Regulation of Herpesvirus Macromolecular Synthesis: Sequential Transition of Polypeptide Synthesis Requires Functional Viral Polypeptides

(herpes simplex 1/ amino acid analogues/polyacrylamide gel electrophoresis)

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ABSTRACT It was previously shown that virus-specific polypeptides made in HEp-2 cells infected with herpes simplex 1 form three groups designated α, β, and γ whose synthesis is coordinately regulated and sequentially ordered. This report shows that one or more functional α polypeptides are necessary to turn on the synthesis of β and γ groups, and conversely, one or more polypeptides in the latter groups turn off the synthesis of α polypeptides. Specifically, infected cells maintained in medium containing either canavanine, an analogue of arginine, or azetidine-2-carboxylic acid, an analogue of proline and hydroxyproline, synthesized α polypeptide at rates comparable to maximal rates in untreated infected cells but did not undergo the normal transition to β and γ polypeptide synthesis. The transition to γ polypeptide synthesis and shut-off of synthesis of earlier polypeptide groups proceeded normally if addition of canavanine was delayed until at least 4 to 5 hr after infection. Addition of canavanine after the onset of β and γ polypeptide synthesis, i.e., between 2 and 3.5 hr after infection, resulted in sustained, simultaneous synthesis of all three polypeptide groups, a phenomenon not seen in untreated infected cells. Canavanine-treated infected cells, synthesizing α polypeptides, recovered the capacity to make β and γ polypeptides after removal of the analogue, but only after a 1- to 2-hr delay compared with infected untreated cells. The data indicate that the on and off controls inherent in the cascade regulation of viral polypeptide synthesis are mediated by one or more polypeptides in each group at transcriptional or post-transcriptional levels.

In preceding reports we showed that the synthesis of about fifty virus-specific polypeptides could be detected in HEp-2 (human epidermoid carcinoma no. 2) cells infected with herpes simplex 1 [HSV-1 (F)] and that these polypeptides formed at least three groups, α, β, and γ, whose synthesis was coordinately regulated and sequentially ordered (1, 2). Evidence for coordinate regulation was based on the observation that the synthesis of polypeptides comprising each group followed similar temporal patterns. Thus, the rates of synthesis of polypeptides of the α group were highest between 3 and 4 hr after infection and thereafter declined. The rates of synthesis of the β group were maximal from 5 to 7 hr after infection and then similarly declined. The γ group was made at increasing rates until at least 12 hr after infection (2). The conclusion that they were sequentially ordered was based on the requirements for their synthesis. The synthesis of α polypeptides required no prior infected cell protein synthesis; they were made immediately after withdrawal of cycloheximide or of puromycin added to the culture medium at the time of infection. The production of β polypeptides required prior synthesis of α polypeptides, and in cultures synchronized with inhibitors of protein synthesis, the synthesis of β polypeptides coincided with a rapid decline in the synthesis of α polypeptides. A similar relationship was observed between β and γ polypeptide synthesis (2).

Few details are available concerning the mechanisms by which the sequential synthesis of coordinately regulated groups is effected, and these relate mostly to the transition from α to β polypeptide synthesis. Specifically, in cells treated with cycloheximide from the time of infection, transcripts arising from 10 to 14% of viral DNA accumulated in the cytoplasm even though the nuclei accumulated transcribe made from at least 50% of the DNA (3). The transcripts retained in the nucleus were not transported into the cytoplasm even after the drug was withdrawn and α polypeptide synthesis began (3), and no synthesis of β polypeptides ensued unless new RNA synthesis was permitted to take place (2). The data suggested that the synthesis of β polypeptides required the participation of α polypeptides in some transcriptional or post-transcriptional event. Direct evidence for a functional involvement of α polypeptides in the synthesis of subsequent polypeptide groups or for functioning of β and γ polypeptide groups in the shut-off of synthesis of α polypeptides was unavailable. In this report we show that the cascade regulation is mediated by one or more polypeptides in each group.

