Characterization of Human TK- Cell Lines Transformed to a TK+ Phenotype by Herpes Simplex Virus Type 2 DNA

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SUMMARY

Human TK- cells carrying the HSV-2 TK gene as a result of transformation with virus DNA express a TK activity of virus origin and maintain the TK+ phenotype when grown in HAT medium. Under non-selective conditions, however, reversion to a TK- phenotype occurs with a significant frequency characteristic of each transformed line. Once reversion has occurred the TK- phenotype appears to be stable, since only very rare instances of TK- to TK+ reversion have been observed. TK- revertants were susceptible to re-transformation by virus DNA, but no reactivation of a silent virus TK gene could be obtained by superinfecting them with a TK- virus mutant. The data presented are consistent with the hypothesis that acquisition of the TK- phenotype is brought about by loss of the virus sequences coding for TK.

INTRODUCTION

We have reported that human cells lacking the enzyme thymidine kinase (TK- cells) can be transformed to a TK+ phenotype by treatment with sheared herpes simplex virus (HSV) DNA, followed by selection of the TK+ transformants in HAT medium (Bacchetti & Graham, 1977). A number of such transformants have been established as cell lines continuously growing in HAT medium and expressing a thymidine kinase of virus origin. This phenotype is maintained by the cell populations as long as the selective pressure for TK+ cells is present. However, when the selective pressure is removed, by growing the transformed lines in non-selective medium, significant differences in the degree of stability of expression of the TK+ phenotype are observed among the various transformants.

The present study is an analysis of this property in several independently transformed lines aimed at understanding the parameters controlling their different behaviour. We have observed that phenotypic conversion from TK+ to TK- occurs under non-selective conditions with a frequency which is variable from one transformed line to another but characteristic of each transformed line and subclones derived from it. Back reversion from TK- to TK+, which has been observed in mouse cells transformed by u.v.-inactivated HSV-1 (Davidson et al. 1973), was only a very rare event in our system. Our results are consistent with the idea that loss of TK activity in transformed cells is most frequently due to loss of the virus TK gene.

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METHODS

Tissue culture media. Three media were used in the present study: (1) non-selective medium α-MEM (Stanners et al. 1971), which allows growth of both TK+ and TK- cells; (2) TK+ selective medium, α-HAT, consisting of α-MEM supplemented with: 0.1 mM-hypoxanthine, 1 μM-aminopterin and 40 μM-thymidine (Littlefield, 1964); (3) counter-selective medium, in which only TK- cells are able to grow, consisting of α-MEM plus 30 μg/ml BrdUrd (α-BrdUrd). All media were supplemented with 10% foetal calf serum (FCS) and antibiotics.

Cells. Human TK- cells, line 143, derived by BrdUrd selection (C. Croce and K. Huebner, personal communication) from the TK+ R970-5 line (Rhim et al. 1975) were used for the transformation experiments as described previously (Bacchetti & Graham, 1977). Spontaneous back reversion of 143 cells to TK+ has never been observed and the reversion rate of the line has been estimated to be less than 10^-8. Ten independent transformants were isolated, grown in HAT medium and assayed for the specific activity and virus origin of their thymidine kinase. Three of these lines (AC1, AC4 and AC5) were also grown for over 200 generations in α-HAT and α-MEM and periodically assayed for their ability to form colonies in α-HAT and α-BrdUrd by plating up to 10^4 cells per dish in 60 mm dishes. After 10 to 12 days of growth at 37 °C the dishes were stained and the surviving colonies counted. Phenotypically TK- revertant clones, isolated from some of the transformed lines by plating them in either α-MEM or α-BrdUrd, were also assayed for their ability to incorporate 3H-dThd and to plate in α-HAT. In the latter case the cells were plated at a concentration of 10^5 cells per 150 mm dish and incubated for 14 days at 37 °C with medium changes every 3 days to remove dead cells.

Virus. HSV-2, strain 219 (Seth et al. 1974) used for the isolation of transforming DNA was propagated in Vero monkey cells. HSV-1 B2006, a TK- derivative of HSV-1 strain Cl 101 (Dubbs & Kit, 1964), was propagated in human 143 TK- cells growing in α-BrdUrd. For infection of the cells, a m.o.i. of 1 p.f.u./cell was used for the 219 strain and a m.o.i. of 2 p.f.u./cell for the B2006 strain.

