Quantification of the Herpes Simplex Virus DNA Present in Biochemically Transformed Mouse Cells and Their Revertants

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SUMMARY

Four cell lines biochemically transformed by u.v.-irradiated herpes simplex virus contain virus DNA fragments ranging from 3 to 22% of the HSV genome. Of five revertant clones selected for 3H-TdR or BrdUrd resistance, four had lost all detectable virus DNA while the fifth, selected for BrdUrd resistance, retained the entire virus fragment but there was a reduction of virus copies per cell from 5 to 1. Three ‘supertransformed’ revertant cell lines contained virus DNA fragments ranging from 12 to 28%. The number of virus DNA fragments per cell ranged from 1 to 5 and clearly indicated that a single copy of the virus thymidine kinase gene is adequate for biochemical transformation. The determination of the base composition of the transforming virus DNA fragment indicated that the transforming DNA has a base composition approximately the same as the HSV genome and does not constitute a low GC virus DNA region. Cross hybridization between HSV-1 transformed cells and HSV-2 DNA is very slight, indicating that the DNA found in clone 139 is not entirely composed of the HSV-1 and HSV-2 common sequences.

INTRODUCTION

Murine cells lacking thymidine kinase activity (tk−) have been biochemically transformed to tk+ by u.v.-irradiated herpes simplex virus types 1 and 2 (HSV-1, HSV-2; Munyon et al. 1971; Davidson, Adelstein & Oxman, 1973). The newly acquired tk activity is of virus origin as shown by its electrophoretic mobility, thermodability and serological reactivity (Thouless & Skinner, 1971; Munyon et al. 1972; Davis & Munyon, 1974). In addition to the virus tk activity, Chadha & Munyon (1975) presented evidence that at least one HSV structural antigen is present in these cells but attempts to rescue infectious virus were unsuccessful. Kraiselburd, Gage & Weissbach (1975) and Davis & Kingsbury (1976) presented evidence that only a fragment of the HSV genome is present in the transformants tested. The genome fragment constituted 9 to 20% of the virus genome and was present in multiple copies per cell.

Chadha, Munyon & Hughes (1977) have isolated a series of tk− revertants of one line of biochemically transformed cells (Cl 139). These cells, isolated after exposure to 3H-thymidine, differed from those previously described by Davidson et al. (1973) due to the stability of the tk− phenotype. The revertants of Davidson et al. (1973) were selected for resistance to BrdUrd and exhibited a high frequency of conversion to the tk+ phenotype.
Table 1. Transformed cell lines and their properties

<table>
<thead>
<tr>
<th>Cell line designation</th>
<th>Transformation class</th>
<th>Selecting marker*</th>
</tr>
</thead>
<tbody>
<tr>
<td>139</td>
<td>Primary</td>
<td>tk+</td>
</tr>
<tr>
<td>163</td>
<td>Primary</td>
<td>tk+</td>
</tr>
<tr>
<td>165</td>
<td>Primary</td>
<td>tk+</td>
</tr>
<tr>
<td>167</td>
<td>Primary</td>
<td>tk+</td>
</tr>
<tr>
<td>171</td>
<td>Primary</td>
<td>tk+</td>
</tr>
<tr>
<td>139 R11</td>
<td>Revertant</td>
<td>3H-TdR-r</td>
</tr>
<tr>
<td>139 R12</td>
<td>Revertant</td>
<td>3H-TdR-r</td>
</tr>
<tr>
<td>139 R13</td>
<td>Revertant</td>
<td>3H-TdR-r</td>
</tr>
<tr>
<td>139 BrdUrd 2</td>
<td>Revertant</td>
<td>BrdUrd-r</td>
</tr>
<tr>
<td>171 BrdUrd 5</td>
<td>Revertant</td>
<td>BrdUrd-r</td>
</tr>
<tr>
<td>139 R11 S7</td>
<td>Supertransformant</td>
<td>tk+</td>
</tr>
<tr>
<td>139 R12 S3</td>
<td>Supertransformant</td>
<td>tk+</td>
</tr>
<tr>
<td>139 R15 S4</td>
<td>Supertransformant</td>
<td>tk+</td>
</tr>
</tbody>
</table>

* Selection was based on the ability to grow in MEM plus 6 × 10⁻⁷ M-methotrexate, 1.6 × 10⁻⁵ M-thymidine, 5 × 10⁻⁵ M-adenosine, 5 × 10⁻⁵ M-guanosine and 1 × 10⁻⁴ M-glycine (tk+) or MEM containing BrdUrd (30 μg/ml) or 3H-TdR (4 μCi/ml).

