JOURNAL OF VIROLOGY, Oct. 1993, p. 5922–5931
0022-538X/93/105922-10$02.00/0

Vol. 67, No. 10

Two Regions of the Herpes Simplex Virus Type 1 UL42 Protein Are Required for Its Functional Interaction with the Viral DNA Polymerase

STEVEN J. MONAHAN,[1] TAMAR F. BARLAM,[2] CLYDE S. CRUMPACKER,[2] AND DEBORAH S. PARRIS[1]*

Department of Medical Microbiology and Immunology and Comprehensive Cancer Center, The Ohio State University, Columbus, Ohio 43210,[1] and Charles A. Dana Research Institute and Harvard-Thorndike Laboratory of Beth Israel Hospital and Department of Medicine, Division of Infectious Disease, Beth Israel Hospital and Harvard Medical School, Boston, Massachusetts 02215[2]

Received 24 March 1993/Accepted 12 July 1993

Two essential gene products of herpes simplex virus type 1, the viral DNA polymerase (pol) and UL42, its accessory protein, physically and functionally interact to form the core of the viral DNA replication complex. Understanding this essential interaction would provide a basis from which to develop novel anti-herpesvirus agents. We previously have shown that when coexpressed in an in vitro transcription-translation system, UL42 stimulates pol activity (M. L. Gallo, D. I. Dorsky, C. S. Crumpacker, and D. S. Parris, J. Virol. 63:5023–5029, 1989). By analyzing various insertion, deletion, and frameshift mutations of UL42 in this system, we found the C-terminal 149 amino acids to be dispensable for the ability of the protein to stimulate pol activity. In addition, two distinct internal regions of UL42 were found to be required for pol stimulation. Regions I and II were defined to lie between amino acid residues 129 and 163 and between residues 202 and 337, respectively. When physical association was examined with antibody to UL42, pol was found to coimmunoprecipitate to the same level when expressed with a UL42 mutant protein lacking region I as that with wild-type UL42. Thus, mere physical association is insufficient for stimulation of pol activity. Deletion of region II reduced or eliminated coimmunoprecipitation with pol. Interestingly, an antibody to pol specific for residues 1216 to 1224 coimmunoprecipitated UL42 when both proteins were synthesized in a baculovirus expression system but not in rabbit reticulocyte lysates. These results indicate that (i) at least a portion of the region recognized by the pol antiserum may be accessible in the pol-UL42 heterodimer and (ii) immunoprecipitation results for products made in different expression systems may vary. Thus, at least two distinct regions of UL42 are essential for functional interaction with pol. Moreover, these results point to a UL42 region I function other than physical association with pol.

Herpes simplex virus type 1 (HSV-1), which replicates in the nuclei of eukaryotic cells, provides a unique model system for eukaryotic DNA replication in that many of the proteins required for genome replication are virus encoded (4), the sequence of the entire 152-kbp genome is known (34), and an abundance of viral mutants (46) lends the system to powerful genetic analysis. Although HSV-1 DNA replication has not been reconstituted in vitro, transient transfection of mammalian cells with plasmids containing an HSV-1 origin of replication (*ori*) and various HSV-1 genes has revealed that seven viral genes are necessary and sufficient to amplify *ori*-containing plasmids (53). Analysis of host-range and temperature-sensitive mutants with defects in each of these genes has confirmed that these seven gene products are essential for viral DNA replication in host cells (for a review, see reference 46). However, it is likely that other viral and/or cellular proteins are required in infected cells for nucleic acid metabolism as well as for cleavage and packaging of unit-length genomes (5, 44).

The proteins involved in DNA replication in well-characterized prokaryotic systems, such as *Escherichia coli oriC* plasmids and the T4 and T7 bacteriophages, form multiprotein complexes held together by protein-protein and/or DNA-protein interactions (32), and such interactions are likely to be involved in eukaryotic DNA replication. Indeed, protein-protein and DNA-protein interactions involving the seven genes required for HSV-1 *ori*-dependent replication have been documented. The product of UL9 is a sequence-specific DNA-binding protein which binds to HSV-1 *ori* sequences (16, 37) and possesses helicase activity important for initiation (1, 17). Three of the gene products (UL5, UL8, and UL52) form a stable protein-protein complex which possesses helicase and primase activities (3, 10), and all three products can be coimmunoprecipitated from HSV-1-infected cells with specific antibody to the UL5 protein (55). UL5 and UL52 are sufficient for helicase-primase enzymatic activities (3, 14), while UL8 may be involved in stabilizing the interaction between primers and the DNA template (48). The remaining three proteins—the DNA polymerase (pol, product of UL30 [27, 42]), a 65-kDa double-stranded DNA-binding protein ($65K_{DBP}$, product of UL42 [33, 41]), and the single-stranded major DNA-binding protein (ICP8, product of UL29 [8, 52])—also have been reported to physically interact (19, 50, 51). Results from our laboratory as well as those from others have demonstrated copurification of UL42 and pol by standard biochemical chromatography and by immunoaffinity chromatography employing monoclonal antibodies (MAbs) to UL42 (9, 19, 50). In some cases, ICP8 also copurified with pol and UL42 (19, 50). Although HSV-1

