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The Essential 65-Kilodalton DNA-Binding Protein of Herpes Simplex Virus Stimulates the Virus-Encoded DNA Polymerase

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The 65-kilodalton DNA-binding protein ($65K_{DBP}$) of herpes simplex virus type 1 (HSV-1), the product of the UL42 gene, is required for DNA replication both in vitro and in vivo, yet its actual function is unknown. By two independent methods, it was shown that the $65K_{DBP}$ stimulates the activity of the HSV-1-encoded DNA polymerase (Pol). When Pol, purified from HSV-1-infected cells, was separated from the $65K_{DBP}$, much of its activity was lost. However, addition of the $65K_{DBP}$, purified from infected cells, stimulated the activity of Pol 4- to 10-fold. The ability of a monoclonal antibody to the $65K_{DBP}$ to remove the Pol-stimulating activity from preparations of the $65K_{DBP}$ confirmed that the activity was not due to a trace contaminant. Furthermore, the $65K_{DBP}$ did not stimulate the activity of other DNA polymerases derived from T4, T7, or *Escherichia coli*. The $65K_{DBP}$ gene transcribed in vitro from cloned DNA and translated in vitro in rabbit reticulocyte lysates also was capable of stimulating the product of the *pol* gene when the RNAs were cotranslated. The product of a mutant $65K_{DBP}$ gene missing the carboxy-terminal 28 amino acids exhibited wild-type levels of Pol stimulation, while the products of two large deletion mutants of the gene could not stimulate Pol activity. These experiments suggest that the $65K_{DBP}$ may be an accessory protein for the HSV-1 Pol.

The genome of herpes simplex virus type 1 (HSV-1) consists of 152 kilobase pairs (kbp) that encodes approximately 70 genes (11). On the basis of analysis of temperature-sensitive mutants (22), a large number of viral genes are required directly or indirectly for the replication of viral DNA, but only seven genes have been identified as essential for the replication of plasmids containing an HSV-1 origin of replication (*ori*) (26). The gene that encodes the HSV-1 DNA polymerase (*pol*) is one of the seven essential genes for *ori*-dependent DNA replication and was initially identified by genetic approaches, which revealed that it was required for DNA replication in vivo (7, 20, 21).

The gene to which *pol* has been assigned encodes a polypeptide with an M_r of approximately 150,000 (8, 14, 15, 19). That Pol activity resides in the polypeptide ascribed to the *pol* gene in the absence of other viral products has been confirmed by using plasmids encoding the HSV-1 *pol* gene, which were transcribed and translated in vitro (3). In addition, an activity with the properties of an HSV-1 Pol has been detected in extracts from *Saccharomyces cerevisiae* cells that carry the HSV-1 *pol* gene under the control of the yeast galactokinase promoter (6).

Several groups that have substantially purified the HSV-1 and HSV-2 Pol have found that purified preparations possess a polymerase and a 3′-to-5′ exonuclease activity (8, 14, 15). In addition to the polypeptide mapping to the *pol* gene, these preparations also contained another protein (8, 15, 24). The Pol-associated protein in HSV-1-infected extracts has been identified as the 65-kilodalton DNA-binding protein ($65K_{DBP}$), the product of the UL42 gene (4, 17). This protein is the serotype equivalent of infected cell-specific polypeptide 34,35 (4), which is associated with the HSV-2 Pol (23, 24). Like *pol*, UL42 is required for HSV *ori*-dependent DNA replication (26) as well as for viral DNA replication in vivo (10, 21).

We were interested in determining whether the Pol-associated protein, $65K_{DBP}$, interacted with Pol in a functional manner. To this end, we have examined the effect of the $65K_{DBP}$ on Pol activity in vitro by two independent methods. In this report, we confirm that Pol isolated from HSV-1-infected cells and free of $65K_{DBP}$ possesses polymerase activity. However, the Pol activity is stimulated up to 10-fold by the addition of purified $65K_{DBP}$ from infected cells. In addition, the $65K_{DBP}$ gene, cloned into a plasmid and transcribed and translated in vitro, is also capable of stimulating the Pol activity from a cloned HSV-1 *pol* gene expressed in the same way. Although the mechanism by which the $65K_{DBP}$ stimulates Pol is not known, the use of such in vitro assays should facilitate a detailed analysis of this function.

