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Evidence That Herpes Simplex Virus DNA Is Transcribed by Cellular RNA Polymerase B

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In herpes simplex virus type 1 (HSV-1)-infected HEp-2 cells, amanitin added before or at various times after infection always reduced viral multiplication. Also, the three waves of transcription of HSV-1 DNA, which led to the synthesis of α, β-, and γ-polypeptides, were all sensitive to amanitin in HEp-2 cells, and the amanitin-sensitive RNA polymerase activities of isolated nuclei were equally sensitive to the inhibitor before and during the infection. On the contrary, HSV-1 DNA transcription was totally unaffected by amanitin in AR1/9-5B cells, a mutant subline of CHO cells that possesses an amanitin-resistant RNA polymerase B. Together, these results strongly suggest that HSV-1 DNA utilizes for its transcription a polymerase undistinguishable from host cell RNA polymerase B with respect to its sensitivity to amanitin.

Honess and Roizman (14, 15) reported that in HEp-2 cells infected with herpes simplex virus type 1 (HSV-1), viral polypeptides form three groups, designated as α, β, and γ, whose synthesis is coordinately regulated and sequentially ordered. It was also reported (19) that the transition from α- to β-polypeptide synthesis requires a new wave of transcription in the presence of functional α-polypeptides. Similarly, the transition from β to γ also requires a new wave of transcription.

Thus far it is not known whether all three waves of transcription, which lead to the synthesis of α-, β-, and γ-polypeptides, are catalyzed by one or more RNA polymerases (EC 2.7.7.6); moreover, the genetic source of this enzyme(s), i.e., whether it is coded by the viral and/or host cell genome, has not been identified.

To investigate these problems, we examined whether all three waves of transcription in HEp-2 cells were sensitive to amanitin, a toxin that allows division of nuclear eukaryotic RNA polymerases into two categories: amanitin insensitive (RNA polymerase A) and amanitin sensitive (RNA polymerase B) (5). The observation that HSV-1 replication is reduced in chicken embryo fibroblasts pretreated with this toxin (17) led to the conclusion that at least one wave of transcription is sensitive to amanitin.

To determine the target of amanitin, i.e., whether it acted on host cell or viral transcriptase, we tested the effect of the toxin on HSV-1 transcription in AR1/9-5B cells, a mutant subline of Chinese hamster ovary (CHO) cells obtained by Chan et al. (6). These cells are not affected by amanitin, since they possess an amanitin-resistant RNA polymerase B; thus it could be predicted that in this host, HSV-1 transcription would be insensitive to amanitin if the virus uses host cell RNA polymerase.

As an indicator of the presence of functional α-, β-, and γ-mRNA's in HSV-1-infected cells treated with amanitin, we chose to examine the electrophoretically separated polypeptides made in these cells, because the products of a transcription wave may be easily identified from the polypeptides belonging to that group. This experimental approach had the advantage that it could be performed in whole cells under physiological control, which would be lost in a cell-free transcribing system (1-3). Since the three transcription waves are coordinately regulated, our experiments were designed so that, in the case of β- and γ-polypeptide mRNA synthesis, an indirect effect due to the action of amanitin on the preceding wave could be excluded.

Although under standard conditions amanitin acts very slowly on in vitro-cultured cells (9), this experimental approach was made possible by our previous observation that a short pretreatment of cell cultures with DEAE-dextran remarkably accelerates the action of amanitins: a 70% inhibition of total RNA synthesis is attained in 2 h in HEp-2 and BHK cells, without an appreciable toxic effect (10).

MATERIALS AND METHODS

Cells. HEp-2 cells were grown in Eagle minimum essential medium (EMEM) containing 10% inactivated fetal bovine serum. Maintenance medium for

infected cells consisted of EMEM containing 1% fetal bovine serum.

The AR1/9-5B subline of CHO cells (6) and CHO cells (16) were grown in α-medium (21) containing 5% inactivated fetal bovine and 5% calf sera. Maintenance medium for infected cells consisted of α-medium containing 1% fetal bovine serum.

Confluent cultures of HEp-2 and AR1/9-5B CHO cells were infected 24 and 36 h after seeding, respectively.