* Corresponding author.

pol expressed in heterologous systems possesses catalytic activity (15, 18, 19, 24–26, 31), UL42 stimulates pol activity four- to sevenfold (18), increases the processivity of pol on primed single-stranded DNA templates (24, 26), and has been defined as a pol accessory protein (18, 24, 26). ICP8 has also been reported to stimulate pol activity twofold (36, 45), and one group has reported that it is required for UL42 to increase processivity of pol (26). Moreover, all three proteins colocalize to the same replication compartments in productively infected cells (2, 11, 22, 23, 43).

It has been shown previously that the carboxy-terminal (C-terminal) 227 amino acids of pol are involved in and sufficient for complex formation with UL42 on the basis of coimmunoprecipitation of in vitro transcription-translation products with pol-specific antibody (13). Two groups have attempted recently to further delineate the C-terminal pol domain which is involved in physical association with UL42. Although Tenney et al. (49) have shown that the last 19 amino acids of pol are not required for functional or physical association with UL42, the C-terminal 19 to 40 residues stabilize the physical association with UL42, although these residues are not required for the pol activity to be stimulated by UL42. By contrast, Digard et al. (12) failed to observe coimmunoprecipitation of truncated pol polypeptides lacking the C-terminal 19 residues with UL42 produced by in vitro translation. Thus, the exact region of pol which is required for complex formation with UL42 remains unclear. Moreover, the domains of UL42 required to functionally interact with pol have not been defined. To determine the domains in UL42 required for both functional and physical association with pol, we have exploited the fact that cotranslation products of UL42 and pol transcripts in rabbit reticulocyte lysates possess up to sevenfold more pol activity than translation products of pol transcripts alone (18). In the present study, we have localized two distinct regions of UL42 required for stimulation of pol activity. Our results demonstrate that one of these domains is particularly sensitive to disruption by mutation and is required for functional but not physical interaction with pol.

MATERIALS AND METHODS

Plasmids. The HSV-1 pol and UL42 genes were subcloned into phagemid vectors in the sense orientation downstream from the T7 RNA polymerase promoter. Plasmid pGEM2-702, which encodes full-length pol, and two amino-terminal deletion mutants, pT7-7.1 and pT7-7.2, which do not encode the first 67 and 27 amino acids, respectively, have been described previously (15). Plasmid pLBN 19A, which encodes full-length UL42 (18), was used as the wild-type UL42 construct, and mutant forms of UL42 were derived from it. All plasmids were routinely propagated in *E. coli* JM109 or HB101 and purified by the use of Quiagen Maxi-Prep columns (Quiagen Inc., Chatworth, Calif.) according to the instructions of the manufacturer.

Construction of UL42 gene mutations. C-terminal deletions of UL42 were expressed from runoff transcripts produced from the wild-type UL42 plasmid either linearized within the UL42 gene or mutagenized via insertion with *Bam*HI linkers (8-, 10-, and 12-mer; New England Biolabs, Beverly, Mass.) to introduce frameshift (fs) mutations. Mutant constructs N459(fs), N248(fs), N206(fs), and N140(fs) encode polypeptides containing wild-type UL42 amino acids from the amino terminus (N terminus) to the residue indicated because of frameshifts created at the *Nru*I, *Not*I, *Hpa*I, and *Nde*I restriction sites, respectively. Plasmids i206 and i140 contain in-frame insertion mutations at the *Hpa*I and *Nde*I sites, respectively.

Deletion mutations were made within the UL42 gene by removing internal restriction fragments. The *d*129–163 mutation was created by digestion of pLBN 19A with *Nde*I and *Aat*II, removal of the single-stranded overhangs with S1 nuclease, and religation. The *d*141–206 mutation was constructed by digestion of pLBN 19A with *Nde*I and *Hpa*I, removal of single-stranded overhangs with S1 nuclease, and religation. The deletion mutation *d*37–282 (formerly designated ΔPst) has been described previously (18). To create the *d*202–337 deletion mutation, the *Bam*HI-to-*Eco*RI fragment from the polylinker of pLBN 19A was removed to delete the *Sst*I site in the vector, and the 398-bp *Sst*I fragment within the UL42 gene was removed to form pLB ΔSst. Since removal of the *Sst*I fragment placed the C-terminal region out of frame, a *Bam*HI linker was introduced at the unique *Sst*I site to create the in-frame *d*202–337 mutation.