MATERIALS AND METHODS

Cells and virus. Vero cells were used for the production of stocks of wild-type HSV-1 (strain KOS) as previously described (16), and baby hamster kidney (BHK) cells were used for the preparation of infected cell lysates used for protein purifications. Cell cultures were maintained as described previously (4). Nuclear lysates were prepared from BHK cells infected at an input multiplicity of infection of approximately 20 PFU per cell and harvested 20 to 22 h postinfection, as previously described (4).

Protein purification. The viral Pol and the $65K_{DBP}$ were purified as described by Gallo et al. (4), except that the running buffer for the DEAE Sephacel column had a pH of 8.20. In addition to the protease inhibitors leupeptin (2 μg/μl) and β-lactoglobulin A (10 μg/μl) previously used, buffers also contained aprotinin (2.0 μg/μl) and pepstatin A (0.5 μg/μl). Briefly, nuclear extracts of HSV-1-infected cells

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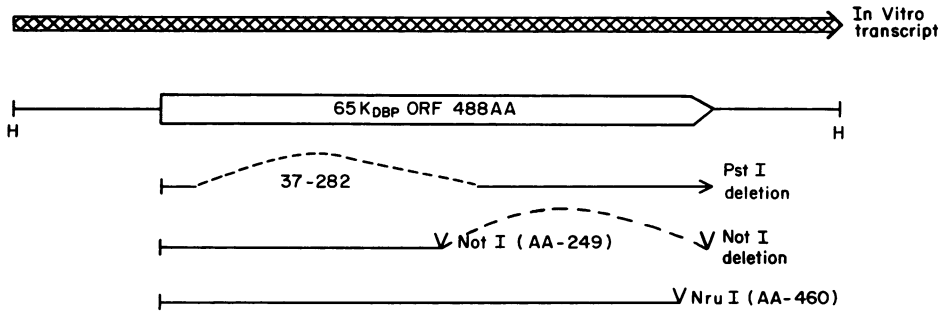

FIG. 1. Schematic drawing of in vitro transcripts of wild-type and mutant polypeptides produced by in vitro transcription-translation of various plasmid constructs. Symbols: ⇛, transcript produced by full-length runoff transcription by the T7 RNA polymerase of the wild-type construct; ⊐, open reading frame (ORF) of the wild-type construct; —, portion of the wild-type polypeptide produced by translation of full-length RNA; ----, internal regions of the wild-type polypeptide or transcript which were deleted. AA, Amino acid.

were subjected to fast protein liquid chromatography through sequential columns of DEAE Sephacel, Blue Sepharose, and Mono Q. All preparations of the 65K$_{DBP}$ used were then subjected to purification by velocity sedimentation through a single glycerol gradient. The purity of each preparation was confirmed by immunoblotting with monoclonal antibody (MAb) 6898, which is specific for the 65K$_{DBP}$ (17), and by assessment of silver-stained sodium dodecyl sulfate-polyacrylamide gels of the material (4). All preparations of the 65K$_{DBP}$ were stored at 4°C and used within 2 weeks. Pol preparations used in these studies were purified in the same way, except that a number of preparations were subjected to two cycles of velocity gradient sedimentation, as indicated in Results.

DNA polymerase assays. HSV-1 Pol activity was measured by the incorporation of [^{3}H]dTTP (specific activity, 55 Ci/mmol) (4) or [^{32}P]dCTP (specific activity, 1,150 Ci/mmol) into trichloroacetic acid-insoluble radioactivity. Activity with a maximally activated calf thymus DNA template (30 μg per reaction) was measured under high-salt conditions (200 mM KCl or 100 mM NH_2SO_4) in a total volume of 100 μl containing 100 mM Tris (pH 8.0), 4 mM $MgCl_2$, 5 mM dithiothreitol, 5 μg of bovine serum albumin, and each of the other three deoxynucleoside triphosphates at 0.2 mM. Reactions were initiated by the addition of 5 to 10 μl of purified enzyme or a dilution thereof. For the assay of in vitro translation products, reactions were initiated with 20 to 30 μl of rabbit reticulocyte lysate.

