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Unusual Regulation of Expression of the Herpes Simplex Virus DNA Polymerase Gene

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During herpes simplex virus infection, expression of the viral DNA polymerase (pol) gene is regulated temporally as an early (β) gene and is additionally down-regulated at late times at the level of translation (D. R. Yager, A. I. Marcy, and D. M. Coen, J. Virol. 64:2217–2225, 1990). To examine the role of viral DNA synthesis in pol regulation, we studied pol expression during infections in which viral DNA synthesis was blocked, either by using drugs that inhibit Pol or ribonucleotide reductase or by using viral mutants with lesions in either the pol or a primase-helicase subunit gene. Under any of these conditions, the level of cytoplasmic pol mRNA was reduced. This reduction was first seen at approximately the time DNA synthesis begins and, when normalized to levels of other early mRNAs, became as great as 20-fold late in infection. The reduction was also observed in the absence of the adjacent origin of replication, ori1. Thus, although pol mRNA accumulated as expected for an early gene in terms of temporal regulation, it behaved more like that of a late (γ) gene in its response to DNA synthesis inhibition. Surprisingly, despite the marked decrease in pol mRNA in the absence of DNA synthesis, the accumulation of Pol polyepitope was unaffected. This was accompanied by loss of the normal down-regulation of translation of pol mRNA at late times. We suggest a model to explain these findings.

Herpes simplex virus (HSV) is a large DNA virus that uses cellular transcriptional and translational machinery for the expression of its genes. HSV gene expression is regulated as an ordered cascade (10, 19, 22). The first genes expressed are the α (immediate-early) genes, which encode regulatory proteins required for the efficient expression of later genes. Next, the β (early) genes, which largely encode DNA replication proteins, are expressed. The levels of early mRNAs and the rates of early protein synthesis peak at about 4 to 7 h postinfection and then decline. Early gene expression is relatively insensitive to inhibition of viral DNA synthesis. In contrast, the expression of γ (late) genes, which largely encode virion proteins, continues to increase until very late in infection and is relatively sensitive to inhibition of viral RNA synthesis. The mechanisms that differentiate the expression of these genes, particularly the early and late genes, are still poorly understood.

The HSV DNA polymerase (Pol) is required for viral replication and serves both as a target for antiviral drugs and as a model eukaryotic DNA polymerase. Previous studies of its regulation have classified it as an early gene (9, 15, 23, 26–28). pol transcription and mRNA accumulation are abolished by the protein synthesis inhibitor cycloheximide, indicating a requirement for the expression of immediate-early proteins (23). The time courses of pol transcription rates and mRNA accumulation resemble those of other early genes involved in nucleotide metabolism and viral replication, such as the thymidine kinase gene (tk) and the major DNA-binding protein gene (ICP8), although pol expression peaks somewhat later before declining (15, 23, 26–28). Both pol transcription and mRNA accumulation, like those of the tk and ICP8 genes, have been reported to be relatively insensitive to inhibitors of viral DNA synthesis, at least up to 6.5 h postinfection (9, 23).

However, the kinetics of Pol protein synthesis differ considerably from those of TK. Whereas the time course of the rate of TK protein synthesis parallels the time course of tk mRNA abundance, Pol protein synthesis peaks before pol mRNA abundance and then shuts off when pol mRNA levels are still high (27). This is due to inefficient translation of pol mRNA at late times in the viral infection (26, 27).

To explore the regulation of pol gene expression further, we asked whether the shutoff of Pol protein synthesis at the level of translation requires viral DNA synthesis. This could be due, for example, to a requirement for a late gene product whose synthesis would depend on viral DNA synthesis. However, in the course of these studies, we unexpectedly found that pol mRNA accumulation depends on viral DNA synthesis. After normalizing for levels of pol mRNA, we did find that DNA replication is required for translational regulation of pol expression. We suggest a model to explain these observations.