Nested deletions within the UL42 open reading frame were created at unique restriction sites by digestion with exonuclease III (United States Biochemical, Cleveland, Ohio), removal of single-stranded ends with mung bean nuclease, and ligation with T4 DNA ligase. Deletion *d*140 was generated from the *Nde*I site of pLBN 19A. The sequences of all constructs in the regions altered or subcloned were confirmed by the dideoxy chain termination method with Sequenase 2.0 according to the instructions of the manufacturer (United States Biochemical).

In vitro transcription and translation. Linearized plasmids were transcribed with T7 RNA polymerase (Promega Biotec, Madison, Wis.) for 1 h at 40°C. Transcripts were translated in rabbit reticulocyte lysates (Promega Biotec) for 1 h at 30°C at a final concentration of 5 to 20 ng of RNA per μl. Cotranslation reaction mixtures contained approximately equal amounts of UL42 and pol RNA, as judged from gels containing serial twofold dilutions of each RNA species. For some experiments, L-[^{35}S]methionine (specific activity, 1,000 to 1,300 Ci/mmol; Amersham, Arlington Heights, Ill.) was added to translation reaction mixtures to a final concentration of 1 μCi/μl. Labelled translation products were analyzed by electrophoresis through denaturing, polyacrylamide gradient (10 to 20%) gels, and the gels were impregnated with En^{3}Hance (DuPont, Boston, Mass.), dried, and visualized by fluorography (33).

pol assays. HSV-1 pol activity was measured as the incorporation of [^{3}H]dTTP (43 to 82 Ci/mmol; ICN or Amersham) into trichloroacetic acid-insoluble radioactivity as described previously (15, 18). To assay in vitro translation products, reactions containing 2 to 10 μg of maximally activated calf thymus DNA as template were initiated with 25 μl of translation reaction mixtures. The relative stability of each UL42 mutant protein was assayed by incubation of labelled translation mixtures under pol assay conditions (with the omission of bovine serum albumin [BSA], deoxynucleoside triphosphates, and template) at 37°C for 30 min, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

Preparation of proteins expressed by recombinant baculoviruses. Recombinant baculoviruses (*Autographica californica*) which express either the HSV-1 UL42 or the HSV-1 pol gene from the polyhedrin promoter were the kind gifts of Mark Challberg (National Institutes of Health, Bethesda, Md.) and Robert Lehman (Stanford University, Stanford, Calif.), respectively. The wild-type strain of baculovirus and the Sf9 insect cell line used to propagate each of the

baculovirus strains were kindly provided by Fred Hink (Ohio State University, Columbus). Sf9 cells were routinely propagated in Grace medium (GIBCO-BRL, Grand Island, N.Y.) supplemented with 10% lactose hydrolysate (Difco Laboratories, Detroit, Mich), 10% tissue culture yeastolate (Difco Laboratories), 100 U of penicillin per ml, 100 μg of streptomycin sulfate per ml, and 10% fetal bovine serum (Flow Laboratories, McLean, Va.) at 28°C and were infected with baculovirus at an input multiplicity of 5 PFU per cell. To prepare proteins for immunoprecipitation, L-[^{35}S]methionine was added at 12 h postinfection to a final concentration of 50 μCi/ml, and high salt nuclear extracts were prepared essentially as described by Gallo et al. (18) from cells harvested at 36 to 48 h postinfection, except that 10 strokes of Dounce homogenization were used to disrupt the cytoplasmic membranes of the Sf9 cells.

Antibodies and coimmunoprecipitation. MAb 6898, specific for UL42, has been described previously (41) and was kindly provided as mouse ascites fluid by Howard Marsden (University of Glasgow, Glasgow, Scotland). The epitope recognized by MAb 6898 has been shown to be immunodominant and maps to amino acids 363 to 369 (35). In addition, a polyclonal antibody to residues from this region of UL42 was raised in rabbits. A 40-amino-acid peptide was synthesized which contained UL42 residues 360 to 377 linked to the amino terminus of a promiscuous T-cell epitope from measles virus (47) via a 4-residue linker consisting of Leu-Gly-Pro-Ser. The linked B- and T-cell epitopes were capable of eliciting an antibody response in rabbits without being coupled to a protein carrier, resulting in antibody 834. A synthetic peptide corresponding to residues 1216 to 1224 of the HSV-1 pol was coupled to BSA (Sigma, St. Louis, Mo.) and injected into rabbits to produce the pol-specific antiserum 771. Both peptides were synthesized at the Peptide and Protein Engineering Laboratory at Ohio State University (Columbus). The specificity of each antipeptide serum was confirmed by immunoprecipitation in the presence and absence of the cognate peptide.