Plasmids. The genes for the HSV-1 Pol and 65K$_{DBP}$ were cloned in the multifunctional vectors Genescribe (United States Biochemical, Cleveland, Ohio) and pGEM2 (Promega Biotec, Madison, Wis.) in sense orientation downstream from the T7 RNA polymerase promoter. Construction of the HSV-1 *pol* plasmid pGEM2-702 has been described previously (3). The 65K$_{DBP}$-containing construct pLBN19A (in pTZ19U) was subcloned from the HSV-1 *Hin*dIII L fragment from plasmid pGX 80 (from B. Matz) (5). It contained 65K$_{DBP}$-coding and -noncoding sequences from the *Bst*EII site located 390 bp upstream of the open reading frame to the *Nsi*I site 329 bp downstream of the open reading frame (12). The *Bst*EII and *Nsi*I sites were removed and changed to *Hin*dIII sites by the addition of linkers to facilitate cloning into the *Hin*dIII site of the polylinker in pTZ19U. All 65K$_{DBP}$ mutant constructs shown (Fig. 1) were obtained, beginning with the pLBN19A construct in which the *Bam*HI or *Pst*I site in the polylinker had been deleted to facilitate cloning. In the ΔPst mutant, the internal 738-bp *Pst*I fragment was deleted, which resulted in an in-frame mutation. In the ΔNot mutant, the gene was deleted from the *Not*I site after amino acid 251 to the *Not*I site 38 bp upstream of the poly(A) recognition site of the 65K$_{DBP}$ gene. For the *Nru* 10 mutant, a 10-bp *Bam*HI linker was inserted at the *Nru*I site to produce an out-of-frame mutation (Fig. 1).

In vitro transcription and translation. Plasmids were linearized in the polylinker downstream from the gene of interest and transcribed in vitro by using T7 RNA polymerase, as described by Dorsky and Crumpacker (3). The start site of transcription for the 65K$_{DBP}$ wild-type (pLBN19A) and mutant gene constructs was 4 bp upstream of the 5′ proximal *Hin*dIII site, and the termination site was 51 bp downstream of the 3′ proximal *Hin*dIII site. The full-length transcript of the wild-type gene produced by runoff transcription with the T7 RNA polymerase is shown in Fig. 1. Mutant transcripts began and ended as shown but contained the internal sequence alterations as indicated (Fig. 1). The purified RNAs were translated in vitro with a commercially prepared rabbit reticulocyte lysate system (Promega). As a positive control for translation, brome mosaic virus RNA (Promega) was used. The quality of translation products was confirmed for each RNA preparation by translation in the presence of [^{35}S]methionine (specific activity, >1,000 Ci/mmol; Amersham Corp. Arlington Heights, Ill.). Polypeptides were separated by electrophoresis through 10% polyacrylamide denaturing gels, treated with En^{3}Hance, and visualized by autoradiography as described previously (17).

Immunodepletion of the 65K$_{DBP}$. Samples (16 μl) of the 65K$_{DBP}$ were incubated at 0°C for 2 h with 4 μl of MAb 6898, specific for the 65K$_{DBP}$ (17), or the appropriate control in a total volume of 20 μl. MAb 6898 and control antibodies were obtained as mouse ascitic fluid and were kindly provided by Howard Marsden (University of Glasgow, Scotland). Protein A-Sepharose (Sigma Chemical Co., St. Louis, Mo.) containing protease inhibitors was subsequently added, and the samples were incubated at 4°C for an additional 1 h. The protein A-immune complexes were removed by centrifugation, and the samples were assayed for their ability to stimulate the viral Pol.

RESULTS

Purified 65K$_{DBP}$ stimulates the viral DNA polymerase. We had observed previously that the viral Pol copurified with the 65K$_{DBP}$ following passage of HSV-1-infected nuclear extracts over three columns but that the two proteins could be separated following subsequent sedimentation through a glycerol gradient (4). However, we occasionally observed

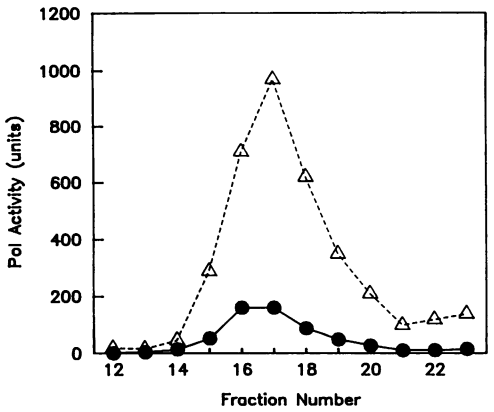

FIG. 2. HSV-1 DNA polymerase activity of glycerol gradient fractions in the presence and absence of the $65K_{DBP}$. The HSV-1 Pol activity of samples (10 μl) of each fraction from a glycerol gradient was assayed in the presence of 200 mM KCl, as described in Materials and Methods, for 2 h at 37°C (●). An equal volume (5 μl) of fraction 10, which contained the $65K_{DBP}$, was assayed with each of the column fractions (△). A unit of activity is defined as the number of femtomoles of [^{3}H]dTMP incorporated per hour per 10-μl Pol sample into trichloroacetic acid-precipitable radioactivity by using an activated calf thymus DNA template.

