Inhibition of Herpes Simplex Virus Type 1 Specific Translation in Cells Treated with Poly(rI).poly(rC)

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SUMMARY
Primary African green monkey kidney cells (GMK) treated with poly(rI).poly(rC) in the presence of DEAE-dextran ('treated cells') developed antiviral resistance and concomitantly released interferon into the medium. Treated and untreated cells were infected with herpes simplex virus type 1 (HSV1) in the presence of cytosine arabinoside (araC), and total RNA was isolated and hybridized with purified radio-labelled HSV1 DNA. The intracellular concentration of virus-specific transcripts was not significantly altered in treated cells, but a smaller proportion of the genome of HSV1 hybridized with the extracted RNA. Transcription was similarly restricted when protein synthesis was inhibited by cycloheximide.

To analyse virus translation, proteins were radiolabelled between 6 and 10 h after infection and were immunoprecipitated with a pool of human sera and run on SDS–polyacrylamide gels. No virus-specific proteins could be detected in treated cells. In contrast about 25 HSV1-induced proteins were found in infected cells and about 22 proteins in cells infected in the presence of araC. In particular, two virus proteins with apparent mol. wt. of 128 000 and 42 500 were immunoprecipitated. Since these two were also detected in cells under conditions where elongation of polypeptide chains was non-specifically retarded, it is unlikely that a similar mechanism was responsible for the impaired growth of HSV1 in our treated cells. We conclude that this impairment probably resulted from regulation at the level of virus translation, probably mediated through interferon.

INTRODUCTION

With most RNA viruses studied, the primary site of interferon action is probably at the level of virus mRNA translation (see review by Friedman, 1977), though this has recently been disputed in relation to vesicular stomatitis virus (Baxt et al. 1977; Marcus & Sekellick, 1978). With the DNA viruses, vaccinia and SV40, there are conflicting views about the main effects of interferon. Thus with SV40, arguments have been put forward in favour and against an effect on SV40 uncoating (Yamamoto et al. 1975; Mozes & Defendi, 1978) and a primary effect on early SV40 transcription (Oxman & Levin, 1971; Brandner & Mueller, 1974). Viruses of the herpes group are also sensitive to interferon, e.g. herpes simplex virus (Glasgow & Habel, 1962; Rasmussen & Farley, 1975) and Epstein–Barr virus (Adams et al. 1975). However, there have as yet been no detailed studies of the interaction of interferon with any member of this virus group.

We report here on the growth of herpes simplex virus type 1 (HSV1) in primary African
green monkey kidney (GMK) cells treated with polyriboinosinic:polyribocytidylic acid complex [poly(rI).poly(rC)] in the presence of diethyl-amino-ethyl (DEAE)-dextran (Dianzani et al. 1968), which are termed ‘treated’ cells in this paper. Although this treatment may have had other effects, it is known that it leads to the formation of more than 100 units of interferon per ml in the supernatant after 18 h (Brandner et al. 1974). We believe that this treatment can be used to study the effects of interferon without the complications which may result from the impurities present in available interferon preparations.

We have studied the functions of the HSV1 genome in treated cells by separating 6-3H-thymidine-labelled DNA from the infected cells by CsCl density equilibrium centrifugation. The formation of virus transcripts was investigated by measuring the reassociation kinetics of total RNA with 3H-labelled HSV1-DNA. Virus translation was monitored by immunofluorescence microscopy and by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of immunoprecipitated HSV1-proteins. We found that in the treated cells, synthesis of virus DNA and protein was impaired, but not synthesis of virus-specific RNA. We conclude that the effect on HSV1 replication was mainly at the level of translation and probably mediated through interferon.

METH.ODS

Cells. Primary cultures of African green monkey kidney (GMK) cells were prepared by conventional techniques. Secondary rabbit kidney (RK) cells were derived from 6-week-old animals.

Preparation of HSV1. HSV1, strain McIntyre (American type culture collection), was propagated (o.1 p.f.u./cell) in RK cells in roller bottles. The virus was harvested after about 48 h, when the c.p.e. was marked. After centrifugation (15000 g, 30 min) the supernatant contained about 10^{6.7} p.f.u./ml when assayed as described by Russell (1962).