Viruses. HSV-1 strains used in these studies were the following: HSV-1(F) (8), which underwent no more than six passages in HEp-2 cells at a multiplicity of 0.02 PFU/cell; and HSV-1(MP) (12), which was passaged in HEp-2 cells infected at 0.01 PFU/cell. In the experiments reported below, the multiplicities of infection, unless otherwise stated, were 10 and 5 PFU/cell for HSV-1(F) and HSV-1(MP), respectively.

Infectivity titrations were performed in HEp-2 cell monolayers incubated at 37°C for 48 h in the presence of 0.16 mg of human gamma globulins per ml of maintenance medium, and the titers were expressed as PFU per milliliter.

The radiolabeling and purification of enveloped virus particles followed the procedure previously described (4).

Solutions. Protein-labeling medium consisted of maintenance medium containing 1/10 the normal concentration of amino acids, 1% dialyzed calf serum, and 2 μCi of ^{14}C-labeled amino acids from *Chlorella* hydrolysate per ml (57 mCi/matom; The Radiochemical Centre, Amersham).

RNA labeling was done in maintenance medium containing 1% dialyzed calf serum and 1 μCi of [^{3}H]-uridine per ml (29 Ci/mmol; The Radiochemical Centre, Amersham) in the presence of a 20-fold excess of unlabeled thymidine.

α- and β-amanitins were dissolved in phosphate-buffered saline lacking calcium and magnesium (PBS-A) at concentration of 1 mg/ml and were further diluted in the media.

Cycloheximide was dissolved in maintenance medium to yield a final concentration of 50 μg/ml (14).

DEAE-dextran pretreatment. DEAE-dextran pretreatment was performed, as previously described (10), just before cultures of either HEp-2 or AR1/9-5B CHO cells were treated with β-amanitin. Briefly, cell monolayers were washed twice with PBS-A and kept in contact with a prewarmed solution containing DEAE-dextran (500 μg/ml, molecular weight 2×10^6; Pharmacia) and glucose (1 mg/ml) in PBS-A for 15 min at 37°C, washed three times with PBS-A, and refed with maintenance medium containing β-amanitin (30 μg/ml). Untreated cultures were pretreated with DEAE-dextran and refed with normal maintenance medium. DEAE-dextran pretreatment was not performed in the experiment in which AR1/9-5B CHO cells were treated with 2 μg of α-amanitin per ml from seeding.

RNA synthesis determination. RNA synthesis was measured by pulse labeling the monolayers with [^{3}H]uridine for 30 min; cells were then harvested in 0.01 M Tris-hydrochloride-buffered saline, pH 7.5, precipitated with cold 10% $HClO_4$, filtered, and counted as previously described (10).

Polyacrylamide gel electrophoresis. Cells in 25 cm^2 Falcon flasks were pulse labeled for 30 min, rinsed with ice-cold PBS-A to stop incorporation, and then stripped from the flasks, denatured, and solubilized by heating at 80°C with a small volume of 50 mM Tris-hydrochloride buffer, pH 7, 2% sodium dodecyl sulfate, and 5% β-mercaptoethanol. Samples were run in a gel slab containing 8.5% acrylamide and cross-linked with *N,N'*-diallyltartardiamide (11). Staining and autoradiography were performed as previously described (11).

The numbers assigned to individual HSV-1 polypeptide bands (VP) and infected cell polypeptides (ICP) followed the designations of Heine et al. (11) and Honess and Roizman (13), respectively. α-Polypeptides (e.g., ICP-4, -0, -27, -35), β-polypeptides (e.g., ICP-6, -8, -36, -38), and γ-polypeptides (e.g., ICP-5, -17, -21, -31) are well represented by ICP-4, -6, and -5, respectively.

Assay of RNA polymerase B activity. Nuclei were isolated from 10^8 uninfected or HSV-1(F)-infected HEp-2 cells, as previously described (7). RNA polymerase activity of about 2×10^6 nuclei was measured in a reaction mixture, in which RNA polymerase B activity is preferentially stimulated (22), containing 0.2 M Tris-hydrochloride buffer, pH 7.9, 0.01 M $MnCl_2$, 0.2 M $(NH_4)_2SO_4$, 0.6 mM each ATP, GTP, and CTP, and 3 μCi of [^{3}H]UTP (52 Ci/mmol; The Radiochemical Centre, Amersham) in a final volume of 0.25 ml and incubated for 10 min at 37°C. Assays were performed in duplicate in the absence or presence of increasing concentrations of α-amanitin from 2.5×10^{-9} to 5×10^{-7} M. RNA polymerase B activity was calculated by subtracting from each activity value the value obtained in the presence of 5×10^{-7} M α-amanitin (18).