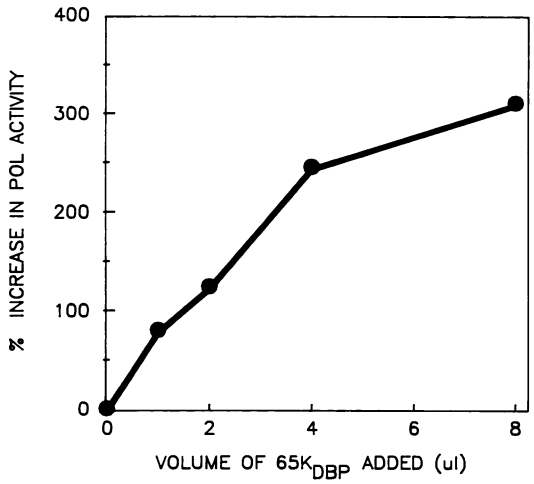

FIG. 3. Titration of polymerase-stimulating activity of the $65K_{DBP}$. A constant amount of HSV-1 Pol (190 U) obtained following two consecutive glycerol gradients was assayed for 30 min at 37°C with increasing volumes of the $65K_{DBP}$ purified as described in Materials and Methods. HSV-1 Pol activity was measured by the incorporation of [^{3}H]dTMP into acid-precipitable form by using an activated DNA template.

poor recovery of Pol activity following the velocity sedimentation. To determine whether the apparent loss of Pol activity was the direct result of removal of the $65K_{DBP}$, we performed reconstitution experiments whereby a portion of the fraction from the gradient containing the $65K_{DBP}$ was added to each fraction prior to assay for Pol activity. When Pol activity was measured in the presence of 200 mM KCl, fractions 15 through 20 contained low but detectable levels of activity (Fig. 2). Fraction 10 from the gradient, which was representative of our purified preparations of the $65K_{DBP}$, contained the $65K_{DBP}$ peak as assessed by immunoblotting with MAb 6898. In addition, it was devoid of other proteins by silver staining and possessed no intrinsic Pol activity above background levels (4; results not shown). However, the addition of the purified $65K_{DBP}$ (fraction 10) increased the activity of fractions 15 through 20, which contain the peak of Pol activity, from four- to eightfold (Fig. 2). We also observed up to a 10-fold increase in Pol activity in the fractions from the bottom of the gradient.

The Pol in fractions 15 through 20 from the glycerol gradient was not pure and contained up to five polypeptide species, as determined by silver staining of sodium dodecyl sulfate-polyacrylamide gels of the fractions, and variably contained trace amounts of the $65K_{DBP}$, as determined by immunoblotting (4; our unpublished results). When this occurred, the Pol fractions were pooled and subjected to a second cycle of velocity gradient sedimentation, which effectively removed these trace contaminants (results not shown). When we assayed a constant amount of Pol, which was subjected to this additional purification step, with increasing volumes of the $65K_{DBP}$, purified by fast protein liquid chromatography and one cycle of velocity sedimentation, we observed a linear increase in the Pol activity (Fig. 3). Thus, the stimulatory effect of the $65K_{DBP}$ was titratable.

Immunodepletion of the Pol-stimulating factor by antibody to the $65K_{DBP}$. The results above indicated that a factor present in the $65K_{DBP}$ preparations was capable of stimulating the activity of the HSV-1 Pol. Although these preparations were highly enriched for the $65K_{DBP}$, as determined by silver staining (4), it was possible that a trace contaminant was responsible for the stimulating activity. Therefore, we performed immunodepletion experiments to determine whether the stimulating factor could be removed by an antibody specific for the $65K_{DBP}$ (Fig. 4). When the $65K_{DBP}$ preparation was incubated first with control ascitic fluid and then with *Staphylococcus aureus* protein A-Sepharose, the resulting supernatant was capable of stimulating Pol activity 4.5-fold over levels observed with bovine serum albumin. However, incubation of the $65K_{DBP}$ preparation with ascitic fluid containing MAb 6898, which is specific for the $65K_{DBP}$ (17), and protein A-Sepharose significantly reduced the ability of the preparations to stimulate the Pol activity. Following the addition of protein A-Sepharose alone, the $65K_{DBP}$ still retained its Pol-stimulating activity (Fig. 4). In other experiments, we observed a linear decline in the ability of $65K_{DBP}$ preparations to stimulate Pol as a function of antibody concentration (results not shown), which confirmed that the stimulation was due to the presence of the $65K_{DBP}$.

