Herpes Simplex Virus Nucleic Acid Synthesis Following Infection of Non-permissive XC Cells

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SUMMARY

DNA hybridization kinetic analysis of cellular DNA following high multiplicity infection of non-permissive XC cells by herpes simplex virus type 1 showed that HSV DNA penetrates to the nucleus of the cell but that the number of virus DNA copies present in each cell quickly begins to decline. There did not appear to be any net virus DNA synthesis and the loss of virus DNA copies continued until there was approximately one per haploid genome equivalent. HSV-2 likewise did not show any detectable virus DNA replication. The residual virus information was stable for more than 48 h. CsCl density gradient analysis of the infected cell DNA suggested an association between the HSV DNA and that of the cells. Network analysis also supported the suggestion that a stable association between the virus DNA and host DNA begins shortly after infection. Cell division resulted in the segregation of the virus DNA but not its loss from the cell population. Virus-specific RNA synthesis was easily detectable and 40 to 50% of a labelled DNA probe was converted to an RNA:DNA hybrid.

INTRODUCTION

XC cells, a line of Rous sarcoma virus-transformed Wistar rat fibroblasts (Svoboda, 1960) are non-permissive for herpes simplex virus type 1 or 2 (HSV-1, HSV-2; Docherty et al. 1973; Garfinkle & McAuslan, 1973). No infectious virus is produced nor is there any visible cytopathic effect following infection. Garfinkle & McAuslan (1973) have shown further that there is transient expression of a virus enzymatic activity (thymidine kinase) and HSV-specific surface antigens in infected cells; however, Docherty et al. (1973) were unable to demonstrate this activity. The nature of the replicative block and the exact step in the infective process at which it acts is unknown, although it is thought to be an early function and Becker et al. (1974) have suggested that HSV not grown in rat cells is unable to adsorb to XC cells. The blocking activity must be fairly specific since the normal rat cell comparable to the XC line is permissive for HSV-1 and HSV-2 and both cells are permissive for pseudorabies virus, another member of the herpesvirus group.

In the experiments reported here, we investigated the kinetics and extent of virus DNA replication and virus RNA production in HSV-1 and HSV-2 infected cells. We found no evidence that adsorption was impaired nor evidence of net virus DNA synthesis as assayed by hybridization of tritiated virus DNA probes to bulk DNA extracted from XC cells infected with HSV-1 or HSV-2. The HSV-1 RNA detected by hybridizing an HSV-1 virus

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DNA probe to HSV-1 infected cell RNA was representative of approximately the same fraction of the genome as seen by others during the late period of a productive infection (Frenkel & Roizman, 1972; Stringer et al. 1977).

**METHODS**

**Cells and viruses.** The XC cells were obtained from Dr Fred Jensen and were maintained on minimum essential medium (MEM) supplemented with non-essential amino acids and 10% foetal calf-serum. The HSV-1 and HSV-2 strains isolated in this laboratory from active lesions are designated JH and RM, respectively. HSV-1 Nii was obtained from B. Hampar. Virus stocks were prepared in a line of normal human fibroblasts (NHF), originating in this laboratory, used at passage levels less than 15 from the primary culture.

**Viral and cellular hybridization probes.** Production of viral probes has been described previously (Davis & Kingsbury, 1976). Briefly, the virus was grown in NHF cells in the presence of 70 μCi/ml [3H]-thymidine (NEN), the virions were banded on CsCl density gradients (average density 1.28 g/ml), lysed with 0.2% sarkosyl and the DNA was banded in CsCl density gradients (average density 1.728 g/ml). The [32P]-cellular DNA was made by growing XC cells in phosphate-free MEM supplemented with 10% foetal calf serum plus 5 mCi of [32P]Pi. After 2 days of growth, the DNA was extracted using the MUP procedure explained below.