MATERIALS AND METHODS

Cells and viruses. African green monkey kidney (Vero) cells were grown in Dulbecco’s modified Eagle’s medium supplemented with calf serum. Viruses used were HSV type 1 strain KOS and the following mutants derived from it: the phosphonoacetic acid (PAA)-resistant derivative PKG7 (11); pol deletion mutant viruses ΔSI and ΔX14 (13); a lacZ insertion mutant containing a mutation in the gene encoding the UL5 subunit of the viral primase-helicase, hr99 (29), generously supplied by Sandra Weller (University of Connecticut Health Center, Farmington); and the ori5 deletion mutant ts7 (17), kindly provided by Priscilla Schaffer (Dana-Farber Cancer Institute, Boston, Mass.).

Viral DNA synthesis inhibitors. Aphidicolin, the gift of M. Suffness (National Cancer Institute, Bethesda, Md.), was...
prepared as described previously (3). Hydroxyurea was obtained from Sigma Chemical Co. (St. Louis, Mo.) and dissolved in water. Ganciclovir was kindly supplied by D. Barry (Burroughs Wellcome, Research Triangle Park, N.C.) and was prepared as previously described for acyclovir (4). PAA was generously provided by S. Schmidt (Abbott Laboratories, North Chicago, Ill.) and prepared as previously described (4).

**RNA isolation.** Vero cells were mock infected or infected with a multiplicity of 5 PFU per cell. For those infections carried out in the presence of drugs, aphidicolin (10 μg/ml), hydroxyurea (10 mM), and ganciclovir (200 μM) were added 1 h postinfection while PAA was added simultaneously with the virus to a final concentration of 0.4 mg/ml. At the indicated times postinfection, cells were washed twice with cold phosphate-buffered saline. After addition of 0.5 to 1 ml of lysis buffer (140 mM NaCl, 1.5 mM MgCl₂, 10 mM Tris-HCl [pH 8.6], 0.5% Nonidet P-40), the cells were scraped into 1.5-ml tubes and the nuclei were pelleted by spinning briefly in a microcentrifuge (30 to 60 s). The supernatant was removed to a new tube. Sodium dodecyl sulfate (SDS) was added to a final concentration of 0.6%, and 10 to 20 μg of protease K was added. After incubation at 55°C for 1 h, the solution was phenol-chloroform extracted. The aqueous phase was rendered 1 M ammonium acetate, and then an equal volume of isopropanol was added. To determine RNA concentration, a portion of the isopropanol suspension was pelleted by centrifugation and resuspended in water, and the A₂₆₀ was determined spectrophotometrically.

**Primer extension analysis.** Ten micrograms of pelleted RNA was resuspended in 30 μl of hybridization mix [80% formamide, 40 mM piperazine-N,N'bis(ethanesulfonic acid) (PIPES; pH 6.7), 0.4 mM NaCl, 1 mM EDTA]. Gel-purified, 5'-3²P-end-labeled primers were added in the following quantities determined empirically to yield products proportional to the amount of RNA added: 50 fmol of pol primer, complementary to residues 52 to 69 of the major pol mRNA (26); 100 fmol of tk primer, complementary to residues 68 to 90 of tk mRNA (11); and 200 fmol of ICP8 primer, corresponding to residues 72 to 92 of ICP8 mRNA (20). The hybridization reaction mixtures were placed at 55°C for 2 h and then precipitated by addition of 70 μl of water, 25 μl of 5 M ammonium acetate, and 375 μl of ethanol. Twenty milliliters of reverse transcription mix (50 mM Tris [pH 7.6], 60 mM KCl, 10 mM MgCl₂, 1 mM each deoxynucleoside triphosphate [dNTP], 1 mM dithiothreitol, 1 U of RNasin per μl, 50 μg of actinomycin D per ml, 0.75 U of avian myeloblastosis virus reverse transcriptase) was added to the precipitated nucleic acids, and the reaction mixture was incubated at 42°C for 1 to 2 h. Reactions were terminated by phenol-chloroform extraction and ethanol precipitation. Precipitated nucleic acids were resuspended and separated by electrophoresis on 10% denaturing polyacrylamide gels. Radioactive species were visualized by autoradiography, using prefetched Kodak XAR-5 film. The amounts of primer extension products were quantified by using either a Betagen β-sc tape, a Molecular Dynamics PhosphorImager, or a Microtek scanner. In the case of pol primer extension products, we measured only the closely spaced major products, not the minor (<10%) species (2, 26). The ratios of pol mRNA primer extension products to the tk or ICP8 mRNA primer extension products from untreated KOS-infected cells versus drug-treated or mutant virus-infected cells were calculated. The fold reduction in pol mRNA relative to other mRNAs was then calculated by dividing the poltk or pol treatment.
pol and tk, at 4 h postinfection. While tk mRNA was only slightly diminished in the presence of the drugs, pol mRNA was more substantially reduced in the presence of all three inhibitors. When quantified and normalized to tk mRNA levels, the reduction in pol mRNA was two- to fourfold, depending on the drug. To ensure that this reduction reflected a decrease in pol mRNA levels and not an increase in tk mRNA levels, ICP8 mRNA, another early gene product, was also measured. This analysis revealed nearly identical results (24). Therefore, inhibition of viral DNA synthesis either by direct inhibition of Pol or by depletion of dNTP pools led to a reduction in pol mRNA relative to two early mRNAs.