Assay for TK activity. Measurements of TK activity in crude cell extracts were carried out as already described (Bacchetti & Graham, 1977). Incorporation of 3H-dThd was measured as acid-precipitable counts in TCA treated cell lysates.

Determination of doubling time and reversion rate. The doubling times of TK+ and TK- subpopulations of the lines were measured as follows: cells growing in α-MEM were plated at low density in the same medium, labelled with 3H-dThd (0.25 μCi/ml, 20 Ci/mmol) every 12 h for 24 h, and fixed in Carnoy’s solution. Following autoradiography, the doubling times were calculated by counting the number of cells in labelled and unlabelled colonies. Similarly, the rate of conversion from TK+ to TK- was calculated by measuring the relative increase in the fraction of unlabelled cells in cultures plated out of α-HAT into non-selective medium and labelled at various time intervals.

RESULTS

HAT resistant colonies arising from monolayers of TK- cells after treatment with HSV-2 DNA could always be established as lines capable of growing continuously in α-HAT and expressing a TK of virus origin (Bacchetti & Graham, 1977). As long as they were kept under selective pressure, the cell populations maintained the TK+ phenotype. However, inherent and characteristic differences among the transformed lines in terms of their stability of expression of TK became apparent when the cells were plated in counter-selective medium.
Table 1. Relative proportion of TK+ and TK- cells in transformed lines*

<table>
<thead>
<tr>
<th>Cell line</th>
<th>No. of passages</th>
<th>% TK+ cells</th>
<th>% TK- cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC1</td>
<td>5</td>
<td>76</td>
<td>24</td>
</tr>
<tr>
<td>AC2</td>
<td>10</td>
<td>88</td>
<td>12</td>
</tr>
<tr>
<td>AC3</td>
<td>10</td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td>AC4</td>
<td>10</td>
<td>99.9</td>
<td>0.06</td>
</tr>
<tr>
<td>AC5</td>
<td>5</td>
<td>99.8</td>
<td>0.2</td>
</tr>
<tr>
<td>DC1</td>
<td>5</td>
<td>92.7</td>
<td>8.3</td>
</tr>
<tr>
<td>DC2</td>
<td>4</td>
<td>77</td>
<td>23</td>
</tr>
<tr>
<td>DC3</td>
<td>5</td>
<td>94.6</td>
<td>5.4</td>
</tr>
<tr>
<td>DC4</td>
<td>6</td>
<td>94.1</td>
<td>5.9</td>
</tr>
<tr>
<td>DC5</td>
<td>5</td>
<td>66</td>
<td>44</td>
</tr>
</tbody>
</table>

* Ten independently transformed lines originating from two transformation experiments (AC and DC lines) were grown in HAT medium and plated in either HAT or BrdUrd medium. The plating efficiency in each medium relative to the sum of the plating efficiencies in both media was taken as a measure of the fraction of TK+ or TK- cells in the population.

Table 2. Specific activity of TK in control and transformed lines*

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin of TK assayed</th>
<th>TK activity (pmol/μg protein)</th>
<th>TK activity (% of control)</th>
<th>% TK- cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>R970-5</td>
<td>human</td>
<td>0.68</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>AC1</td>
<td>HSV-2</td>
<td>0.52</td>
<td>76</td>
<td>24.0</td>
</tr>
<tr>
<td>AC2</td>
<td>HSV-2</td>
<td>0.31</td>
<td>46</td>
<td>12.0</td>
</tr>
<tr>
<td>AC3</td>
<td>HSV-2</td>
<td>0.83</td>
<td>122</td>
<td>1.0</td>
</tr>
<tr>
<td>AC4</td>
<td>HSV-2</td>
<td>0.39</td>
<td>57</td>
<td>0.06</td>
</tr>
<tr>
<td>AC5</td>
<td>HSV-2</td>
<td>0.35</td>
<td>51</td>
<td>0.20</td>
</tr>
</tbody>
</table>

* TK activity in crude cell extracts from human TK+ cells (R970-5) or several independent transformants expressing the HSV-2 TK (AC lines) was assayed as already described (Bacchetti & Graham, 1977), and is expressed as pmol of thymidine phosphorylated in 45 min at 37 °C per μg of protein. Under the conditions used the TK assayed is essentially the cytosol form of the enzyme (whether human or virus). The mitochondrial form of TK is virtually undetectable even in 143 TK- cells. The % of TK- cells was calculated from the plating efficiency in medium containing BrdUrd relative to the sum of the plating efficiencies in α-HAT and α-BrdUrd.