Pol stimulation by the $65K_{DBP}$ is not due to a generalized effect on components of the reaction mixture. It was possible that the ability of the $65K_{DBP}$ to stimulate the HSV-1 Pol was due to a nonspecific effect on the template or other component of the reaction mixture. If stimulation of Pol by the $65K_{DBP}$ was due to an alteration in the template (such as nuclease activation) or a relatively nonspecific effect on the reaction mixture (as would be provided by a condensing agent or a polyamine), the $65K_{DBP}$ would be expected to stimulate the activities of other polymerases. With maximally activated calf thymus DNA as the template, we tested the effect of the $65K_{DBP}$ on several commercially available polymerases and found that it did not stimulate the activity of T4 DNA polymerase, modified T7 DNA polymerase (Sequenase), or *E. coli* Klenow (large fragment DNA polymerase I) in the presence of 200 mM KCl, the salt concentration at which the HSV-1 Pol was assayed (Table 1). Stimulation was also not detected under the assay conditions optimal for each polymerase activity (results not shown). Thus, we were able to rule out the possibility that the

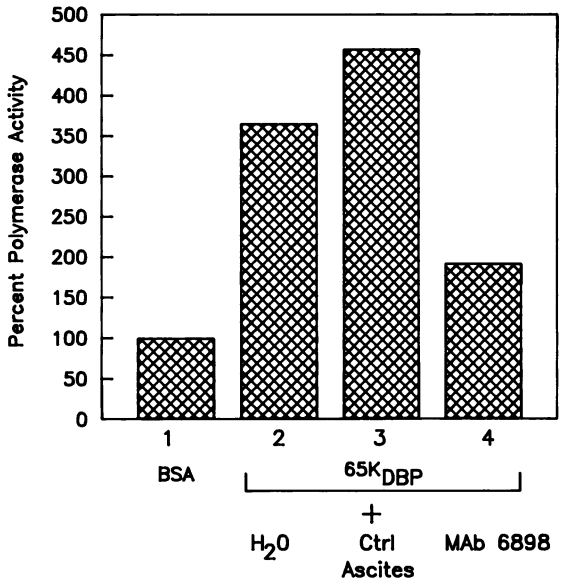

FIG. 4. Immunodepletion of the Pol-stimulating activity of purified $65K_{DBP}$ with MAb 6898. A constant amount of HSV-1 Pol (79 U) obtained following sedimentation through two glycerol gradients was assayed in the presence of 10 μg of bovine serum albumin (BSA) (column 1) or in the presence of 10 μl of immunodepleted $65K_{DBP}$ (columns 2 through 4). Purified $65K_{DBP}$ (16 μl) was incubated with 4 μl of water (column 2), 4 μl of control (ctrl) ascitic fluid (column 3), or 4 μl of MAb 6898 ascitic fluid, specific for the $65K_{DBP}$ (column 4), as described in Materials and Methods. Immune complexes were removed by the addition of protein A-Sepharose followed by low-speed centrifugation. The supernatant represented the immunodepleted $65K_{DBP}$ used.

stimulatory effect of the $65K_{DBP}$ on the HSV-1 Pol was due to nonspecific alterations of the reaction mixture.

Stimulation of Pol by in vitro translation products of the $65K_{DBP}$ gene. As an independent approach to determining the ability of the $65K_{DBP}$ to stimulate the Pol, we employed an in vitro assay system free of other viral proteins. Each gene was cloned into specialized phagemid vectors downstream of the T7 RNA polymerase-responsive promoter. Transcripts generated in vitro with T7 RNA polymerase were purified and subsequently translated in vitro with a commercially prepared rabbit reticulocyte lysate. Dorsky and Crumpacker (3) previously demonstrated that when plasmid pGEM2-702, which contains the *pol* gene, is transcribed and translated in vitro, it yields products which possess DNA polymerase activity on an activated calf thymus DNA template. We confirmed that in vitro translation products generated from pGEM2-702 possessed low but detectable Pol activity (Table 2). By contrast, in vitro translation products from pLBN19A, containing the $65K_{DBP}$ gene, or from brome mosaic virus RNA contained no inherent Pol activity. When transcripts generated from the $65K_{DBP}$ and *pol* plasmids were cotranslated, the products possessed seven times more Pol activity than Pol alone (Table 2).