This study examines a series of revertants of clone 139 cells to compare the virus DNA present in cells selected for resistance to BrdUrd and 3H-thymidine. The results suggest that BrdUrd selected revertants may or may not lose the virus DNA fragment whereas all of the 3H-TdR selected revertants have no detectable virus genetic information. It also examines an additional group of biochemically transformed cells to explore the consistency of the amount of virus DNA present in biochemically transformed cells. In addition, it examines a group of biochemically transformed cells derived from revertants of previously transformed clones. Such cell lines are referred to as supertransformants in this study.

METHODS

Cells and viruses. Table 1 lists the designations and properties of the HSV transformed, revertant and supertransformed cells used in this study. The properties of these cells and the derivation of the parental cell lines have been described elsewhere (Munyon et al. 1971; Chadha et al. 1977). The media used for propagation of each cell type have been described by Davis & Kingsbury (1976). All the revertant clones were propagated in the BrdUrd medium described for the parental Ltk⁻ cells.

The HSV-1 hybridization probes were prepared from virions of the JH and MacIntyre strains. These strains show complete nucleotide sequence homology to the KOS strain used in the transformation experiments (Sugino & Kingsbury, 1976). The HSV-2 probe was prepared from strain Z570.

Extraction of cellular DNA and production of hybridization probes. Cellular DNA was prepared from cell monolayers as described elsewhere (Davis & Kingsbury, 1976). After precipitation in cold ethanol the DNA was always centrifuged in addition to spooling. This precaution was taken to ensure quantitative recovery of the cellular DNA.

Radioactive virus DNA for use as hybridization probes was prepared as described earlier (Davis & Kingsbury, 1976). The HSV-1 JH and HSV-1 MacIntyre probes used in this study had specific activities of 4.4 × 10⁶ cpm/μg and 9.6 × 10⁶ cpm/μg respectively. The Z570 probe had a specific activity of 5.5 × 10⁶ cpm/μg.

All the DNA used in hybridization experiments was sheared at 0 °C for 2 min with a
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Branson W140 sonifier prior to use. Fragment length was approximately 350 nucleotides as determined by velocity sedimentation in alkaline sucrose gradients.

Measurement of DNA-DNA reassociation kinetics. To give a total vol. of 500 μl, sheared 3H-labelled HSV DNA was added to a reaction mixture containing 0.48 M-phosphate buffer, pH 6.8 (PB), 1 mM-EDTA and 2.2 to 3 mg/ml of sheared cell DNA. After denaturation at 115 °C for 5 min in a sealed glass vial the reaction mixture was quickly brought to the incubation temperature (70 °C) and a zero time sample was taken. Heated (65 °C) mineral oil (200 μl) was added to the top of the reaction mixture to prevent evaporation during the time of incubation. Successive samples were taken at predetermined times. Each sample was immediately diluted into 2.5 ml 0.14 M-PB containing 0.4% SDS and passed through a hydroxyapatite column equilibrated to 60 °C. The 0.14 M-PB eluent containing the single strands was collected and the double strands (reassociated DNA) bound to the column were eluted with 0.3 M-PB. The column effluents were adjusted to equal volumes and PB concentrations. They were then counted in Aquasol-2 (NEN) in a liquid scintillation counter. Percent reassociation was calculated by subtracting the background and dividing the counts in the 0.3 M-PB effluent by the total number of counts in the sample.

Thermal elution of DNA hybrids. In a total reaction volume of 500 μl, 1.5 x 10^-3 μg of HSV-1 probe (specific activity, 9.6 x 10^6 ct/min/μg) was reacted with 3.5 mg of biochemically transformed cell DNA. The reaction was allowed to proceed to a C₀t at which the virus fragment present in the transformed cell had reacted almost to completion. The reaction mixture was then diluted to 0.14 M-PB and applied to a hydroxyapatite column at 60 °C. The column was washed four times with 7 ml 0.14 M-PB containing 0.4% SDS followed by two additional 7 ml vol. of 0.14 M-PB. The column temperature was lowered to 40 °C and the thermal elution started after equilibration of the column with elution buffer (5 M-NaClO₄ plus 0.14 M-PB). The column temperature was raised in 3 °C increments. After temperature equilibration the column was washed with 5 ml of elution buffer at column temperature. Following the thermal elution the column was washed with 0.3 M-PB to ensure that all the DNA had been eluted. The samples were counted after the addition of 4.5 ml of water and 10 ml of Aquasol-2.