**Extraction of DNA.** Bulk DNA was extracted by the 8 M-urea, 0.24 M-phosphate buffer (MUP) technique of Britten et al. (1974). The cells were lysed in the above solution by addition of 1% SDS and the DNA sheared by multiple passage through a 26 gauge needle or high speed blending. The lysate was passed over a large column of hydroxyapatite (HA) and the column extensively washed with MUP plus 1% SDS followed by MUP alone then by 0.12 M-phosphate buffer (equimolar concentrations of mono and dibasic Na+ phosphate, pH 6.8: PB). The purified DNA was eluted from the column with 0.48 M-PB and dialysed extensively against 10⁻⁴ M-EDTA. After dialysis the DNA was lyophilized to dryness and resuspended in 0.48 M-PB. DNA from purified cell nuclei was prepared in the same way following the isolation of the nuclei by the NP40 technique of Lerner et al. (1971). The high mol. wt. DNA utilized in the network formation experiments was extracted by the phenol extraction procedure described previously (Davis & Kingsbury, 1976). In every case following the ‘spooling’ of the DNA from cold ethanol, the fluid was centrifuged to ensure quantitative recovery of the cellular DNA.

**Extraction of RNA.** Cells were lysed in 0.15 M-NaCl, 0.015 M-EDTA and 1% SDS. The lysate was extracted with a 50:50 re-distilled phenol-chloroform mixture followed by two extractions with chloroform. The nucleic acid was precipitated overnight at -20 °C in 2.5 vol. of 95% ethanol and the precipitate removed by centrifugation. The DNA and small (less than 5S) RNA was removed by the 3 M-sodium acetate, 5 mM-EDTA procedure of Palmiter (1973). The remaining RNA was extracted once more in chloroform, precipitated in ethanol, centrifuged and resuspended in H₂O. Such RNA preparations were always > 95% alkali-labile.

**Measurement of DNA-DNA reassociation kinetics.** Approximately 5×10⁶ XC cells were infected with HSV-1 at a multiplicity of 3 p.f.u./cell or HSV-2 at a multiplicity of 0.1 p.f.u./cell for each determination. At various times after the 30 min adsorption period the cells were washed with saline, trypsinized from the glass, pelleted and washed with saline, lysed and the DNA extracted using the MUP procedure outlined above. The DNA was sheared and concentrated to 1 to 2 mg/ml. Small amounts of [3H]-labelled HSV DNA probe (sp. act. 8×10⁶ ct/min/μg) and [32P]-labelled uninfected XC DNA (sp. act. 5×10⁴ ct/min/μg) were
added and a sample was taken to determine the final DNA concentration and the fraction of the undenatured probe DNA that would not stick to HA. The DNA was denatured by heating to 115 °C for 3 min in a sealed vial. The reaction was quickly brought to the incubation temperature of 65 °C and a zero time sample taken. Samples were taken at various times during the reaction and assayed on HA columns for the percentage of total probe counts that were in double stranded form.

Measurement of RNA-DNA reassociation kinetics. Approximately 1 × 10⁶ XC cells were infected with HSV-I at a multiplicity of 3 p.f.u./cell, and following 10 h of infection, the RNA was extracted as described above. The RNA was hybridized to the HSV-I probe in a vol. of 400 μl containing 1·1 mg of RNA in 0·48 M-PB. For these reactions the probe DNA was denatured by itself, quick-cooled in an ice bath and added to the RNA. A zero-time sample was taken and the reaction was brought to the incubation temperature of 65 °C. Samples taken from the reaction were assayed for hybrid formation on HA columns.

Hydroxyapatite (HA) column chromatography. The samples removed from the reactions were immediately diluted into 2·0 ml of 0·14 M-PB containing 0·4% SDS and passed over a HA column equilibrated to 60 °C. The column was washed with 12 ml of 0·14 M-PB, 0·4% SDS to remove the single stranded nucleic acids and then washed with 7 ml of 0·3 M-PB to remove the reassociated double stranded material. The column effluents were adjusted to equal volumes and PB concentrations and counted in Aquasol-2 (NEN).