Poly(rI).poly(rC) treatment. Confluent Petri dish (150 cm²) cultures of GMK cells were treated with 20 μg/ml of poly(rI).poly(rC) (from Dynatec, Nuertingen) together with 400 μg/ml of DEAE-dextran (Pharmacia, Freiburg) in serum-free Eagle’s minimal essential medium (E-MEM). The supernatant was removed 15 min later, the cells were washed with serum-free medium and fresh medium containing 5% foetal bovine serum (FBS) was added. After an 18 h incubation period, the cultures were infected with HSV1.

Extraction of RNA from HSV1-infected cells. RNA was isolated according to Levin et al. (1969). After three extractions with freshly distilled phenol at 66 °C, the RNA was precipitated with ethanol, treated with DNase (40 μg/ml, from Boehringer, Mannheim) for 60 min at 37 °C, re-extracted with phenol, again precipitated with ethanol, and dialysed against 0.1 × SSC (0.15 M-NaCl, 0.015 M-trisodium citrate) supplemented with 1 mM-EDTA.

Preparation of HSV1-3H-DNA. One roller bottle culture containing 8 × 10^7 RK cells was infected with 5 p.f.u./cell of HSV1 in serum-free medium. The virus suspension was withdrawn after 2 h and 100 ml of medium containing 5% FBS were added; 9 h later the medium was replaced by 30 ml medium containing 30 mCi 6-3H-thymidine (25 Ci/mmol, from Amersham-Buchler, Braunschweig). The cells were harvested 6 h later and DNA was isolated according to Colter et al. (1962). Purification of phenol-treated DNA was performed by successive precipitation with ethanol, incubation with bovine pancreatic RNase (25 μg/ml, from Boehringer, Mannheim) at 37 °C for 45 min, further phenol treatment and dialysis against 0.1 × SSC containing 1 mM-EDTA. To separate virus DNA (density 1.726 g/ml) from the cellular DNA (1.70 g/ml), CsCl (0.765 g/ml of DNA solution) was added and the solution was centrifuged at 34000 rev/min in a Beckman Ti 60 rotor for 48 h at 25 °C. The peak fractions at a density of 1.726 g/ml were pooled and CsCl density gradient fractionation was repeated. CsCl was removed by dialysis against 0.1 × SSC. The resulting 3H-HSV1 DNA exhibited 1.9 × 10^{6} ct/min/μg.

Estimation of virus DNA synthesis in HSV1 infected cells. Petri dish cultures (150 cm²)
of GMK cells were treated with poly(rI), poly(rC) and 18 h later the cultures were infected with HSV1 as described in Results. Two h before cell harvest, the volume of medium was reduced to 5 ml and 6-3H-thymidine (40 μCi/ml) was added. Total DNA was isolated, and after CsCl equilibrium density centrifugation, the radioactivity and absorbance profiles of virus and cellular DNA were determined.

Reassociation kinetics of 3H-HSV1-DNA incubated with total RNA from HSV1-infected cells. 3H-HSV1 DNA was fragmented by sonication to 6 to 7S in size. By adding unlabelled salmon sperm DNA or rabbit liver RNA, the final concentration of nucleic acids was adjusted to about 40 A260/ml. Reassociation was performed in 50 μl siliconized glass capillaries (Brend micropipettes). Incubation mixtures contained 0.020 μg/ml to 0.200 μg/ml of 3H-HSV1 DNA and/or 0.09 to 1.6 mg/ml of total cell RNA in PIPES buffer [10 mM-piperazine-N,N'bis(2-ethane sulphonic acid), 300 mM-NaCl, 1 mM-EDTA, pH 6.7]. The sealed capillaries were heated to 115 °C for 8 min to denature the DNA and RNA and then quickly chilled to −5 °C. Reassociation incubation was performed at 72 °C. At the end of the incubation period (2 to 26 days), the capillaries were chilled in ice. The contents were transferred into 100 μl S1 buffer (30 mM-sodium acetate-acetic acid, 300 mM-NaCl, 2.5 mM-ZnSO4, pH 4.6) containing 8 μg salmon sperm DNA and then frozen at −70 °C. The extent of reassociation was estimated by S1 nuclease (EC 3.1.4.2.) degradation of the remaining single-stranded (ss) DNA that had not reassociated (at 45 °C for 2 h). S1 nuclease was prepared from Aspergillus oryzae (Vogt, 1973), donated by Dr B. Hirt, Lausanne; 50 μl of 0.3 % bovine serum albumin and 5 ml of 5 % trichloroacetic acid (TCA) were added and the samples filtered through Whatman GF/C glass fibre filters and washed twice with 10 ml cold TCA (5 %). Percent hybridization was calculated by subtraction of the S1 nuclease-resistant fraction of samples without previous incubation for reassociation from the S1 nuclease-resistant fraction of reassociated samples.

