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Regulation of Herpesvirus Macromolecular Synthesis

I. Cascade Regulation of the Synthesis of Three Groups of Viral Proteins[1]

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Based on evidence that 50% of herpes simplex 1 DNA is transcribed in HEp-2 cells in the absence of protein synthesis we examined the order and rates of synthesis of viral polypeptides in infected cells after reversal of cycloheximide- or puromycin-mediated inhibition of protein synthesis. These experiments showed that viral polypeptides formed three sequentially synthesized, coordinately regulated groups designated α, β, and γ. Specifically: (i) The α group, containing one minor structural and several nonstructural polypeptides, was synthesized at highest rates from 3 to 4 h postinfection in untreated cells and at diminishing rates thereafter. The β group, also containing minor structural and nonstructural polypeptides, was synthesized at highest rates from 5 to 7 h and at decreasing rates thereafter. The γ group containing major structural polypeptides was synthesized at increasing rates until at least 12 h postinfection. (ii) The synthesis of α polypeptides did not require prior infected cell protein synthesis. In contrast, the synthesis of β polypeptides required both prior α polypeptide synthesis as well as new RNA synthesis, since the addition of actinomycin D immediately after removal of cycloheximide precluded β polypeptide synthesis. The function supplied by the α polypeptides was stable since interruption of protein synthesis after α polypeptide synthesis began and before β polypeptides were made did not prevent the immediate synthesis of β polypeptides once the drug was withdrawn. The requirement of γ polypeptide synthesis for prior synthesis of β polypeptides seemed to be similar to that of β polypeptides for prior synthesis of the α group. (iii) The rates of synthesis of α polypeptides were highest immediately after removal of cycloheximide and declined thereafter concomitant with the initiation of β polypeptide synthesis; this decline in α polypeptide synthesis was less rapid in the presence of actinomycin D which prevented the appearance of β and γ polypeptides. The decrease in rates of synthesis of β polypeptides normally occurring after 7 h postinfection was also less rapid in the presence of actinomycin D than in its absence, whereas ongoing synthesis of γ polypeptides at this time was rapidly reduced by actinomycin D. (iv) Inhibitors of DNA synthesis (cytosine arabinoside or hydroxyurea) did not prevent the synthesis of α, β, or γ polypeptides, but did reduce the amounts of γ polypeptides made.

Parallel studies on viral RNA and proteins made in HEp-2 cells productively infected with herpes simplex 1 (HSV-1, human herpesvirus 1) have suggested the existence of controls regulating the synthesis and accumulation of these viral macromolecules (5, 6, 10, 20, 21). The pertinent data may be summarized briefly as follows.

(i) The stable RNA transcripts accumulating in the infected cell arise from approximately 50% of the DNA (5). Transcripts arising from at least 40 to 43% of the DNA have been detected in polyribosomes prepared by two different procedures (21; M. Kozak and B. Roizman, manuscript in preparation). Assuming that all the stable RNA transcripts direct protein synthesis, it may be estimated that the informational content of HSV-1 is sufficient to specify the sequence of a maximum of 55,000 amino acids. In a recent publication we reported (10) the identification of 47 infected cell polypeptides (ICP) which fulfilled at least one of several criteria for virus specificity. Since no rapid posttranslational cleavages could be demonstrated either by comparison of short and long radiolabeling intervals or by the use of inhibitors of proteolytic enzymes, we regarded these polypeptides as primary gene products and esti-

[1] Dedicated to the memory of Gordon Plummer.

mated that they could account for approximately 75% of the transcribed viral DNA.

(ii) Evidence presented elsewhere showed that viral DNA templates specify two sets of RNA transcripts differing in abundance and in the extent of adenylation (5) implying that the signals for adenylation and abundances of transcripts are encoded in the template and expressed post-transcriptionally. Moreover, on the basis of measurements of rates of synthesis of viral ICP throughout the virus growth cycle, it became apparent that the polypeptides formed multiple classes differing in the temporal patterns of their synthesis throughout infection. Recognition of these "temporal" classes implied the existence of coordinate regulation of viral polypeptide synthesis.

Among the questions which remain unanswered are the nature of the factors determining RNA abundance, the nature of the polypeptides specified by the abundant and scarce RNAs, and the factors determining the patterns of coordinate synthesis of viral polypeptides. The purpose of this series is to attempt to delineate the interrelationships between viral RNA and protein synthesis. The studies described here concerned three questions. The first was whether the syntheses of ICP were totally independent of each other or whether the synthesis of some ICP required prior synthesis of others. Since an interdependence was found, the second question was whether the interdependence was mediated solely by the presence of ICP made earlier in the course of the reproductive cycle or whether an additional requirement such as new RNA synthesis was involved. Thirdly, the question arose whether the patterns of synthesis of early groups of ICP were determined by the synthesis of later groups of ICP. The experiments described in this paper were based on the observation that transcription of viral DNA does not require prior viral protein synthesis and that the amount of DNA template transcribed in the presence of inhibitory concentrations of cycloheximide (6) or puromycin (S. Silverstein and B. Roizman, unpublished data) corresponds to at least the amount observed in untreated cells incubated for the same length of time. Operationally, the experiments dealt with the nature of the viral polypeptides made after treatment of infected cells with inhibitors of protein synthesis at different intervals during the reproductive cycle.

MATERIALS AND METHODS

Solutions and chemicals. Maintenance medium for infected cells was mixture 199 supplemented with 1% calf serum. Labeling medium consisted of mixture 199 containing one-tenth the usual amounts of leucine, isoleucine, and valine and supplemented with 1% dialyzed calf serum. Labeled amino acids were purchased from Schwartz/Mann, Orangeburg, N.Y., and were as follows: L-[U-^{14}C]isoleucine, L-[U-^{14}C]leucine, and L-[U-^{14}C]valine, all with specific activities of approximately 300 mCi/mmol. Puromycin dihydrochloride and cytosine arabinoside were purchased from Nutritional Biochemicals Co., Cleveland, Ohio, and cycloheximide (Actidione) and hydroxyurea from Calbiochem. Actinomycin D (Dactinomycin) was a gift from Merck Sharpe and Dohme, Research Laboratory, Rahway, N.J.