The pol and UL42 transcripts (0.5 to 3 μg of RNA) were translated in 70 μl of rabbit reticulocyte lysate as described above. After removal of a 7-μl aliquot, 3 volumes of B2 buffer (10 mM Tris-HCl [pH 8.0], 10% glycerol) were added. To this solution, an equal volume of 2× Zweig's buffer (0.2 M Tris-HCl [pH 8.0], 20% glycerol, 1.0% Nonidet P-40, 1.0% sodium deoxycholate, 0.4 mM phenylmethylsulfonyl fluoride) was added. Samples were precleared by incubation at room temperature with a 0.1 volume of a 50% (wt/vol) slurry of protein A-Sepharose CL-4B (Sigma) in 1× Zweig's buffer containing 0.02% sodium azide. Supernatants were split into two 1.5-ml tubes and gently mixed with 20 to 25 μl of antiserum overnight at 4°C. Immune complexes were recovered after the addition of a 0.2 volume of protein A-Sepharose for 1 h at 4°C. The Sepharose beads were washed four times with buffer containing 0.1 M Tris-HCl (pH 8.0), 0.5 M NaCl, and 1.0% 2-mercaptoethanol. Bound protein was eluted by being boiled for 3 min in 25 μl of dissociation buffer (0.125 M Tris-HCl [pH 6.8], 2% SDS, 2% glycerol, 5% 2-mercaptoethanol, 0.004% bromophenol blue) and was analyzed by SDS-PAGE.

RESULTS

Analysis of pol constructs. In a previous report, we observed a four- to sevenfold stimulation of pol activity when HSV-1 pol RNA transcribed in vitro was cotranslated in rabbit reticulocyte lysates with wild-type UL42 RNA compared with the activity of translation products of pol RNA alone (18). Although a UL42 mutant RNA encoding a product lacking the 28 C-terminal residues resulted in wild-type levels of pol stimulation, the failure of large amino-terminal (N-terminal) and C-terminal deletion mutants of UL42 to stimulate pol activity in that study (18) suggested that this system would be useful in further delineating the domains in UL42 required for functional interaction with pol. Nevertheless, the sensitivity of the system to screen large numbers of UL42 mutations was hindered by the low and often variable activities of translation products of wild-type pol RNA.

TABLE 1. pol activities of two amino-terminal deletion mutants of UL30 coexpressed in vitro with UL42[a]

UL30 construct	Polypeptide composition	Pol activity (units)[b]		Stimulation index[c]
		−UL42	+UL42	
pGEM2-702	1–1235	116 ± 62	1080 ± 674	9.3
pT7-7.2	28–1235	418 ± 121	2416 ± 277	5.8
pT7-7.1	68–1235	311 ± 109	2251 ± 185	7.2

[a] UL30 RNA transcribed from linearized plasmid was translated alone or with UL42 RNA in rabbit reticulocyte lysates for 60 min at 30°C.

[b] Units of pol activity were defined as the femtomoles of [^{3}H]dTTP incorporated into activated calf thymus DNA at 37°C for 30 min. Activities are expressed as means ± standard deviations. pol assays were performed as triplicates for two independent experiments with pGEM2-702 and pT7-7.2 and three experiments with pT7-7.1.

[c] (Mean pol activity + UL42)/(mean pol activity − UL42).

It has been suggested that pol production may be regulated in HSV-infected cells posttranscriptionally through an attenuation mechanism resulting from secondary-structure formation at the N-terminal end of pol RNA (54). Because secondary-structure formation also could affect the ability of RNA to be translated in vitro, we tested two N-terminal deletion mutants of pol for their abilities to be stimulated by the wild-type UL42 in the in vitro transcription-translation system (Table 1). Transcripts from these mutant pol constructs previously were reported to be translated with greater efficiency than those of the wild-type, and translation products possessed pol activity (15). The results of the present study demonstrate that the wild-type pol construct that we previously used, pGEM2 702, which encodes the full-length (1,235-amino-acid) protein, yielded only barely detectable pol activity alone, although in this particular assay the activity was stimulated 9.3-fold when the RNA was cotranslated with wild-type UL42 RNA (Table 1). Both mutant pol transcripts which encode proteins in which the first 27 (pT7-7.2) or 67 (pT7-7.1) amino acids are deleted yielded higher pol activities than the wild-type when translated alone, confirming an earlier report (15). Furthermore, the pol activities of the pT7-7.1 and pT7-7.2 transcripts were stimulated 7.2- and 5.8-fold, respectively, upon cotranslation with UL42 transcripts (Table 1). We also observed less variability from experiment to experiment in pol activities produced by transcripts of these mutated pol genes than in those produced by transcripts of the wild-type gene. On the basis of the somewhat higher stimulation indices observed with the pT7-7.1 construct, we selected it for further functional domain mapping studies.