3H-DNA/DNA reassociation kinetics analysed by using S1 nuclease show a significant deviation from ideal second order kinetics and can be corrected by applying the formula

$$\frac{C_{i}}{C_{0}} = \left[\frac{1}{1 + k C_{0} t}\right]^{n}$$

where n = 0.453 (Smith et al. 1975); (C0, total concentration of DNA; Ci, concentration of ssDNA at time t in moles of nucleotides/litre; k, association rate constant in m⁻¹×s⁻¹).

By transformation, this gives

$$\frac{C_{i}^{1/n}}{t - C_{i}^{1/n}} = \frac{1}{K^{n}} \frac{1}{C_{0} t}$$

For the initial points of each reassociation kinetic, the corrected ratio of single-strand to double-strand fraction was plotted against the reciprocal C0t-value. From the slope of the resultant straight line, the association rate K was determined and the reassociation curves were calculated from equation A. C0t is the product of DNA concentration and time in seconds (Britten et al. 1974); C1t is the product of RNA concentration and time (mol. s/l).

Immunofluorescence microscopy and antisera. In all experiments, the Petri dish cultures contained glass coverslips (24 × 24 mm). These were washed in phosphate-buffered saline (PBS) and fixed in acetone:methanol 1:1 for 5 min at −18 °C. Formation of HSV1 antigens was assayed by indirect complement immunofluorescence with sera from rabbits infected with HSV1 (donated by Dr H.-W. Doerr, Heidelberg). Alternatively, human anti-HSV1 sera were used in an indirect immunofluorescence test: the sera were selected for a positive immunofluorescence reaction with GMK cells infected with HSV1 and simultaneously treated with cytosine-β-arabinofuranoside (araC; 40 μg/ml) in order to detect a high serum titre against HSV1 proteins made in the absence of virus DNA synthesis. Six of these human sera with a CF-titre of 1:80 to 1:320 were pooled and the IgG-fraction
was purified by DEAE-Sephasel (Pharmacia, Freiburg) column separation (Joustra & Lundgren, 1969).

**Labelling of proteins.** Subconfluent Petri dish (28 cm²) cultures of GMK cells (approx. 2 x 10⁶ cells) were treated as described above and infected with HSV₁ (1 to 10 p.f.u./ml). The virus suspension was removed 1 h later and the cells were incubated from 6 to 10 h p.i. in 0.2 ml of medium, free of unlabelled L-methionine and containing 100 μCi of L-³⁵S-methionine (sp. act. 890 Ci/mmol; Amersham-Buchler, Braunschweig). After labelling, the monolayers were washed, lysed and further fractionated into a NP₄₀ soluble 'cytoplasmic extract' and a 'nuclear pellet' as described by Mann *et al.* (1977).

**Immunoprecipitation.** The soluble cytoplasmic extract was further clarified by centrifugation for 2 min at 12000 g. For each experimental parameter 100 μl of the extract containing the same amount of TCA-precipitable ct/min (about 6 x 10⁶ ct/min for a typical experiment) were first incubated with 30 μl of a human HSV₁-negative serum (tested by complement fixation and by indirect immunofluorescence in a dilution of 1:5) for 16 h at 0 °C. The IgG-protein was then removed by the addition of 50 μl of protein-A-Sepharose suspension [1 g protein-A-Sepharose (Pharmacia, Freiburg) per 5 ml TNE-buffer (10 mM-tris-HCl, pH 7.6; 100 mM-NaCl; 1 mM-EDTA; 1% Na-azide)]. After incubation for 45 min at 0 °C the resulting complex was pelleted for 2 min at 12000 g. This procedure was essential to reduce the amount of non-specific precipitation. The supernatant was then incubated for 16 h at 4 °C with 40 μl of the IgG-fraction of the pooled human HSV₁ antisera. After incubation, 50 μl protein-A-Sepharose were added. The mixture was pelleted at 12000 g for 2 min and washed twice with 500 μl of a buffer containing 10 mM-tris-HCl, pH 7.6, 1 M-NaCl, 1 mM-EDTA, 0.5% NP₄₀ and 1% deoxycholate, and finally twice with 500 μl of a buffer containing 1 mM-tris-HCl, pH 7.6; 10 mM-NaCl, 0.1 mM-EDTA. The antigen-antibody-protein-A-Sepharose complex was resuspended in 50 μl of electrophoresis sample buffer (0.0625 M-tris-HCl, pH 6.8; 2% SDS; 4% mercaptoethanol; 10% glycerol), boiled for 5 min at 100 °C and centrifuged at 12000 g for 2 min. A sample of the supernatant was counted in a toluene-based scintillation fluid for radioactivity and 1 to 15 μl samples per slot were applied on analytical SDS-polyacrylamide gels.