Cells. Human epidermoid carcinoma no. 2 (HEp-2) cells were grown in Eagle minimal essential medium (EMEM) supplemented with 10% calf serum, 0.001% ferric nitrate, and 1% sodium pyruvate.

Viruses. All of the experiments described in this paper were done with the F prototype of HSV-1 [HSV-1(F)].

Labeling of proteins synthesized by infected and uninfected cells. Confluent HEp-2 cell monolayers in tissue culture flasks (approximately 10^6 cells per flask) were exposed to 20 PFU of virus per cell unless otherwise noted (in 2.0 ml of maintenance medium) or were mock infected with 2.0 ml of this medium. Inoculated cultures were incubated with constant agitation for 1 h at 37 C, and therafter virus or mock inocula were decanted. The monolayers were then rinsed with 5.0 ml of maintenance medium per flask, replenished with the same volume of maintenance medium, and reincubated at 37 C. For labeling, the cultures were rinsed and then replenished with labeling medium containing [^{14}C]leucine, [^{14}C]isoleucine, and [^{14}C]valine (0.3 to 1.0 μCi of each amino acid per ml). 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 C in a small volume of 2% SDS, 5% β-mercaptoethanol, 0.05 M Tris-hydrochloride (pH 7.0) for subsequent electrophoresis on polyacrylamide gels.

The times at which labeled precursors were added and removed, etc., are stated in hours after the exposure of cells to virus.

Use of inhibitors. Inhibitors of protein synthesis, either puromycin or cycloheximide, were added to cell culture medium to yield final concentrations of 50 μg/ml. After treatment the medium containing the inhibitors was decanted, and monolayer culture containing 10^6 HEp-2 cells was rinsed four times with 5.0-ml portions of maintenance medium maintained at 37 C. The total duration of the rinsing procedure did not exceed 10 min. These concentrations of inhibitors were sufficient to reduce amino acid incorporation by 98 to 99%. It is particularly pertinent to note, in relation to the experiments described below, that the addition and immediate removal of these inhibitors followed by the rinsing procedure was not in itself deleterious to cells. In control experiments done with both infected and uninfected cells, the polypeptides made before and after this series of procedures could not be differentiated either in number or in their rates of synthesis. Inhibitors of DNA synthesis,

either cytosine arabinoside or hydroxyurea, were added to cell culture medium to yield final concentrations of 50 μg/ml. Actinomycin-D was used at a final concentration of 10 μg/ml. The inhibitors of DNA and RNA synthesis were removed from the culture medium before the addition of medium containing ^{14}C amino acids.

Polyacrylamide gel electrophoresis. The electrophoretic, staining, and autoradiographic techniques were as described previously (8, 22). The polyacrylamide gel electrophoresis was done in a discontinuous buffer system (3, 16) modified by the inclusion of SDS. The polyacrylamide slabs were cross-linked with *N,N'*-diallyltartardiamide (Aldrich Chemical Co., Milwaukee, Wis.) in place of the same weight of *N,N'*-methylenebisacrylamide (1, 8).

Densitometry of stained gels and autoradiograms and computer-aided analysis of absorbance tracings. Absorbance measurements of the autoradiographic images were made in a Gilford recording spectrophotometer equipped with a gel scanner attachment. The voltage output from the photocell was also recorded by a General Automation 16/45 digital computer and displayed as absorbance (voltage) plotted as a function of distance migrated (time of scan) on the oscilloscope screen of a Tektronix model 4010 control console. The computer was programmed to expand regions of the initial profile as determined by the operator and to compute the areas under the tracing for individual bands defined by vertical lines set parallel to the absorbance (voltage) axis. The use of computer-aided planimetry for the quantitation of herpesvirus protein synthesis was described in greater detail elsewhere (10).

RESULTS

Patterns of synthesis of virus-specific polypeptides in infected cells. Analysis of the polypeptides pulse labeled at various times during the reproductive cycle and separated by electrophoresis in polyacrylamide gels showed that they differ in time and overall pattern of synthesis (10). For the purposes of this paper, this fact is also apparent from the autoradiograms of untreated infected cell lysates shown in Fig. 1 and 2. Based on more detailed analyses of such materials, we concluded that ICP formed at least five temporal classes designated as A, B, C, D, or E (Fig. 11 of ref. 10). Polypeptides of class A were synthesized at gradually increasing rates throughout infection. Polypeptides in classes B, C, and D were synthesized at rates which increased at first and thereafter remained constant (B) or decreased (C and D). Polypeptides in class E were synthesized at gradually diminishing rates throughout the observation period.

In this paper we shall be concerned with ICP representative of these classes and some of their properties are relevant to this study. ICP 34, 35, and a polypeptide designated as host 21 have been chosen as representatives of host polypeptides. As reported earlier (10), they do not fulfill any of the criteria for virus specificity. In infected cells, their temporal patterns of synthesis are those of class E and at at no time do their rates of synthesis exceed those seen in uninfected cells. The properties of all but one of the virus-specific ICP (i.e., ICP 0) cited as examples in the present study have been summarized previously (10). Briefly, ICP 4, 6, and 8 comigrate in polyacrylamide gels with several minor components of the herpes virion and are members of temporal class C. ICP 5 is a member of