**Time course of effects of PAA.** A more detailed analysis was performed with the Pol inhibitor PAA by examining pol, tk, and ICP8 mRNAs at various times postinfection by primer extension analysis (Fig. 2A). The amounts of primer extension products were quantified and were plotted for each mRNA relative to the maximal amount expressed (this being 4.5 h postinfection in the absence of drug) (Fig. 2B). PAA appeared to slightly reduce (~20%) the amount of tk and ICP8 mRNA at 3 and 4.5 h postinfection compared with the no-drug controls, but by 9 and 10.5 h there was substantially more tk and ICP8 mRNA in PAA-treated cells than in untreated cells. In contrast, although PAA had little effect on pol mRNA at 1.5 h (24) or 3 h (Fig. 2), at 4.5 h, which corresponds to the onset of viral DNA synthesis, it reduced this species two- to threefold compared with the no-drug control. This was similar to the results with the other inhibitors at 4 h (Fig. 1).

Relative to tk mRNA, pol mRNA was reduced twofold in the presence of PAA at 4.5 h postinfection. At later times, however, the reduction relative to tk mRNA became severe. For example, at 9 h in the absence of PAA, there was a greater pol signal than tk signal, but in the presence of PAA, there was a much greater tk signal than pol signal (Fig. 2). This corresponds to a 20-fold reduction in the ratio of pol mRNA to tk mRNA. A similar pattern was observed when pol mRNA was compared with ICP8 mRNA (Fig. 2), with a ~10-fold reduction at 9 h.

These effects were specific to inhibition of HSV DNA synthesis because the pol, tk, and ICP8 mRNA profiles of a PAA-resistant mutant, PKG7, were similar in the presence or absence of the drug (24). Moreover, a similar time course experiment carried out with hydroxyurea gave results similar to those for PAA in KOS-infected cells (24), demonstrating that the relative decrease in pol mRNA levels during infection was common to both DNA synthesis inhibitors.

**Replication-negative mutants exhibit decreases in pol mRNA.** To examine the role of DNA replication in pol expression further, we studied replication-negative mutants of HSV, thereby avoiding the use of drugs. The first mutants examined were mutants ΔS1 and ΔX14, which contain deletions in pol gene protein-coding sequences. These mutants do not replicate in Vero cells and produce truncated Pol proteins (13). Figure 3 compares the expression of pol and tk mRNAs by these two mutants with that of KOS in the absence or presence of PAA. The time point used in this experiment was 8 h postinfection, at which time the effect of PAA on the pol/tk mRNA ratio is fairly severe (Fig. 2 and 3). The deletion mutants expressed a pol/tk mRNA ratio similar to that produced by the wild-type virus in the presence of PAA, with pol/tk mRNA ratios <10% of that of KOS in the absence of drug.