**Polyacrylamide gel electrophoresis and radiofluorography.** SDS-PAGE was performed by the method of Laemmli (1970). After electrophoresis, the gels were prepared for fluorography as described by Bonner & Laskey (1974). *Escherichia coli* RNA polymerase β- and β'-chain (mol. wt. 150000 and 160000), phosphorylase a (94000), bovine serum albumin (68000), ovalbumin (43000), α-chymotrypsinogen (25000) and cytochrome c (12500) served as markers for mol. wt. determinations.

**RESULTS**

**Inhibition of HSV₁ DNA synthesis by poly(rI).poly(rC)**

The replication cycle of HSV₁ is about 25 to 30 h in GMK cells infected at a multiplicity of 10 p.f.u./cell (data not shown). Cells were stimulated to synthesize interferon with poly(rI).poly(rC) and DEAE-dextran as described in Methods. Controls were treated with DEAE-dextran only. DNA was labelled for 10 to 12 h and for 20 to 22 h after infection with ⁶³⁵H thymidine. Total DNA was isolated and the cell and virus DNA were separated by CsCl equilibrium density centrifugation as shown in Fig. 1 for the 20 to 22 h label. Fig. 2 demonstrates quantitatively the specific radioactivity incorporated in cell and virus DNA. We observed a complete inhibition of virus DNA synthesis in the treated GMK cells, whereas in mock-infected cells cellular DNA synthesis was not significantly altered.

In contrast to poly(rI).poly(rC) treatment of GMK cells, we observed that treatment
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Fig. 1. Profiles of host cell DNA and HSV1 virus DNA labelled with 6-3H-thymidine (20 to 22 h p.i.) from poly(rI).poly(rC)-treated monkey cells analysed by CsCl density centrifugation. MI, mock infected; pI:pC, poly(rI).poly(rC).

Fig. 2. Incorporation of 6-3H-thymidine in host cell DNA and virus DNA separated by CsCl density centrifugation as shown in Fig. 1. The radioactivity profiles were normalized for 1 A260 unit of total DNA. MI, mock infected; pI:pC, poly(rI).poly(rC), Cell DNA; virus DNA.
Self-annealing of \(^{3}H\)-HSV\(_1\) DNA and calibration of the reassociation kinetics

Since, in treated cells, very small amounts of virus-specific RNA might need to be detected, we optimized the sensitivity and accuracy of our hybridization assay. The results of self-annealing of 1 to 10 ng per 50 \(\mu\)l of \(^{3}H\)-HSV\(_1\) DNA in the presence of varying amounts of DNA and RNA from uninfected cells yielded a sigmoidal reassociation curve (Fig. 3a) with a relative \(C_{\alpha1}\) of 0.19 mol. s/l (\(Na^{+} = 0.3M\)), amounts corresponding to 0.44 mol. s/l under standard conditions with 0.12 M-phosphate (Britten & Smith, 1970).

Calibration experiments with a constant input of \(^{3}H\)-HSV\(_1\) DNA, hybridized in the presence of increasing amounts of unlabelled HSV\(_1\) DNA, gave the typical deviation from ideal second order kinetics (Smith et al. 1975) which was corrected as described in Methods; the resulting graphically obtained ratios of the \(C_{\alpha1}\) values relative to the input \(^{3}H\)-HSV\(_1\) DNA coincided fairly well with the theoretically expected values (Fig. 3b). On the basis of these calibration experiments, an input of 1 to 2 ng (1900 to 3800 ct/min) of \(^{3}H\)-HSV\(_1\)
Analysis of HSV1-specific RNA in treated and untreated GMK cells in the presence of araC