RESULTS

HSV-1(F) yield in HEp-2 cells in the presence of β-amanitin. In this experiment, the HEp-2 cells were infected with HSV-1(F) at a multiplicity of 1 PFU/cell. The cells were treated with β-amanitin (10 μg/ml) (10) from 1 h before infection until harvest. The HSV-1(F) yield at 24 h was reduced about 20 times as compared with that of untreated cells (Fig. 1). Viral yield in cell cultures that received β-amanitin at 1, 3, 6, or 9 h after infection was progressively less impaired.

Effect of amanitin on the synthesis of α-, β-, and γ-polypeptides in HEp-2 cells. In this series of experiments, the sensitivity to amanitin of HSV-1(F) RNA synthesis was evaluated from an analysis of infected-cell polypeptides (see introduction).

Figure 2 shows the autoradiogram of electrophoretically separated polypeptides from lysates of HSV-1(F)-infected HEp-2 cells pulse labeled at different times after infection and treated with β-amanitin (30 μg/ml) from 1 h after infection until harvest. Control cells were mock-treated with DEAE-dextran only. In the

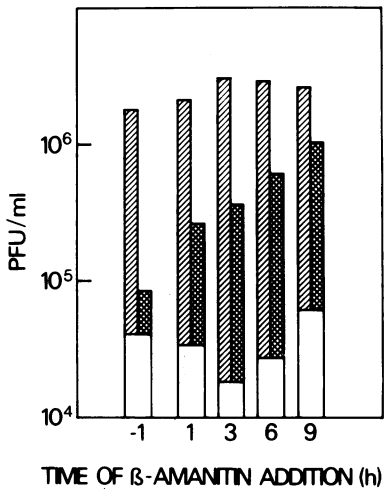

FIG. 1. *HSV-1(F) yield in HEp-2 cells treated with β-amanitin (10 μg/ml) from 1 h before or 1, 3, 6, or 9 h after infection until harvest. Duplicate cultures were frozen 24 h after infection, and virus was then titrated as described in Materials and Methods. Symbols: (▨) untreated cells; (▩) β-amanitin-treated cells; (□) cell cultures frozen at the time of addition of β-amanitin. The first empty column on the left represents cells frozen immediately after adsorption.*

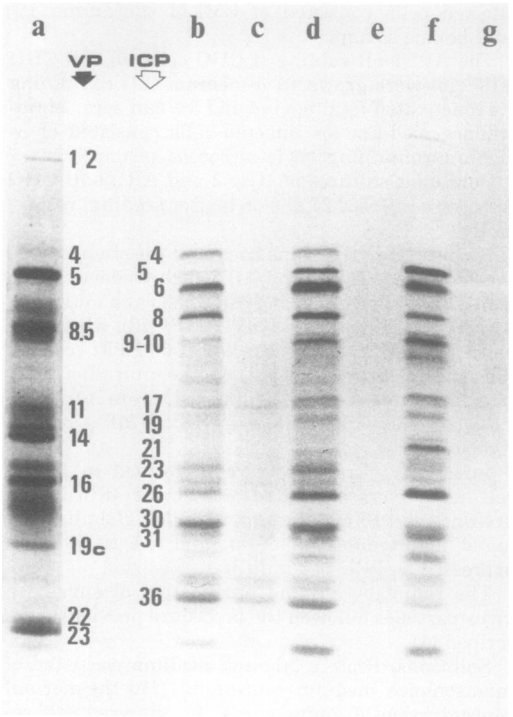

FIG. 2. *Autoradiogram of a 8.5% polyacrylamide gel slab containing electrophoretically separated polypeptides from HSV-1(F)-infected HEp-2 cells untreated (b, d, f) or treated (c, e, g) with β-amanitin (30 μg/ml) from 1 h after infection until harvest, i.e., at the end of the labeling period. (b, c) Cells labeled from 3.5 to 4 h after infection; (d, e) cells labeled from 5.5 to 6 h after infection; (f, g) cells labeled from 9.5 to 10 h after infection; (a) autoradiogram of virion polypeptides (VP) of HSV-1 labeled with ^{14}C-labeled amino acids. VP and infected-cell polypeptides (ICP) are numbered according to Heine et al. (11) and Honess and Roizman (13).*

amanitin-treated cells, a dramatic disappearance of almost all labeled polypeptides was obtained, indicating that the synthesis of mRNA precursors for α-polypeptides was inhibited directly by β-amanitin, whereas the disappearance of β- and γ-polypeptides might have been caused either directly by amanitin or by the absence of α-polypeptides.

