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Evidence for a Herpes Simplex Virus-Specific Factor Controlling the Transcription of Deoxypyrimidine Kinase

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A cistron-specific, enzyme-forming-capacity method was used to study the control of herpes simplex virus (HSV)-specific deoxypyrimidine kinase (dPyK) mRNA synthesis. A virus-specific factor was formed by a primary infecting virus, and this factor effected the transcription of dPyK mRNA of a superinfecting virus in the presence of cycloheximide, suggesting that the factor acted in "*trans*" and was a diffusible one. After the addition of actinomycin D to prevent further transcription and upon removal of cycloheximide, the dPyK mRNA was allowed to express into dPyK activity. A factor from HSV-1 could effect the transcription of dPyK mRNA of both HSV-1 and HSV-2. Amino acid analogs, canavanine or ethionine, inhibited the action of this factor, suggesting that a protein was involved. This protein factor was shown to belong to the alpha (or immediate-early) group of HSV-specific polypeptides in productively infected cells.

Physical, enzymatic, and immunological studies indicate that herpes simplex virus types 1 and 2 (HSV-1, HSV-2) induce a new deoxypyrimidine kinase (dPyK; exhibits both thymidine kinase and deoxycytidine kinase activities) in lytic infection (6, 9, 10, 12). The dPyK appears to be virus coded, since viral mutants have been isolated which are unable to induce this enzyme activity (6, 9) or which synthesize incomplete polypeptides of the enzyme (17). In recent years, increasing interest has been drawn to this enzyme as a marker for studies of transformation of mammalian cells (8, 11, 13). However, the control of expression of this enzyme is poorly understood. In the present communication, an attempt was made to search for a factor(s) effecting the transcription of dPyK mRNA. The synthesis of mRNA was measured by an enzyme-forming-capacity method (14), in which the dPyK mRNA synthesized in the presence of cycloheximide was allowed to express into dPyK activity after the addition of actinomycin D (to inhibit further transcription) and the subsequent removal of cycloheximide. In the following series of experiments, the controlling factor was formed by a primary infecting virus acting in "*trans*" on the transcription of dPyK mRNA of a superinfecting virus in the presence of cycloheximide. A dPyK-deficient viral mutant, B2006 (9), was utilized as the primary infecting virus; therefore, the dPyK activities assayed were only those expressed by the superinfecting virus.

MATERIALS AND METHODS

Virus and cell culture. Wild-type viruses used included HSV-1, strain KOS, and HSV-2, strain 186. HSV-1 dPyK-deficient mutant B2006 was developed by Saul Kit and obtained from Robert Hughes of Roswell Park Memorial Institute. The cytosol thymidine kinase-deficient LMTK$^-$ cells were developed by Saul Kit and obtained from Karl Freeman of McMaster University (8).

Thymidine kinase assay. The thymidine kinase activity of the dPyK was assayed as described by Leung et al. (12). The reaction mixture consisted of 12 mM ATP, 5 mM Mg^{2+}, 0.15 M KCl, 0.01 M Tris-hydrochloride (pH 8.0), 0.1 mM [^{3}H]deoxythymidine (New England Nuclear), and 50 μl of enzyme extract in a final volume of 125 μl. The enzyme was incubated for 15 min at 37°C, and the reaction was terminated by the addition of 25 μl of 50% trichloroacetic acid. The [^{3}H]dTMP formed was separated from [^{3}H]deoxythymidine by chromatography on DE-81 paper (Whatman).

Virus infection. Confluent LMTK$^-$ cells in 100-mm petri dishes were used. At zero time the cells were infected with dPyK-deficient virus, B2006, and the virus was adsorbed for 1 h at 37°C. The unadsorbed virus was removed, and 10 ml of miminum essential (F-17) medium supplemented with 5% fetal calf serum was added. After another 3 h at 37°C (i.e., 4 h postinfection), the monolayers were superinfected with either HSV-1 or HSV-2 in the presence of 50 μg of cycloheximide per ml. One hour later the unadsorbed virus was again removed. The culture was fed with medium containing 50 μg of cycloheximide per ml. Three hours later (i.e., 8 h postinfection), the medium was removed, and the monolayer was washed five times with medium. The washing was done within 5 min, and medium containing actinomycin D at 5 μg/ml was then added immediately to the monolayer. The culture was incubated for another 4 h (i.e., 12 h postinfection) at 37°C. The monolayers were harvested by scraping the cells from the culture dish with a rubber policeman. The cells were washed three times in 0.01 M Tris-hydrochloride (pH 7.5)-0.1 M KCl. Then they

were swollen in 0.01 M Tris-hydrochloride (pH 8.0)–0.01 M KCl–0.001 M $MgCl_2$. The cells were homogenized in a Dounce homogenizer. KCl and thymidine were added to final concentrations of 0.15 M and 0.2 mM, respectively. The extract was centrifuged in 100,000 × *g* for 1 h at 4°C, and the supernatant was used for the enzyme assay.