Total RNA was isolated from Petri dish cultures of treated GMK cells, which were infected with HSV1 18 h later in the presence and absence of araC (40 μg/ml). The RNA was hybridized in solution with 3H-HSV1 DNA. Controls consisted of infected and uninfected cells pre-treated with DEAE-dextran only. Formation of virus proteins was monitored by immunofluorescence microscopy of coverslip cultures grown in the same Petri dishes, and virus antigen synthesis was inhibited by more than 98% by poly(rI), poly(rC). When compared with control cells infected for 10 h, the pool size of virus RNA was drastically diminished in treated cells (Fig. 4b). However, the extent of this could be predicted since formation of and transcription from newly made virus DNA continued in the infected control cells, whereas in treated cells, synthesis of new DNA was prevented (see above). If formation of virus DNA was inhibited by araC, comparable amounts of HSV1 RNA were detected in these and in treated cells (Fig. 4b). However, when treated cells were infected...
in the presence of araC, the pool size of virus RNA was reproducibly higher (two- to three-
fold), as shown in Fig. 4(a), indicating accumulation of early virus transcripts under these
conditions. Late in infection (22 h p.i.) we did not observe such an accumulation of virus-
specific RNA (data not shown).

RNA isolated 10 h p.i. from GMK cells drove 45 to 50% of the 3H-HSV1 DNA into
a DNA–RNA hybrid (Fig. 4b). When GMK cells were infected in the presence of araC,
early the same saturation level of annealed DNA (40 to 45%) was reached (Fig. 4b).
However, in cells treated with poly(rI). poly(rC) the portion of the virus genome transcribed
in the presence (Fig. 4a) or absence of araC (Fig. 4b) was smaller than in non-treated
controls. Such HSV1 RNA exhibited a restriction of saturation level to 30 to 35%, after
correction for superimposed self-annealing of the 3H-HSV1 DNA.

Analysis of HSV1-specific RNA pool size in the presence of cycloheximide

To see whether a translational block would affect accumulation of virus RNA and restrict
the transcription of the virus genome, as found in poly(rI), poly(rC)-treated cells, we assayed
the formation of HSV1 RNA in the presence of cycloheximide (40 µg/ml). An accumulation
of virus RNA is frequently observed in other virus–cell systems (Esteban & Metz, 1973;
Ben-Porat et al. 1974; Brandner & Mueller, 1974; Jean et al. 1974; Kozak & Roizman, 1974).
We found that cycloheximide had only a slight effect on the pool size of HSV1 RNA com-
pared with cells infected in the presence of araC (Fig. 4a) or pre-treated with poly(rI).
poly(rC) (Fig. 4b). In the presence of cycloheximide we observed restricted transcription of
the HSV1 genome, as shown by other investigators (Kozak & Roizman, 1974; Swanstrom
& Wagner, 1974; Swanstrom et al. 1975). The DNA–RNA hybridization curve reached a
saturation level at 30 to 35% of annealed DNA (Fig. 4b). Thus, after induction of interferon
by poly(rI). poly(rC), the transcription pattern of the HSV1 genome may be restricted in a
similar way to that caused by treatment with cycloheximide.

Immunoprecipitation of HSV1 proteins synthesized in the presence of araC

In coverslip cultures of GMK cells infected with HSV1, the proportion of virus-positive
cells varied in different batches from 55 to 75%. In the presence of araC the values ranged
from 35 to 60%. Without exception, in all infected (1 p.f.u./cell) cultures treated with
poly(rI). poly(rC) and assayed 10 h p.i., the number of cells positive for HSV1 antigens was
at least 98% less than in untreated control cells.

Since the sensitivity of the immunofluorescence assay may not be sufficient to detect tiny
amounts of HSV1 proteins, we also analysed the virus-specific translation by SDS–PAGE of
L-35S-methionine-labelled proteins. Since host protein synthesis is only partially switched
off in HSV1-infected (10 h p.i.) GMK cells, we enhanced the sensitivity of the method by a
preceding immunoprecipitation step (see Methods). We detected about 25 proteins from
the cytoplasmic extract of infected cells (infected cell protein, ICP) which were infected with
1 p.f.u./cell of HSV1 and labelled from 6 to 10 h p.i. with L-35S-methionine. In the presence
of araC under the same conditions, the pattern of ICPs was identical with the exception of
three proteins (Fig. 5). Two major bands appeared in the protein profiles of the infected and
araC-treated cells after immunoprecipitation with the human serum pool: ICP 128 000,
the major HSV1 envelope protein, and ICP 42 500, presumably a non-structural protein.