C-terminal deletions in UL42. We first determined how much of the C-terminal region of the UL42 protein was required for its ability to stimulate pol activity. The C-terminal mutant forms of the UL42 protein (Fig. 1) were generated in three ways: (i) by cleavage with the restriction

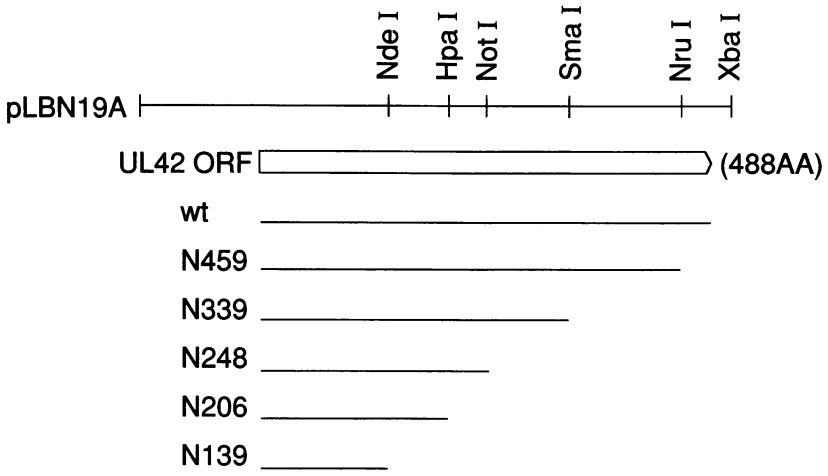

FIG. 1. Generation of C-terminal deletion mutants of UL42. Schematic diagram of the UL42 gene cloned into the phagemid vector pTZ 19U downstream of the T7 RNA polymerase promoter. Transcripts encoding C-terminal deletions were produced by (i) linearizing within the open reading frame (ORF) at the restriction sites indicated to produce runoff transcripts or (ii) inserting linkers at these sites to introduce frameshift mutations after the amino acid (AA) indicated. wt, wild type.

enzyme indicated within the open reading frame of the wild-type gene, followed by production of runoff transcripts; (ii) by deletion of sequences encoding the C-terminal end; or (iii) by insertion of linkers of various sizes out of frame at the indicated sites. Because the last method produces C-terminal tails of nonrelated sequence, linkers were inserted to create two different out-of-frame mutations. The activities of the products of the pT7-7.1 transcripts translated alone (pol control), with the wild-type UL42 transcripts (normalized to 100%), or with each of the UL42 mutant transcripts are indicated in Table 2. The results indicate that UL42 proteins containing the first 139 (140 if generated via frameshift), 206, or 248 N-terminal residues, regardless of the method by which they were generated, failed to stimulate pol activity above that observed in the absence of mutant UL42 protein. In fact, pol activities were approximately twofold less in the presence of these mutant proteins than in the absence of any UL42 transcript. The reduction in pol activity to 50% of that in translation mixtures containing pol RNA alone is consistent with competition of UL42 RNAs for limited translation machinery which we observed previously (18). In fact, we observed a reduction by approximately twofold or more in the amount of pol polypeptide synthesized in the presence of either wild-type or mutant UL42 RNA, compared with that observed in the absence of competing RNA (results not shown). A C-terminal mutant protein generated by truncation of the UL42 gene at the *Sma*I site (N339) produced wild-type levels of pol stimulation, as did the N459 mutations (Table 2). These results demonstrate that the C-terminal 149 amino acids of UL42 are dispensable for the protein's ability to stimulate pol activity.

TABLE 2. pol stimulation by C-terminal deletion mutants of UL42

Construct	pol stimulation (%)[a]	
	Truncation[b]	Frameshift mutation[c]
Wild type	100[d]	100[d]
N459	110	81
N339	100	ND[e]
N248	11	8
N206	13	7
N139	7	11

[a] pol stimulation was calculated as the ratio of pol activity measured for in vitro cotranslation products of pol and mutant UL42 RNAs to that measured for cotranslation of pol and wild-type UL42 RNAs. pol (UL30) RNA was transcribed from linearized pT7-7.1 and, when translated alone, yielded an average activity of 239 units.

[b] Mutant UL42 RNAs were generated by linearizing within the UL42 open reading frame.

[c] Mutant UL42 RNAs were generated from frameshift mutations linearized downstream of the UL42 gene.

[d] (Activity of pol and mutant UL42 translation products/activity of pol and wild-type UL42) × 100.

[e] ND, not done.

The failure of mutant UL42 proteins to stimulate pol activity could have been due to the instability of mutant proteins. Therefore, the relative stability of each mutant protein was measured after expression in rabbit reticulocyte lysates. The mutant proteins were incubated under pol reaction conditions (+) and compared with proteins in reactions held at 0°C in the absence of pol reaction buffer (−). Figure 2 shows the profile of polypeptides in some representative samples as analyzed by SDS-PAGE. The mutant polypeptide species are indicated by the small open circles. The results indicate that some of the mutations generated by linearizing the DNA template within the UL42 open reading frame were relatively unstable under pol reaction conditions (compare lane 4 with lane 5 and lane 10 with lane 11). The frameshift mutations generating the same UL42 N-terminal sequences, however, were stable (compare lanes 6 and 7 and lanes 12 and 13). These results suggest that UL42 proteins possessing C-terminal truncations of greater than 149 residues are unstable but can be stabilized by the presence of a polypeptide of nonrelated sequence at the C terminus. The stable polypeptides produced by the N140(fs), N206(fs), and N248(fs) frameshift mutations yielded no stimulation of pol activity (Table 2), and we conclude that these sequences are insufficient for functional interaction with pol.