RESULTS
Rationale of the experimental approach

A small amount of radioactive HSV DNA when denatured and allowed to reassociate will do so at a characteristic rate. This reassociation rate is not directly affected by the presence of an unrelated DNA, such as mouse DNA. If there are virus sequences present in the mouse DNA, however, the rate of reassociation of the HSV DNA will be increased in proportion to the amount of virus DNA present. As described by Britten & Kohne (1968), the extent of reassociation of any set of homologous sequences in a hybridization mixture may be described by the equation:

\[
\frac{t}{f_{ss0}} = 1 + kC_{0t},
\]

where fss₀ is the fraction of the DNA remaining single stranded at time t, C₀ is the total DNA concentration in solution and k is proportional to the second order rate constant. If all the different sequences in the virus DNA probe are present at equal concentrations, a plot of t/fss₀ against C₀t will be a straight line with slope k. If a fraction of the virus DNA is at a higher concentration this plot will give a biphasic curve with an initial (driven) component corresponding to those sequences present in a higher concentration and a second component corresponding to those present at a lower concentration.
Table 2. Amount and number of copies of virus DNA present in HSV-transformed cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>% of the HSV genome present</th>
<th>HSV fragment</th>
<th>No. of copies/ haploid genome</th>
</tr>
</thead>
<tbody>
<tr>
<td>163</td>
<td>3 ± 3</td>
<td>3000-6000</td>
<td>1-2</td>
</tr>
<tr>
<td>165</td>
<td>24 ± 3</td>
<td>3800</td>
<td>1.6</td>
</tr>
<tr>
<td>167</td>
<td>3 ± 3</td>
<td>3000-6000</td>
<td>1-2</td>
</tr>
<tr>
<td>171</td>
<td>17 ± 3</td>
<td>4000</td>
<td>1.5</td>
</tr>
<tr>
<td>139 R11 S7</td>
<td>14 ± 3</td>
<td>7200</td>
<td>0.83</td>
</tr>
<tr>
<td>139 R15 S4</td>
<td>12 ± 3</td>
<td>1800</td>
<td>3.3</td>
</tr>
<tr>
<td>139 R12 S3</td>
<td>28 ± 3</td>
<td>2000</td>
<td>3.0</td>
</tr>
<tr>
<td>171 BrdUrd 5</td>
<td>0 ± 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>139 BrdUrd 2</td>
<td>9 ± 3</td>
<td>6000</td>
<td>1.0</td>
</tr>
<tr>
<td>139 R11</td>
<td>0 ± 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>139 R12</td>
<td>0 ± 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>139 R15</td>
<td>0 ± 3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

If the quantity of input virus DNA (probe) is sufficiently small, then the probe makes an insignificant contribution to the rate of reassociation of the virus sequences found in a transformed cell. Under these conditions the transforming virus DNA can be considered a cellular gene and the rate of probe reassociation in the driven reaction is in proportion to the rate of cellular gene reassociation. In the experiments reported here, we have based the determination of the number of virus copies per cell on this observation and the data is presented in that format. The computation of \( C_{ot} \) is based on the total cellular DNA concentration in the reaction and is plotted against the rate of reassociation of the virus probe which represents a series of nucleotide sequences found in part as a component of that cellular DNA constituent. The number of copies present per cell is determined by comparing the rate of the driven portion of the probe reaction with the rate of the reaction of the cellular unique sequences.