Infection with labelled virus. Labelled HSV-I virions were produced as described for the hybridization probes. They were purified by centrifugation on a 15 to 40% sucrose density gradient at 39000 g for 40 min in a Spinco SW 41 rotor. The visible virus band was removed, diluted with 0·15 M-NaCl and used to infect approx. 5 × 10⁷ XC cells for each time point. A multiplicity of 10 p.f.u./cell was used. At various times after infection, the cells were washed with saline and lysed in 3 ml of 0·15 mg/ml Pronase, 0·15 M-NaCl, 0·015 M-EDTA and 0·1% sarkosyl. This lysate was incubated for 2 h at 37 °C and then added directly to CsCl (mean density of 1·728 g/ml) either with or without shearing. The gradients were centrifuged in a Spinco Ti 50 fixed angle rotor at 44000 rev/min for at least 36 h at 15 °C. The gradient fractions were collected directly on to small paper squares, dried, washed in cold 10% TCA followed by 95% ethanol, than dried and counted.

Network analysis. Roller bottle cultures of XC cells were infected at multiplicities of 2 to 3 p.f.u./cell prior to confluency. After a 30 min adsorption the cells were fed with complete medium and incubated for a pre-determined time. Following incubation the cells were washed with saline and removed from the glass by trypsinization. Following two additional washes with saline the cells were resuspended in 0·15 M-NaCl, 10⁻³ M-EDTA, lysed by the addition of 1% SDS and digested with Pronase. This was followed by a gentle phenol extraction.

The formation of DNA networks was similar to that reported by Varmus et al. (1973) and Bellett (1975). High mol. wt. DNA at a concentration of 1·25 mg/ml in 0·48 M-PB was denatured at 110 °C for 4 min followed by reannealing at 60 °C to obtain a Cₐ of 100 mol. s/l. The mixture was diluted twofold in 0·48 M-PB at 60 °C and centrifuged in a Spinco SW 50·1 rotor at 114000 g for 20 min at 10 °C. The supernatant was removed, dialysed, and lyophilized prior to suspension in 10⁻³ M-EDTA and shearing. The pellet was suspended directly in 10⁻³ M-EDTA and sheared.

The amount of HSV DNA present in the supernatant and pellet fractions was estimated by DNA hybridization kinetics as described above and elsewhere (Davis & Kingsbury, 1976). The number of virus copies present in each fraction was calculated by correction of the determined number of virus copies per cell to account for the portion of the cellular
Table 1. Copies of HSV DNA in infected cells

<table>
<thead>
<tr>
<th>Time p.i. (h)</th>
<th>Virus C₄t₄*</th>
<th>Number of virus DNA† copies/ haploid genome</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 5 (nuclear only)†</td>
<td>465±200</td>
<td>4·3±2·0</td>
</tr>
<tr>
<td>3</td>
<td>750±200</td>
<td>3·0±0·3</td>
</tr>
<tr>
<td>6</td>
<td>1363±200</td>
<td>1·5±0·3</td>
</tr>
<tr>
<td>12</td>
<td>2058±200</td>
<td>0·97±0·3</td>
</tr>
<tr>
<td>24</td>
<td>2085±200</td>
<td>0·96±0·3</td>
</tr>
<tr>
<td>HSV-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>7974±1000</td>
<td>0·25±0·3</td>
</tr>
<tr>
<td>6</td>
<td>9504±1000</td>
<td>0·21±0·3</td>
</tr>
<tr>
<td>12</td>
<td>15980±3000</td>
<td>0·13±0·3</td>
</tr>
</tbody>
</table>

* Corrected for the GC content of HSV DNA (see text).
† Calculated on the basis of a C₄t₄ of the host unique sequences of 2000. Copies = \( \frac{2000}{\text{C₄t₄ virus probe}} \).
‡ Nuclear preparation as described in Methods.

Fig. 1. Computer generated least squares best fit plots of the kinetics of hybridization of HSV-1 probe with DNA extracted from infected XC cells at (a) 0, (b) 3, (c) 6, and (d) 24 h p.i. The C₄t values are corrected for salt concentration and expressed as C₄t equivalent to the rate in 0·12 MPB. The reaction conditions are as described in Methods. Generally, 2 mg/ml of XC DNA was reacted with 2 to 4 x 10⁻⁴ μg/ml of ³H-HSV DNA. Radioactive ³²P-labeled XC DNA was added to each reaction at a concentration of 1 μg/ml and the rate of XC DNA reassociation was followed. The derivation of %R is explained in the text.
RESULTS

Location and number of copies of HSV DNA in infected XC cells

The initial experiments in this study were designed to determine whether the replicative block in the XC cell was due to defective transport of the virus DNA into the cell nucleus. The results of these experiments are shown in Table 1. Virtually all of the cell-associated HSV DNA reached the nucleus by 30 min after the adsorption period (30 min). Therefore, the remainder of the work to be described here was done on DNA extracted from whole cells.