Immunoprecipitation analysis of HSV1 proteins in treated GMK cells

The immunoprecipitation technique was applied to poly(rI). poly(rC)-treated GMK cell
cultures infected with HSV1 (1 p.f.u./cell) in the presence or absence of araC and labelled
from 6 to 10 h p.i. with L-35S methionine. Control cells were mock-infected or not treated
Fig. 5. SDS–polyacrylamide gel electrophoresis of virus-specific proteins from HSV1-infected (b) and araC-treated (c) GMK cells after immunoprecipitation with a human serum pool. (a) Mock-infected cells (b) HSV1-infected cells; (c) cells infected in the presence of araC (40 µg/ml). GMK cells were infected with 1 p.f.u./cell and labelled with [35S]methionine from 6 to 10 h p.i. Immunoprecipitation was carried out as described in Methods. Clearly detectable virus-specific proteins not immunoprecipitable from cytoplasmic extracts of uninfected cells are indicated with arrows. (The apparent mol. wt. of these proteins are noted on the right hand side.) The figure shows the radiofluorogram of a 6 to 12.5 % gradient gel; 1 µl of the lysed antigen–antibody complex of each parameter was applied to the gel.

with poly(rI).poly(rC). Cytoplasmic extracts were prepared and identical amounts of TCA-precipitable radioactivity (6 x 10⁶ ct/min) were analysed per parameter. Following immunoprecipitation, the antigen–antibody–protein-A–Sepharose complex was lysed and 5 µl fractions were tested for radioactivity as described above. As shown in Table 1 (lines 4 to 9), no radioactivity exceeding the background level could be immunoprecipitated from cytoplasmic extracts prepared from infected, treated GMK cells. A subsequent separation by SDS–polyacrylamide slab gels confirmed this result. No virus-specific ICPs were detected, regardless of the presence or absence of araC (Fig. 6, slots e and f); in contrast, samples isolated from the untreated controls showed a pattern of ICPs similar to that described in the previous section (Fig. 6, slots c and d).
Table 1. Immunoprecipitation with a human serum pool of HSV1-specific antigens from cytoplasmic extracts of GMK cells treated with different metabolic inhibitors and labelled from 6 to 10 h p.i. with $^{35}$S-methionine

<table>
<thead>
<tr>
<th>Line*</th>
<th>Treatment†</th>
<th>Sp. act. (ct/min/mg protein $\times 10^{-7}$)</th>
<th>TCA-precipitable ct/min used for immunoprecipitation assay ($\times 10^{-6}$)‡</th>
<th>Total immunoprecipitated precipitates by SDS-PAGE see:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 MI</td>
<td>HSV1</td>
<td>6.9</td>
<td>12.0</td>
<td>3.0</td>
</tr>
<tr>
<td>2 HSV1</td>
<td>araC</td>
<td>4.8</td>
<td></td>
<td>29</td>
</tr>
<tr>
<td>3 HSV1</td>
<td>araC</td>
<td>5.4</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>4 MI</td>
<td>HSV1</td>
<td>3.3</td>
<td></td>
<td>1.6</td>
</tr>
<tr>
<td>5 MI</td>
<td>araC</td>
<td>3.0</td>
<td></td>
<td>1.7</td>
</tr>
<tr>
<td>6 HSV1</td>
<td>araC</td>
<td>3.2</td>
<td></td>
<td>3.4</td>
</tr>
<tr>
<td>7 HSV1</td>
<td>araC</td>
<td>2.8</td>
<td>6.0</td>
<td>2.4</td>
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<tr>
<td>8 HSV1</td>
<td>araC</td>
<td>3.8</td>
<td></td>
<td>1.6</td>
</tr>
<tr>
<td>9 HSV1</td>
<td>araC</td>
<td>2.1</td>
<td></td>
<td>1.4</td>
</tr>
<tr>
<td>10 MI</td>
<td>CH</td>
<td>0.6</td>
<td>0.6</td>
<td>0.30</td>
</tr>
<tr>
<td>11 HSV1</td>
<td>CH</td>
<td>0.4</td>
<td></td>
<td>0.36</td>
</tr>
</tbody>
</table>

* Lines 1 to 3 and lines 4 to 11 represent two experiments performed with different batches of GMK cells.
† MI, mock-infected; HSV1, infected with 1 p.f.u./cell; araC, cytosine arabinoside (40 µg/ml); pl: pC, poly(rI): poly(rC); CH, cycloheximide (0.2 µM).
‡ For experimental details of immunoprecipitation assay see Methods.
§ The antigen-antibody-protein-A-Sepharose complex was lysed in 50 µl sample buffer.