Amino acid analogs. For studies with amino acid analogs, canavanine (Sigma) was added to arginine-free medium and ethionine (Sigma) was added to methionine-free medium. The monolayer was washed five times with Hanks medium before or after treatment with an amino acid analog.

Analysis of alpha polypeptides. The synthesis of alpha polypeptides was monitored by pulse-labeling the virus-infected cells with 10 μCi of [^{35}S]methionine per ml in methionine-free medium supplemented with 5% dialyzed fetal calf serum. At the end of the labeling period, the cells were harvested, washed, and lysed by 1% sodium dodecyl sulfate–1% 2-mercaptoethanol. The cell extracts were analyzed by electrophoresis on a 7.5 to 15% gradient slab gel. The gel was then dried and processed for autoradiography. The alpha polypeptide ICP4 (VP175) was located on the photographic film, and the corresponding area of the gel was removed. The gel slice was solubilized by 10% NCS (Amersham) and counted in a toluene-based scintillation fluid.

RESULTS

Kinetics of appearance of dPyK in lytic infection occurs about 6 to 10 h postinfection. This time period of appearance of dPyK is compatible with that of a delayed-early beta polypeptide, according to the scheme of Honess and Roizman (4). To ascertain the transcriptional classification of the dPyK, an experiment was done in which LMTK$^-$ cells were infected with HSV-1 (KOS) and incubated in the presence of cycloheximide for the initial 4 h after infection. The cycloheximide was then removed by washing, and the kinetics of the appearance of dPyK was examined. The dPyK began to appear 4 h after washing out the cycloheximide, and the maximum level of activity was reached 2 h later. The addition of actinomycin D at the time of washing out the cycloheximide completely abolished any dPyK activity. On the other hand, by pulse-labeling the infected cells with [^{35}S]methionine and analyzing the cell extract by polyacrylamide gel electrophoresis, an alpha polypeptide, ICP4 (VP175), began to be expressed between 1 and 2 h after removal of the cycloheximide and reached a maximum level by 3 h (data not shown). This study confirms the findings of Garfinkle and McAuslan (3), which suggest that the PyK is a beta polypeptide.

Effect of superinfection on dPyK activity. Previous studies indicated that superinfection of HSV-infected cells with either HSV-1 or HSV-2 resulted in a reduced yield of infectious virus. Coinfection of the cells by the wild type and by the dPyK-deficient mutant, B2006, resulted in a diminished dPyK activity induced by wild-type virus (2, 15). However, reexamination of the effect of superinfection on the dPyK activity revealed a time-dependent effect (Fig. 1). The LMTK$^-$ cells were first infected by B2006 at 5 PFU/cell. At various times after primary infection, the cells were superinfected with 5 PFU of either HSV-1 or HSV-2 per cell. The cells were harvested at 7 h after the superinfection, and the dPyK was analyzed. A drastic reduction of dPyK activity was seen when the superinfection was done either very early or after midcycle of the primary infection. However, a lesser decrease in dPyK activity was observed when the superinfection occurred between 2 and 4 h after the primary infection began. The data suggest that although superinfection generally results in a reduction of dPyK activity, a function expressed about 3 h into the primary infection counteracts the suppressive effect.

Virus-specific function controlling dPyK activities. Although the interference effect of superinfection did affect the observed dPyK activity, the high sensitivity of the enzyme assay permitted the detection of low levels of enzyme