We then examined a third replication-negative mutant virus, hr99. This mutant contains a lesion in the UL5 gene,
which encodes a subunit of the HSV primase-helicase (29). At 9 h postinfection, this mutant also exhibited a pol/tk mRNA ratio similar to that of KOS in the presence of PAA (Fig. 4). Thus, both pol and UL5 mutants that are replication negative exhibited decreases in pol mRNA accumulation.

The decrease in pol mRNA does not depend on oriL. One mechanism that we could envision to account for our findings was based on the close proximity (55 bp) of the start site of pol transcription to one of the HSV origins of replication, oriL (26). We hypothesized that use of this origin might stimulate transcription of the pol gene, as suggested by Tomalski et al. (21). According to this hypothesis, when DNA synthesis was inhibited, the decrease in pol mRNA levels would be due to lack of transcriptional stimulation by activation of oriL. To examine this possibility, we analyzed pol mRNA levels during infection with the oriL deletion mutant ts+7, which is still replication competent, presumably as a result of use of its two copies of the other HSV origin, oriS (17). If use of the adjacent oriL were required to stimulate pol transcription, then relative pol mRNA levels should be lower in ts+7-infected cells than in KOS-infected cells, especially at late times, and the same in ts+7-infected cells in the presence or absence of PAA.

Figure 5 shows the pol, tk, and ICP8 mRNA levels at various times following infection by ts+7 in the presence and absence of PAA. Comparison of Fig. 5 with Fig. 2A shows that in the absence of PAA, the relative levels of pol mRNA in ts+7-infected cells were similar to those in KOS-infected cells throughout the time course. This result was confirmed by direct comparisons of mRNAs from KOS-infected cells and ts+7-infected cells (24). As was true for wild-type strain KOS (Fig. 2), a severe decrease in pol mRNA levels relative to those for tk and ICP8 was seen in the presence of PAA despite the absence of a functional oriL. Again, the ratio of pol mRNA to tk or ICP8 mRNA decreased by 20- or 10-fold, respectively, in the presence of PAA. Therefore, the use of the adjacent oriL had no apparent effect on relative pol mRNA levels.

Interestingly, although the deletion on ts+7 removes sequences from the putative pol and ICP8 promoters (20, 26), the overall level of pol and ICP8 mRNAs, normalized to the tk level, were not noticeably decreased relative to KOS. However, the deletion (17) removes parts of two Sp1 consensus sequences ca. 70 bp from each start site and replaces them with a single very similar sequence. Thus, it may be that in ts+7, the same Sp1 binding site serves to activate transcription of both the ICP8 and pol genes.

**Effects of inhibition of DNA synthesis on Pol protein synthesis.** Having established the effects of inhibiting viral DNA synthesis on pol mRNA accumulation, we then sought to determine the effect on Pol protein synthesis. We first examined the amount of Pol and TK protein that accumulated during infections by KOS, KOS in the presence of PAA, and the hr99 primase-helicase mutant. Infected cell protein samples were prepared at 18 h postinfection and fractionated by SDS-polyacrylamide gel electrophoresis, and TK and Pol were visualized by Western blotting (immunoblotting). To obtain a more quantitative assay, three different amounts of each protein sample were analyzed; the intensity of the autoradiographic signal reflected the amount of protein loaded on the gel (Fig. 6). When Pol protein levels were normalized to TK protein levels, very little difference was seen in the amount of Pol protein produced under the different conditions. For example, there was no apparent difference in the relative amounts of Pol protein harvested from hr99- and KOS-infected cells and only a slight decrease harvested from cells infected with KOS in the presence of PAA. These differences were far less substantial than the difference in pol mRNA levels in the presence or absence of PAA (compare the areas under the curves in Fig. 2B for pol and tk mRNAs).

