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Inhibitory effect of papaverine on RNA and protein synthesis of vesicular stomatitis virus

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Summary

Papaverine, an inhibitor of cAMP phosphodiesterase, reduced yields of infectious vesicular stomatitis virus in HEp-2 cells approximately 100-fold if added to cultures at a concentration of 30 μM before and after virus infection. The extent of papaverine-induced suppression of viral growth was dependent on drug dose and treatment regimen. Cells progressively recovered their viral permissive state after removal of drug. The cyclic nucleotide, cGMP, nullified the inhibitory effect of papaverine if added to cells during drug treatment. Pulse labeling experiments with [35S]methionine showed that papaverine compromises production of all virus-specific proteins in infected cells without adversely affecting host cell protein synthesis. Treatment of cells with papaverine strongly inhibited the production of viral RNA and both cellular RNA and DNA. It was found that VSV causes an immediate but transient stimulation of DNA synthesis in HEp-2 cells which is prevented by papaverine treatment. This drug also selectively blocked primary transcription of VSV in vivo and to a lesser extent in vitro RNA polymerase activity of the virion-bound transcriptase. The finding that papaverine has a strong inhibitory effect on viral biosynthesis including early transcription suggests that VSV replication may depend on host factors that regulate intracellular levels of cyclic nucleotides such as cAMP.

vesicular stomatitis virus, papaverine, cyclic AMP, phosphodiesterase inhibitor

Introduction

The rhabdovirus, vesicular stomatitis virus (VSV), has an unusually broad host range that may reflect, in part, an ability to utilize host regulatory factors which are
common to most eukaryotic cells of higher animals. For example, we have recently shown that inhibitors of cellular prostaglandin biosynthesis prevent VSV replication at the level of viral RNA and protein synthesis (Mukherjee and Simpson, 1984, 1985). These studies also demonstrated that cells can recover their permissive state for productive viral infections after removal of such inhibitors.

The involvement of cyclic nucleotides and their modulators in the replication of animal viruses has been reported earlier. Exogenous addition of cAMP to cell cultures has been shown to inhibit replication of measles virus (Robbins and Rapp, 1980). We have recently demonstrated that cAMP can partially reverse the inhibition of VSV replication by indomethacin, a drug which targets various enzyme systems including phosphodiesterase (Mukherjee and Simpson, 1984). Miller and Carrigan (1982) showed that treatment of neural cells with papaverine, a potent agonist of phosphodiesterase which elevates intracellular cAMP levels, caused a marked but reversible suppression of measles virus growth. Others have found that RNA but not protein synthesis of measles virus can be inhibited by papaverine in neural and non-neural cells (Yoshikawa and Yamanouchi, 1984). In this communication, it is shown that papaverine prevents both viral RNA and protein synthesis in HEp-2 cells infected with VSV and acts at an early stage of the viral growth cycle.

Materials and Methods

Virus stocks and cell cultures

The Indiana strain of VSV was grown in cultures of BHK-21 cells for preparation of working stocks (Mukherjee and Simpson, 1984). Cultures of BHK-21 or HEp-2 cells used in this study were maintained in reinforced MEM (RMEM) supplemented with 10% newborn calf serum (NCS). Infectivity titrations were performed by plaque assay in BHK-21 monolayer cultures as described earlier (Simpson et al., 1979).

Virus used for primary transcription assays was labeled in its RNA by growth for 36 h in BHK-21 cells with [3H]uridine (specific activity 27.9 Ci/mmol) at 20 μCi/ml and purified by established methods (Bean and Simpson, 1973).

Assays for viral transcriptase activity

The method of Baltimore et al. (1970) was used with slight modification for measuring in vitro transcriptase activity of the virion-associated RNA polymerase of VSV. Reaction mixtures (100 μl) contained final concentrations of 64 mM Tris-HCl (pH 8), 80 mM NaCl, 8 mM MgCl2, 0.2 mM DTT, 0.05% Triton-N101, 1 mM each of ATP, GTP and CTP, 10 μCi of [3H]UTP (specific activity 14 Ci/mmol), appropriate amounts of papaverine (see Results) and purified virus (15 μg viral protein, Lowry units).