It was possible that the increase in Pol activity, which we observed in the in vitro cotranslations of *pol* and the $65K_{DBP}$ RNA compared with those of *pol* RNA alone, was the result of more efficient translation of the *pol* transcripts in the presence of the $65K_{DBP}$ gene products rather than a direct effect of the $65K_{DBP}$ gene product on the Pol activity. To rule this out, we included [^{35}S]methionine in the in vitro translation reactions and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis the labeled polypeptides produced when RNA species were present alone or together (Fig. 5). The results demonstrate no qualitative difference in the *pol* gene-specific polypeptides produced in the presence or absence of the $65K_{DBP}$ transcripts (Fig. 5, compare lanes 3 and 4). However, the levels of each translated product in lysates in which both RNAs were present were reduced by approximately 50%. These results indicate that the increase in Pol activity observed in the in vitro cotranslations was unlikely to be caused by an increase in the in vitro efficiency of *pol* gene translation. They also suggest that the $65K_{DBP}$ in vitro translation products stimulated the Pol activity directly. In fact, because of the excess amount of RNA and the actual decrease in *pol* translation products in the cotranslated mixtures of RNA, the ability of the $65K_{DBP}$ to stimulate Pol may be up to two times greater than that observed.

To further confirm that stimulation of Pol specifically required a functional $65K_{DBP}$ polypeptide, we tested a variety of $65K_{DBP}$ mutations in this assay. The plasmid constructs which were transcribed and translated in vitro are shown in Fig. 1. The polypeptides which were produced from the gene transcripts coincided with their predicted sizes, which were based on nucleotide sequence (results not shown). As observed for the wild-type $65K_{DBP}$ gene, no polymerase activity was detected in the in vitro translation products from any of the $65K_{DBP}$ gene constructs (Fig. 6; results not shown). When the *Not*I deletion mutant which contained the 249 amino-terminal amino acids of the wild-type $65K_{DBP}$ gene was cotranslated with *pol* transcripts, it failed to stimulate Pol activity. Likewise, the *Pst* deletion mutant (ΔPst), which produced a polypeptide in which 245 amino acids were deleted from the amino-proximal region,

TABLE 1. Effects of $65K_{DBP}$ on the activities of various DNA polymerases

DNA polymerase[a]	Pol activity[b]		Fold stimulation
	$-65K_{DBP}$	$+65K_{DBP}$	
HSV-1	9.9 ± 0.7	57 ± 1.6	5.8
T4	210 ± 15	190 ± 32	0.91
T7	120 ± 7.3	120 ± 7.7	1.0
Klenow	240 ± 62	240 ± 16	1.0

[a] Commercial preparations of DNA polymerases T4, T7 (modified, Sequenase), and Klenow and HSV-1 DNA polymerase, purified as described in Materials and Methods through two glycerol gradients, were diluted and assayed in the absence or presence of 10 μl of $65K_{DBP}$ for 30 min at 37°C by using standard high-salt reaction conditions and activated calf thymus DNA template.

[b] Assays were performed in triplicate; values are averages plus or minus the standard deviation. A unit of activity was defined as the activity required for the incorporation of 1 fmol of [^{3}H]dTMP per h into trichloroacetic acid-insoluble form.

TABLE 2. DNA polymerase activity of in vitro translation products

RNA used[a]	Pol activity[b]
None	30
BMV	7.3
65K	<1
pol	110
65 + *pol*	770

[a] 4 μl of the RNA obtained from in vitro transcription of 8 μg of linearized plasmic DNA. BMV, Brome mosaic virus.

[b] A unit of polymerase activity is defined as the activity required for incorporation of 1 fmol of [^{32}P]dCMP into an activated calf thymus DNA template at 37°C/h per ml of rabbit reticulocyte lysate.

