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Quantitation of the Viral DNA Present in Cells Transformed by UV-Irradiated Herpes Simplex Virus

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Two cell lines biochemically transformed by UV-irradiated herpes simplex virus (HSV) each contain virus DNA. A comparison of the kinetics of reassociation of ^{3}H-labeled HSV DNA in the presence and absence of either clone 139 (HSV-1 transformed) or clone 207 (HSV-2 transformed) DNA showed that the presence of transformed cell DNA increased the rate of reassociation of approximately 10% of the viral genome while having no effect on the remaining 90%. The $C_0t_{\frac{1}{2}}$ of this reaction was approximately 1,000 in each cell type, as compared to approximately 3,000 for the cellular unique sequences. These results suggest the presence of four to six copies of a 10% fragment of the virus DNA per cell. The DNA from a hamster fibroblast cell line morphologically transformed by UV-irradiated HSV-2 (333-8-9) did not affect the rate of reassociation of HSV-2 DNA, indicating that these cells had less than 3% of a viral genome present.

Herpes simplex virus types 1 (HSV-1) and 2 (HSV-2) have been used to transform both the morphological and biochemical properties of cells in tissue culture (3, 4, 6, 7, 13). Infection by UV-irradiated HSV-1 or HSV-2 is able to induce the formation of foci of morphologically transformed cells in primary cultures of hamster fibroblasts (6). A small proportion of these cells carries HSV antigens, as demonstrated by immunofluorescence, and is capable of causing tumors in hamsters.

Both murine and human cell lines lacking the enzyme thymidine kinase (tk) have acquired tk activity after infection by UV-irradiated HSV (13) (biochemically transformed cells). The electrophoretic mobility of the tk present in HSV biochemically transformed cells is different from that of the normal mouse or human enzyme (12). In addition, like the tk induced by HSV-2 infection, the tk activity in HSV-2-transformed cells is thermolabile when compared with the tk activity from wild-type cells or from cells transformed by HSV-1 (5). An HSV structural antigen also appears to be present on the surface of the HSV-transformed cells (K. Chada, personal communication).

Attempts to rescue infectious virus from the biochemically transformed cells by cocultivation with permissive cells, by UV irradiation, by growth in the presence of bromodeoxyuridine or iododeoxyuridine, and by amino acid starvation have proven unsuccessful (Davis, unpublished data; W. Munyon and E. Kraiselburd, personal communication). The continued presence of viral information is suggested by the presence of viral tk and antigens. Therefore, any viruses present in the transformed cells are either defective or repressed for some lytic functions.

Analysis of the kinetics of DNA reassociation has proven to be the most sensitive technique now available for detection of the small amount of viral DNA present in cells transformed by DNA viruses. This technique was initially employed to show that mouse cell lines transformed by simian virus 40 contained one to two copies of the simian virus 40 genome (8) and more recently to show that adenovirus 2-transformed rat cells contained only part of an adenovirus genome (14).

This study details the kinetics of reassociation of HSV DNA with DNA from cells transformed by HSV. The HSV biochemically transformed cells contain approximately 10% of an HSV genome. There was no detectable HSV DNA in a line of morphologically transformed cells.

MATERIALS AND METHODS

Cells and viruses. The parental Ltk cells and Ltk cells biochemically transformed by HSV-1 (clone 139) and by HSV-2 (clone 207) were obtained from W. Munyon and are described by Munyon et al. (13) and Davis and Munyon (5). NHF cells are diploid human foreskin fibroblasts originating in this laboratory. Line 333-8-9 is HSV-transformed hamster fibroblasts

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obtained from F. Rapp. BHK 21-C13 is a tissue culture line of hamster fibroblasts. HSV-1 JH was passaged from a primary isolate, HSV-2 Z570, isolated by A. Nahmias and obtained from M. Fiala. Studies from this laboratory (Sugino and Kingsbury, submitted for publication) have shown that these virus strains have a complete nucleic acid homology to those used to transform the above cells.

All cell cultures were grown at 37 C in Eagle minimum essential medium plus nonessential amino acids and 5 or 10% fetal calf serum with the following supplements: Ltk cells, 30 μg of bromodeoxyuridine per ml; clones 139 and 207, 6×10^{-7} M methotrexate, 1.6×10^{-5} M thymidine, 5.0×10^{-5} M adenosine, 5.0×10^{-5} M guanosine, and 1×10^{-4} M glycine.