Therefore, we decided to separate the synthesis of each group of polypeptides by treating the infected cells with cycloheximide (50 μg/ml), according to the method of Honess and Roizman (14). When cycloheximide is added to the cells immediately after virus adsorption (zero time) until 7 h after infection, α-mRNA's selectively accumulate in the cytoplasm, and only α-polypeptides are made immediately after removal of cycloheximide (14).

Amanitin, present during cycloheximide exposure, suppressed or reduced the appearance of α-polypeptides (e.g., ICP-4) depending on the length of amanitin treatment (Fig. 3), thus giving further evidence that amanitin directly affected the synthesis of mRNA's for α-polypeptides. On continued incubation of cells after removal of cycloheximide, the rate of synthesis of β-polypeptides (e.g., ICP-6) remarkably increased; at that time γ-polypeptides (e.g., ICP-5) appeared and α-polypeptides declined. In the presence of amanitin, added shortly before removal of cycloheximide and present until harvest, β-polypeptide production was severely reduced (Fig. 3). In this experiment, amanitin was added at the time of removal of cycloheximide and therefore could not reduce the production of α-polypeptides, whose RNAs were synthesized during cycloheximide exposure and translated as soon as cycloheximide was washed out. Since it could be excluded that β-polypeptide reduction was a consequence of a decreased α-polypeptide level, we concluded that amanitin inhibited the synthesis of mRNA's for β-polypeptides directly. This experiment is in agreement with previously reported findings that β-mRNA's are synthesized in the presence of functional α-polypeptides (15).

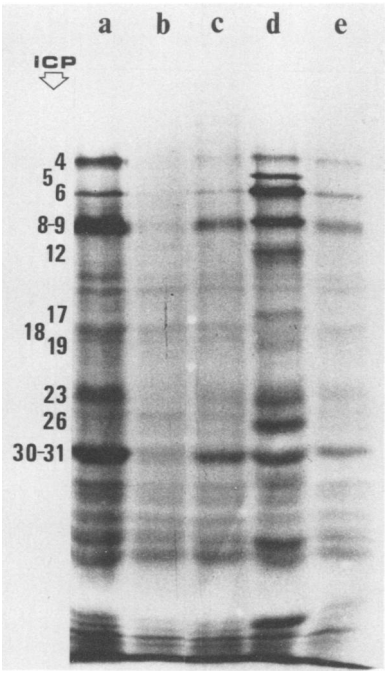

FIG. 3. *Autoradiogram of HSV-1(F)-infected HEp-2 cell polypeptides electrophoretically separated on a polyacrylamide gel slab. All these cultures were exposed to cycloheximide from 0 to 7 h after infection and then washed. (a–c) Cells labeled from 7 to 7.5 h after infection; (a) untreated cells; (b) cells treated with β-amanitin (30 μg/ml) from 2 to 7.5 h after infection; (c) cells treated with β-amanitin (30 μg/ml) from 4 to 7.5 h after infecton; (d, e) cells labeled from 9 to 9.5 h after infection and incubated between 6 and 9.5 h after infection in the absence (d) or presence (e) of β-amanitin (30 μg/ml).*

Evidence for the action of amanitin on γ-polypeptide synthesis was obtained from the following experiments. Cells were pulse labeled at 2 to 2.5 h after infection or after a further 5-h incubation in the absence or presence of β-amanitin (Fig. 4); during this 5-h incubation the synthesis of β-polypeptides reached its maximum and then declined, whereas that of γ-polypeptides continued at increasing rates. In the presence of amanitin, γ-polypeptide synthesis (e.g., ICP-5) was totally prevented.

In a further experiment cells were exposed to cycloheximide between 3.5 and 7 h after infection and further incubated for 3 h after the removal of cycloheximide (Fig. 4); during this last incubation, β-polypeptide synthesis declined while γ-polypeptide synthesis continued at a high rate. In the presence of amanitin, which was added at the time of removal of cycloheximide, the rate of synthesis of γ-polypeptides was decreased. It should be noted that in both experiments amanitin was added when β-polypeptide synthesis was at its maximum or already declining and γ-polypeptide synthesis had already been switched on; therefore the inhibitory effect of amanitin on γ-polypeptide RNA synthesis might not be attributed to the absence of β-polypeptides necessary for the synthesis of γ-mRNA's.