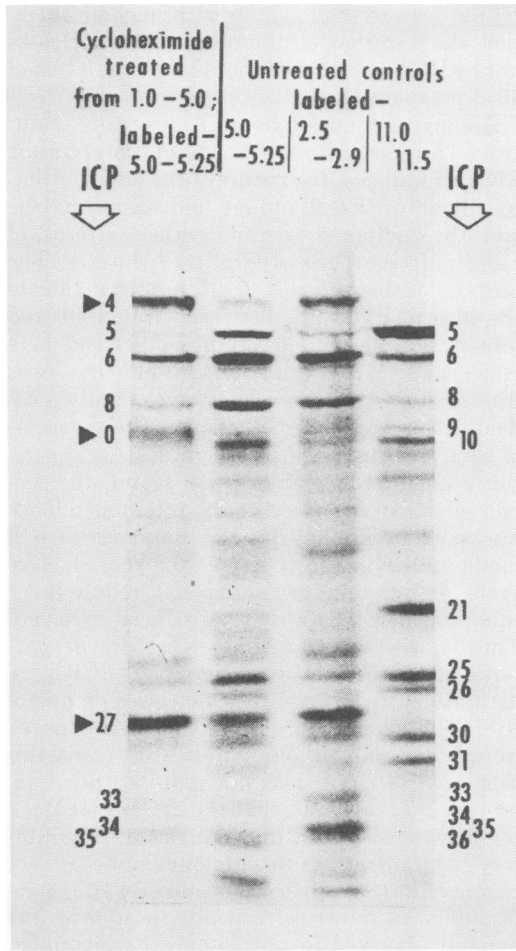

FIG. 1. *Autoradiograms of a 7.5% polyacrylamide gel slab containing electrophoretically separated polypeptides from HSV-1 (F1)-infected cells labeled with ^{14}C-amino acids from 5 to 5.25 h postinfection, i.e., immediately after removal of cycloheximide added at 1 h postinfection, and from 5 to 5.25, 2.5 to 2.9, and 11.0 to 11.5 h postinfection in untreated cells. Enumeration of ICP (other than ICP 0) is as previously reported (10). The arrows to the left of the figure identify the virus-specific ICP which are synthesized at high rates after the removal of cycloheximide.*

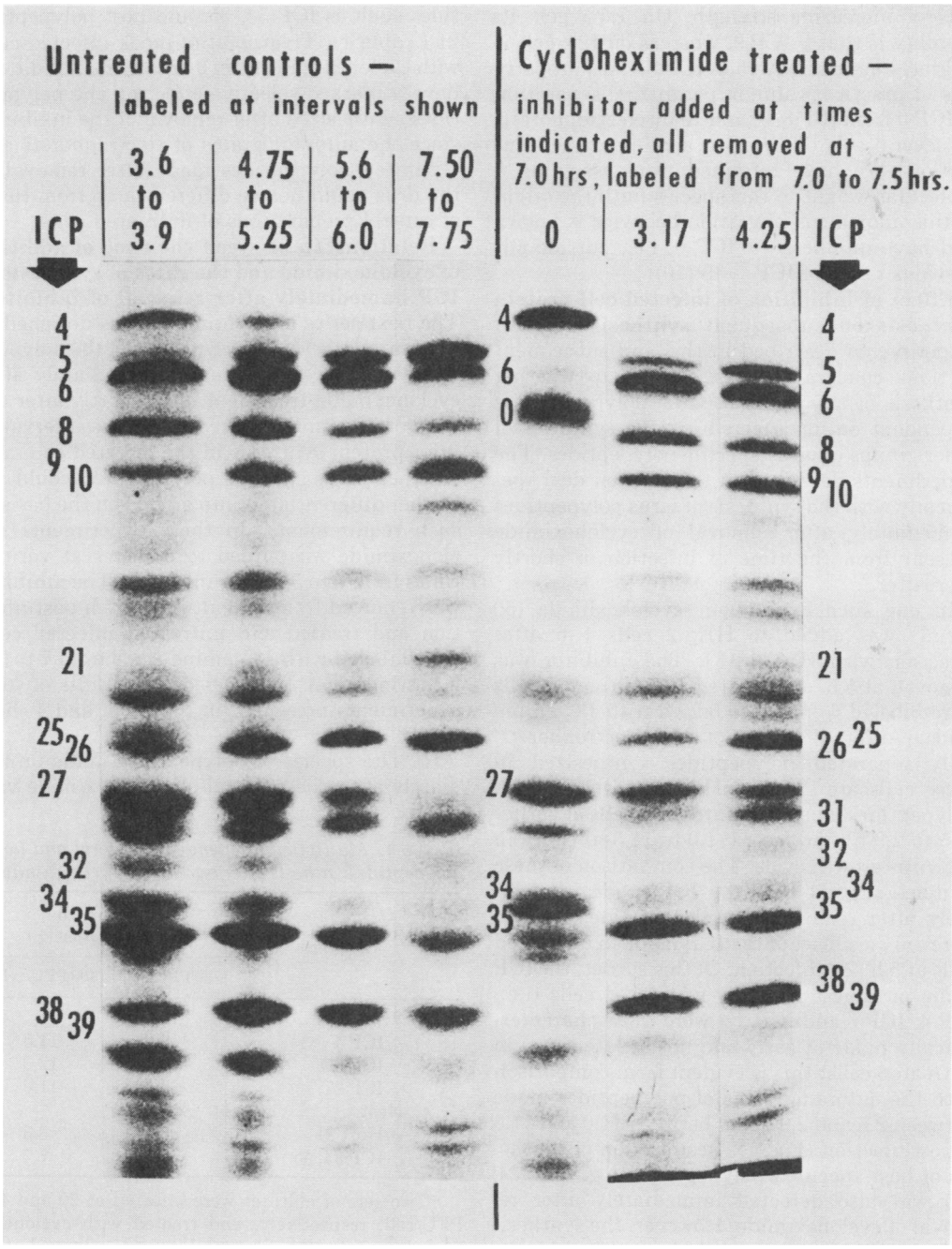

FIG. 2. *Autoradiogram of a polyacrylamide gel slab containing electrophoretically separated polypeptides pulse labeled in untreated control cultures with* ^{14}*C-amino acids at various intervals after infection (four samples on left of figure), and from 7.0 to 7.5 h postinfection immediately after the removal of cycloheximide added at either 0, 3.17, or 4.25 h postinfection, respectively (three samples on right of figure).*

class A and comigrates with the major polypeptide (VP 5, ref. 7, 10) of the virus capsid. ICP 0 was not detected in our previous analyses of untreated infected cells. It exhibits an anomalous migration behavior unique among proteins labeled for short intervals in treated or untreated cells in that it is retarded more than ICP of similar mobilities (e.g., ICP 8, 9, and 10) on

gels of increasing strength. On 7.5% gels its mobility is that if ICP 9, whereas on 12% gels it migrates more slowly than ICP 8. This property was of practical value in permitting separation of ICP 0 from ICP 8, 9, and 10 by electrophoresis on several gels differing in acrylamide concentration. We have refrained from assigning a molecular weight to this species until the origin of this anomalous migration behavior is known and have numbered it ICP 0, i.e., outside our previous range of ICP 1-49 (10).