One possible explanation for this result was that viral DNA synthesis is required for the normal shutoff of translation of pol mRNA (26, 27) as well as for accumulation of pol mRNA. To determine whether this was the case, cells were pulse-labeled with [35S]methionine for 2 h starting at 4 h or 7
h postinfection with KOS in the presence or absence of PAA to permit measurements of rates of Pol and TK protein synthesis at these times. At the end of the radioactive pulse, lysates were prepared from infected cells and reacted with anti-Pol or anti-TK antisera under conditions that immunoprecipitated these proteins quantitatively. In parallel, RNA was isolated and the amounts of pol and tk mRNAs were measured by primer extension. The expected decrease in pol mRNA relative to tk mRNA was observed when replication was inhibited. For example, as shown in Fig. 7A, at 9 h in KOS-infected cells in the absence of drug, there were similar pol and tk primer extension signals, whereas in the presence of PAA, the pol signal was much weaker than the tk signal (a ≥12-fold decrease in the pol/tk mRNA ratio).

Remarkably, the ratio of Pol to TK protein synthesis was similar in the presence or absence of PAA late in infection (Fig. 7B), even though there was much less pol mRNA in the presence of PAA (Fig. 7A). During KOS infection in the absence of PAA, the translational efficiency of pol mRNA (amount of protein synthesized per mRNA molecule) decreased substantially between early and late times postinfection (Table 1), consistent with previous results (27). In contrast, during KOS infection in the presence of PAA, there was no decrease in translational efficiency of pol mRNA (Table 1). Similar results were obtained by comparing pol deletion mutant AS1 with KOS (25). These results confirmed our hypothesis that when DNA synthesis is inhibited, the shutoff of pol mRNA translation is relieved. This would account for the normal levels of Pol protein observed (Fig. 6) despite the severe decrease in pol mRNA.

**DISCUSSION**

In this study, we found that when HSV DNA synthesis was inhibited by using a variety of drugs or mutants, pol mRNA accumulation decreased dramatically relative to other early mRNAs. However, this decrease in pol mRNA did not result in decreased accumulation of Pol protein. Rather, under conditions in which DNA synthesis was inhibited, the normal shutoff of translation of pol mRNA late in infection was relieved, thereby offsetting the decrease in mRNA accumulation. We discuss these results in terms of current understanding of the cascade of HSV gene expression and in terms of the possible mechanisms and biological significance of the rather complicated regulatory phenomena that we have uncovered.

**pol mRNA is early temporally but late in dependence on DNA synthesis.** As shown previously (15, 23, 26–28) and reiterated here, pol is an early (β) gene in terms of its time course of expression, especially in terms of the decline in transcription rate (28). mRNA accumulation, and protein synthesis after 7 h postinfection. The peak of pol mRNA accumulation comes later than that of ICP8 mRNA, in agreement with the assignment of these genes to the β2 and β4 groups, respectively, in one classification (19) and to the late β and early β groups, respectively, in another classification (28). (However, we disagree with the assignment of tk mRNA to the β2 group [19], as we find that tk mRNA and protein synthesis more closely parallels that of ICP8 than

**Table 1. Changes in translational efficiency of KOS pol mRNA**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(Pol synthesis/pol mRNA)/(TK synthesis/tk mRNA)*</th>
<th>Change during infectionb</th>
</tr>
</thead>
<tbody>
<tr>
<td>No drug</td>
<td>0.34</td>
<td>4.7-fold decrease</td>
</tr>
<tr>
<td>PAA</td>
<td>0.44</td>
<td>1.3-fold increase</td>
</tr>
</tbody>
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* Cells were infected with KOS in the presence or absence of PAA (0.4 mg/ml). At 4 h (early) or 7 h (late) postinfection, cells were pulse-labeled with [35S]methionine; 2 h later, they were harvested and assayed for Pol and TK protein synthesis and for pol and tk mRNAs. Protein synthesis was quantified by densitometry of autoradiograms of SDS-polyacrylamide gels of immunoprecipitated proteins, and mRNAs were quantified by analysis of primer extension products. The values obtained were used to calculate the ratios in arbitrary units.

b The change in ratio of Pol protein synthesis per pol mRNA to TK protein synthesis per tk mRNA from early to late in infection.
that of pol [Fig. 2 and 5 and reference 24], similar to transcription rate measurements of Zhang and Wagner [28], who placed both tk and ICP8 in the early β group."