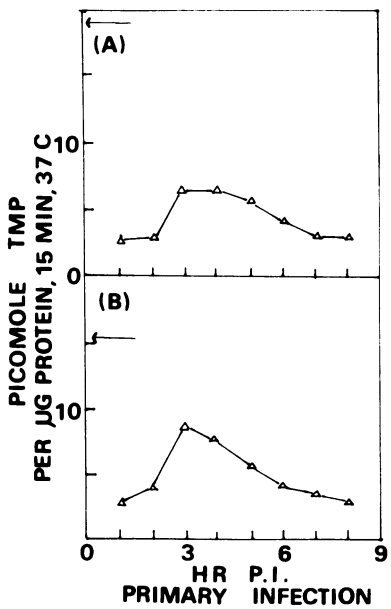

FIG. 1. *Effect of coinfection of a wild-type and a dPyK$^-$ virus mutant on dPyK activity. The cells were infected with B2006 at 5 PFU/cell at zero time. At various times after primary infection, the cells were superinfected with (A) HSV-1 or (B) HSV-2 at 5 PFU/cell. Seven hours later the cells were harvested, and cell extracts were assayed for dPyK activity. The arrow indicates the level of enzyme activities induced by strain KOS or 186 in parallel experiments in which the cells were not previously infected with B2006. P.I., Postinfection.*

activities observed in a cistron-specific, enzyme-forming-capacity method. Cells primarily infected with the dPyK-deficient mutant B2006 were then superinfected 3 h later with wild-type HSV-1 or HSV-2 in the presence cycloheximide. The enzyme-forming capacity of the synthesized mRNA was allowed to be expressed into dPyK activity upon removal of cycloheximide. Actinomycin D was then added to prevent further transcription; therefore, the dPyK activity assayed was formed from mRNA synthesized in the presence of cycloheximide only.

Consecutive treatment with cycloheximide and actinomycin D effectively blocked the formation of dPyK in cultures infected with wild-type virus (Table 1 rows 6 and 10) but not in those infected earlier with B2006 virus. When primary infection occurred, about 51 or 42% of the dPyK activities was observed in superinfection with HSV-1 or HSV-2, respectively (rows 7 and 11), as compared with the activities induced by wild-type virus in productive infection without prior primary infection or drug treatment (rows 3 and 9). Actinomycin D together with cycloheximide, added at superinfection, abolished the induced dPyK activities (rows 8 and 12).

A dose-dependent relationship was observed between the input of the superinfecting virus and the enzyme-forming capacity of the dPyK mRNA (Fig. 2A and B). However, for the primary infection, the relationship tended to be linear only between 1 and 5 PFU/cell (Fig. 2 C and D). The enzyme-forming capacity soon leveled off between 5 and 10 PFU/cell, and no further increase was observed with further increases in the input virus multiplicities.

Inhibition of the virus-specific function by amino acid analogs. To ascertain whether the function involved in the control of dPyK mRNA synthesis requires functional protein, amino acid analogs were used in the superinfection. Addition of canavanine, an arginine analog, during the primary infection reduced the dPyK activities observed to 36% those of the untreated culture (Table 2). Addition of canavanine during the actinomycin D phase of superinfection also inhibited the dPyK activity to 16%, suggesting that the dPyK activity itself was also inhibited

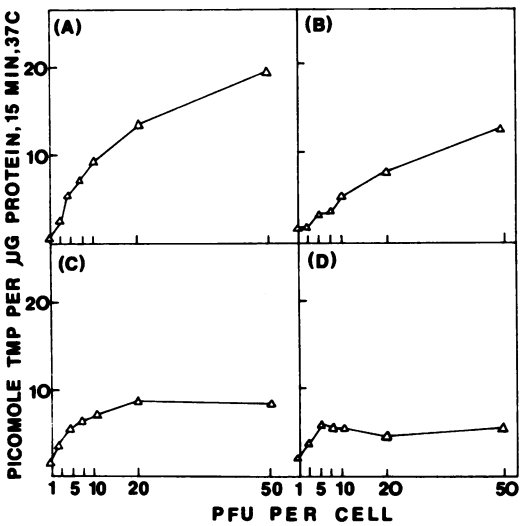

FIG. 2. *Effect of input multiplicities on the induction of dPyK activities. (A and B), Multiplicity of infection (MOI) of primary infecting B2006 was at 5 PFU/cell; the MOI of superinfecting HSV-1 (A) or HSV-2 (B) varied. (C and D) MOI of the superinfecting HSV-1 (C) or HSV-2 (D) was at 5 PFU/cell; the MOI of the primary infecting B2006 varied.*

TABLE 1. *Induction of dPyK activities from a superinfecting virus by a primary infecting virus*