Effect of inhibition of infected cell protein synthesis on subsequent synthesis of ICP. Experiments described in this and subsequent sections concern the question as to whether synthesis of any group of virus polypeptides is dependent on, or affected by, the synthesis of other groups of virus-specific polypeptides. The experiments described in this section deal specifically with the synthesis of virus polypeptides immediately after removal of cycloheximide present from the time of infection or shortly thereafter.

In one such experiment cycloheximide (50 μg/ml) was added to HEp-2 cells 1 h after infection with HSV-1 (F). The inhibitor was removed at 5 h, and treated and untreated cells were labeled from 5.0 to 5.25 h with ^{14}C-amino acids. Autoradiograms of the electrophoretically separated polypeptides synthesized in these cells are shown in Fig. 1, together with polypeptides labeled in untreated cells at earlier (2.5 to 2.9 h) and later (11.0 to 11.5 h) times in the virus growth cycle. The comparison of these samples showed that the ICP made immediately after removal of cycloheximide at 5.0 h were only a subset of the ICP made in untreated cells at 5 h postinfection. Of this subset, the ICP made in largest amounts in treated cells (i.e., ICP 4, ICP 0 and ICP 27) were those characteristically made at early and not at late times in untreated cells; this is evident from comparison with the autoradiograms of polypeptides made between 2.5 and 2.9 h and between 11.0 and 11.5 h postinfection (Fig. 1). Resumption of synthesis of host-specified polypeptides (e.g., ICP 34, 35) was also detected immediately after removal of cycloheximide. However, the synthesis of host polypeptides and those specified by the virus and belonging to this subset could be readily differentiated by varying the multiplicity of infection. Increasing the multiplicity of infection in the presence of cycloheximide from 20 to 400 PFU/cell resulted in increased initial rates of synthesis of ICP 4, 0, and 27 immediately after removal of the drug and in decreased initial rates of synthesis of host cell polypeptides such as ICP 34, 35, and host polypeptide 21 (Table 1). Treatment of mock-infected cells with cycloheximide from 0 or 1 until 5.0 h did not have a marked selective effect on the polypeptides synthesized after removal of the inhibitor, since the autoradiograms of electrophoretically separated polypeptides made after removal of the drug could not be differentiated from those of control preparations of untreated cells.

Relationship between the time of addition of cycloheximide and the rates of synthesis of ICP immediately after removal of inhibitor. The next series of experiments was designed to determine whether the synthesis of the polypeptides which were not made immediately after cycloheximide treatment at or shortly after the time of infection required a specific interval of prior protein synthesis in the infected cell, and whether virus-specific polypeptides could be further differentiated into groups on the basis of such requirements. In these experiments cycloheximide was added to cultures at various times from 0 to 5 h after infection. The inhibitor was removed from all cultures at 7 h postinfection and treated and untreated infected cells were labeled with ^{14}C-amino acids from 7 to 7.5 h postinfection. Analyses of the results of such experiments presented in Fig. 2, 3, and 4 show the following.

(i) The species of polypeptides made immediately after the removal of cycloheximide var-

TABLE 1. *Multiplicity dependence of viral and host polypeptide synthesis after removal of cycloheximide*[a]

Polypeptides	Total amino acid incorporation (%)	
	20 PFU/cell	400 PFU/cell
Viral		
ICP 4	8.3	14.5
ICP 0	10.5	12.3
ICP 27	5.6	11.0
Host		
Host 21	5.8	3.3
ICP 34, 35	8.1	7.2

[a] Two sets of cultures were infected at 20 and 400 PFU/cell, respectively, and treated with cycloheximide from 0 to 12 h. The inhibitor was removed at 12 h and the cultures were labeled with ^{14}C-amino acids from 12 to 12.5 h. The polypeptides of total cell lysates were then separated by electrophoresis on a polyacrylamide gel slab, and the labeled polypeptides were quantitated by computer-aided planimetry of absorbance tracings from the autoradiogram of the electrophoretically separated polypeptides. The total incorporation of labeled amino acids in the two samples were approximately the same.

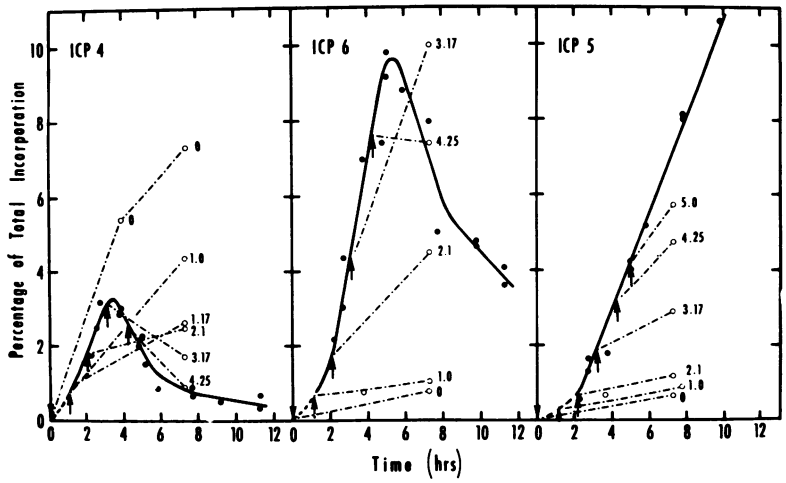

FIG. 3. *The synthesis of polypeptides representative of groups α (ICP 4), β (ICP 6), and γ (ICP 5) in cycloheximide-treated and in untreated infected cells. The amounts of polypeptide made are expressed in terms of their percentage contribution to the total incorporation of ^{14}C-amino acids into protein during 0.25-to 0.5-h labeling intervals. The filled circles connected by solid lines represent the synthesis of polypeptides at different times after infection in untreated control cultures. The open circles represent the synthesis of polypeptides immediately after the removal of cycloheximide. Cycloheximide was added at the times indicated by arrows to the control curves and the arabic numbers next to the open circles. Corresponding times of addition and removal are interconnected by the dashed lines. The data were derived from planimetry of autoradiograms similar to those shown in Fig. 2. Similar data were obtained for other polypeptides of each group, notably for ICP 0 and ICP 8, used as examples of α and β polypeptides and shown in Fig. 4. In the data shown in this and preceding figures, the samples subjected to electrophoresis contained equal amounts of proteins. Although the data are expressed in percentages of total amino acid incorporation, they reflect real differences in molar rates of synthesis. Specifically, in the case of samples exposed to cycloheximide from 0 to 7.0 h postinfection and labeled from 7 to 7.5 h, in four experiments the specific activities of treated samples were all >50% of untreated cultures labeled at the same time, whereas 4- to 10-fold differences were observed in the percentage of incorporation due to α polypeptides in these same experiments.*