The multiple polypeptide species produced by the wild-type and mutant transcripts have been observed previously (13, 15, 18) and most likely are due to initiation of translation

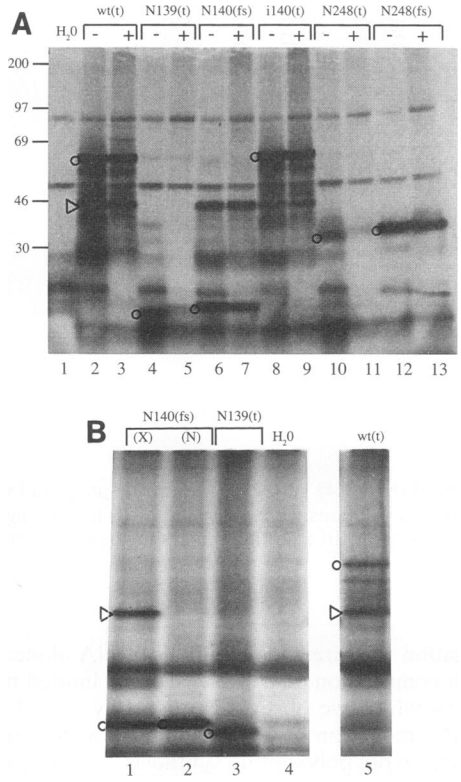

FIG. 2. (A) The stability of the C-terminal deletion mutants of UL42. Transcripts encoding representative deletion mutants from Table 1 were translated in rabbit reticulocyte lysates in the presence of [^{35}S]methionine and either frozen immediately (−) or incubated under polymerase assay conditions (+) prior to SDS-PAGE on 10 to 20% gradient polyacrylamide gels. Mutants are designated by the last UL42 amino acid before truncation (t), frameshift (fs), or in-frame insertion mutation (i). The position of each full-length product is denoted by a circle. (B) Internal initiation products of UL42 expressed downstream from frameshift mutations. Transcripts produced from pLBN 19A linearized downstream of the UL42 gene (lane 5) or at the internal *Nde*I site (lane 3) were translated as described for panel A. Transcripts were produced from template encoding a frameshift after residue 140 and linearized with *Xba*I (X) downstream of the UL42 gene (lane 1) or at the *Not*I (N) site within the gene but downstream of the frameshift mutation (lane 2); transcripts were translated and analyzed by SDS-PAGE as described for panel A. The product denoted by was produced via initiation at the third AUG of the UL42 gene. wt, wild type.

from internal AUG codons. Indeed, their apparent molecular masses are consistent with that hypothesis. We observed an unexpected species with an apparent molecular mass of 44 kDa (small arrow) in translation products from the N140(fs) mutant in which the transcriptional template was linearized at the *Xba*I site in the polylinker downstream of the UL42 gene (Fig. 2B, lane 1). This species had a size apparently larger than that predicted for the mutant protein, and it comigrated with a product that was predicted to be initiated from the third AUG codon in the wild-type protein. Since the third methionine codon (amino acid 158) is located downstream of the frameshift mutation in N140(fs), internal translation initiation from this site would yield a protein of wild-type sequence from amino acids 158 to 488, but lacking the N terminus. To determine whether this product was due to initiation of translation from the third AUG codon, we cleaved DNA from the N140(fs) mutant at the *Not*I site (wild-type residue 248) instead of at the *Xba*I site (Fig. 2B, lane 2). Although cleavage at the *Not*I site was not predicted to affect the length of the product resulting from N140(fs), it would have shortened a product produced from internal initiation at the third methionine codon. Although the size of the predicted frameshifted product did not change, there was a loss in the larger polypeptide species, predicted to arise from internal initiation (Fig. 2B, compare lanes 1 and 2). Therefore, it seems likely that the larger polypeptide species observed for the N140(fs) mutant results from the internal initiation of translation at residue 158, downstream from the frameshift mutation. Despite the presence of substantial levels of a wild-type C-terminal peptide in translation products from the N140(fs) mutant RNA generated from template cleaved with *Xba*I, we observed no stimulation of pol activity. Thus, the polypeptide corresponding to residues 158 to 488 of UL42 also is insufficient for functional interaction with pol.