As demonstrated by Sharp, Pettersson & Sambrook (1974), interpretation of hybridization data presented in the format of \( 1/fss \) vs. \( C_{ot} \) is very difficult and misleading. If the virus sequences in the transformed cells represent the entire virus genome, then a plot of \( 1/fss \) vs. \( C_{ot} \) will be a straight line. If only a fragment of the virus genome is present, then such a plot will deviate from linearity (second order kinetics). As pointed out by Sharp et al. (1974), it is particularly difficult to determine the deviation from second order kinetics when less than half of the virus genome is not represented in the transformed cell. Frequently, under such conditions, it is possible to draw a straight line through the experimental points when in reality they deviate from linearity. The conclusion from such a plot would be that the whole genome was present and not a fragment. For this reason, it is valuable to analyse the data in the conventional percent reassociated (in this case percent virus probe) against \( C_{ot} \). As mentioned above, \( C_{ot} \) refers to the total cellular DNA since the virus information is being treated as a cellular gene.

The reassociation of \( ^{32}P \)-labelled mouse DNA in the presence of a 100-fold excess of unlabelled mouse DNA gives a value of 3000 mol s/l for the \( C_{ot} \) of the unique sequences. A similar reassociation experiment with HSV alone yields a value of 0.2 mol s/l for the \( C_{ot} \). In reconstruction experiments, one complete virus copy per mouse haploid genome gives a \( C_{ot} \) of 3000 for the virus sequences relative to the cellular DNA concentration (Fig. 5). Similarly, a doubling of the virus gene concentration halves the \( C_{ot} \) value relative to the mouse DNA concentration. Therefore, the simple division of the mouse unique \( C_{ot} \) by the
Fig. 1. Kinetics of hybridization of $^{3}$H-labelled HSV-1 DNA with DNA from cells biochemically transformed with HSV-1. (a) The reassociation of $3.3 \times 10^{-3} \mu g$ of $^{3}$H-labelled HSV-1 DNA (specific activity, $9.6 \times 10^6$ ct/min/ug) per ml was determined in the presence of 3 mg DNA per ml from clone 165 cells (○○○) or from Balb/c DNA (■■■). The dashed (---) lines represent theoretical curves in which points were plotted at $C_{at}$ values corresponding to experimental points. (a) 25% HSV fragment represented 2 copies/cell; (b) 22% HSV fragment represented 1-6 copies/cell; (c) 20% HSV fragment represented 2 copies/cell; (d) 20% HSV fragment represented 1 copy/cell. The $C_{at}$ values have been corrected for salt concentration and represent $C_{at}$ equivalents ($E \ C_{at}$) in 0.12 M-PB. (b) Same reaction as (a) (○○○) except the data is plotted as % hybridization v. $E \ C_{at}$. The self reaction has been subtracted at each time point. A reconstruction experiment of 2 virus copies/cell (○○○) performed under the same hybridization conditions is included for reference.

An alternative calculation method has been provided by Sharp et al. (1974) who derived a composite expression to deal with the problems associated with the reassociation kinetics of virus DNA fragments in transformed cells. Equation (4) of Sharp et al. (1974) allows the derivation of theoretical curves based on the fraction of the virus DNA present and number of copies as well as solving for these factors from experimental curves.

**Analysis of primary transformants**

Four independently isolated primary biochemical transformants (clones 163, 165, 167 and 171) were examined for the presence of HSV-1 DNA. The data obtained is summarized in Table 2. Clones 163 and 167 contained approximately 3% of a virus genome, bordering on cellular $C_{at}$ which shows the half reaction of the driven probe reaction, provides the number of virus gene copies per haploid genome.
I W. M. Sugino, K. C. Chadha and D. T. Kingsbury

Fig. 2. Kinetics of hybridization of 3H-labelled HSV-1 DNA with DNA from cells biochemically transformed with HSV-1. (a) The reassociation of $3.3 \times 10^{-3}$ μg of 3H-labelled HSV-1 (specific activity, $9.6 \times 10^6 \text{ cpm/μg}$) per ml was determined in the presence of 3 mg DNA per ml from clone 171 cells (○—○) or from Balb/c DNA (■—■). A theoretical curve for a 17% HSV fragment represented at 1.5 copies/cell is shown (○—○). The conditions of hybridization were as described in the text. The $C_{ot}$ values have been corrected for salt concentration and represent $C_{ot}$ equivalents in 0.12 M-PB. (b) Same reaction as (a) (○—○) except the data is plotted as % hybridization v. $E C_{ot}$. The self reaction has been subtracted at each time point. A reconstruction experiment as in Fig. 1 (b) is also included.