For determining primary transcription in vivo, the method of Bean and Simpson (1973) was used with only minor changes and HEp-2 cell monolayers (2 × 10⁶ cells) were infected with purified ³H-labeled VSV at an input of approximately 5 × 10⁵ cpm (3 × 10⁶ pfu) per 60 mm Petri dish culture.
Electrophoresis of viral proteins and RNA species

For determination of viral protein synthesis, HEp-2 cells infected with VSV were pulse-labeled with \([^{35}\text{S}]\)methionine under different regimens of drug treatment as described in the figure legends. Washed cells, lysed in Laemmli buffer (0.0625 M Tris-HCl, pH 6.8/2% SDS/10% glycerol), were sonicated for 5 min at 4°C and combined with \(\beta\)-mercaptoethanol and 0.025% bromphenol blue before boiling for 5 min. The dissociated proteins were analyzed by standard discontinuous SDS–PAGE in 10% acrylamide resolving gels (Laemmli, 1970). The \(^{35}\text{S}\)-labeled proteins were detected by autoradiography using Kodak XAR-5 X-ray film exposed with a Cronex lightning-plus intensifying screen for 7–10 days at \(-80^\circ\text{C}\).

For analysis of viral RNAs, infected HEp-2 cells were pulse-labeled with inorganic \([^{32}\text{P}]\)phosphate in phosphate-free RMEM at different times during infection and according to the drug regimens described under Results. Total RNA was extracted from washed monolayers solubilized with STE buffer containing 0.5% SDS. After the lysate was aspirated three times through a 22 gauge needle, the RNA was extracted with STE-saturated phenol and precipitated with 95% ethanol at \(-20^\circ\text{C}\) (Bean and Simpson, 1973). RNA preparations dissolved in STE buffer with 8 M urea and 20% glycerol were stored at \(-20^\circ\text{C}\) until analyzed in 1% agarose acid–urea slab gels by the method of Lynch et al. (1979). For autoradiography, gels fixed in 50% methanol were exposed to Kodak XAR-5 film with a Cronex lightning-plus intensifying screen at \(-80^\circ\text{C}\) for 7 days.

Chemicals

Papaverine was purchased from Sigma Chemical Corporation (St. Louis, MO). The \([^{3}\text{H}]\)thymidine (specific activity = 62 Ci/mmol), \([^{3}\text{H}]\)UTP (specific activity = 14 Ci/mmol), and \(^{32}\text{P}\) (carrier-free), were obtained from ICN Pharmaceuticals (Irvine, CA). The \([^{35}\text{S}]\)methionine (specific activity = 1450 Ci/mmol) was purchased from Amersham Corporation (Arlington Heights, IL).

Results

Inhibition of virus yields by papaverine

Preliminary experiments were carried out to establish conditions for maximum inhibition of VSV growth by papaverine in HEp-2 cells. Although papaverine was recently shown to inhibit measles virus replication by more than 99% in neural cell cultures treated at a dose level of 7 \(\mu\text{M}\) for 6 days (Miller and Carrigan, 1982), we found that HEp-2 cells required exposure to 30 \(\mu\text{M}\) papaverine (before and after infection) to obtain a 98% reduction of virus yields (Table 1). Use of lower doses or omitting drug after infection was less effective in reducing virus yields. Prolonged drug treatment of HEp-2 cells for more than 2 days produced cytotoxic effects. In all subsequent experiments, HEp-2 cells were treated with drug at a concentration of 30 \(\mu\text{M}\) and for periods not exceeding 48 h.

The reversibility of the drug effect was investigated by pretreating cells with papaverine, infecting with virus, and further incubating cells for 24 h before removal
### TABLE 1

**YIELDS OF INFECTIOUS VSV IN HEp-2 CELLS TREATED WITH PAPAVERINE ACCORDING TO DIFFERENT REGIMENS**

| Treatment with papaverine | Virus yield at 24 h (log<sub>10</sub> pfu/ml) | Percent inhibition |
|---------------------------|---------------------------------------------|--------------------|
| -24 h                     | 0 time                                      |                    |
| 10 μM                     | none                                        | 7.92               | 58.5               |
| 10 μM                     | 10 μM                                       | 7.91               | 59.5               |
| 20 μM                     | none                                        | 7.72               | 74.0               |
| 20 μM                     | 20 μM                                       | 7.64               | 78.0               |
| 30 μM                     | none                                        | 7.60               | 80.0               |
| 30 μM                     | 30 μM                                       | 6.48               | 98.5               |
| RMEM, 2% NCS              | none                                        | 8.30               | 0                  |
| RMEM, 2% NCS              | 10 μM                                       | 7.95               | 55.5               |
| RMEM, 2% NCS              | 20 μM                                       | 7.92               | 58.5               |
| RMEM, 2% NCS              | 30 μM                                       | 7.30               | 90.0               |