The results of the hybridization of labelled HSV DNA to infected XC cell DNA extracted at various times after infection are shown in Fig. 1 and 2. These curves have been corrected for minor variations in salt and DNA concentrations by setting the Cot ½ of the host unique sequences, as determined by the reassociation of the ³²P-labelled normal XC DNA probe, to 2000. This is an arbitrary value, but it is in good agreement with the results of Rice (1971) and represents an average value of our own experiments. Under the conditions of these experiments, the unique sequences of the XC cell genome comprise about 60% of the total DNA. The virus reassociation curves are expressed in terms of % R which is the percentage of the virus probe that has reassociated at a given Cot. This value differs from the simple percentage of the probe, which is double stranded, by two corrections. (1) The raw percentage of total counts bound to HA is divided by 1 minus the fraction of the undenatured probe that will not bind to HA. The non-binding fraction was always less than 10% of the total probe activity. (2) Due to the problem of a high zero-time fraction (snap-back) in HSV DNA (D. T. Kingsbury & D. E. Kohne unpublished data), this value must be further corrected by subtracting from it the percentage that sticks to HA in the zero-time fraction and dividing this by 1 minus the zero-time fraction, usually 8%. The data is then used to generate a computer determined least squares best fit curve using the formula

\[
\frac{1}{F_a(t)} = 1 + KC_{0t}
\]
Fig. 3. CsCl density gradient profiles of 3H-labelled input virus DNA at 6 and 19 h after infection of XC cells. Infected cell DNA (○—○) was extracted and mixed with 32P-labelled XC DNA (□—□). A portion of each mixture was sheared and the sheared and unsheared sample added to CsCl density gradients and centrifuged to equilibrium in Spinco Ti 50 rotor at 44,000 rev/min. Density increases from right to left as shown. The 0 h sample served as a trapping control and demonstrated that under these conditions virus DNA can be completely separated from cellular DNA. In each case the 32P-XC DNA serves as a marker to determine the density profile of the gradient.

where \( f_s(t) \) is the fraction of the probe that is single stranded at time \( t \), \( K \) is an experimentally determined rate constant equal to \( 1/C_0t_{1/2} \), \( C_0 \) is the initial DNA concentration in the reaction (in mol. of nucleotide/l) as determined by the \( A_{260} \) value and \( t \) is the time in seconds from the start of the reaction (Britten & Kohne, 1968). By varying the value of \( K \), the best fit is found by the computer and a \( C_0t_{1/2} \) value for the virus probe in each reaction is derived and used to determine the number of virus DNA copies that are present per haploid host genome equivalent in each reaction. Because of the high sp. act. of the probe used in these experiments, the probe self-reaction was always less than 10%.

To determine the number of virus DNA copies present, the \( C_0t_{1/2} \) of the host unique sequences is divided by the \( C_0t_{1/2} \) of the virus reassociation reaction. The virus \( C_0t_{1/2} \) must first be corrected for the underestimate caused by the high guanine-cytosine content of the HSV DNA. The correction factor for the GC content is a linear extrapolation of the data of Wetmur & Davidson (1968) to the 69% GC content of HSV (Graham et al. 1972) and has a value of 1.36. The experimental \( C_0t_{1/2} \) values are multiplied by this number to give \( C_0t_{1/2} \) values.
Herpes simplex virus in XC cells