MATERIALS AND METHODS

Viruses and Cells. All experiments were done with F prototype of HSV-1 and HEp-2 cell monolayers grown in plastic flasks (10⁶ cells per flask). After infection with 20 plaque-forming units per cell, the cultures were maintained at 37° in mixture 199 supplemented with 1% calf serum. The cells were labeled with 0.3-1.0 μCi of L-[U-14C]leucine, L-[U-14C]-isoleucine, and L-[U-14C]valine, all with specific activities of approximately 300 mCi/mmol (Schwarz/Mann BioResearch, Orangeburg, N.Y.), per ml of mixture 199 containing one-tenth the usual amounts of these amino acids and supplemented with 1% dialyzed calf serum. At the end of the labeling period the cells were rinsed with ice-cold phosphate-buffered saline to terminate amino-acid incorporation and then were stripped from the flask, denatured, and solubilized by heating at 80° in a small volume of 2% sodium dodecyl

Abbreviations: HSV-1, herpes simplex virus 1, human herpesvirus 1; HEp-2, human epidermoid carcinoma no. 2; VP, viral structural polypeptides; ICP, infected cell polypeptides.* Paper no. 3 in the series. Paper no. 2 is ref. 3.
sulfate, 5% 2-mercaptoethanol, 50 mM Tris·HCl (pH 7.0) for subsequent electrophoresis on polyacrylamide gels.

Times at which inhibitors, amino-acid analogues, and labeled precursors were added and removed, etc., are stated in hours after the exposure of cells to virus.

Amino-Acid Analogues and Inhibitors of Protein Synthesis. Cycloheximide and amino-acid analogues (L-canavanine, Sigma Chemical Co., St. Louis, Mo., and L-azetidine-2-carboxylic acid, Calbiochem, La Jolla, Calif.) were added to infected and uninfected cells in unmodified maintenance medium at times and final concentrations stated in text and legends. The concentrations of arginine, proline, and hydroxyproline in this medium were 0.33, 0.54, and 0.076 mM, respectively. The drugs were removed from treated cultures by rinsing monolayer cultures four times with 5-ml portions of unmodified maintenance medium at 37°C.

Polyacrylamide Gel Electrophoresis. Procedures for electrophoretic separation of polypeptides in gel slabs including sodium dodecyl sulfate and consisting of acrylamide cross-linked with N,N'-diallyltartardiamide, as well as for autoradiography of the dried gels, have been described (1, 4).

Densitometry of Autoradiograms and Computer-Aided Analysis of Absorbance Tracings. Absorbance measurements of autoradiographic images, made with a Giflord recording spectrophotometer, were displayed on the oscilloscope screen of a Tektronix model 4010 control console of a General Automation 16/45 digital computer. The computer was programmed to expand regions of the profile as determined by the operator and to compute areas under the tracing for individual polypeptide bands (1). Measurements of the synthesis of individual polypeptides are expressed either as percent of total ongoing synthesis or as relative rate of synthesis. If the integrated absorbance of each separated band is \( A_1 \), \( A_2 \), \( A_3 \), then for a given labeling interval, \( t \), the synthesis of polypeptide \( i \) as a percent of ongoing synthesis is obtained from:

\[
\frac{(A_i) \times 100}{\sum (A_1, A_2, A_3)}
\]

Since the overall rate of protein synthesis varies throughout infection or under different culture conditions, comparisons of the synthesis of a given polypeptide at different times must take account of these changes. In practice, these changes were monitored by measuring variations in incorporation of labeled amino acids into acid-precipitable material over the desired range of conditions and expressed as a fraction \( K_i \) of the incorporation obtained in untreated mock-infected cells labeled for an equivalent interval. The relative rate of synthesis was then calculated from \( K_i \times \) percent of ongoing synthesis.