ied depending upon the time at which cycloheximide was added. The longer the interval between initiation of infection and exposure to the drug, the closer was the resemblance of the electropherograms of polypeptides made immediately upon removal of the drug to those made in untreated infected cells incubated for the same length of time (Fig. 2). Moreover, an equivalent duration of cycloheximide treatment over different intervals of the virus growth cycle did not necessarily produce similar patterns of synthesis of ICP after removal of the inhibitor. This is readily apparent from comparisons of the polypeptides synthesized after a treatment from 1 to 5.0 h (Fig. 1) with those synthesized after a treatment from 3.17 to 7.0 h (Fig. 2). On the basis of these observations we conclude that an interval of infected cell protein synthesis prior to the addition of the drug is required for the production of certain virus-specific polypeptides immediately after the removal of cycloheximide. This interval need not be immediately prior to the amino acid pulse; in fact, it may be followed by several hours of exposure to cycloheximide prior to the amino acid pulse.

(ii) Quantitative analyses of the rates of synthesis of ICP as a function of the time of addition of cycloheximide (Fig. 3 and 4) permitted recognition of three groups of virus-specific polypeptides. These groups, designated α, β, and γ, differed in their requirements for a prior interval of protein synthesis for their own synthesis to take place immediately after treatment with cycloheximide.

(iii) Polypeptides of the α group (e.g., ICP 4, ICP 0) were synthesized immediately upon removal of cycloheximide added at the time of infection or a short interval later (Fig. 2, 3, and 4). The initial rates of synthesis of α polypeptides, i.e., immediately after removal of the inhibitor, increased with the duration of the drug treatment, and with prolonged treatment exceeded the maximum rates observed in untreated infected cells (Fig. 3). Initial rates of synthesis of α polypeptides were progressively reduced by delaying the time of addition of cycloheximide (Fig. 3 and 4). α Polypeptides were synthesized in untreated cultures at the highest rates from 3 to 4 h postinfection (Fig. 3). Based on the temporal patterns of their synthe-

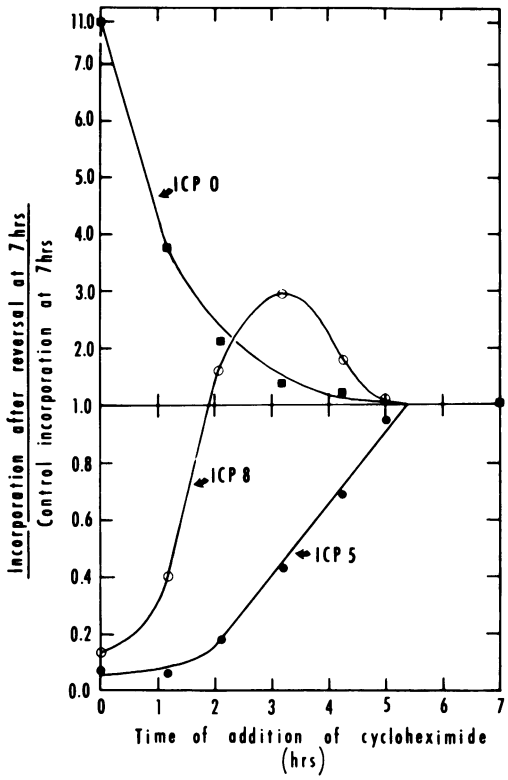

FIG. 4. *Initial rates of synthesis of α (ICP 0, filled squares), β (ICP 8, open circles), and γ (ICP 5, filled circles) polypeptides observed on removal of cycloheximide added at different times and removed simultaneously at 7.0 h postinfection. The data are expressed as the ratio of the percentage of total radioactivity incorporated in the polypeptide immediately after removal of cycloheximide (cycloheximide was removed at 7.0 h and cultures were labeled from 7.0 to 7.5 h) to the percentage of incorporation into the same polypeptide in untreated cultures labeled from 7.5 to 7.75 h. Data points were plotted as a function of the time of addition of the inhibitor and are based on analyses of autoradiograms similar to that shown in Fig. 2. Almost identical plots were obtained for other polypeptides of each group, notably for ICP 4 and ICP 6 used as examples of α and β polypeptides and shown in Fig. 3.*

sis they were previously (10) classified as either class C (ICP 4) or were unclassified (ICP 0, 27). α Polypeptides resembled host polypeptides in two respects. Both α and host polypeptides were made upon removal of cycloheximide added at the time of infection, i.e., their synthesis did not require prior infected cell protein synthesis. In addition, the initial rates of synthesis of both host and α polypeptides were reduced in cultures exposed to cycloheximide at successively later intervals after infection. α Polypeptides differed from host polypeptides in that, as pointed out earlier in the text, the initial rates of synthesis of α polypeptides increased with the multiplicity of infection, whereas the rates of synthesis of host polypeptides decreased as the multiplicity of infection was increased. Also, whereas the initial rates of synthesis of α polypeptides increased with the duration of treatment (beginning before 1.0 h), the initial rates of synthesis of host proteins decreased with the duration of the drug treatment.

