rates vary by a factor of 2 to 3. The reasons for this are not obvious, but it is clear that while positive controls are of value in demonstrating the approximate sensitivity of the experiment, they should not be relied upon to indicate accurately the sequence copy number in experimental hybridizations. Despite these reservations the following conclusions can be drawn.

**D₂₁ cell DNA**

Annealing of probes I and G+H was considerably accelerated by D₂₁ cell DNA, but annealing of probe E (H+M) was unaffected. Therefore the adjacent EcoR₁ sequences I and G (see Fig. 1) are represented in cell line D₂₁. The annealing of probe J was slightly increased in the presence of D₂₁ DNA, but this result is on the borderline of significance. Since fragment J lies adjacent to fragment I, it is nevertheless tempting to suppose that cell line D₂₁ contains an HSV-2 sequence which includes a small part of fragment J. No other probe fragment showed an increased annealing rate in the presence of D₂₁ DNA.
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Fig. 3. Kinetic hybridization of probe DNA fragments in the presence of D2₅ cell DNA. Probe DNA sequences were hybridized in the presence of 2.5 mg/ml D₂₅ DNA (○—○○), 2.5 mg/ml LMTK⁻ cell DNA (●—●) or 2.5 mg/ml LMTK⁻ cell DNA plus 0.075 µg/ml HSV-2 DNA (■—■). Probe fragments N and O were not included in this analysis.

D₂₅ cell DNA

The results are similar to those obtained with D₂₁ DNA except that no effect was seen on the annealing of probe fragment J and, in addition to fragments I and G + H, sequences in fragment L are also represented in D₂₅ DNA.

Although the data are not extensive it is clear that the annealing of probes I and G + H in the presence of D₂₁ or D₂₅ DNA is comparable with the annealing kinetics of the positive controls. We conclude that the majority of the I and G sequences are represented in D₂₁ and D₂₅ cells and that the copy number is low; one or two copies per haploid genome. D₂₁ cells contain a sequence which includes part of fragment J and extends through fragment I into fragment G (map co-ordinates approx. 0.2 to 0.4), while D₂₅ cells contain sequences from the adjacent fragments I and G and also a sequence from the non-adjacent fragment L.

Thymidine kinase-negative revertant cells

Revertants of cell line D₂₁ have been derived by selection in BrdUrd. These cells, designated D₂₁R₁, D₂₁R₂, D₂₁R₃, contain no detectable thymidine kinase and lack the ability to compensate the defects of ts-N102 and ts-208 (Minson et al. 1979) and thus lack all the known virus-specific functions of the parental cell line. Hybridization of probes I and G + H in the presence of revertant cell DNA showed convincingly that these cell lines do not contain these sequences (Fig. 4), and are therefore generated by deletion of the virus specific sequences from the cell genome.

Location of the HSV-2 thymidine kinase gene

The HSV-2 sequences common to cell lines D₂₁ and D₂₅ are those represented by EcoR₁ fragments I and G, and it follows that the HSV-2 thymidine kinase gene must lie within this region (map co-ordinates approx. 0.2 to 0.4). The HSV-1 kinase gene has been mapped approx. at map co-ordinate 0.3 (Wigler et al. 1977; Morse et al. 1978; Stow et al. 1978). The equivalent position in HSV-2 DNA would be close to the junction of EcoR₁ fragments I and G, and evidence that the type 2 kinase gene maps near this junction is provided by
Fig. 4. Probe sequences I and G + H were hybridized in the presence of 2.5 mg/ml LMTK- cell DNA (●–●), 2.5 mg/ml LMTK- cell DNA plus 0.075 μg/ml HSV-2 DNA (■—■) or in the presence of 2.5 mg/ml DNA from cell lines D21R1, D25R2 or D25R3. The data obtained using DNA from the three revertant cell lines at each sampling point lie within the limits defined by the horizontal bars.