**RESULTS**

Evidence that transition from \( \alpha \) to \( \beta \) polypeptide synthesis requires functional early polypeptides

The hypothesis that functional \( \alpha \) polypeptides are required for the synthesis of subsequent groups of polypeptides predicts that substitution of one or more amino acids by its analogue should prevent the transition from early to late polypeptide synthesis. In these experiments, canavanine and azetidine-2-carboxylic acid were added, at final concentrations indicated in Fig. 1, panel A, to HEp-2 cell cultures at 1 hr after infection. The autoradiograms of labeled polypeptides from lysates of infected cells treated with analogues (panel A) are shown together with autoradiograms of major virion polypeptides (autoradiogram 1), of polypeptides labeled in untreated infected cells from 9.75 to 10 hr (autoradiogram 2), and of polypeptides labeled at the same time immediately after removal of cycloheximide present in the medium from the time of infection. In untreated infected cells at 9–10 hr, the synthesis of \( \alpha \) polypeptides (e.g., ICP 4, 0, and 27) could not be detected; the rates of synthesis of \( \beta \) polypeptides (e.g., ICP 6 and 8), although still high, were declining; whereas \( \gamma \) polypeptides, which migrate with major structural polypeptides (e.g., ICP 5, 10, 21, and 26), were being made at increasing rates (autoradiogram 2 and 0 mM canavanine; see also Fig. 2). The polypeptides made after removal of cycloheximide added at 0 hr (autoradiogram 3) consisted largely of viral \( \alpha \) polypeptides and residual host cell polypeptides (e.g., ICP 34 and 35), as shown previously (2). The main feature of the data is the analogue dose-dependent prolongation of \( \alpha \) polypeptide synthesis concomitant with a drastic reduction in the synthesis of \( \gamma \) polypeptides. \( \beta \) Polypeptides (e.g., ICP 6 and 8) were less markedly reduced, but in the presence of 2.8 mM canavanine they were made in lower amounts than in untreated infected cells. It is noteworthy that both analogues blocked \( \gamma \) polypeptide synthesis and that the effective concentration of each was 10- to 30-fold greater than that of the unmodified L-amino acid present in the medium. All subsequent experiments were done with canavanine at a concentration of 2.8 mM.

Two comments may be made in connection with the infected cell polypeptides identified in Fig. 1. First, all \( \alpha \) polypeptides, whether produced in untreated infected cells, after cycloheximide treatment, or in the presence of amino-acid analogues, undergo post-translational modification. Specifically, these polypeptides become phosphorylated (unpublished data) and migrate more slowly in polyacrylamide gels. The relationship between phosphorylation and reduced electrophoretic mobility is not clear, since the electrophoretic mobility of phosphorylated \( \beta \) polypeptides remains unchanged (e.g., ICP 6 and 39). With respect to Fig. 1, panel A, the difference in the position of ICP 4 labeled after cycloheximide treatment (autoradiogram 3, panel A) and in the presence of analogues is due to the differences in the duration of labeling intervals, since after a chase the bands comigrate. Second, treatment of uninfected HEp-2 cells with canavanine despresses ICP 22 (Fig. 1C and D, autoradiograms 1 and 2), which was previously seen after removal of cycloheximide from infected but not from uninfected HEp-2 cells (2). This polypeptide was previously thought to be an \( \alpha \) polypeptide on the basis of its increasing rate of synthesis with increasing multiplicities of infection after removal of cycloheximide from infected cells (2, 3). This polypeptide is also induced by incubation of uninfected cells at supraoptimal temperatures (38.5°C) (Cassai, Honess, and Roizman, unpublished data) and cannot now be regarded as being specified by the virus.

Evidence that cessation of early polypeptide synthesis requires the presence of functional late polypeptides