Primary infecting virus	Superinfecting virus	Treatment with:		Sp act[a]
		Cycloheximide	Actinomycin D	
B2006		−	−	0.4 (1.3%)[b]
B2006		+	+	0.6 (2.6%)
None	HSV-1	−	−	22.7 (100.0%)
None	HSV-1	−	+	0.9 (3.9%)
None	HSV-1	+	−	2.4 (10.5%)
None	HSV-1	+	+	0.3 (1.3%)
B2006	HSV-1	+	+	11.6 (51.1%)
B2006	HSV-1	+Actinomycin D	+	0.8 (3.5%)
None	HSV-2	−	−	16.2 (100.0%)
None	HSV-2	+	+	0.5 (3.1%)
B2006	HSV-2	+	+	6.8 (41.9%)
B2006	HSV-2	+Actinomycin D	+	0.7 (4.3%)

[a] dPyK activity is expressed by its thymidine kinase activity. Specific activity is given in picomoles of dTMP formed per microgram of protein in 15 min at 37°C.

[b] Numbers in parentheses are percentages of dPyK activities as compared with that of an untreated control.

TABLE 2. *Effect of amino acid analogs on the induction of dPyK*

Amino acid analog	Time of treatment			Sp act[a]
	Primary infection	Cycloheximide phase of superinfection	Actinomycin D phase of superinfection	
No addition	−	−	−	15.1 (100%)[b]
Canavanine	+	−	−	5.4 (35.7%)
Canavanine	−	−	+	2.4 (15.9%)
Canavanine	−	+	−	14.6 (96.7%)
Ethionine	+	−	−	5.2 (34.4%)
Ethionine	−	−	+	13.8 (91.4%)
Ethionine	−	+	−	14.2 (94.0%)

[a] Activity of dPyK is expressed by its thymidine kinase activity. Specific activity is given in picomoles of dTMP formed per microgram of protein in 15 min at 37°C.

[b] Numbers in parentheses are percentages of dPyK activities as compared with that of controls not treated with amino acid analogs.

by canavanine. Although inhibition of enzyme activity was observed, the effect of canavanine on the function of inducing dPyK RNA was not due to the residual effect of canavanine left in the cells even after extensive washing. This is evident since canavanine treatment during the cycloheximide phase alone had little effect on the dPyK activity observed. Ethionine, a methionine analog, also inactivated the function supplied by the primary infection for the transcription of dPyK mRNA, since only 34% of the dPyK activity compared to the untreated control was observed. However, ethionine has little or no effect on the dPyK itself. The above study indicated that a functional protein factor is required for the expression of dPyK mRNA.

Classification of the protein factor in the alpha group. The kinetics of appearance of the factor involved in the induction of dPyK was investigated. To synchronize the expression of the polypeptide groups, the primary infection of B2006 was carried out in the presence of cycloheximide for 4 h. Removal of cycloheximide by washing resulted in a burst of synthesis of alpha polypeptides, followed by beta and gamma polypeptides (4). In this experiment, cycloheximide was added at various times after washing to stop further synthesis of polypeptides induced by the primary infecting virus. Superinfection took place simultaneously with cycloheximide treatment. Three hours after the cycloheximide treatment and superinfection, the cells were again washed, and actinomycin D was added to allow the enzyme-forming capacity of the dPyK mRNA to express into dPyK activity. Figure 3 shows that the ability of the primary infecting virus to effect the transcription of the superinfecting dPyK mRNA reached a maximum between 2 and 3 h after the first washing. As controls, the synthesis of alpha polypeptides of the primary infecting virus, as presented by the kinetics of synthesis of ICP4, was followed by pulse-labeling the infected cells with [^{35}S]methionine. The amount of ICP4 synthesized also reached the maximum level by 2 h after the first washing. Moreover, a parallel experiment was done using the wild-type HSV-1 as the primary infecting virus and without further superinfection. The activity of the dPyK as a representative of the beta polypeptide from the primary infection was shown to begin increasing by 4 h after washing and reached the maximum level by 6 h. The data indicated that the protein factor expressed by the primary infection belongs to the alpha group.

DISCUSSION

This communication presents evidence that an HSV-specific protein factor effects transcription of dPyK mRNA. To analyze the level and mode of control of dPyK synthesis, the experiments were performed in a superinfection system. The cistron-specific, enzyme-forming-capacity method allowed a factor(s) from the primary infecting virus to effect the synthesis of dPyK mRNA of a superinfecting virus in the presence of cycloheximide. Upon removal of cycloheximide, the enzyme-forming capacity of dPyK mRNA was allowed to express into dPyK enzyme activity. Actinomycin D was added simultaneously with removal of cycloheximide to prevent any transcription of dPyK mRNA effected by factors from either the primary infecting virus or the superinfecting virus. Such a factor was found in the present study, since the synthesis of dPyK mRNA occurred only with prior primary infection. Since the factor was derived from the primary infecting virus and effected the synthesis of mRNA of the superinfecting virus, this observation suggested that the factor is a diffusible one and acts in "*trans*." Amino acid analogs inhibited the expression of