As shown in Table 1, the fraction of TK- cells, that is, cells able to survive in medium containing BrdUrd, varied greatly from one line to another even at early passages after transformation. As already reported (Bacchetti & Graham, 1977), when TK+ subclones were derived from each of the lines by plating single cells in HAT medium, it was observed that, in general, they maintained the degree of stability characteristic of the parent cells. This suggests that the rate with which cells revert to a TK- phenotype is an inherited trait. The specific activity of the virus enzyme (Table 2) was also variable among the transformed lines and in most cases lower than the specific activity of the cytosol human enzyme expressed in the grandparental TK+ line R970-5. No correlation was, however, apparent between levels of enzymic activity and the degree of stability of the TK+ phenotype.

Three of the transformed lines were analysed in more detail for the stability of expression of the virus enzyme. The lines (AC1, AC4 and AC5) were grown for over 200 generations in either α-HAT selective medium or α-MEM non-selective medium and at intervals the relative fractions of TK+ and TK- cells were determined by measuring cell survival in medium containing HAT or BrdUrd. As shown in Fig. 1a, the AC1 line grown in HAT medium contained initially 25% TK- cells, that is cells which were able to form colonies in BrdUrd.
Prolonged growth under selective pressure for TK\(^+\) cells decreased the fraction of BrdUrd survivors, that is, it appeared to stabilize the transformed character of the line. On the other hand, growth in non-selective \(\alpha\)-MEM resulted in an increase in the percentage of negative cells, in this case up to about 45\%. The same pattern was exhibited by another line, AC5 (Fig. 1b), except that in this case the initial percentage of negative cells was only 0.2\% and the increase in the fraction of these cells in the population grown in \(\alpha\)-MEM reached a plateau value of about 80\%. The third transformed line analysed in this manner (AC4, data not shown) did not show any significant variation in the relative percentages of TK\(^+\) and TK\(^-\) cells, whether it was grown in \(\alpha\)-HAT or \(\alpha\)-MEM for up to 200 generations. In either case no more than 1 to 2\% TK\(^-\) cells were present in the population, indicating that this line is relatively stable for virus TK expression.

Studies similar to ours, performed on mouse TK\(^-\) cells carrying the HSV-1 TK gene (Davidson et al. 1973), have indicated that the transformed cells can revert to a TK\(^-\) phenotype without losing the virus DNA sequences coding for TK and that TK\(^-\) cells are capable of re-acquiring the TK\(^+\) phenotype by reactivating the expression of the virus gene. Such an ability to modulate gene expression could result in a balance between the rates of reversion (from TK\(^+\) to TK\(^-\)) and back-reversion (from TK\(^-\) to TK\(^+\)) which, in turn, could give rise to the plateau in the fraction of TK\(^-\) cells observed in the AC\(1\) and AC\(5\) lines growing in \(\alpha\)-MEM. Experiments were therefore performed to determine whether such modulation of gene expression occurred in our system. Nine TK\(^-\) revertants were isolated by selection in \(\alpha\)-BrdUrd from AC\(1\), AC\(4\), and AC\(5\). Of these, eight lines failed to back revert to TK\(^+\) (no survivors from approx. \(10^8\) cells plated in \(\alpha\)-HAT) and one line exhibited only very low back reversion (1 survivor in \(2 \times 10^6\) cells). Since BrdUrd might have a mutagenic effect resulting
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Table 3. Reversion from TK\(^+\) to TK\(^-\) during growth in non-selective medium*

<table>
<thead>
<tr>
<th>Labelling period (h)</th>
<th>0-24</th>
<th>24-48</th>
<th>48-72</th>
<th>72-96</th>
<th>96-120</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC(_1) line</td>
<td>1.3</td>
<td>12.5</td>
<td>21.2</td>
<td>32.6</td>
<td>41.2</td>
</tr>
<tr>
<td>AC(_5) line</td>
<td>12.9</td>
<td>17.4</td>
<td>20.5</td>
<td>24.9</td>
<td>29.0</td>
</tr>
</tbody>
</table>

* Cells were plated out of α-HAT into α-MEM and labelled for 24 h with \(^3\)H-dThd at various time intervals. The % of TK\(^-\) cells was determined by scoring labelled and unlabelled cells following autoradiography.

in loss of TK activity or might select specifically for cells which have lost the ability to modulate the expression of TK, TK\(^-\) clones were also derived from transformants by plating single cells in non-selective medium. Several clones were isolated, labelled with \(^3\)H-dThd and screened after autoradiography. Parallel cultures of the clones which were negative for \(^3\)H-dThd incorporation were then plated in α-HAT to test their ability to re-express the TK gene. No HAT survivors were obtained from \(2 \times 10^4\) cells plated, indicating that under these conditions re-expression of the TK gene did not occur at a frequency higher than 0.005%.