Sensitivity to amanitin of RNA polymerase activity in isolated nuclei. The activity of the amanitin-sensitive RNA polymerase was assayed in isolated nuclei from uninfected and from 3- and 8-h HSV-1(F)-infected HEp-2 cells in the presence of increasing concentrations of

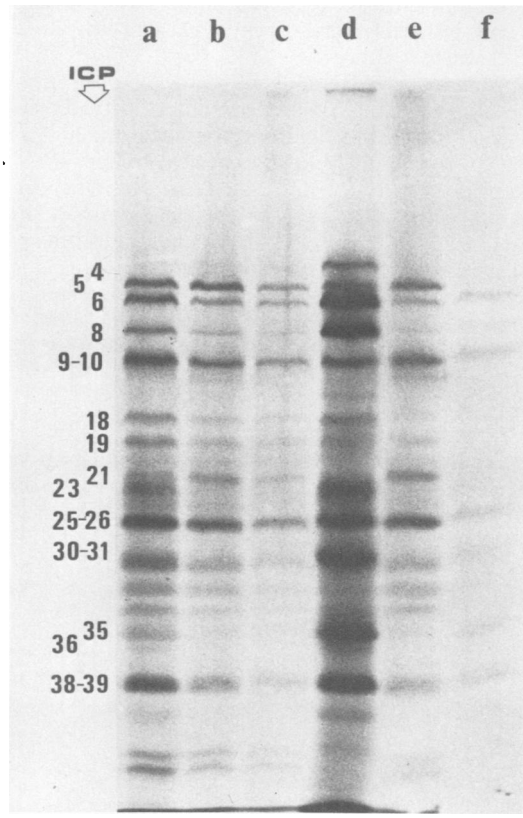

FIG. 4. *Autoradiogram of polypeptides from HSV-1(F)-infected HEp-2 cells electrophoretically separated on a polyacrylamide gel slab. (a–c) Cells exposed to cycloheximide from 3.5 to 7 h after infection; (a) cells pulse labeled immediately after removal of cycloheximide; (b, c) cells labeled after a further 3-h incubation in the absence (b) or presence (c) of β-amanitin (30 μg/ml); (d) cells labeled from 2 to 2.5 h after infection; (e, f) cells incubated in the absence (e) or presence (f) of β-amanitin (30 μg/ml) from 2 to 7.5 h after infection and labeled from 7 to 7.5 h after infection.*

α-amanitin. Amanitin-sensitive RNA polymerase activities in infected and in uninfected cell nuclei did not differ appreciably in the degree of sensitivity to amanitin (Fig. 5).

Effect of amanitin on viral polypeptide synthesis in HSV-1(MP)-infected AR1/9-5B CHO cells. The sensitivity to amanitin of viral protein synthesis was measured in AR1/9-5B CHO cells treated with 2 μg of α-amanitin per ml from seeding throughout the whole experiment, i.e., for about 40 h, a time period sufficiently long to attain 80% RNA synthesis inhibition in parental CHO cells. The presence of α-amanitin did not influence the viral polypeptide pattern at 3 h after infection (Fig. 6). It is noteworthy that in AR1/9-5B CHO cells, viral protein synthesis occurred earlier and all the viral proteins were already present in these cells at this time.

Moreover, AR1/9-5B CHO cells were pretreated with DEAE-dextran before the administration of high doses of β-amanitin (30 μg/ml), a treatment that did not at all affect RNA synthesis in the uninfected cells. At the same time, cells were exposed to cycloheximide to demonstrate early viral polypeptides. In amanitin-treated cells the pattern of polypeptides was undistinguishable from that obtained in untreated cells. These results indicate that HSV-1 transcription is totally unaffected by amanitin in amanitin-resistant cells.