Table 2. Distribution of DNA in network experiments

<table>
<thead>
<tr>
<th>DNA</th>
<th>Total DNA</th>
<th>HSV DNA*</th>
<th>Copies/cell HSV DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% network</td>
<td>% supernatant</td>
<td>% network</td>
</tr>
<tr>
<td>Human placenta+ 4 copies HSV</td>
<td>77</td>
<td>23</td>
<td>15</td>
</tr>
<tr>
<td>XC DNA 0.5 h p.i.</td>
<td>87</td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td>XC DNA 1.5 h p.i.</td>
<td>67</td>
<td>33</td>
<td>13</td>
</tr>
<tr>
<td>XC DNA 16 h p.i.</td>
<td>75</td>
<td>25</td>
<td>36</td>
</tr>
<tr>
<td>XC DNA 24 h p.i.</td>
<td>88</td>
<td>12</td>
<td>50</td>
</tr>
</tbody>
</table>

* Percentage virus DNA is corrected for 15% trapping evident in the human placenta HSV control reaction. This value is added to the supernatant value and subtracted from the network value.

that are directly comparable to the host values for the virus copies/haploid genome determination.

The number of virus DNA copies present at various times after infection is summarized in the last column of Table 1. The values for the HSV-1 infected cells indicate that there is a rapid loss of virus copies in excess of 1 copy per haploid genome between 0 and 6 h post infection (p.i.). This loss stops at the single copy level after 6 h p.i. and remains at this level for 24 h p.i. This would indicate that little, if any, HSV-1 DNA replication takes place in XC cells. The HSV-2 data also argue against virus DNA replication although the maintenance of virus copies over time is not as clear as for HSV-1. However, virus sequences can still be detected 12 h after infection even though the input multiplicity was very low.

To investigate this maintenance phenomenon further, a single bottle of XC cells was infected with HSV-1 at a multiplicity of 3 p.f.u./cell and the cells passed at 24 h p.i. and then again 3 days later to give 15 bottles of cells. These cells were harvested at confluency, their DNA extracted and assayed as above for HSV-1 sequences. A small reaction was detectable, but it was not sufficient to calculate reliably the number of virus genomes present or determine whether a whole copy was still present. It was clear, however, that the single copy per cell present at 24 h p.i. was not maintained through the division and growth of the cells. The limit of detection in these experiments is approx. 1 copy per 20 cells (Davis & Kingsbury, 1976).

Physical state of virus DNA in infected cells

Since the virus DNA has a substantially higher density in CsCl gradients than the host DNA, it should be possible to determine whether the virus sequences are associated with the host DNA by infecting XC cells with 3H-thymidine labelled HSV-1, gently extracting the DNA and observing whether the virus label is found at host or virus density in CsCl gradients. The gradient profiles are shown in Fig. 3 and suggest some form of association. The unsheared DNA extracted immediately after infection shows very little virus label running with the host marker. At 6 h p.i. two virus label peaks were seen in the unsheared DNA gradient, at virus and host densities. Upon shearing this DNA, the virus label shows a single peak at virus density with some trail-off into the host density region. At 19 h p.i. virtually all of the 3H-labelled material runs with the host marker in the unsheared preparation while, after shearing, about half runs at virus density. The remainder of the 3H-labelled material may be host DNA which has incorporated re-cycled labelled thymidine released by the breakdown of superfluous virus DNA.
Fig. 4. Computer generated plot of the hybridization of $^3$H-labelled HSV-1 DNA to bulk cellular RNA extracted from infected XC cells 10 h p.i. The reactions were done as described in Methods. The percentage of reactable probe converted to a double stranded form (%R, see text) was assayed by hydroxyapatite chromatography.

An additional means to test the physical state of the HSV genome in infected cells is 'network analysis' (Bellett, 1975; Varmus et al. 1973). If the HSV remaining in the cell after infection is associated with the chromosome, then HSV sequences will be found attached to the repeated sequences of the XC DNA, whereas if the HSV DNA is free, there will be no such association. When high mol. wt. mammalian DNA is denatured and reannealed, the majority of the DNA forms a very high mol. wt. complex resulting from the reassociation of the repeated sequences found on many different stands of the DNA (network). Centrifugation results in the fractionation of the DNA into a pellet composed of the repeated sequences and the DNA associated with them, and supernatant consisting of unique sequences found only distantly from the repeated sequences and separated by chance breakage. By using specific hybridization probes the relative amounts of virus DNA in each fraction are easily determined.