(iv) Polypeptides of the second group, β, were not synthesized immediately on removal of cycloheximide added before 1.0 h postinfection (Fig. 2, 3, and 4). Additions of cycloheximide later than 1.5 h postinfection resulted in increasing initial rates of synthesis of β polypeptides. In certain instances, for example, in cultures treated with cycloheximide beginning between 3.0 and 5.5 h until 7 h postinfection, the initial rates of synthesis exceeded those observed in untreated cells. In untreated cultures, β polypeptides were synthesized at highest rates from 5 to 7 h postinfection (Fig. 3). Based on the temporal patterns of their synthesis, they were classified as belonging to class C (e.g., ICP 6 and 8) or D (e.g., ICP 36) in our previous study (10).

(v) The third group of polypeptides, γ, likewise required an interval of infected cell protein synthesis before they were synthesized (Fig. 2); they differed from β polypeptides in the duration of this interval and in the initial rates of synthesis after removal of drug. Thus, γ proteins were not made immediately on removal of cycloheximide added before about 2 h postinfection. Between 2 and 5 h postinfection, the later the exposure of infected cells to cycloheximide, the higher were the initial rates of synthesis of γ polypeptides (Fig. 3 and 4). In cells exposed to cycloheximide between 5 h postinfection or later and until 8 h postinfection, the initial rates of synthesis of γ group polypeptides were the same as those in untreated controls. Unlike those of α and β polypeptides, the initial rates of synthesis of γ polypeptides in no instance exceeded those observed in untreated infected cells. On the basis of the kinetics of their synthesis, γ polypeptides (e.g., ICP 5, 17, 21, 31, etc.) were previously (10) classified as belonging to class A, i.e., in untreated infected cells they were made at progressively higher rates until at least 12 h postinfection.

Effects of actinomycin-D on the synthesis of α, β, and γ polypeptides. In the preceding section we established that the synthesis of β and γ group virus polypeptides required prior infected cell protein synthesis. The experiments described in this section were designed to deter-

mine whether the production of α proteins was the sole requirement for the synthesis of β and γ polypeptides. In one such experiment 12 replicate monolayers of HEp-2 cells were infected at multiplicities of 10 to 20 PFU/cell. Cycloheximide was added to six of these cultures at 1.0 h postinfection and removed at 7.0 h. Two treated and two untreated infected cultures were labeled with ^{14}C-amino acid from 7.0 to 7.5 h. At the time of removal of cycloheximide, actinomycin-D (10 μg/ml) was added to each of two treated and two untreated cultures and these cultures, together with those not exposed to actinomycin-D, were labeled from 9.5 to 10.0 h postinfection. Labeled polypeptides were separated by electrophoresis on polyacrylamide gel slabs and quantitated as described in Materials and Methods. The results of these analyses for several polypeptides characteristic of α, β, and γ groups of virus-specific polypeptides and for selected host polypeptides are summarized in Table 2.

The salient features of the data may be summarized as follows. (i) Upon removal of cycloheximide, in the absence of actinomycin-D, the rates of synthesis of α and host polypeptides declined concomitantly with increases in the rates of synthesis of β and γ polypeptides (columns 4 and 5). (ii) Addition of actinomycin-D to cultures previously not exposed to cycloheximide resulted in two effects. First, actinomycin-D prevented or reduced the normal decline in the rates of synthesis of α, β, and host polypeptides observed in untreated cultures at that time. Second, it caused a rapid decline in the rates of synthesis of γ polypeptides relative to those of untreated cultures. (iii) Actinomycin-D added at the time of removal of cycloheximide reduced the decline in the rate of synthesis of α and host polypeptides and at the same time precluded the synthesis of β and γ polypeptides (column 6). The data indicate that the synthesis of β and γ polypeptides after removal of cycloheximide added at 0 or at 1 h postinfection required RNA synthesis in the presence of α polypeptides. They also show that the decline in the synthesis of α polypeptides in untreated cultures was not due to the action of α polypeptides alone. Also, throughout the period tested the ongoing syn-

TABLE 2. *Effects of actinomycin D on synthesis of polypeptides in untreated infected cells and after removal of cycloheximide*

Group: polypeptide	Amounts of polypeptide synthesized[a]					
	(1)	(2)	(3)	(4)	(5)	(6)
	Untreated cells		Actinomycin D from 7.0 to 9.5 h Pulse 9.5 to 10.0 h	Cycloheximide treated from 1.0 to 7.0 h		
	Pulse 7.0 to 7.5 h	Pulse 9.5 to 10.0 h		Pulse 7.0 to 7.5 h	Pulse 9.5 to 10.0 h	Actinomycin D from 7.0 to 9.5 h Pulse 9.5 to 10.0 h
α: ICP 4	2.2	1.3[b]	1.6	7.4	2.9	6.8
α: ICP 0			1.6	13.9	1.5	5.1
β: ICP 6	13.7	7.1	10.6	3.0	5.4	2.5
β: ICP 8	9.8	5.1	5.8	1.7[b]	3.7	1.7[b]
γ: ICP 5	12.5	13.6	6.7	1.5[b]	2.8	1.1[b]
Host: ICP 34, 35	9.5	4.2	9.7	14.9	7.7	10.1
Relative incorporation of ^{14}C-amino acids into total protein in sample[c]	1.54	1.27	1.16	1.68	1.00	1.38

[a] Data were obtained by analysis of absorbance tracings of autoradiograms of electrophoretically separated polypeptides. The percentage of total incorporation in each polypeptide was determined by computer-aided planimetry of the absorbance tracing of the autoradiogram (10). These percentages were then corrected for differences in the total incorporation of labeled amino acids so that amounts of polypeptide synthesized in different samples could be compared.

[b] Values are maximum estimates for amounts of these minor components.

[c] Relative incorporation of ^{14}C-amino acids into total protein in various samples, expressed as the ratios of the total integrated absorbance of autoradiograms from electrophoretically separated samples. Note that the amount of polypeptide synthesized is the product of the relative incorporation into the sample and the percentage of total integrated absorbance due to a given polypeptide. These data are shown to indicate the extent of variation in total amino acid incorporation observed in these samples.

thesis of γ polypeptides was more rapidly affected by actinomycin D than the synthesis of host or of viral α and β polypeptides.