It is clear from this data that clone 165 contains a fragment which constitutes approximately 22% of the virus genome and reassociates with a $C_{ot}$ of 3800 (Fig. 1 b). By dividing the cellular unique sequence $C_{ot}$ by the virus DNA $C_{ot}$, the number of virus copies, relative to the cellular unique sequences, is obtained. Fig. 1 (a) also contains a theoretical plot based on the mathematical formula of Sharp et al. (1974) for a 22% virus fragment which is represented an average of 1.6 times per cell. It is clear that the theoretical curve is in close agreement with the experimental results.

Fig. 2 (a and b) shows the results obtained in identical experiments with DNA extracted from clone 171. As determined from this data, clone 171 contains a 17% virus fragment which is represented 1.5 times per cell.
DNA in herpes simplex virus transformed cells

Fig. 3. Kinetics of hybridization of 3H-labelled HSV-1 DNA with DNA from cells biochemically transformed with HSV-1 followed by selection for tk- by resistance to BrdUrd. (a) The reassociation of $3.3 \times 10^{-15}$ µg of 3H-labelled HSV-1 DNA (specific activity, $9.6 \times 10^{6}$ ct/min/µg) per ml was determined in the presence of 2.91 mg DNA per ml from 139 BrdUrd cells (■) or from Balb/c DNA (■). A theoretical curve for a 9 % HSV fragment represented at 1 copy/cell is shown (○). The conditions of hybridization were as described in the text. The $C_{ot}$ values have been corrected for salt concentration and represent $C_{ot}$ equivalents in 0.12 M-PB. (b) Same reaction as (a) (■) except the data is plotted as % hybridization vs $C_{ot}$. The self reaction has been subtracted at each time point. A reconstruction experiment of 2 virus copies/cell (○) is included for reference.

Examination of revertant cell lines

Five cell lines which had lost their thymidine kinase activity, originally acquired by HSV-1 transformation, were examined for the continued presence of the transforming virus DNA fragment. As shown in Table 2, four of the five cell lines (clones 171 BrdUrd 5, 139 R11, 139 R12 and 139 R15) had lost all detectable virus DNA (< 3 % of the virus genome). Clone 171 BrdUrd 5 was selected as a BrdUrd resistant revertant of clone 171 described above. The other three lines were selected as 3H-thymidine resistant clones of the 139 cell line examined in an earlier study (Davis & Kingsbury, 1976; Kraiselburd et al. 1975).

Clone 139 has been examined extensively in a variety of studies and contains 9 % of the HSV-1 genome represented at 5 copies per cell. These figures are based on approximately 50 independent determinations using six different HSV-1 probe preparations. Clone 139 BrdUrd 2 was the only revertant which maintained a detectable DNA fragment. The data shown in Fig. 3(a and b) demonstrates that the 9 % fragment found in the parental cells was maintained but that there had been a reduction in the number of copies per cell from 5 to 1. Fig. 3(a) also includes a theoretical curve for the reaction of a 9 % fragment represented once per cell; this curve is almost identical to the experimental data.

Examination of supertransformed cell lines for HSV DNA

Chadha et al. (1977) derived a series of cell lines by superinfecting revertants obtained from clone 139 cells. These lines were termed supertransformants and were obtained at
Fig. 4. Kinetics of hybridization of \(^{3}H\)-labelled HSV-1 DNA with DNA from cells biochemically transformed with HSV-1. The reassociation of 2.5 \times 10^{-3} \mu g of \(^{3}H\)-labelled HSV-1 DNA (specific activity, 4.4 \times 10^{6} \text{cpm/\mu g}) per ml was determined in the presence of 2.05 mg DNA per ml from 139 R15 S4 cells (○) or from human placenta DNA (■). A theoretical curve for a 12% HSV fragment represented at 3.3 copies/cell is shown (□ -- □). The conditions of hybridization were as described in the text. The \(C_{ot}\) values have been corrected for salt concentration and represent \(C_{ot}\) equivalents in 0.12 M-PB.