The rate of forward reversion from TK\(^+\) to TK\(^-\) of two transformed lines, AC\(_1\) and AC\(_5\), was measured by plating cells out of α-HAT into α-MEM and labelling with \(^3\)H-dThd at various times thereafter. The fraction of TK\(^-\) cells was determined by counting unlabelled and labelled cells following autoradiography (Table 3). From the rate of increase in the proportion of TK\(^-\) cells, reversion rates of 0.11 and 0.042 per cell per generation were calculated for lines AC\(_1\) and AC\(_5\), respectively. Our results show that back reversion (from TK\(^-\) to TK\(^+\)) is too rare in comparison to the forward reversion (from TK\(^+\) to TK\(^-\)) to account for the plateau in the percentage of TK\(^-\) cells observed in the AC\(_1\) and AC\(_5\) lines (Fig. 1). Instead, it appears from measurements of generation times of TK\(^+\) cells and TK\(^-\) revertants (Fig. 2) that the plateau might result from an equilibrium between TK\(^+\) and TK\(^-\) cells due to differential growth rates of the two subpopulations (Fig. 2) and reversion from TK\(^+\) to TK\(^-\) (Table 3).

The reversion rates calculated from the data in Table 3 indicate that the probability of a transformed cell to lose the TK\(^+\) phenotype at each cell division, even in the absence of selective or mutagenic agents such as BrdUrd, is significantly higher than the mutation frequencies observed for mammalian cells (Chasin, 1974).

Acquisition of the TK\(^-\) phenotype could be brought about by several mechanisms: (1) physical loss of the virus DNA sequences coding for TK, (2) suppression of their activity, or (3) gene mutation. Because of the high reversion frequencies observed, mutation in either the structural TK gene or in control genes appears rather unlikely, especially as back reversion to TK\(^+\) almost never occurs. Although permanent suppression of the virus TK gene cannot be ruled out by our present data, the results of two different types of experiments would argue in favour of loss of the virus gene in TK\(^-\) cells.

Firstly, transformation of the phenotypically TK\(^-\) revertant clones to a TK\(^+\) phenotype can be readily achieved by a second treatment with virus DNA at efficiencies similar to those obtained in the initial transformation (our unpublished data). Thus, if the TK gene is merely suppressed, this suppression does not apparently prevent re-transformation, i.e., it does not affect the activity of a second TK gene. Secondly, superinfection with a TK\(^-\) mutant of HSV does not reactivate a silent virus TK gene possibly still present in the TK\(^-\) revertants, as it
Fig. 2. Cell doubling times were measured for the TK+ and TK− subpopulations of the AC1 line as described in Methods, ○—○, TK+ cells; ●—●, TK− cells.

has been found to do in other systems (Buttyan & Spear, 1977). As illustrated in Fig. 3b, superinfection of the AC4 transformed line with the TK− B2006 virus results in a three-fold stimulation of the activity of the endogenous virus gene, in agreement with data obtained in transformed mouse lines (Lin & Munyon, 1974; Leiden et al. 1976; Kit & Dubbs, 1977). However, no reactivation of TK activity was obtained upon superinfection of TK− revertants with the B2006 virus (Fig. 3a), an observation which is again consistent with the hypothesis of virus gene loss.

DISCUSSION

Analysis of several human lines carrying the HSV-2 TK gene has indicated that the transformed cells, though capable of continuous growth in selective medium, can revert to a TK− phenotype when grown in non-selective or counter-selective conditions. The reversion rate varies among the different transformants but appears to be characteristic of each independent line and its subclones. For some lines the frequency of reversion to the TK− phenotype is as high as 0.1 per cell per generation, even under non-selective conditions. This suggests that in our system loss of the TK+ phenotype is a spontaneous event rather than an effect induced by the mutagenic action of the counter-selective agent BrdUrd.