Extraction of cellular DNA. DNA was prepared from cell monolayers removed from the culture flask by scraping and washed once with saline. Cell pellets (5 to 6 cm^3) were resuspended in 5 volumes of 0.15 M NaCl and 10^{-3} M EDTA and lysed by the addition of 1% sodium dodecyl sulfate, followed by incubation at 37 C for 1 h in the presence of 300 μg of self-digested Pronase per ml. The DNA was then shaken with an equal volume of freshly distilled phenol and chloroform (1:1). The aqueous phase was removed and extracted with an equal volume of chloroform, and the DNA was spooled after the addition of 2 volumes of cold 95% ethanol. The DNA was redissolved in 0.05 M Tris and 10^{-3} M EDTA, pH 7.7. The sodium ion concentration was raised to 0.2 M by the addition of 5 M NaCl, and the DNA was incubated at 37 C for 2 h in the presence of 200 μg of RNase per ml, followed by 2 h of Pronase digestion (100 μg/ml). This was followed by additional extractions in phenol-chloroform and chloroform followed by spooling from cold ethanol. The DNA was resuspended in 10^{-3} M EDTA, sheared, lyophilized, resuspended at the desired concentration, and dialysed against 5×10^{-3} M EDTA. DNA concentrations are based on the optical density at 260 nm. ^{32}P-labeled cellular DNA was prepared from Ltk cells grown in phosphate-free minimum essential medium containing 50 μCi of carrier-free [^{32}P]phosphate per ml harvested and extracted as above.

Shearing of DNA. The DNA was sheared at 0 C under an N_2 atmosphere for 2 min with a Branson sonifier. 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 of 500 μl, sheared ^{3}H-labeled HSV DNA was added to a reaction mixture containing 0.48 M phosphate buffer (pH 6.8) (PB), 5×10^{-3} M EDTA, and 3 to 5 mg of sheared cell DNA per ml. After denaturation at 115 C for 5 min in a sealed glass vial, the reaction mixture was quickly brought to the incubation temperature (65 or 75 C), and a zero time sample was taken. Successive samples were taken at predetermined times. Each sample was immediately diluted into 2.5 ml of 0.14 M PB containing 0.4% sodium dodecyl sulfate 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 0.30 M PB. The column effluents were adjusted to equal volumes and PB concentrations. They were then counted in PCS (Amersham/Searle) in a liquid scintillation counter. Percent reassociation was calculated by subtracting background and dividing counts in the 0.3 M PB effluent by the total number of counts in the sample.

Preparation of the ^{3}H-labeled HSV (type 1 and 2) DNA probes. Confluent NHF monolayers were infected at a multiplicity of 1 to 10, and the virus was labeled for 48 h in minimum essential medium without calf serum and containing 100 μCi of [^{3}H]thymidine per ml. The infected cells were then placed at 4 C overnight, and the virus was purified the following day. The purification involved removal of cell debris by spinning at 8,000 × *g* for 15 min, followed by a pelleting of the virus by centrifugation at 60,000 × *g* for 60 min. The resulting virus pellets were banded in a CsCl density gradient by centrifugation for 48 h. This treatment removed the outer viral membrane with any adhering contaminants and yielded primarily naked nucleocapsids. These nucleocapsids were disrupted by treatment with 2% Sarkosyl at 56 C. The resulting mixture was again subjected to CsCl gradient centrifugation for 48 h. After the density and radioactive profile were checked, the viral DNA peak was removed, dialyzed, and sheared for use. Each viral probe preparation was monitored for possible contamination by human DNA by hybridization to human placental DNA and by analytical CsCl gradients of both sheared and unsheared DNA.

RESULTS

Strategy of the experimental approach. The HSV-transformed cells were examined for the presence of HSV DNA by determining the effect of cell DNA on the rate of reassociation of ^{3}H-labeled HSV DNA. A very small amount of highly radioactive viral "probe" DNA was mixed with a large excess of unlabeled parental (Ltk) or transformed cell DNA. The mixture was dissociated into single strands, and the rate of conversion of the label into a double-stranded form was followed. The rate of reassociation of any nucleotide sequence is proportional to its concentration in the hybridization mixture; therefore, viral sequences present in the cellular DNA will increase the reassociation rate of that portion of the probe homologous to those sequences. Labeled probe sequences not represented in the cellular DNA will see a much lower concentration of homologous sequences and will, therefore, reassociate more slowly.