Fig. 6. Analysis of HSV1-specific proteins synthesized in treated GMK cells by SDS-PAGE after immunoprecipitation with a human serum pool. (a) Mock-infected cells; (b) mock-infected cells treated with poly(rI): poly(rC) and araC; (c) HSV1-infected cells; (d) infected cells in the presence of araC; (e) treated cells infected with HSV1; (f) treated cells infected in the presence of araC. The pAGMK cells were infected with HSV (1 p.f.u./cell), labelled with L-$^{35}$S methionine from 6 to 10 h p.i. and extracted. The major immunoprecipitable virus-specific proteins are indicated by arrows and by the corresponding apparent mol. wt. The figure shows the upper part of the radiofluorogram of a 6 to 15% gradient gel with 5 µl of the lysed antigen–antibody complex of each extract applied.
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Fig. 7. Immunoprecipitation of HSV1-specific proteins synthesized in the presence of 0.2 μM-cycloheximide analysed by SDS–PAGE. (a) Mock-infected cells (MI); (b) infected cells (1 p.f.u./cell). The GMK cells were labelled with L-35S-methionine from 6 to 10 h p.i. in the presence of 0.2 μM-cycloheximide. Extraction and immunoprecipitation were carried out as described in Methods; 15 μl of the lysed antigen-antibody complex of each extract were applied to a 7.5% SDS gel.

Immunoprecipitation of HSV1 proteins synthesized in the presence of cycloheximide

Recently Yau et al. (1978) showed that the effects of interferon and of very low concentrations of cycloheximide were very similar with respect to synthesis of the proteins of encephalomyocarditis, vesicular stomatitis and murine leukaemia viruses. They concluded that one of the primary mechanisms of interferon action may be a non-specific retardation of one or more elongation steps. Therefore we were interested to see whether cycloheximide might similarly influence HSV1-specific protein synthesis in GMK cells.

Petri dish cultures of GMK cells were infected with HSV1 (1 p.f.u./cell) in the presence of a low concentration of cycloheximide (0.2 μM). The cells were labelled with L-35S-methionine from 6 to 10 h p.i. and prepared for immunoprecipitation. Parallel coverslip cultures were examined at 10 h p.i. for virus-specific antigens, and a diffuse, faint but significantly positive fluorescence was seen in about 15% of the cells. However, the overall polypeptide synthesis rate (measured as incorporation of TCA-precipitable radioactivity per mg protein) was also reduced to 35% of the rate in uninfected and to 25% of that in infected control cells (Table 1, lines 4 and 10, 6 and 11). On the other hand, in poly(rI):poly(rC)-treated cells, the protein synthesis rate was not significantly altered (Table 1, lines 5 and 8). Nevertheless, even in the presence of cycloheximide (0.2 μM), the synthesis of ICP 128000 and ICP 42 500 could be demonstrated (Fig. 7): none was found in samples from poly(rI).
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poly(rC)-treated and HSV1-infected cells, containing ten times as much TCA-precipitable radioactivity (Fig. 6, slot e and f). Thus it is unlikely that a similar mechanism was responsible for the impaired growth of HSV1 in our treated cells.

DISCUSSION

Primary GMK cells can be used to study the action of interferon or poly(rI).poly(rC) (Oxman & Levin, 1971; Brandner & Mueller, 1974; Brandner et al. 1974), as again demonstrated in the study. Host macromolecular synthesis was not significantly impaired by the treatment with poly(rI).poly(rC) and DEAE-dextran which we have used.

HSV1 DNA replication was found to be inhibited by more than 98% in treated cells. Regardless of whether poly(rI).poly(rC) first affected virus transcription or translation, inhibition of virus translation by cycloheximide led to a similar inhibition of HSV1 DNA synthesis (Roizman & Roane, 1964; Swanstrom et al. 1975). Note that the suppression of cellular DNA synthesis by HSV1 infection was not abolished in the treated cell, which suggests that the suppression was caused by a constituent of the infecting virus particles, as Fenwick & Walker (1978) have recently postulated for HSV2. If this inhibition was effected by a HSV1 immediate-early or early protein, at least a partial derepression under poly(rI).poly(rC) treatment would have been expected.