The principal technical problem in analysing network experiments is to have a proper control for non-specific trapping of free DNA into the network. Table 2 contains the data obtained in trapping control experiments. When HSV-1 DNA was added to high mol. wt. human DNA followed by network formation, 77% of the human DNA was found in the network pellet while 15% of the virus DNA was in the network. This control figure of 15% was used in the remainder of the experiments described here and was deducted from the percentage of the virus DNA found in the network and added to the supernatant fraction. This procedure leads to a slight underestimate of the percentage of the DNA in the network.

Table 2 also contains the results of network analysis of XC cells infected with HSV-1 examined at various times post infection. In all of these experiments, 67 to 88% of the cellular DNA entered the network. The amount of HSV DNA in the network was dependent on the time after infection. At 0.5 h no HSV DNA was found in the network pellet. At 1.5 h the number of virus copies per cell had begun to decline as demonstrated earlier; however, 13% of the remaining sequences were found in the network. By 16 h p.i. 36% of
the remaining sequences were in the network and after 24 h 50% of the sequences sedimented with the network.

**Transcription and translation of viral gene products**

It has been suggested by others that at least some HSV antigens and enzymes are produced in XC cells following infection (Garfinkle & McAuslan, 1973). Thus, at least limited transcription of the virus genome takes place. To determine the extent of transcription, RNA extracted from HSV-1 infected XC cells after 10 h of infection was assayed for the ability to drive denatured HSV-1 DNA probe into RNA-DNA hybrids. The reaction was assayed on hydroxyapatite columns as described in Methods and the results are shown in Fig. 4. The meaning of % R is the same as described earlier and the extent of probe self reaction in these experiments was less than 5%; the reaction seems to give a minimum of 45 to 50% of the total genome represented in the RNA. In other experiments (data not shown) transcription could be detected as early as 1 h p.i. and had disappeared by 18 h.

**DISCUSSION**

The infection of XC cells by herpes simplex virus clearly proceeds through virus DNA transcription but is blocked at a step essential for normal replication of HSV DNA. Either the function of a virus protein is inhibited or some host component necessary for replication is inactive or non-existent. Transcription of the virus genome seems to be sufficient to supply message for most, if not all, virus functions although the quantity of RNA made is very small. This underproduction of RNA transcripts may simply reflect the lack of virus gene amplification resulting from DNA replication.

The expression of the virus genetic information appears to be transient as shown by Garfinkle & McAuslan (1973), and suggested in the present study. The shut-off of transcription occurs despite the continued presence of virus DNA. A possible explanation of this is that the virus DNA associated with that of the host is incapable of supporting continued transcription and that all the expression of virus genes results from transcription of free virus DNA. Alternatively, virus DNA sequences could be degraded rapidly at late times. The kinetics of expression of virus genes in HSV-1 infection observed by Garfinkle & McAuslan (1973) showed a peak at 12 h p.i. for both thymidine kinase activity and HSV-1 antigens on the cell surface. The multiplicities used in their experiments were higher (10 p.f.u./cell) than in this study (3 p.f.u./cell) suggesting that free virus copies may persist longer after higher multiplicity infections. The input label gradients presented here support this last point since free virus DNA is present at 6 h p.i. using multiplicities of 10 p.f.u./cell.

The association of HSV with the XC cell genome may provide an explanation for the observations of Garfinkle & McAuslan (1973) that in CsCl density gradients of DNA from infected cells labelled for 4 h after infection, the label ran slightly denser than DNA from uninfected cells. We have also observed such a shift toward virus density and interpret it as resulting from limited replication associated with the insertion of unlabelled HSV DNA into the host genome. This covalent attachment between virus and cellular DNA is suggested from the present studies where virus DNA was demonstrated in the host density region of cell gradients and in repeated sequence networks.

None of the properties described here appears to be the explanation for the non-permissiveness of the XC cell for HSV. The block occurs after virus RNA transcription but before virus DNA replication. There is obviously some condition directly or indirectly concerned with DNA replication that is absent or blocked in the XC cell.
REFERENCES


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