As described by Britten and Kohne (1), the extent of reassociation of any set of homologous sequences in hybridization mixture may be described by the equation:

$$\frac{1}{f_{ss}(t)} = 1 + k\,C_0 t \qquad \text{(i)}$$

where f_{ss} (t) is the fraction of DNA remaining single stranded at time t; C_0 is the total DNA concentration in solution (in moles of nucleotide per liter), and k is the second-order rate constant. If all of the different sequences in a hybridization mixture are present at equal concentrations, a plot of $1/f_{ss}$ (t) versus C_0t will be a straight line with slope k.

Examination of the biochemically transformed cells for HSV DNA. The base line control for these experiments was the kinetics of reassociation of a small amount of the ^{3}H-labeled HSV-2 DNA probe (specific activity, 8×10^6 counts/min per μg) in the presence of 5 mg of Ltk DNA per ml. As seen in Fig. 1, the experimental points fell on the straight line predicted by equation (i).

In the presence of 5 mg of clone 207 DNA (HSV-2-transformed Ltk cells) per ml, the experimental points for probe reassociation no longer fell on a straight line but, instead, fit a biphasic curve (Fig. 1). The initial rate of probe reassociation is much higher than the rate of probe reassociation in the presence of Ltk DNA. At higher C_0t values, the rate of probe reassociation in the presence of clone 207 DNA changed to approximately that exhibited in the presence of Ltk DNA. The addition of transformed cell DNA to the hybridization mixture increased the concentration of sequences homologous to only a restricted portion of the viral probe. Therefore, HSV DNA corresponding to a fraction of the viral genome was present in the cells biochemically transformed by HSV-2.

The fraction of the virus probe represented in the genome of the transformed cell is equal to the fraction of the probe reassociated ($1-f_{ss}$) after the initial (driven) portion of the reaction is completed (C_0t, approximately 10^4), corrected for the amount of probe reassociation observed in the Ltk reaction at the same C_0t (self-reassociation of probe). This calculation was performed for three experimental points in each of two experiments. The HSV-2-transformed cells contained approximately 10% of a viral genome. Similar experiments performed with cells transformed by HSV-1 are shown in Fig. 2. A fragment of HSV-1 DNA consisting of 7 to 11% of the viral genome was present in the cells biochemically transformed by HSV-1. The results were essentially the same when the experiment was repeated at 75 C. Reconstruction experiments at different numbers of viral copies per cell have been performed to evaluate the sensitivity of the detection. The results of these experiments were consistent with the findings presented here. At the high C_0t values used in this study, it is possible to easily differentiate between the fraction of a genome present at one copy or more per cell and one genome present in a fraction of the cells.

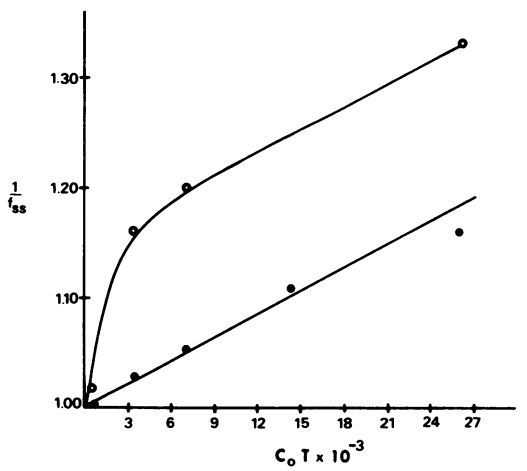

FIG. 1. *Kinetics of hybridization of ^{3}H-labeled HSV-2 DNA with DNA from cells biochemically transformed by HSV-2. The reassociation of 4×10^{-2} μg of ^{3}H-labeled HSV-2 DNA (specific activity, 8×10^6 counts/min per μg) per ml was determined in the presence of 5 mg of DNA per ml from clone 206 cells (O) or from the parental Ltk cells (●). The conditions of hybridization were as described in the text. The C_0t values have been corrected for salt concentration and represent C_0t equivalents in 0.12 M PB.*

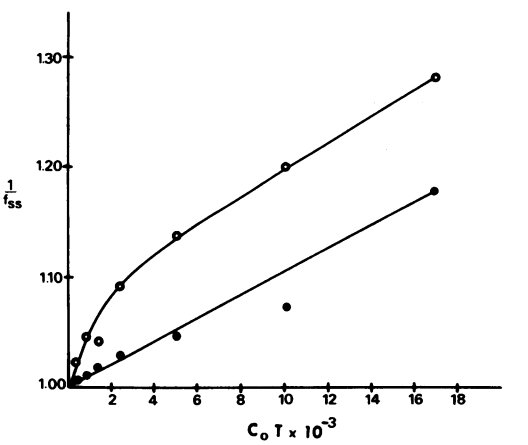

FIG. 2. *Kinetics of hybridization of ^{3}H-labeled HSV-1 DNA with DNA from cells biochemically transformed by HSV-1. The reassociation of 4×10^{-2} μg of ^{3}H-labeled HSV-1 DNA (specific activity, 1×10^7 counts/min per μg) was followed in the presence of 3 mg of DNA per ml from clone 139 cells (O) or from the parental Ltk cells (●). The conditions of hybridization were as described in the text. The C_0t values have been corrected for salt concentration and represent C_0t equivalents in 0.12 M PB.*