Internal insertion and deletion mutations. As a means of further defining the critical domains of UL42 required for its functional interaction with pol, we created several in-frame insertion and deletion mutations in UL42 and tested them for stability (data not shown) and their abilities to stimulate pol activity as described above (Fig. 3). Some of the deletion mutants contain insertions of novel amino acids, as indicated, as a result of the mutation itself or as a result of the insertion of additional sequences to place an out-of-frame deletion mutation back in frame. All of the mutant forms indicated were stable under pol reaction conditions (results not shown). We confirmed that the *d*37–282 deletion mutant, used in a previous study with the wild-type pol construct (18), failed to stimulate the activity of the pT7-7.1 pol construct (Fig. 3). Several other smaller deletion mutants within this region of UL42 also failed to stimulate pol activity, including two nonoverlapping mutations, *d*129–163 and *d*202–337. Insertion of four novel amino acids after residue 206 (i206) had little effect on the ability of the protein to stimulate pol activity compared with the wild-type UL42 protein. However, an insertion of four residues after amino acid 140 (i140) completely abolished the pol-stimulating activity of UL42. Because the sequence inserted into i140 encodes two proline residues, which could have had profound effects on secondary and tertiary structures at some distance from the site of insertion, we created a more subtle mutation at the site by deleting only residue 140. Interestingly, *d*140 stimulated pol activity, but consistently only at half the level observed for the wild-type UL42. Taken together, these results indicate that at least two separable domains are required for UL42 to stimulate pol activity. One of these, which will be referred to as region I, maps between amino acids 129 and 163 and is sensitive to pertubations produced by both insertions and deletions in this region. The other domain, denoted region II, maps between residues 202 and 337.

Coimmunoprecipitation of pol and UL42 proteins. Because the ability of UL42 to stimulate pol activity may be the result of multiple attributes or functions of the UL42 protein, we investigated a second property of UL42: its ability to physically associate with pol. Others have previously used a coimmunoprecipitation assay with pol antiserum or antiserum to UL42 to determine the domains of pol required for physical association with UL42 (12, 13, 49). It seems likely that interfering with pol-UL42 complex formation would abolish UL42's pol-accessory functions. To assess whether the region I or II deletions abolished the ability of UL42 to

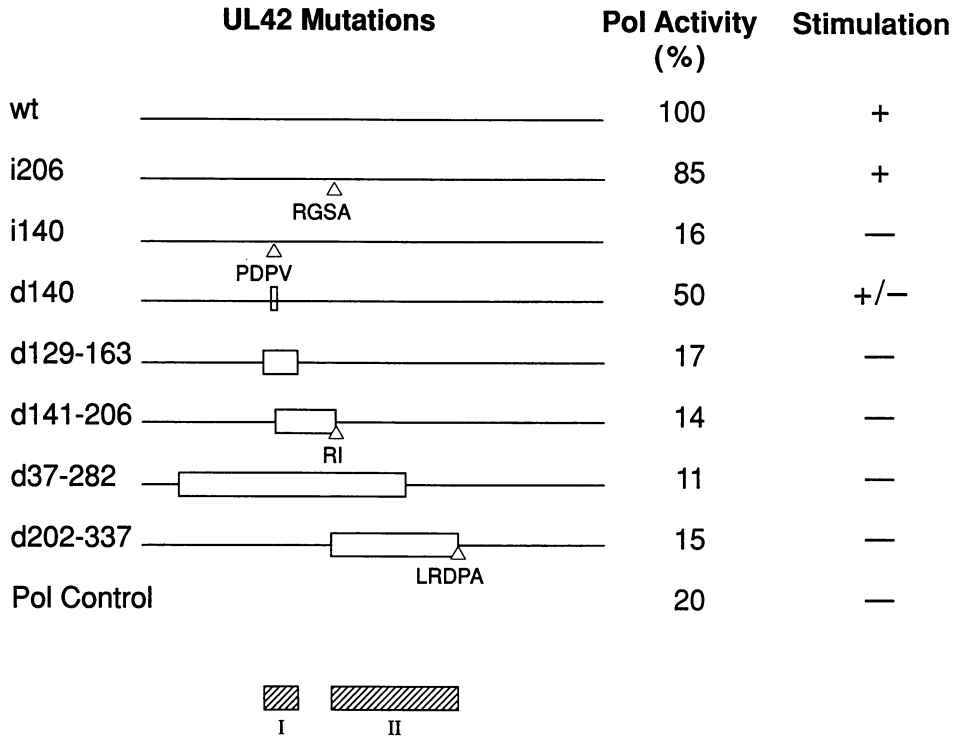

FIG. 3. Stimulation of pol activity by insertion and deletion mutants of UL42. Schematic diagram of insertion mutants made by inserting in-frame linkers at convenient restriction sites and deletion mutants constructed as described in Materials and Methods. The pol activities of in vitro translation products produced by coexpression of RNA encoding UL30 (from pT7-7.1) and each UL42 mutant are reported as percentages of the activity produced by pol and wild-type (wt) UL42 transcripts. UL42 mutants which stimulated pol activity to greater than 50% of the level measured for wild-type UL42 were considered positive (+). Activities are the averages of two to six experiments.