Fig. 5. Kinetics of hybridization of \(^{3}H\)-labelled HSV-1 DNA with mouse DNA plus 2 copies/cell of the complete HSV-1 genome. The reassociation of 3.3 \times 10^{-3} \mu g of \(^{3}H\)-labelled HSV-1 DNA (specific activity, 9.6 \times 10^{6} \text{cpm/\mu g}) per ml was determined in the presence of 7 mg/ml of mouse DNA plus 0.7 \mu g of \(^{3}P\)-labelled HSV-1 DNA. A DNA molecular weight of 2 \times 10^{12}/cell was used to calculate the quantity of virus DNA needed to give 2 virus copies/cell.

frequencies much lower than primary transformants of tk cells. Table 2 shows the results of hybridization experiments done with DNA extracted from these cells. Three supertransformed cells were examined for HSV-1 DNA and each contained a large virus DNA fragment. Fig. 4 illustrates the kinetics of hybridization with DNA extracted from one of these cell lines. Clone 139 R11 S7 contained a single copy of a fragment constituting 14% of the
DNA in herpes simplex virus transformed cells

Fig. 6. Thermal elution profile of the DNA hybrids formed between 3H-labeled HSV-1 probe and biochemically transformed cell DNA. (a) Clone 139. 3H-Labelled HSV-1 DNA (specific activity, \(9.6 \times 10^4 \text{ ct/min/} \mu \text{g}\)) at \(3.3 \times 10^{-3} \mu \text{g} \text{ per ml}\) was reacted with \(7 \text{ mg/ml}\) of Cl 139 DNA to a \(C_{ot}\) of \(5000 \text{ mol s/} \text{l}\). The reaction mixture was then diluted to \(0.14 \text{ M-PB}\) and \(8 \times 10^3 \text{ ct/min of } ^{32}\text{P}-labeled\) denatured and reannealed HSV-1 (JH strain) DNA was added. This mixture was then applied to a hydroxyapatite column for the thermal elution as described in Methods. (b) Clone 139 RI1 S7. Same as for (a) except the 3H-labelled probe was reacted with \(7 \text{ mg/ml}\) of Cl 139 RI1 S7 DNA to a \(C_{ot}\) of \(25088 \text{ mol s/} \text{l}\). 3H-Labelled HSV-1 (●—●); ^{32}\text{P}-labelled HSV-1 (○—○).

virus genome while clone 139 R12 S3 contained three copies of a 28% virus fragment and clone 139 R15 S4 contained a 12% fragment represented 3.3 times/cell (Fig. 4).

A reconstruction experiment in which 2 copies/cell of the complete virus genome were added to a normal mouse DNA reaction was done to test the accuracy of the copy/cell determinations. As shown in Fig. 5, the \(C_{ot}\) of the virus reaction is 2800 mol s/l which corresponds to approximately 2.1 copies/cell. These results suggest that the observed differences in fragment copies per cell are valid experimental observations.

Determination of the thermal stability of the transforming DNA fragment

Fig. 6 shows the thermal elution profiles obtained by melting the DNA hybrids formed between an HSV-1 DNA probe and DNA extracted from various transformed cells. In each case, there was an approximately 1 °C depression of the elution mid-point \((E_m)\) of the cell virus hybrid when compared to a control of ^{32}P-labeled HSV-1 which was melted and reannealed under identical conditions. In these experiments, the 3H-labeled HSV-1 probe was reacted with the transformed cell DNA to a \(C_{ot}\) at which the virus fragment present in the transformed cells had almost completely reacted. After the addition of ^{32}P-labeled HSV marker, the entire mixture was passed through a hydroxyapatite column and the thermal elution done on the mixture of double-stranded DNA. The results of these experiments showed that the HSV-1 probe was reacting with a specific HSV fragment and not nonspecifically with regions of cellular DNA. Additionally, they showed that the hybridization conditions constituted such stringent criteria that only stable HSV hybrids were formed. In these thermal elution experiments, the hybridization kinetics were such that no more than
20\% of the labelled DNA hybrids could be the result of probe self reaction. Therefore, the sharp melting profiles had to result from well matched hybrids between the probe and the transforming fragment.

**Type specificity of the DNA fragment in clone 139 cells**

HSV-1 and HSV-2 share approximately 50\% of their nucleotide sequences but only 20 to 30\% of the total sequences are carefully matched (Sugino & Kingsbury, 1976). Since both HSV-1 and HSV-2 are able to transform cells biochemically, it is not unexpected that these functions might be coded for in the common regions. Clone 139 DNA was reacted with an HSV-2 hybridization probe (strain Z570) under the conditions described in Methods. The results of these experiments indicated that the DNA fragment which reacted 9\% with the HSV-1 probe reacted only 3.5\% with the HSV-2 probe. The kinetics of the reaction were similar to the HSV-1 reaction.