Determination of the number of virus copies in the biochemically transformed cells. Since the rate of reassociation of any given nucleotide sequence is a function of its concentration in the hybridization mixture, for any nucleotide sequence, the C_0t value at which half reassociation ($C_0t_{1/2}$) of that sequence is reached is inversely proportional to the concentration of that sequence in the hybridization mixture (1). Therefore,

$$C_T = \frac{(C_0t_{1/2})_L}{(C_0t_{1/2})_T} \times C_L \qquad \text{(ii)}$$

where C_L and C_T are the respective concentrations in copies per haploid cell genome of the unique sequences (single copy) present in Ltk DNA and the HSV fragment represented in the transformed cells. $(C_0t_{1/2})_L$ and $(C_0t_{1/2})_T$ are the C_0t values at half reassociation of the unique sequences and of the fragment present in the transformed cells.

In the preceding experiments, the $C_0t_{1/2}$ of the viral fragment present in the transformed cells was obtained directly from the experimental curves after subtracting the contribution due to probe self-reassociation. The $C_0t_{1/2}$ of the unique sequences present in Ltk DNA was determined by following the reassociation of ^{32}P-labeled Ltk DNA in the presence of a 100-fold excess of cold cellular DNA. The $C_0t_{1/2}$ was approximately 3,000, in good agreement with the value for L cell DNA (D. Kohne, personal communication). The results of these calculations are shown in Table 1. The HSV fragments in the biochemically transformed cells were present at a multiplicity of four to six copies per cell.

Examination of the morphologically transformed cells for HSV DNA. DNA was prepared from the 333-8-9 cell line of Duff and Rapp (6), a line that was transformed by UV-irradiated HSV-2 and selected on the basis of focus formation. At the time of isolation, this cell line exhibited viral antigens on the cell surface of approximately 3% of the cells. The DNA used in this study was prepared from cells at passage 100. At this passage no membrane or cytoplasmic viral antigens were demonstrable. These findings were confirmed in another laboratory (B. Hampar, personal communication).

The kinetics of reassociation of a ^{3}H-labeled HSV-2 probe were determined in the presence of a vast excess of unlabeled transformed cell DNA and as a control in the presence of DNA extracted from a line of nontransformed hamster fibroblasts (BHK 21, C13). As shown in Fig. 3, there was no detectable increase in the rate of probe reassociation in the presence of 333-8-9 DNA. If any HSV-2 DNA sequences were present in these cells, there was less than 3% of the HSV-2 genome.

DISCUSSION

Careful examination of cells biochemically transformed by either serological type of HSV indicates that multiple copies of a fragment of the virus genome is present. The HSV fragment present in both the HSV-1 (139)- and HSV-2 (207)-transformed cell lines is from 7 to 11% of the HSV genome. In both cell lines, this virus DNA fragment is present at a multiplicity of four to six copies per cell. These results are in general agreement with those of E. Kraiselburd (personal communication), who has detected approximately six copies of 15% of the HSV-1 genome in the clone 139 cells used in this study.

The presence of approximately the same fraction of the HSV genome in two independent lines of biochemically transformed cells suggests that viral coded functions in addition to the tk gene must be present either for the maintenance of the transforming DNA or for the control of the expression of the viral tk gene. The expression of the tk gene in the biochemically transformed cells appears to be under viral control. The enzyme activity in the biochemically transformed

TABLE 1. *Amount and number of copies of viral DNA present in HSV-transformed cell lines*

Cell Line	% of the HSV genome present	HSV fragment $C_0t_{1/2}$	$C_0t_{1/2}$ L cell DNA	No. of copies/haploid genome[a]
207[b]	10 ± 2	1,000 ± 200	2,800 ± 200	3.0
139[c]	9 ± 3	1,200 ± 200	2,800 ± 200	2.5
333-8-9[d]	0 ± 3			

[a] The number of copies per haploid genome has been corrected for the rate increase that accompanies high guanine plus cytosine DNA. The correction factor of Wetmur and Davidson (15) was used.
[b] Ltk cells biochemically transformed by HSV-2.
[c] Ltk cells biochemically transformed by HSV-1.
[d] Hamster fibroblasts morphologically transformed by HSV-2.