HEp-2 cell monolayers were incubated with or without papaverine at the concentrations indicated in RMEM (2% NCS) at 37°C for 24 h before infection with VSV (m.o.i. = 10). The infected cultures were washed after virus adsorption (0 time) and fresh RMEM (lacking serum) with or without papaverine was added. After further incubation at 37°C for 24 h, the cultures were frozen/thawed three times and the clarified lysates were tested for virus content by plaque assay in BHK-21 cells.

### TABLE 2

**PROGRESSIVE LOSS OF INHIBITORY EFFECT OF PAPAVERINE ON INFECTIOUS VIRUS PRODUCTION IN HEp-2 CELLS AFTER REMOVAL OF DRUG**

| Initial drug treatment of cells (-24 h → 24 h p.i.) | Drug content of recovery medium added at 24 h p.i. | Virus yields (log<sub>10</sub> pfu/ml) at different times during recovery period: |
|----------------------------------------------------|--------------------------------------------------|--------------------------------------------------|
|                                                    |                                                  | 0 h                                              | 6 h  | 12 h | 24 h |
| Papaverine                                         | 30 μM                                            | 5.7                                             | 5.8  | 5.9  | 6.3  |
| Papaverine                                         | none                                             | 5.8                                             | 6.7  | 7.2  | 7.9  |
| None                                               | none                                             | 8.4                                             | 8.3  | 8.4  | 8.4  |

Monolayer cultures of HEp-2 cells were incubated for 24 h at 37°C with RMEM/2% NCS containing or lacking 30 μM papaverine. The cultures were infected with VSV (m.o.i. = 10) and incubated for another 24 h with medium of the same composition used for pre-treatment. At 24 h post-infection (p.i.), appropriate sets of cultures received fresh medium containing or lacking papaverine after which incubation was continued for 0, 6, 12 or 24 h. Fresh medium was not added to untreated infected cultures at 24 h p.i. owing to the extensive cytopathic alterations that occurred by that time. The virus yields of all cultures were determined in BHK-21 cells by standard plaque assay.
TABLE 3

EFFECT OF cGMP ON PAPAVERINE INHIBITION OF VSV IN HEp-2 CELLS

| Drug treatment regimen | Postinfection period | Virus yield | Percent control |
|------------------------|----------------------|-------------|----------------|
| 24 h pre-treatment period |                     |             |                |
| Papaverine             | Papaverine           | 6.36        | 1.3            |
| Papaverine             | Papaverine + cGMP    | 6.64        | 1.5            |
| Papaverine + cGMP      | Papaverine           | 7.92        | 50.0           |
| Papaverine + cGMP      | Papaverine + cGMP    | 8.53        | 200.0          |
| cGMP                   | None                 | 8.17        | 87.0           |
| None                   | None                 | 8.23        | 100.0          |

At 24 h before infection, cultures of HEp-2 cells received fresh RMEM/2% NCS with or without 30 µM papaverine and 5 mM cGMP as indicated above. After pretreatment, cells were inoculated with VSV (m.o.i. = 10), washed after virus adsorption and further incubated at 37°C with RMEM (no serum) with or without the supplements indicated. Virus yields (log_{10} pfu/ml) were measured by plaque assay.

biosynthesis had occurred during the postinfection drug treatment period (data not shown). We also tested the effect of known modulators of cyclic nucleotide metabolism for their ability to counteract the viral inhibitory effect of papaverine. The most significant effect was obtained when cells were exposed to cGMP during treatment with drug (Table 3). Cyclic GMP totally negated the papaverine block of viral replication if it was present during the entire drug treatment period. Inhibition of measles virus growth in vitro by papaverine is similarly antagonized by exogenous addition of cGMP (Miller and Carrigan, 1982). Although papaverine is known to affect intracellular levels of Ca^{2+} ions which in turn can regulate phosphodiesterase activity (Carpendo et al., 1975), we found that altering the calcium concentration of the culture medium had no apparent effect on inhibition of VSV by this drug (data not shown).