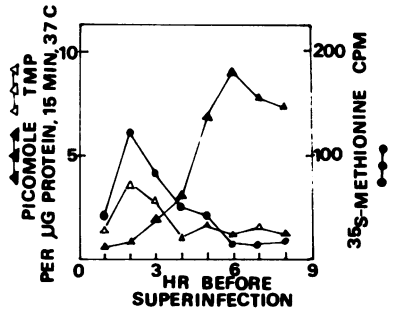

FIG. 3. *Kinetics of the expression of protein factor by primary infecting virus. The cells were first infected with B2006 at 5 PFU/cell for 4 h in the presence of cycloheximide. The cells were then washed at zero time and incubated with minimum essential medium at 37°C. At various times after the wash, cycloheximide was again added, and the cells were superinfected with strain KOS at 5 PFU/cell. Three hours after superinfection the cells were washed, and actinomycin D was added for another 3 h. The monolayer was then harvested, and cell extracts were analyzed for dPyK as expressed by its thymidine kinase activity. The open triangles indicate the thymidine kinase activity induced. In a parallel experiment, similar cultures were labeled with [^{35}S]methionine for 1 h at various times after the first wash. Cells were then harvested after pulse-labeling and analyzed by polyacrylamide gel electrophoresis and autoradiography. The radioactivity corresponding to an alpha polypeptide, ICP4, was determined by cutting out the gel slices for scintillation counting, and these values are shown by closed circles. In another parallel experiment, similar cultures were infected with strain KOS in the presence of cycloheximide for 4 h. The cells were washed and incubated at 37°C with minimum essential medium. At various times after washing, the cells were harvested and cell extracts were assayed for dPyK; the activity is shown by closed triangles.*

the factor, suggesting that a functional protein(s) is required. Although superinfection exclusion did occur in HSV-infected cells, the protein factor(s) of HSV-1 is able to effect at least the transcription of dPyK mRNA of HSV-2.

The expression of HSV-specific polypeptides in productive infection occurs in a sequential and coordinated pattern (4). Three groups of polypeptides are found according to their time of appearance after initiation of infection. These polypeptides have been named the alpha (or immediate-early) polypeptides, which are synthesized first, followed by the beta (or delayed-early) polypeptides and the gamma (or late) polypeptides. The synthesis of alpha mRNA, but not beta or gamma RNA, occurs in the presence of cycloheximide (1, 7) and does not require prior viral protein synthesis (4). The synthesis of beta polypeptides requires the presence of a functional alpha polypeptide(s) (5). New RNA synthesis is also required, since the addition of actinomycin D inhibits the expression of beta polypeptides (4). However, whether the effect of an alpha polypeptide(s) on the expression of beta polypeptides is at the transcriptional or post-transcriptional level is not known. The above observations could, alternatively, be interpreted as indicating that cycloheximide inhibits the synthesis of a host cell component which is required for the transcription of beta genes and that the effect of an alpha polypeptide(s) is on the post-transcriptional modification or transport of the beta mRNA. In the present study, the dPyK was confirmed to be a member of the beta polypeptides. The protein factor controlling the synthesis of dPyK mRNA was shown to belong to the alpha group. Since the alpha polypeptide(s) supplied by a primary infecting virus is able to effect the synthesis of dPyK mRNA in the presence of cycloheximide, it indicated that the role of the alpha polypeptide(s) in this assay system was primarily to exert a positive effect on transcription. However, whether the alpha polypeptide(s) will also act on the post-transcriptional level of the beta mRNA synthesis cannot be ruled out by the present experiment.

In a different context, several laboratories have shown that cytosol thymidine kinase-deficient cells can be biochemically transformed to a dPyK-positive phenotype by HSV. The expression of HSV dPyK in these cells can be increased severalfold upon superinfection by an HSV dPyK-deficient mutant (8, 11, 13). However, because of the limitation of that system, the nature of the factor(s) supplied by the superinfecting virus and the level of regulation are not known. The factor(s) supplied by the dPyK-deficient mutant in those studies, in all respects, is probably similar to the alpha polypeptide(s) assayed in this study. The approach used in this study might be applied to examine the mechanism of regulation of HSV genes in transformed cells.

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