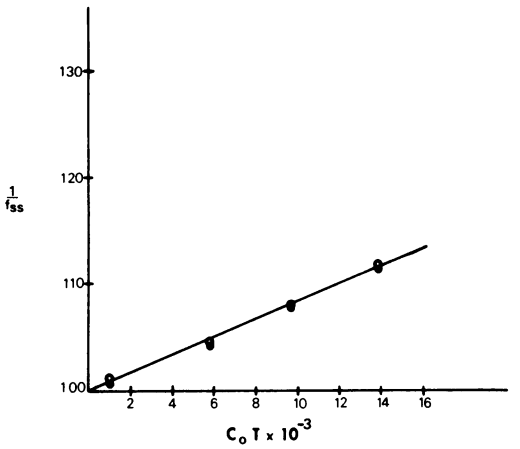

FIG. 3. *Kinetics of hybridization of ^{3}H-labeled HSV-2 DNA with DNA from hamster cells morphologically transformed by HSV-2. The reassociation of 4 × 10^{-2} μg of ^{3}H-labeled HSV-2 DNA (specific activity, 8 × 10^6 counts/min per μg) was determined in the presence of 5 mg of 333-8-9 DNA (○) per ml or 5 mg of BHK21C13 DNA (●) per ml. The conditions of hybridization were described in the text. The C_0t values have been corrected for salt concentration and represent C_0t equivalents in 0.12 M PB.*

cells is present in a different portion of the cell cycle than in normal L cells and continues to be expressed at a maximal rate long after the cells have reached confluence and DNA synthesis has ceased. Furthermore, infection of the biochemically transformed cells by a tk-less mutant of HSV causes an increase in tk activity, rather than the inhibition observed when normal L cells are infected by this mutant (10). This is consistent with the hypothesis that there is an HSV gene product which exerts a positive control on the level of expression of the HSV tk gene. The minimum size of the HSV fragment present in the biochemically transformed cells may, therefore, be determined by the distance between a control gene and the structural gene for tk. A maximum limit on the size of the fragment might then be determined by the placement of genes contributing to cytopathology. Another possibility is that a host restriction enzyme determines the size of the HSV fragment.

The presence of multiple copies of the HSV fragment in the biochemically transformed cells can be most easily explained by the possible need for a greater dosage of a relatively inactive viral tk to maintain the cells under selective conditions. During log-phase growth, the biochemically transformed cells have less tk activity than do normal L cells (10), notwithstanding the probable presence of multiple copies of the viral tk gene. A somewhat simpler explanation for the presence of multiple copies of a specific fragment of the HSV genome in the biochemically transformed cells is that the HSV particles effective in transformation contain multiple copies of this fragment. HSV-defective particles with a repeated fragment of the genome have been observed (N. Fraenkel, personal communication).

In sharp contrast to the biochemically transformed cells, a line of morphologically transformed hamster fibroblasts (333-8-9) did not contain a detectable amount of HSV DNA. These cells originally selected on the basis of focus formation were shown to be highly oncogenic when injected into newborn hamsters (6). At the time of isolation, 333-8-9 exhibited HSV-specific antigens (6) and HSV-specific RNA (2) complementary to approximately 11% of the genome. The studies reported here demonstrate that at the present passage level of these cells there is little or no viral DNA present. The limit of detection applied in these studies is between 2 and 5% of the HSV genome.

In addition to the lack of HSV-2 DNA in 333-8-9 cells, there was no detectable immunofluorescence when these cells were stained for viral antigens. The same cells were examined by B. Hampar, who also was unable to detect any cytoplasmic or membrane-associated HSV antigens (personal communication). The loss of antigens after repeated passage of HSV-transformed cells has also been reported by Li et al. (9).

It appears likely that during passage of the HSV-transformed hamster cells, the virus information originally present was lost. This result suggests that the HSV role in oncogenic transformation of cells may be different than that of other DNA tumor viruses where the continued presence of the viral DNA is required. An alternative explanation for these observations, of course, is the possibility that the morphologically transformed cells were not transformed by HSV but only carried HSV antigens for a number of passages. An examination of additional transformed cells and careful study of their passage history may help to clarify this mechanism.

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