**Effect of papaverine on synthesis of viral proteins**

To determine the effect of drug on viral protein synthesis, papaverine-treated cells were pulse-labeled with \[^{35}\text{S}]\text{methionine during infection and total radiolabeled proteins were analyzed in SDS-PAGE gels as illustrated in Fig. 1. The production of viral proteins was totally blocked in cells infected immediately after drug treatment (Fig. 1, lane 5) even though cellular protein synthesis was not apparently reduced (cf. Fig. 1, lane 4). Under the conditions employed in these experiments, papaverine reduced yields of infectious virus to 1% of infected control cultures (data not shown). Calcium had no sparing effect on the inhibition of viral protein synthesis by this drug (Fig. 1, lane 6).**

**Nucleic acid synthesis in papaverine-treated cells**

Our inability to detect VSV proteins in drug-treated cells suggested that either the
Fig. 1. Polyacrylamide gel analysis of $^{[35]S}$methionine labeled polypeptides detected in HEp-2 cells treated with papaverine and infected with VSV. Confluent cell monolayers were exposed to 30 μM papaverine in RMEM (2% NCS) for 24 h at 37°C. Control cultures were mock-treated with drug-free medium. Treated cultures were infected with VSV (80 pfu/cell) and incubated at 37°C with or without drug at the same concentration used during pretreatment. All infected cultures were pulse-labeled with $^{[35]S}$methionine (25 μCi/ml) from 3 to 5 h after infection in RMEM lacking serum. In some cultures, CaCl$_2$ (0.1 mM) was present in the labeling medium. Preparation of cell lysates and resolution of radiolabeled proteins by electrophoresis and autoradiography were as described in Materials and Methods. The autoradiogram shows polypeptide components of: $^{35}$S-labeled purified VSV particles (Lane 1); mock-treated infected cells (Lane 2); mock-treated infected cells incubated with 0.1 mM CaCl$_2$ (Lane 3); papaverine treated mock-infected cells (Lane 4); papaverine treated infected cells without (Lane 5) or with (Lane 6) CaCl$_2$; mock-treated uninfected cells (Lane 7).

production or processing of viral mRNAs might be impaired. When papaverine-treated HEp-2 cells were analyzed for RNA species produced during the early stages of infection, none of the expected VSV mRNAs or genome-length 42S RNA were detected (Fig. 2). Synthesis of cellular ribosomal RNA was also prevented by this drug (Fig. 2, lane 4).

The effect of papaverine on host DNA synthesis was also examined. Fig. 3 illustrates that $[^3]$H]thymidine uptake in drug-treated cells was totally blocked whether or not they were infected. An interesting aside of these experiments was the finding that immediately after infection with VSV, there was an appreciable stimulation of $[^3]$H]thymidine incorporation by comparison with uninfected cells (Fig. 3). Although the true significance of this phenomenon is unknown, it appears to be related to virus uptake since it was multiplicity dependent and did not occur when
Fig. 2. Gel electrophoresis of RNA species synthesized in HEp-2 cells treated with papaverine and infected with VSV. Cells pretreated with papaverine (30 μM) for 24 h at 37°C were infected with VSV (10 pfu/cell), incubated with fresh medium containing drug, and labeled with $^{32}$P (25 μCi/ml) from 2 to 4 h p.i. Total RNA was extracted and analyzed in acid-urea agarose gels (Materials and Methods). RNA species from: untreated, virus infected cells (Lane 1); infected cells treated with papaverine (Lane 2); mock-treated uninfected cells (Lane 3); uninfected cells treated with papaverine (Lane 4). Arrows indicate the position of 28S and 18S ribosomal RNA, respectively.

infected cells were incubated in the cold (data not shown). Collectively, the foregoing experiments established that papaverine blocks host nucleic acid synthesis but not protein synthesis under conditions where no VSV-coded polypeptides or RNA species are produced.

**Drug effect on viral transcription**

In the absence of viral mRNA synthesis, it was of interest to determine whether papaverine directly affects viral transcription. We tested for primary transcription in vivo in drug-treated cells by measuring the association of radiolabeled input viral genomic RNA with RNase resistant complexes during the first 2 h of infection (see Materials and Methods). Fig. 4 shows that primary transcription of VSV in cells
Incubation Time (hrs.)