We believe, from our calibration experiments, that we could accurately estimate virus DNA and RNA by reassociation in solution. In araC-treated cells virus transcription was restricted to involve only about 40 to 45% of the total genome. Although Swanstrom et al. (1975) found different results, our observations agree with many other investigators who have shown by other methods that the majority of HSV1 genome can be transcribed (Harris & Wildy, 1975; Clements et al. 1977) and translated (Powell et al. 1975; Roizman et al. 1975) in the absence of virus DNA replication.

Estimation of the HSV1 RNA pool size in poly(rI).poly(rC)-treated cells was one of the two main objectives of this study. The results clearly indicate that the pool size of virus RNA was not significantly impaired in treated cells. On the contrary, we repeatedly observed that, relatively early after infection, virus RNA even increased in treated cells compared to cells infected in the presence of araC. However, since the pool size at a given moment is the result of RNA formation and decay, we cannot decide which was affected by poly(rI).poly(rC) treatment. Impaired decay could result from binding of mRNA to polysomes followed by an inhibition of subsequent processing. To simulate this situation, we used cycloheximide in a transcription experiment, but we observed no increase or decrease in RNA pool size. On the other hand, we observed that the overall RNA synthesis rate, measured as incorporation of 5,6-3H-uridine, decreased in the presence of cycloheximide by about 45% compared to the untreated control (data not shown). Therefore it seems likely that the observed hybridization rates reflect two opposing effects of cycloheximide, accumulation of RNA on the one hand and diminished synthesis of HSV1 RNA on the other.

The annealing kinetics of RNA isolated from infected cells pre-treated with poly(rI).poly(rC) were similar to those of cells infected in the presence of cycloheximide. Both curves reached a saturation level of 30 to 35%, implying similar restriction of the HSV1 genome transcription. Unfortunately, available data on HSV1 genome expression in the presence of cycloheximide are contradictory (Kozak & Roizman, 1974; Swanstrom et al. 1975; Clements et al. 1977; Jones et al. 1977). Nevertheless, our findings with cycloheximide (cf. Clements et al. 1977) support the assumption that, under conditions of interference, one or more regulator proteins, which normally caused the transcription of an additional portion of the HSV1 genome, are not synthesized.

The second main objective of this study was to estimate the effect of poly(rI).poly(rC)
on HSV1 genome translation by immunofluorescence microscopy and immunoprecipitation of virus-specific proteins. We found that the number of HSV1 antigen-positive cells was reduced by more than 98%. We also found HSV1 proteins in cells infected in the presence of araC, in agreement with other investigators, who have shown that most HSV1 proteins can be translated in the absence of virus DNA replication (Honess & Roizman, 1974; Powell et al. 1975).

Immunoprecipitation of L-35S-methionine-labelled HSV1 proteins, with subsequent SDS–gel electrophoresis and radiofluorography, confirmed the immunofluorescence data. Our main interest was that virus-specific proteins could be detected in cells infected with low multiplicities in the presence of araC. At 10 h p.i. we immunoprecipitated, with a human sera pool, about 25 proteins that did not occur in uninfected cells; the same proteins were found if araC was present during infection. This was evidently not the case with extract from poly(rI).poly(rC)-treated cells. Interestingly our human serum pool predominantly precipitated proteins with apparent mol. wt. of 128000 and 42500 from araC-treated, infected cell extracts. Efficient antigenic determinants and/or an enhanced synthesis in the presence of araC may both be responsible for these pronounced immunoprecipitations. Both ICPs belong to the β-class; ICP 128000 is the major envelope protein and ICP 42500 is presumably a non-structural virus-specific protein, probably the virus-specific deoxythymidine kinase (Thouless & Wildy, 1975; Courtney et al. 1976; Morse et al. 1978). Also from immunoprecipitation experiments, we concluded that non-specific retardation of an elongation step of polypeptide synthesis, such as results from a low concentration of cycloheximide (Yau et al. 1978), may be a subordinate rather than the primary mechanism of the effect of poly(rI).poly(rC) treatment of HSV1-infected GMK cells.

In conclusion, we have shown that poly(rI).poly(rC) treatment induces an antiviral state in cells; on infection with HSV1, these cells retain the ability to transcribe the virus genome, but virus-specific translation is efficiently suppressed and, as a result, DNA replication is also inhibited. This interpretation is based, however, on the assumption that the virus RNA (or at least the portion of the immediate-early transcripts) made in poly(rI).poly(rC)-treated GMK cells is biologically active. Our results demonstrate that the genome expression of this DNA-containing virus is inhibited at a post-transcriptional level, probably by primary inhibition of virus translation by interferon.

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