DISCUSSION

The experiments reported in this paper show that in HSV-1-infected HEp-2 cells, amanitin reduced or suppressed the appearance of all the viral polypeptides (α, β, and γ). Since the only effect of amanitin known to date is its inhibitory effect on transcription, it seems very likely that the three waves of HSV-1 DNA transcription that lead to the synthesis of α-, β-, and γ-polypeptides are all catalyzed by amanitin-sensitive RNA polymerase(s). This result correlates well with previous reports (2, 3) that in isolated nuclei HSV-1 RNA synthesis is inhibited by α-amanitin. The finding that the amanitin-sensitive RNA polymerase assayed in isolated nuclei is equally sensitive to amanitin before and during infection may suggest that

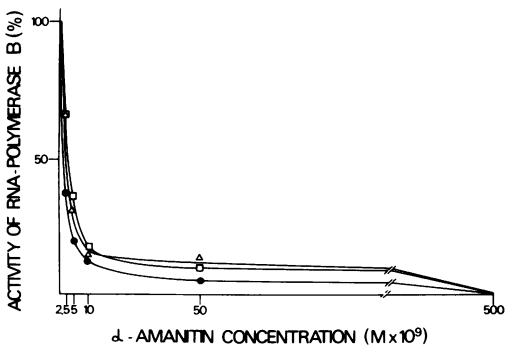

FIG. 5. *Effect of increasing concentrations of α-amanitin on RNA polymerase B activity in 2 × 10⁶ nuclei isolated from HEp-2 cells infected for 3 (□) or 8 (△) h, with HSV-1(F) or from uninfected cells (●). The average values obtained in the absence or presence of 5 × 10⁻⁷ M α-amanitin were, respectively, 11.2 or 4.2 pmol/ml for uninfected nuclei, 11.8 or 4.5 pmol/ml for 3-h-infected nuclei, 9 or 2.4 pmol/ml for 8-h-infected nuclei.*

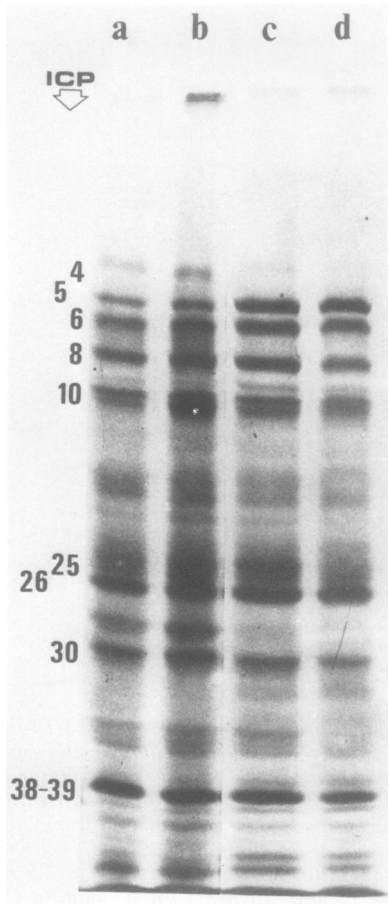

FIG. 6. *Autoradiogram of electrophoretically separated HSV-1(MP)-infected AR1/9-5B CHO cell polypeptides. (a, b) Cells exposed to cycloheximide from 0 to 4 h after infection and labeled from 4 to 4.5 h after infection soon after removal of cycloheximide; (a) untreated cells; (b) cells treated with β-amanitin (30 μg/ml) from 1.5 to 4.5 h after infection; (c, d) cells labeled from 2.5 to 3 h after infection; (c) untreated cells; (d) cells treated with α-amanitin (2 μg/ml) from seeding until harvest.*

HSV-1 DNA-transcribing enzyme does not differ from host cell RNA polymerase B as far as sensitivity to amanitin is concerned.

The results obtained in HSV-1-infected AR1/9-5B CHO cells show that the synthesis of all the viral polypeptides is totally unaffected by amanitin if added to cells before or during the infection cycle, thus showing that HSV-1 DNA transcription is insensitive to amanitin when cells possess an amanitin-resistant RNA polymerase B. The different effect of amanitin on HSV-1 DNA transcription in the two kinds of cells correlates well with sensitivity of RNA polymerase B of the two hosts to amanitin, thus suggesting that transcription of the HSV-1 genome is strictly dependent on host cell transcriptase, and in particular that HSV-1 DNA utilizes host cell RNA polymerase B for its transcription. These results do not rule out the possibility that during the infection cycle the host RNA polymerase B can be modified at a site that does not affect its sensitivity to the drug.

Our results are consistent with the report by Sheldrick et al. (20) that fully deproteinized HSV-1 DNA is infectious and therefore able to utilize host RNA polymerase.

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