- After Virus Adsorption

Fig. 3. Effect of papaverine on $[^3]$H]thymidine uptake in HEp-2 cells. Subconfluent (ca. 80%) monolayers of HEp-2 cells in 60 mm plates ($3 \times 10^6$ cells) were treated for 24 h at 37°C with RMEM (2% NCS) containing or lacking 30 $\mu$M papaverine. After treatment, the cultures were washed with BSS and either mock-infected (GBSS) or infected with VSV at an input multiplicity of 10 pfu/cell. Virus was adsorbed at room temperature for 30 min (0 time) and fresh medium with 30 $\mu$M papaverine was added to those cultures pretreated with drug while all others received RMEM without supplements. Separate cultures from each of the experimental groups were pulse-labeled with 1 $\mu$Ci/ml of $[^3]$H]thymidine for 60 min at the time intervals indicated during a 10 h incubation at 37°C. Isotope incorporation was stopped by chilling cultures on ice and acid-insoluble radioactivity was measured (Evans and Simpson, 1980). $[^3]$H]Thymidine uptake is shown as a function of time of pulse-labeling following virus adsorption for drug-treated infected cells (A), drug-treated uninfected cells (A), mock-treated infected cells (○) and mock-treated uninfected cells (●).

Fig. 4. Effect of papaverine on primary transcription of vesicular stomatitis virus in monolayer cultures of HEp-2 cells. Cultures were exposed to RMEM (2% NCS) with or without 30 $\mu$M papaverine for 24 h at 37°C. The drug treated cultures were temperature equilibrated at 4°C and inoculated with gradient-purified VSV radiolabeled in its RNA with $[^3]$H. The virus input was approximately 10 pfu per cell or $4 \times 10^5$ cpm. Cells were washed with cold BSS after virus adsorption and incubated at 37°C with pre-warmed (37°C) serum-free medium containing or lacking papaverine (30 $\mu$M). Individual cultures were extracted for total RNA at different time intervals and the percentage of ribonuclease resistant radioactivity after annealing was determined (Materials and Methods). Percent RNase-resistant $[^3]$H counts as a function of time of incubation for untreated infected cultures (○) and cultures treated before and after infection with drug (●).

treated with papaverine before and after infection was largely prevented during the early stages of infection.

To determine whether the impairment of primary transcription seen in these experiments might involve a direct action of drug on viral transcriptive complexes, we tested the activity of the virion transcriptase in in vitro reaction mixtures containing different concentrations of papaverine. As shown in Table 4, transcriptase activity was incompletely inhibited in reaction mixtures with drug concentrations of 30 $\mu$M or higher. Considering that drug levels greater than 30 $\mu$M are toxic for the HEp-2 cells used in this study, this partial inhibition of in vitro viral transcriptase activity obtained suggests that the stronger block of VSV transcription induced in vivo may not necessarily reflect a direct effect of papaverine on viral transcriptive complexes under the conditions used.
TABLE 4
EFFECT OF PAPAVERINE ON IN VITRO TRANSCRIPTASE ACTIVITY OF VSV

| Drug concn. (μg) a | Measurement of [3H]UTP uptake as | Percent inhibition |
|-------------------|---------------------------------|-------------------|
|                   | cpm/μg virus protein | pmol |                  |
| 100               | 610                | 43.5 | 77.2             |
| 75                | 803                | 57.3 | 70.1             |
| 50                | 1007               | 79.0 | 62.3             |
| 30                | 1194               | 85.2 | 55.3             |
| 20                | 1248               | 89.1 | 53.1             |
| 10                | 1266               | 90.4 | 52.6             |
| 5                 | 1376               | 98.2 | 48.5             |
| 1                 | 1886               | 134.7| 23.4             |
| 0                 | 2668               | 190.5| 0                |

Complete transcriptase reaction mixtures (100 μl) containing purified VSV particles with papaverine at the final concentrations listed were initiated as described in Materials and Methods. After a 30 min incubation at 37°C, the reactions were stopped by adding 1 ml aliquots of cold 10% TCA. After further chilling for 30 min, the precipitates were collected on 0.22 μm S/S filter disks and TCA-insoluble radioactivity was measured in a scintillation counter. 

a Concentration per ml of reaction mixture.