In the preceding section we showed that the addition of analogues early in infection prevented the transition from early to late protein synthesis, an indication that functional early proteins were required to effect this transition. In this section, we present experiments that show that the "shut-
Fig. 1. Autoradiograms of infected cell polypeptides (ICP) electrophoretically separated on polyacrylamide gels. Panel A: The relationship between the concentration of analogues added to cultures of infected HEp-2 cells and the transition from α to β and γ polypeptide synthesis. The times of addition of analogues and labeling intervals are as shown. Also shown are the following: autoradiogram 1, virion polypeptides (VP) of HSV-1 labeled with [14C]amino acids; autoradiogram 2, lysates from infected untreated cells; autoradiogram 3, infected cell lysates labeled from 9.75 to 10.0 hr immediately after removal of cycloheximide (50 μg/ml) added at the time of infection. Two autoradiograms of the lysates from infected cells treated with 2.8 mM canavanine are shown to facilitate comparisons with autoradiograms 1–3. The one to the left contains a higher concentration of labeled protein. Panels B and C: The relationship between the time of addition of canavanine (2.8 mM) and the transition from α to β and γ polypeptide synthesis. Panel D: The transition from α to β and γ polypeptide synthesis after removal of 2.8 mM canavanine from infected cell cultures. Autoradiograms 1 and 2 of panels C and D show labeled polypeptides from lysates of mock-infected HEp-2 cells treated with 0 (autoradiogram 1) or 2.8 mM (autoradiogram 2) canavanine from 0 to 7.5 hr after mock infection and labeled with [14C]amino acids from 7.5 to 8.0 hr. The designations of virion polypeptides (VP) and infected cell polypeptides (ICP) are as described (1, 2, 4).

off” of early polypeptide synthesis requires functional late polypeptides. In the first series of experiments (Fig. 1, panel B), canavanine was added to infected cells at times shown above the autoradiogram and the cultures were labeled from 9.5 to 10 hr after infection. In the second (Fig. 1, panel C), canavanine was added at 0, 1.25, 2.75, or 4.25 hr and the polypeptides were labeled at intervals thereafter until about 20 hr after infection. Fig. 2 shows the rates of synthesis of representative α (ICP 4), β (ICP 6), and γ (ICP 5) polypeptides obtained from this experiment. The data show the following: (i) Addition of canavanine before 1.5 hr after infection precluded γ polypeptide synthesis, and of the β polypeptides, only ICP 6 was detected. By contrast, the α polypeptides (ICP 4, 0, and 27) were made for more than 18 hr at a relatively constant rate, close to the maximal rate observed in untreated cells, against a diminishing background.
of host cell polypeptide synthesis (Fig. 1C and Fig. 2A and B). As a consequence, by 18 hr after infection, ICP 4 and 0 accounted for 10 and 12%, respectively, of total protein synthesis. (ii) Addition of canavanine after 4 hr after infection did not interfere with the normal transition from early to late protein synthesis with concomitant shut-off of α and β polypeptide synthesis. However, the rate of synthesis of γ polypeptides was reduced (Fig. 2D). (iii) Addition of canavanine between 2.75 and 3.5 hr after infection resulted in simultaneous and sustained synthesis of α, β, and γ polypeptides. Compare, for example, the electrophoretic profiles of polypeptides from cultures exposed to canavanine at 2.75 hr in panel C of Fig. 1 and at 3.5, 1, and 5.5 hr after infection in panel B.

Recovery of viral protein synthesis after withdrawal of amino-acid analogues

The purpose of these studies was to compare the synthesis of β and γ polypeptides after withdrawal of canavanine with the normal pattern observed in untreated infected cells. In these experiments, the cultures were treated with canavanine from 0 to 8 hr after infection. The cells were then labeled, for intervals shown above corresponding autoradiograms in Fig. 1, panel D, at different times before and after removal of the analogue. These autoradiograms were also analyzed with respect to rates of synthesis of several polypeptides in each group (not shown). The main features of the results are as follows: (i) Infected cells exposed to canavanine for 8 hr after infection were still capable of synthesizing β and γ polypeptides once the drug was withdrawn. (ii) The synthesis of α polypeptides (ICP 4) continued for a longer interval than in cells synchronized by exposure for several hours to cycloheximide from the time of infection. (iii) Initiation of synthesis of β polypeptides (e.g., ICP 6 and 8) was delayed for 1–2 hr, and moreover, the peak rates of synthesis of these polypeptides were lower than in untreated controls. (iv) The initiation of synthesis of γ polypeptides was also delayed; in addition, their rates of synthesis increased less rapidly than in untreated infected cells.