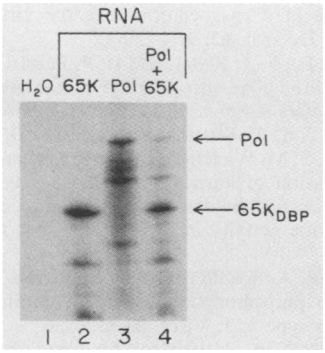

FIG. 5. In vitro translation products of the $65K_{DBP}$ and Pol RNA transcribed in vitro. RNA transcribed in vitro by runoff transcription of linearized plasmid DNAs with T7 RNA polymerase was incubated with rabbit reticulocyte lysate supplemented with amino acids (50 μM) and [^{35}S]methionine (10 μCi) in a final volume of 10 μl at 30°C for 2 h. Translation products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis through a 10% polyacrylamide gel followed by autoradiography. Lane 1, H_2O-translated control; lane 2, $65K_{DBP}$ RNA (1 μg); lane 3, Pol RNA (1 μg); lane 4, 1 μg each of $65K_{DBP}$ and Pol RNA.

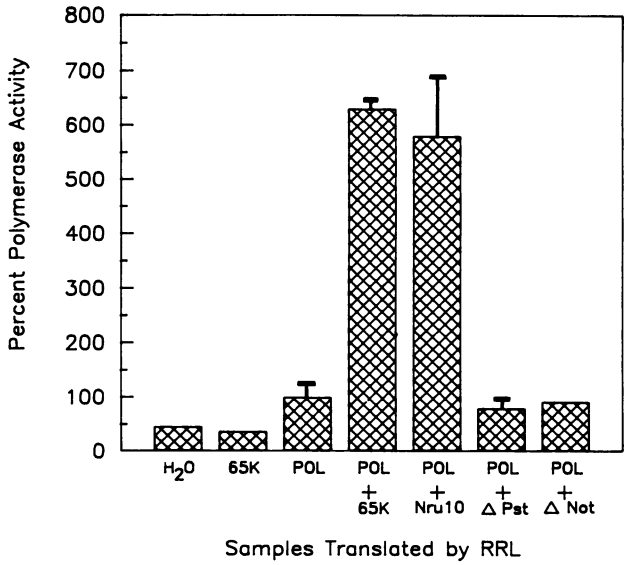

FIG. 6. Polymerase-stimulating activity of $65K_{DBP}$ mutant gene products. DNA polymerase activity of in vitro translation products. Rabbit reticulocyte lysates (30 μl) supplemented with amino acids were incubated with water (H_2O), wild-type $65K_{DBP}$ RNA (65K), Pol RNA (POL), Pol and wild-type $65K_{DBP}$ RNA (POL + 65K) Pol and *Nru* 10 RNA (POL + Nru10), Pol and Δ*Pst* RNA (POL + Δ Pst), and Pol and Δ*Not* RNA (POL + Δ Not). Cotranslations contained 4 μg of each RNA, while translations with single species of RNA contained 4 μg of the appropriate RNA. The HSV-1 Pol activity was assayed for 30 min at 37°C as described in the legend to Fig. 2 and was compared with that produced by in vitro translation products of Pol RNA alone (790 U/ml of rabbit reticulocyte lysates).

did not stimulate the activity of the *pol* translation products. However, both the in-frame (results not shown) and out-of-frame linker insertions at the *Nru*I site (located prior to the 28 carboxy-terminal amino acids) yielded translation products which were as effective as the wild-type $65K_{DBP}$ products in stimulating the HSV-1 Pol activity. Thus, the first 460 amino acids of the $65K_{DBP}$ are sufficient for the stimulatory activity. The results of the *Not*I translation products further suggest that the amino-terminal 249 amino acids are not sufficient for stimulation.

DISCUSSION

We have observed that the $65K_{DBP}$ is capable of stimulating the activity of the virus-encoded Pol by reconstitution of purified proteins from infected cells as well as by using proteins expressed in an in vitro transcription-translation system. This activity may have been overlooked in the past because the amount of $65K_{DBP}$ required to achieve maximum Pol activity is small and because most Pol preparations which have been studied previously contained some contaminating $65K_{DBP}$. In our experience, our ability to detect stimulation is dependent upon having a Pol preparation devoid of the $65K_{DBP}$. In fact, when little or no stimulation of Pol has occurred, additional purification of the Pol ultimately led to our ability to stimulate its activity with the $65K_{DBP}$. Likewise, immunodepletion of the $65K_{DBP}$ preparations with MAb specific for the $65K_{DBP}$ reduces their ability to stimulate Pol in a titratable fashion. However, because of the extremely small amounts of protein present in our purified Pol and $65K_{DBP}$ preparations, we have not been able to determine the stoichiometry of the interaction.