Interchangeability of cycloheximide and puromycin. In the preceding experiments we used cycloheximide as the inhibitor of protein synthesis. To determine whether the observed effects of cycloheximide were indeed the consequence of inhibition of protein synthesis, the polypeptides synthesized in infected cells after the removal of cycloheximide were compared with those synthesized after the removal of puromycin. The inhibitors were each added to replicate cultures at 1.0 h and removed at 7.0 h postinfection. These cultures as well as untreated cultures were labeled from 7.0 to 7.5 h with ^{14}C-amino acids in the same fashion as in the experiment with cycloheximide-treated cells summarized in Table 2. Preferential synthesis of α-polypeptides and resumption of synthesis of host protein was observed immediately after the removal of either cycloheximide or puromycin, and the time courses of the subsequent synthesis of β and γ proteins were entirely comparable. The only differences between the results obtained with each of the two inhibitors were the lower initial level of overall protein synthesis after the removal of puromycin and higher initial rates of synthesis of β polypeptide ICP 6 than those observed in cycloheximide-treated cells.

Effects of inhibitors of DNA synthesis on synthesis of α, β, and γ polypeptides. It has been previously reported that viral structural proteins are made in the presence of inhibitors of DNA synthesis (2, 14, 15, 19, and A. Buchan and S. M. Luff, personal communication). In connection with the present studies, it was of interest to determine whether these inhibitors affected the rates of synthesis of the three groups of polypeptides (α, β, and γ).

Replicate cultures of HEp-2 cells were infected at multiplicities of 20 PFU/cell and incubated in the absence of inhibitors and in the presence of cytosine arabinoside and hydroxyurea, respectively. At 5.5 h postinfection the inhibitors were removed and all cultures were labeled with ^{14}C-amino acids from 5.50 to 6.0 h postinfection. Samples of total cell lysates were then subjected to electrophoresis on a polyacrylamide gel slab. Analysis of the absorbance tracings of the autoradiograms of the labeled polypeptides separated from these samples showed that the inhibitors of DNA synthesis did not prevent the synthesis of α, β, or γ polypeptides. However, the amounts of γ polypeptides made in the treated cultures were one half to one third lower than the amounts made in untreated cells (Fig. 5).

DISCUSSION

In this paper we present evidence for the following. First, the polypeptides specified by herpes simplex virus may be divided into three groups designated as α, β, and γ, whose synthesis is coordinately regulated. Second, we discern two kinds of regulatory processes. The first of these turns "on" the synthesis of the three groups of polypeptides sequentially. The second regulatory process turns "off," or reduces the rate of synthesis, first of the α polypeptides and subsequently of the β polypeptides. The emergent pattern is that of cascade regulation. It is

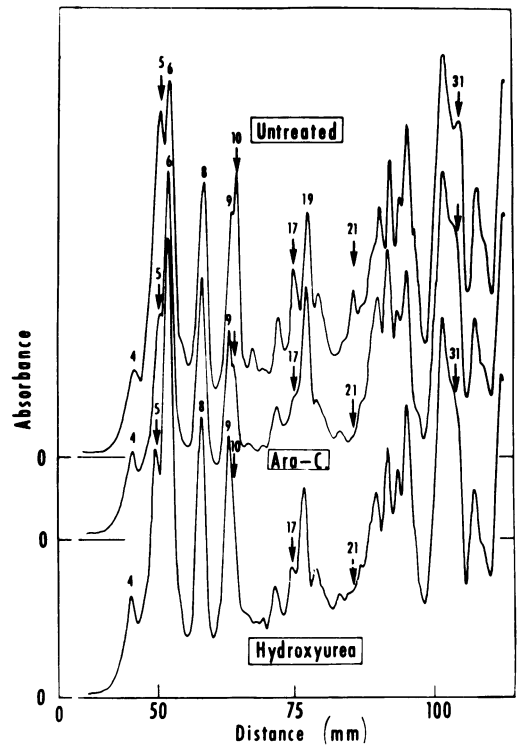

FIG. 5. *Absorbance tracings of autoradiograms of electrophoretically separated polypeptides from infected cells labeled with ^{14}C-amino acids from 5.5 to 6.0 h after infection in untreated cells (top tracing) and in cells treated from 0 to 5.5 h with 50 μg of cytosine arabinoside per ml (Ara-C, middle tracing) or 50 μg of hydroxyurea per ml (bottom tracing). Selected ICP are identified by their numbers; those annotated with arrows directed downward were reduced in treated cultures. Absorbance scales for the three tracings are directly comparable. The zero absorbance values for middle and top tracings are indicated by short lines to the ordinate.*

convenient to discuss each of these regulatory processes separately.

Sequential turning "on" of viral polypeptide synthesis. The salient features of the data presented in this paper are that the onset of synthesis of α, β, and γ polypeptides have different requirements. α Polypeptides are synthesized at maximum rates at 3 to 4 h postinfection in untreated cells. The turning "on" of the synthesis of α polypeptides does not require prior viral protein synthesis. Rakusanova et al. (18) also noted the synthesis of a selected subset of polypeptides after removal of cycloheximide from pseudorabies virus-infected cells. The synthesis of β protein does require infected cell protein synthesis, presumably the synthesis of α polypeptides. In untreated cells, β protein synthesis rises to a maximum at 5 to 7 h postinfection. Production of γ polypeptides is also dependent on prior virus protein synthesis. However, the interval required before γ proteins can be synthesized is longer than that required for β proteins. During this interval appreciable amounts of both α and β polypeptides are synthesized. The production of γ proteins may therefore involve either a delayed action of one or more α polypeptides, or the participation of one or more β polypeptides by themselves or with α polypeptides.

In the preceding paragraph we have emphasized only requirements for protein synthesis. We should reiterate that although inhibitors of DNA synthesis do not reduce α- and β-polypeptide synthesis, they do reduce the synthesis of γ polypeptides. We conclude that DNA synthesis is not an obligate requirement for the production of any of the three groups of virus polypeptides. However, the synthesis of progeny virus DNA may serve to amplify the production of γ polypeptides.

Functions of α polypeptides and their role in the production of β polypeptides. Of the currently identified α polypeptides only one, ICP 4, is clearly identified as a structural component of the virus by our present criteria (10). It comigrates with VP 4, a minor virion polypeptide, which is incorporated into virions much less efficiently than major structural components. On the basis of this low efficiency of incorporation, we previously suggested it may have a function other than that of a structural polypeptide (10). The remaining polypeptides in the α group have no known electrophoretic counterparts in the virion.