"In contrast, when sensitivity to inhibition of DNA synthesis is considered, pol mRNA accumulation is more akin to that of a late gene than that of an early gene. In our hands, the peak expression of early genes such as tk and ICP8 is decreased somewhat by inhibition of DNA synthesis, not enhanced as commonly considered, although the expression of these early genes continues for longer times. However, pol mRNA accumulation decreased substantially when DNA synthesis was inhibited relative to that of tk and ICP8. This finding would appear to conflict with its assignment by Holland et al. (9) as an early mRNA on the basis of its relative insensitivity to the DNA synthesis inhibitor arabinosylthymine. This apparent discrepancy may be due to the isolation of polyribosomal RNA by Holland et al. (9), which may have underrepresented total cytoplasmic pol RNA in untreated cells relative to treated cells (26; this report). Our results are consistent with those of Weinhimer and McKnight (23), who noted that PAA tended to lower the level of pol mRNA at 8, 10, and 12 h postinfection without significantly altering the patterns of tk and ICP8 mRNA accumulation.

"How should pol be classified, then—as an early or a late gene? Because pol mRNA and Pol poly peptide behave temporally as early gene products, because Pol poly peptide synthesis is, in fact, insensitive to inhibition of DNA synthesis, and because most, if not all, other replication proteins are early gene products, we favor the continued classification of pol as an early (β) gene. Nevertheless, its unusual behavior emphasizes the variability in behavior among different members of the same kinetic class (19, 22, 26).

"Possible mechanisms of pol regulation. One can envision many different mechanistic models that could account for our results. One such model is that pol mRNA accumulation is sensitive to inhibition of viral DNA synthesis because pol transcription is up-regulated upon viral DNA replication. Arguing against this model are results showing that pol transcription measured by pulse-labeling decreases at times when DNA synthesis and late gene transcription remain high (28) and that the pol transcription rate measured by nuclear runoff is insensitive to inhibition by PAA (23). Also, one possible mechanism for transcriptional up-regulation, activation of ori1, was ruled out by our studies of the ori1 deletion mutant ts+7. Additionally, this model would not explain the dependence of transcriptional down-regulation of pol mRNA on viral DNA synthesis.

Because many of the drugs and mutations that we used to inhibit DNA synthesis inactivate Pol function, a second model to explain our results would entail autogenous negative regulation of pol translation, akin to the situation in bacteriophage T4, in which T4 DNA polymerase binds to its own mRNA and negatively regulates its translation (1). However, given that the primase-helicase mutant hr99 exhibits the same synthesis of Pol poly peptide as do pol mutants, there is no need to invoke autogenous regulation. It is still possible to contrive scenarios that would explain the data via autogenous regulation by Pol; however, we do not presently favor such a model.

"A third model is that a late gene product is responsible both for stabilizing pol mRNA, thereby increasing its steady-state levels, and for decreasing net pol mRNA translation efficiency. Specifically, if a late gene product binds pol mRNA, it may simultaneously prevent degradation of the mRNA and inhibit its translation. It could act on a fraction of pol mRNA, thereby totally sequestering this fraction from nuclease and ribosomes, or it could act equally on all pol mRNAs, thereby reducing the probability that they would encounter nuclease or ribosomes. This model has the appeal of simplicity since a single event explains both observed phenomena, the change in pol mRNA levels and the change in pol mRNA translational efficiency in the absence of replication.

"Does the regulation have adaptive significance? A final question is, what could be the selective advantage, if any, of the unusual regulation of pol expression that we have observed, especially since the net result of inhibition of DNA synthesis is the same amount of Pol protein? A related question is why much of the regulation appears to be posttranscriptional. It may be that Pol accumulates with a different time course in the presence or absence of DNA synthesis and that this both makes a difference and is facilitated by posttranscriptional mechanisms. A second speculation is that this regulation is not confined to pol expression but operates for a number of other HSV genes, such as those encoding other relatively nonabundant replication proteins, and that the effects on these genes lead to different amounts of protein synthesized. Further studies to determine how widespread this regulation is and its mechanisms will likely be necessary to answer questions regarding its adaptive significance to HSV.

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