To produce HSV-1 Pol which was certain to be devoid of the $65K_{DBP}$ or any other viral protein, we used the in vitro transcription-translation system first utilized by Dorsky and Crumpacker (3) to obtain Pol activity. Despite the presence of a large number of proteins in the rabbit reticulocyte lysate, we were able to demonstrate up to a sevenfold increase in Pol activity when translations included both the *pol* and the $65K_{DBP}$ transcripts compared with activity in those with *pol* transcripts alone (Table 2 and Fig. 6). This level of stimulation was similar to that obtained by reconstitution of the $65K_{DBP}$ and Pol purified from infected cells (Fig. 2). Furthermore, the in vitro transcription-translation assay required a functional $65K_{DBP}$ in order for stimulation of Pol activity to be observed (Fig. 6). Thus, these results from two independent systems provide strong evidence that the stimulation of Pol activity was due to the presence of the $65K_{DBP}$.

The ability of the $65K_{DBP}$ to stimulate Pol cannot be attributed to a generalized effect on the template, since we did not observe stimulation of several procaryotic DNA polymerases. In addition, the $65K_{DBP}$ stimulates the HSV-1 Pol activity on a variety of templates, including activated calf-thymus DNA, primed circular single-stranded DNA, and multiply primed homopolymeric DNA (unpublished results). The question of whether the stimulation of activity by the $65K_{DBP}$ is specific for the HSV-1 Pol is currently under investigation.

O'Donnell et al. (13) previously reported that the activity of their purified HSV-1 Pol on a primed M13 DNA template could be stimulated by factors in extracts of infected cells. Although those authors found that stimulation was not due to the presence of ICP8, the stimulating activity was not identified. Our results indicate that the $65K_{DBP}$ may have been responsible for the observed stimulation. However, we cannot exclude the possibility that other accessory proteins stimulate Pol or that other viral or host factors may facilitate the ability of the $65K_{DBP}$ to stimulate Pol.

The ability of a viral protein to stimulate the activity of the DNA polymerase encoded by another human herpesvirus, Epstein-Barr virus, has been reported (1). The stimulatory effect could be observed only with low levels of Pol activity. Those investigators further demonstrated that the maximum

stimulation achieved was approximately eightfold but varied with the degree of contamination of their polymerase preparation. Recently, it was found that MAb R3, reactive to the Epstein-Barr virus diffuse early antigen component, which is encoded by BMRF1 (18), could inhibit the activity of Epstein-Barr virus DNA polymerase preparations (9). Although BMRF1 possesses no significant amino acid homology to the HSV-1 $65K_{DBP}$, the similarity of its genomic location to that of HSV-1 UL42 has been noted (12). Thus, it seems likely that BMRF1 and the $65K_{DBP}$ are analogous proteins.

We have been unable to detect any ATPase activity or primase activity in our preparations of the $65K_{DBP}$, which suggests that it is not part of a helicase-primase complex. This is in agreement with results of Crute et al. (2), who have been able to reconstitute helicase-primase activity with the proteins encoded by UL5, UL8, and UL52 without the addition of the $65K_{DBP}$. Thus, the means by which the $65K_{DBP}$ stimulates Pol activity has not been determined. However, other possible mechanisms include increased processivity of the Pol, increased rate of elongation by Pol, inhibition of the 3′-to-5′ exonuclease activity of the Pol, increased recycling of Pol upon termination of chain elongation, other Pol accessory functions which affect the dimerization of *pol*, and the efficiency with which Pol engages the template. We are currently examining these possible activities using defined templates, such as primed M13 DNA. The $65K_{DBP}$ also may be involved in rolling circle replication, recombination, or another viral function. Viral replicative machinery, including the $65K_{DBP}$, has been shown to be necessary and sufficient for genome isomerization (25), and therefore, multiple roles for the $65K_{DBP}$ may be possible. Whether the Pol-stimulating activity observed in vitro truly reflects the activity of the $65K_{DBP}$ during in vivo replication of the viral DNA must await the detailed study of viral mutants with defects in this gene.

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