Discussion

Maitland & McDougall (1977) have provided evidence that the HSV-2 thymidine kinase gene is not co-linear with the type I gene and lies within the map co-ordinates 0.53 to 0.65. Our data are not consistent with this conclusion. Two cell lines which carry the type 2 kinase gene have in common a maximum of sequences lying within map co-ordinates 0.2 to 0.4. The failure of EcoRI digests of HSV-2 DNA to donate the kinase gene to LMTK- cells implies that the gene lies close to an EcoRI restriction site within this region; namely the junction of the I and G fragments at a map position of approx. 0.3. Our data are therefore consistent with co-linearity of the type I and type 2 kinase genes. A similar conclusion has been drawn from an examination of the thymidine kinase specified by HSV type 1 × type 2 intertypic recombinants (I. A. Halliburton, personal communication).

The hybridization data are insufficient to allow allocation of map positions to ts-N102 and ts-208. Since D21 and D26 cells both complement ts-208, this mutation must lie within EcoRI fragments I and G. The ts-N102 mutant is complemented by D24 and not D26, and D24 cells apparently contain sequences extending further leftwards than those in D26 cells. The ts-N102 mutant may therefore lie to the left of the kinase gene, close to the junction of fragments J and I (map position approx. 0.2).
Table 1. Transformation of LMTK− cells to a kinase-positive phenotype using sheared or restricted HSV-2 DNA*

<table>
<thead>
<tr>
<th>Expt</th>
<th>Treatment of DNA</th>
<th>Dose/dish (μg)</th>
<th>Proportion of dishes with colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sheared</td>
<td>2.5</td>
<td>14/16</td>
</tr>
<tr>
<td>2</td>
<td>Sheared</td>
<td>0.5</td>
<td>8/10</td>
</tr>
<tr>
<td>2</td>
<td>Sheared</td>
<td>0.05</td>
<td>2/10</td>
</tr>
<tr>
<td>3</td>
<td>HindIII restricted</td>
<td>2.5</td>
<td>8/8</td>
</tr>
<tr>
<td>2</td>
<td>HindIII restricted</td>
<td>0.5</td>
<td>7/10</td>
</tr>
<tr>
<td>2</td>
<td>HindIII restricted</td>
<td>0.05</td>
<td>3/10</td>
</tr>
<tr>
<td>1</td>
<td>EcoRI restricted</td>
<td>2.5</td>
<td>0/10</td>
</tr>
</tbody>
</table>

* DNA was sheared to a mol. wt. of $15 \times 10^6$, and HindIII or EcoRI digested DNA was examined by gel electrophoresis before use. Experimental methods have been described in detail elsewhere (Minson et al. 1978).

None of the three tk− revertants of cell line D21 contains the HSV-2 sequences found in the parental line. These revertants have therefore arisen by deletion of the virus-specific sequences and this is consistent with the finding that selection against the thymidine kinase gene results in simultaneous loss of the non-selected virus-specific markers. Although only one set of revertants has been examined for HSV-2 DNA sequences, other kinase positive lines (D2s and D2g) revert to a kinase-negative phenotype with the same frequency as D21 cells (reversion frequency is $10^{-3}$ to $10^{-4}$), suggesting that the mechanism of reversion is the same. The stability of the kinase-negative phenotype following selection of revertants led Bacchetti & Graham (1979) to a similar conclusion, that reversion resulted from loss of HSV sequences.

Finally, it is of interest that cell line D2s contains HSV-2 sequences derived from different parts of the virus genome. EcoRI fragment L is separated by sequences of at least $30 \times 10^6$ mol. wt. from the kinase gene and since this cell line was obtained by using as inoculum DNA of mol. wt. $15 \times 10^6$ it is unlikely that the sequences found in this cell line are derived from a single infecting molecule. However, the frequency of acquisition of the thymidine kinase gene in transformation experiments is, at best, about 1 cell in $10^6$, so it would be surprising if two separate sequences were acquired by independent events by a single cell. The low efficiency of transformation implies either that only a small proportion of calcium phosphate crystals in the inoculum has the properties required for a DNA vector or that only a small proportion of the cells are ‘transformable’ at any one time. In either instance, if two different sequences are co-precipitated with calcium phosphate, selection for one sequence will simultaneously select for the other. This view is supported by the experiments of Wigler et al. (1979).

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REFERENCES


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