Discussion

The use of papaverine in this study as a reversible inhibitor of viral replication further implicates the participation of endogenous host factors in biosynthetic activities of vesicular stomatitis virus during infection of permissive cells. Pharmacologically, papaverine (6,7-dimethoxy-1-veratryl-isoquinoline) is an alkaloid derivative of Papaver poppies which acts as a smooth muscle relaxant by inhibiting phosphodiesterase, causing an increase in intracellular levels of 3',5'-monophosphate (cyclic AMP) (Triner et al., 1970; Carpendo et al., 1975). Recent studies on the possible role of cyclic nucleotides as modulators of acute and persistent infections with measles virus have established that elevation of cAMP levels either by exogenous addition of this nucleotide (Robbins and Rapp, 1980) or by treatment of cells with papaverine (Miller and Carrigan, 1982; Yoshikawa and Yamanouchi, 1984) renders cells reversibly nonpermissive for measles virus replication. Yoshikawa and Yamanouchi (1984) reported that papaverine blocks measles virus in vitro at the level of viral RNA synthesis although their ability to detect relatively low levels of measles P protein in neural and non-neural cells treated with this drug would indicate that inhibition of viral transcription in this system was not absolute. Other workers have shown that papaverine can selectively inhibit production of intracellular matrix (M) protein in measles infected neural cells without negatively affecting synthesis of other viral structural proteins detected by immunofluorescent staining (Miller and Carrigan, 1982). It would thus appear that host cell differences influence the extent to which papaverine interferes with virus-specific macromolecular synthesis observed in these systems.
The results of the present investigation contrast with those cited above since we found that VSV biosynthesis, including early transcriptive events, is strongly compromised by papaverine treatment of cells. This profound inhibitory effect parallels that observed in our recent studies on the ability of indomethacin, an antagonist of fatty acid cyclooxygenase, to reversibly block VSV transcription, RNA synthesis and protein synthesis in HEp-2 cells (Mukherjee and Simpson, 1985). We also recently reported (Mukherjee and Simpson, 1984) that cAMP and specific modulators of cyclic nucleotide metabolism such as theophylline, can partially negate the inhibitory effect of indomethacin which itself can block phosphodiesterase activity at higher concentrations (Flower and Vane, 1974). It is possible, therefore, that the inhibition of viral transcription in VSV-infected cells treated with either papaverine or indomethacin involves common host factors required for viral mRNA synthesis in vivo. Of relevant interest is our recent discovery of a host membrane protein factor from normal cells that can significantly restore in vitro RNA synthesis of functionally defective viral nucleocapsids recovered from VSV-infected cells treated with indomethacin (Mukherjee and Simpson, in preparation). Whether this viral transcription enhancing factor can counteract the inhibitory effect of papaverine on VSV remains to be determined.

The reversibility of the inhibition of virus replication by papaverine seen in this and other investigations (Miller and Carrigan, 1982; Yoshikawa and Yamanouchi, 1984) indicates that compromised host factors required for productive infections can be restored upon removal of drug. Alternatively, the elevation of intracellular cAMP resulting from exposure of cells to this drug may arrest the cell cycle (Coffino et al., 1975) and require a return to normal levels of this nucleotide before cells recover their permissive state for viral replication. That cAMP plays an important role in the inhibitory effects seen in this study is strongly suggested by the ability of cGMP to block papaverine action (Table 3). However, since papaverine has complex pharmacological and biochemical properties which affect other enzyme systems besides cAMP phosphodiesterase (see Ferrari, 1974), it is entirely possible that the viral inhibitory effect obtained in this study may entail additional cellular targets.

The fact that papaverine was found to inhibit both cellular DNA and RNA synthesis without adversely affecting production of host proteins would suggest that translation of existing cellular mRNA species is not blocked by this metabolic antagonist. These experiments also revealed the interesting finding that VSV causes an early stimulation of host DNA synthesis immediately after infection (Fig. 3). This phenomenon may relate, in part, to the association of VSV-specific leader RNA species with the nuclear compartment recently demonstrated by other workers (Kurilla et al., 1982) and may possibly represent an obligatory event that occurs prior to the shutoff of host macromolecular synthesis postulated to be caused by these early viral transcripts (Weck et al., 1979; McGowan et al., 1982).

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