DISCUSSION

In this paper we have shown that the addition of amino-acid analogues at different times after herpesvirus infection produces selective inactivation of "on" and "off" regulatory functions. Specifically, the data show that the function of one or more α polypeptides is required to turn "on" synthesis of subsequent polypeptide groups, since cells maintained in media containing amino-acid analogues from the time of infection made predominantly α polypeptides for at least 20 hr; the transition to later polypeptide groups did not take place. Moreover, the presence of functional polypeptides from subsequent groups is required to shut off the synthesis of α polypeptides, as evident from the fact that addition of analogues at about 3 hr after infection resulted in sustained simultaneous synthesis of all three polypeptide groups. Since the mRNA specifying α polypeptides appears to be relatively stable (2), the shut-off of α polypeptide synthesis is probably regulated at a post-transcriptional and possibly translational level.

We previously reported a rapid decline in both host and α polypeptide synthesis after the synthesis of β polypeptides (2). In the experiments described in this paper, we observed a progressive reduction in the synthesis of host polypeptides in cells treated with canavanine from the time of infection. In these cells, α polypeptides continued to be synthesized at undimin-

![Fig. 2. Quantitative analyses of the synthesis of selected host and viral polypeptides in the presence and absence of amino acid analogues.](image-url) (A) ●, Changes in the overall rate of protein synthesis in infected cells treated with 2.8 mM canavanine from the time of infection, expressed as the percent change in specific activity of treated cells relative to untreated uninfected cells pulse-labeled for 30-min intervals with [14C]amino acids. ○, Relative rates of synthesis of host polypeptides ICP 34 and 35 (see ref. 1). (B) The relative rates of synthesis of a representative α polypeptide (ICP 4) in untreated cells (solid curve) and in cells treated with canavanine added at 0 and 1.25 hr (●), 2.75 hr (○), and 4.25 hr (▲) after infection. The synthesis of ICP 4 as percent of ongoing protein synthesis in cells treated from 0 hr is shown by (●); correction of these data for differences in overall rates of protein synthesis (panel A) yields the relative rates of synthesis shown by (○). (C) The relative rate of synthesis of a representative β polypeptide (ICP 6) in untreated cells (solid curve) and in cells treated with canavanine added at 0 and 1.25 hr (●), 2.75 hr (○), and 4.25 hr (▲). (D) The relative rate of synthesis of a representative γ polypeptide (ICP 5) in untreated cells (solid curve) and in cells treated with canavanine added at 0 and 1.25 hr (●), 2.75 hr (○), and 4.25 hr (▲). The relative rates of synthesis of α, β, and γ polypeptides in untreated infected cells are based on numerous measurements made in connection with experiments cited in this and previous papers (1, 2).
ished rates. These data suggest either that both $\alpha$ and $\beta$ polypeptides are involved in the inhibition of host polypeptide synthesis and that this function of $\alpha$ polypeptides is not affected by analogue substitution, or that the inhibition of host polypeptide synthesis results from two independent steps of which only one is mediated by polypeptides synthesized after infection. Whatever the mechanism for the preferential inhibition of host cell protein synthesis, prolonged exposure of cells to canavanine from the time of infection provides an alternative procedure to cycloheximide treatment (2) for obtaining cells synthesizing predominantly viral $\alpha$ proteins.

Attention should also be drawn to the observation that after removal of analogues added at the time of infection there is both a delay in the onset and a decrease in the maximum amount of $\beta$ and $\gamma$ polypeptide synthesis. Although these data could be interpreted in terms of interference between functional and nonfunctional $\alpha$ polypeptides, we cannot presently exclude alternative explanations, such as impairment of essential host functions during the exposure to canavanine.

Amino-acid analogues have been elegantly used in studies by Jacobson et al. (5, 6) to demonstrate that the translational product of poliovirus RNA is a single polypeptide. The data presented in this paper show that amino-acid analogues are versatile tools for analysis of the relationships between time of synthesis, structure, and function of viral polypeptides and, in particular, for determining their roles in regulatory events.

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