Data obtained from experiments utilizing actinomycin-D indicated that RNA synthesis in the presence of α polypeptides is required for β (and γ) polypeptide synthesis. Since RNA transcribed in the presence of cycloheximide is complementary to the same amount of viral DNA as that transcribed in the absence of the drug, i.e., at least 44% (6), the interpretation of this requirement is not immediately apparent. However, it is clear that mRNAs for α polypeptides are functionally stable, and they either accumulate in the presence of cycloheximide, for as long as 12 h (Table 1), or their translation is enhanced by the accumulation of a factor whose synthesis is stimulated in the presence of cycloheximide as suggested by Penman et al. (17). By the same considerations, functional mRNA for β polypeptides does not accumulate in cells treated with cycloheximide from the time of infection. Therefore, at least one role of α polypeptides involves the production or maintenance of functional β polypeptide mRNA. We cannot exclude the possibility that α polypeptides also function at the level of translation of functional β and γ mRNAs.

In untreated infected cells α protein synthesis declines after about 4 h postinfection. This observation and the data from cycloheximide-treated cells (Fig. 3 and 4) show that the continuous synthesis of α polypeptides is not required to sustain their role in subsequent protein synthesis.

Functions of β polypeptides and their role in γ polypeptide synthesis. Polypeptides of the β group are both minor structural components of the virion (e.g., ICP 6, ref. 10) and nonstructural components (e.g., ICP 36). Based on the similarity between the time course of accumulation of β polypeptides (this can be derived from Fig. 4 for ICP 6) and that of a number of herpesvirus specified enzymes (e.g., thymidine kinase, DNA polymerase, DNase; see ref. 9 and 11), our expectation is that these enzymes are members of the β group. In contrast, γ polypeptides are typically major structural components of the virus (e.g., ICP 5).

The role of β polypeptides in the production of γ polypeptides is not certain because we cannot at this time entirely dissociate the synthesis of β polypeptides from those of the α group. If β proteins do have such a role, their synthesis is not continuously required to sustain it.

Turning "off" of α and β polypeptide synthesis. In the preceding sections we dealt with requirements for the production of α, β, and γ polypeptides. We now wish to draw attention to the finding that, after the synthesis of α and β proteins has begun, the subsequent time course of their synthesis is regulated by the

appearance of later groups of viral polypeptides. Specifically, the production of β polypeptides clearly determines the normal decline in α polypeptide synthesis. Thus, when β polypeptide synthesis is prevented either by cycloheximide treatment alone or by cycloheximide followed by actinomycin D, the rates of synthesis of α polypeptides exceed those in untreated cultures, and the interval of their synthesis is extended. Moreover, the synthesis of α polypeptides is rapidly depressed when β polypeptides are made but before appreciable amounts of γ polypeptides are detected. The data exclude the possibility that α polypeptides directly determine the course of their own biosynthesis.

The mechanism by which β group polypeptides affect the synthesis of α polypeptides is not known. However, it is of interest to note that the synthesis of the host polypeptides monitored in this study was also depressed by the synthesis of β polypeptides. This decline was more rapid than that in the presence of α proteins alone.

The rates of synthesis of β polypeptides can also exceed the maximum rates achieved in untreated cells when cycloheximide is added after β polypeptide synthesis has begun but before γ polypeptides are made in large amounts. In this instance the hypothesis that the decline in rates of synthesis in untreated cells is due to the production of γ polypeptides is less compelling and requires further testing.

Implications of coordinate regulation. The observation that viral polypeptides are regulated in a coordinate fashion has a number of implications. First, the different requirement for the production or stability of mRNAs for α, β, and γ polypeptides implies either that these groups have differential but coordinate requirements for their transcription or transport, or that systematic differences in elements of their structure underlie differences in stability of transcripts not engaged in protein synthesis. In the first instance, β mRNAs may be synthesized as a single polycistronic mRNA precursor, which have been reported in herpesvirus-infected cells (23), or monocistronic β-polypeptide mRNAs may possess cognate sequences for the initiation of their transcription. Similar considerations would apply to mRNAs for γ polypeptides. The second point is based on the demonstration that the "shut-off" of α and β polypeptides is not due to inhibition of synthesis of their respective mRNAs since actinomycin-D reduces or prevents this "shut-off." It follows that the coordinate depression of α and β polypeptide synthesis is achieved by a post-transcriptional mechanism, also requiring some common elements in the structure of mRNAs within each group to serve as a basis for the discrimination required of this mechanism. The third point is based on the effects of actinomycin-D on ongoing protein synthesis. Although we cannot be certain that measurements of the longevity of protein synthesis in the presence of actinomycin-D are not complicated by indirect effects of the inhibitor, actinomycin-D does discriminate between the synthesis of different groups of polypeptides. The synthesis of γ polypeptides diminishes rapidly in the presence of actinomycin-D, whereas that of α and β polypeptides, although not identical in their stability, are in general less sensitive to the inhibitor. These data also suggest differences between mRNAs for α, β and those for γ polypeptides. If the above reasoning is correct, the operation of the coordinate "on" and "off" controls which we have outlined requires that mRNAs for polypeptides within each group share at least one and possibly several common features of their structure which may determine either their synthesis, transport, or stability, and possibly regulation at the level of their translation or functional half-life.

Nomenclature for infected cell polypeptides. The recognition of at least 47 virus-specific polypeptides whose synthesis is coordinately regulated, necessitates a system of nomenclature which both identifies them and provides a succinct description of their properties. We have used three descriptive terms, i.e., Arabic numbers from 1 to 47, which identify the relative electrophoretic mobility of the polypeptides, the designation S (structural) and NS (non-structural) specifying their presence or absence in the virion, and the Greek letters, α, β, and γ, defining the coordinately regulated group to which they belong. Thus, ICP 5 [S; γ], ICP 4 [S; α], and ICP 36 [NS; β] are readily identifiable in the context of this and our preceding paper (10). This nomenclature will be employed and extended in the future as additional properties of these polypeptides are elucidated.

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