**DISCUSSION**

Examination of a number of biochemically transformed cell lines indicates that the active transforming region of the HSV-1 genome consists of a very limited portion of the DNA, in the order of 3 to 4\%. This amount of genetic information is equivalent to 3 to 4 million daltons of DNA, approximately the size of the small transforming papovaviruses. The large amount of virus DNA in cell lines 171, 139 and 165 may only indicate that the establishment of the transformed state by u.v.-irradiated viruses involves the random selection of a DNA fragment. The apparent upper limit of 25\% of the genome may just be a reflection of the u.v. damage inflicted on the DNA when the virus was inactivated. As Sharp et al. (1974) have pointed out, if the transformed cells contain only a partial copy of the virus genome, or if some parts of the virus genome are present at a much higher frequency than others, the observed rate of reannealing of the labelled probe in the presence of transformed cell DNA will be an average of the rates of reannealing of each independent segment of the virus DNA.

In the experiments reported here, the determination of the percentage of the virus genome present in the transformed cell DNA is the only one done with great confidence. Determining the actual number of virus copies per haploid genome is very difficult. If the virus DNA present in the transformed cell is homogeneous, then the determinations of virus copies per haploid genome are fairly accurate. It is possible, however, that part of the virus genome is present at a different concentration than the rest; in this case, it will be almost impossible to detect this difference, especially if part of the virus fragment is very small compared to the rest or if the fragments have similar concentrations. If the virus fragments are large enough to detect and have large concentration differences, then a multiphasic hybridization curve would result.

The results obtained with the revertant cell lines suggest that 'revertants' arise by two mechanisms, the loss of the virus DNA or some form of regulation of the expression of the virus DNA. The data of Davidson et al. (1973) demonstrated that transformed cells exposed to different selective media could undergo repeated rounds of tk suppression and reactivation. The results presented here support that observation by demonstrating the presence of the virus DNA in the BrdUrd resistant cells. The data presented here agrees with the earlier observation by Kraiselburd et al. (1975) that some cell lines lose the entire virus fragment.

It is difficult to interpret the significance of the loss of approximately 4 virus copies per cell in the case of the 139 BrdUrd 2 revertant. It is clear that the entire virus fragment is present in these cells; however, the revertant clone has only one fragment per cell instead of the five found in the parent line. It is apparent from the results obtained with the clone 171
cells that a single copy of the virus tk gene per cell is adequate for maintenance of the transformed state rather than multiple copies as originally thought (Davis & Kingsbury, 1976). The loss of a portion of the transforming DNA from a cell with the maintenance of the balance suggests that the multiple virus copies exist either independently or as a tandemly duplicated single unit. Preliminary observations have suggested that the majority of the virus DNA in clone 139 cells is not integrated into the host chromosomal DNA (Moore & Kingsbury, unpublished observations) but is most likely replicating as extrachromosomal units.

In every supertransformed cell line examined, the size of the DNA fragment maintained was much larger than that in the parental cell. This observation is even more puzzling in view of the fact that only a small virus fragment may be necessary. The revertant cell lines from which the supertransformants are derived are highly resistant to retransformation (Chadha et al. 1977) and the large DNA fragment found in the supertransformed cells may be the result of the requirements for an additional virus gene product in the revertant cells. It should be mentioned that these were all clones derived from the same parent cell line and all had lost any detectable virus DNA.

These studies, like some earlier work (Sugino & Kingsbury, unpublished observations), suggest that the maximum required fragment of HSV DNA for either the biochemical or morphological transformation of cells is in the order of 3 to 4% of the virus genome. These results suggest that obtaining the required transforming genes in pure form and utilizing that DNA as a hybridization probe may be the only reliable assay for the HSV DNA present in transformed cells and human tumours. Likewise, it is clear that the transforming genes responsible for biochemical transformations are not part of a large region of ‘common sequences’ shared by HSV-1 and HSV-2. Whether the actual transforming genes are common cannot be determined from this data. However, these results suggest that type specific probes may be necessary for tumour screening even though strain specific probes are not (Sugino & Kingsbury, 1976).

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