Davidson et al. (1973) have reported that mouse TK− cells transformed to TK+ by u.v.-inactivated HSV-1 can suppress and reactivate the virus gene at rather high frequency. In these cells, expression of the virus TK is apparently controlled by other viral, rather than
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Fig. 3. $^3$H-dThd incorporation measured as acid precipitable counts in: (a) •, 143 TK- cells infected with 1 p.f.u./cell of HSV-2 strain 219 (TK-); ○, 143 TK- cells infected with 2 p.f.u./cell of HSV-1 strain B2006 (TK-); ▲, ▲, TK- revertants infected with strain B2006 (2 p.f.u./cell); (b) ○, AC4 TK+ cells infected with strain B2006 (2 p.f.u./cell); •, AC4 TK+ cells, mock infected.

cellular, gene products (Lin & Munyon, 1974; Leiden et al. 1976; Buttyan & Spear, 1977; Kit & Dubbs, 1977). The DNA-transformed human cells carrying the HSV-2 TK gene which we have analysed behave quite differently. Although the expression of the endogenous virus gene in TK+ transformants appears to respond to stimulation by a superinfecting TK- virus, only rare instances of back reversion to a TK+ phenotype by TK- revertants and no reactivation of a silent virus gene by superinfection with a TK- mutant of HSV, have been observed in our system. Absence of back reversion to TK+ has been recently reported also by Minson et al. (1978) for TK- revertants of mouse cells transformed by HSV-2 DNA.

In studies somewhat similar to ours, Steinberg et al. (1978) have observed that revertants of SV40-transformed rat cells can be re-transformed as efficiently as primary rat cells if they have lost all or part of the virus genome, but are resistant to re-transformation if they still contain it without expressing it. Chadha et al. (1977) have also obtained re-transformation (albeit with a reduced efficiency) of TK- revertants of mouse cells transformed by HSV-1; in this case too, no detectable amounts of HSV DNA were present in the TK- cells. By analogy, the normal efficiency of re-transformation obtained with our TK- revertants might be taken as an indication that the virus DNA sequences coding for TK have been lost by the cells. Although direct evidence to support this hypothesis can be derived only from DNA re-association studies, all our data so far suggest that under non-selective conditions the virus gene can be lost with relatively high frequency from transformed cells and does not remain in a latent reactivable state in phenotypically TK- cells. Other explanations, such as suppression of gene activity by mutation within the structural gene or cis acting mutations outside it, are of course possible, though it is not clear why such mutations should occur with so high a frequency.

To account for both the high frequency of reversion to TK- and the rarity of back rever-
sion we suggest that in our system reversion occurs primarily by segregation of the cellular chromosome carrying the virus TK gene. Chromosome loss as a mechanism of phenotypic reversion has also been suggested recently for SV40-transformed rat cells (Steinberg et al. 1978). Since the human cells we have used as recipient for virus DNA have a quasi tetraploid karyotype, loss of certain chromosomes could probably occur without loss of cell viability. Particular chromosomes, moreover, might be lost more readily than others, depending for example on their ploidy in the cell. Thus the differences in the degree of stability of TK expression among transformants and the fact that subclones generally inherit the stability of the parental line might depend on the chromosomal location of the virus gene. Finally, chromosome loss might result in a reduction of growth rate which would explain the observation that some TK− revertants divide more slowly than their TK+ parents.

This model is supported by related studies reported by others. Firstly, Donner et al. (1977) and Smiley et al. (1978) have shown by somatic cell hybridization that in HSV-1 transformed human and mouse cells the virus TK gene is associated with one or a few chromosomes. Analysis of several independent transformants has not been carried out as yet but the possibility that the TK gene might be associated with different chromosomes in each transformed line seems likely. Secondly, Pellicer et al. (1978) have recently shown that in mouse cells transformed by restriction endonuclease fragments of HSV-1 DNA the virus TK gene is present as a single copy integrated into different sites in the genome of different transformants. Although chromosome segregation would account for most of the properties of our transformed lines, other mechanisms for the loss of TK activity are possible and indeed may also occur perhaps with lower frequency. More definitive proof that segregation is the dominant process will require the determination of the virus DNA content in the transformants and revertants as well as the identification of the chromosomal integration sites of the virus gene in different lines.

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