stimulate pol activity by abolishing physical interaction with pol, we utilized a coimmunoprecipitation assay to detect complex formation. pol and UL42 transcripts were cotranslated and immunoprecipitated with antibody 834, directed to UL42 residues 360 to 377, or with antibody 771, directed to pol residues 1216 to 1224 (Fig. 4). The specificity of the UL42 antipeptide serum 834 is demonstrated by the precipitation of a 63-kDa product from translations of the wild-type UL42 RNA (lane 2) as well as the 61- and 49-kDa products

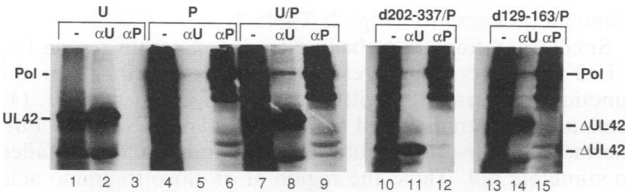

FIG. 4. Immunoprecipitation of the pol-UL42 complex formed in vitro. Translation products were produced from UL42 RNA (U), pol RNA (P), UL42 and pol RNA expressed together (U/P), UL42 region I mutant with pol RNA (*d*129–163/P), and UL42 region II mutant with pol RNA (*d*202–337/P). Translation products were either electrophoresed without immunoprecipitation (−) or were immunoprecipitated with polyclonal antibody 834 to UL42 (αU) or antibody 771 to pol (αP). Translation products not treated with antibody (lanes 1, 4, 7, 10, and 13) represent one-fifth of the sample immunoprecipitated. The linotronix print was derived from the same exposure of two gels run in parallel and was obtained via scanning and manipulating with Adobe Photoshop 2.01 software (Adobe Systems, Inc., Mountain View, Calif.).

from the region I and region II UL42 mutants (lanes 14 and 11). Likewise, the pol antibody 771 immunoprecipitated its 140-kDa full-length cognate protein and several other pol-related species (lane 6). Although the UL42 antibody possesses no cross-reactivity with pol, we have found that a very low level of pol adheres nonspecifically to protein A-Sepharose, in the absence of antibody and even upon extensive preclearing with protein A-Sepharose (results not shown). This nonspecific adherence accounts for the low level of pol polypeptide which is immunoprecipitated in the absence of UL42 (Fig. 4, lane 5). When transcripts encoding pol and the region I mutant, *d*129–163, were coexpressed and immunoprecipitated with antibody to UL42, pol was coimmunoprecipitated (lane 14) to levels comparable to those observed with wild-type UL42 (lane 8). Thus, region I does not appear to be required for pol-UL42 complex formation.

By contrast, we observed only an extremely low level of pol polypeptide to be immunoprecipitated with the UL42 region II mutant protein with antibody 834 (Fig. 4, lane 11). Although the level of pol polypeptide which is precipitated in the presence of the region II mutant is similar to the nonspecific level observed, the relatively low sensitivity of this assay precludes a definitive determination of the role of region II in complex formation. Nevertheless, both regions are required for functional interaction between UL42 and pol.

As can be seen in Fig. 4, UL42 could not be coimmunoprecipitated from pol-UL42 cotranslation products with the pol antibody 771 directed to residues 1216 to 1224. The ability of antibodies specific to this region to coprecipitate

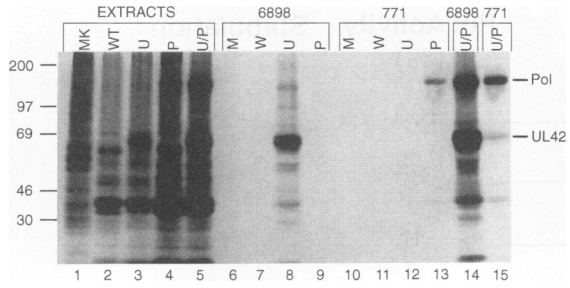

FIG. 5. Coimmunoprecipitation of the pol-UL42 complex expressed from baculovirus recombinants. Nuclear extracts were prepared from Sf9 cells metabolically labelled from 12 to 48 h postinfection with 50 μCi of [^{35}S]methionine per ml, which were mock infected (M) or infected with wild-type *A. californica* (W), a pol-containing recombinant (P), a UL42-containing recombinant (U), or both recombinants (U/P). Nuclear extracts were electrophoresed without immunoprecipitation (lanes 1 to 5) or were immunoprecipitated with MAb 6898 to UL42 (lanes 6 to 9 and 14) or polyclonal antibody 771 to pol (lanes 10 to 13 and 15). Nuclear extracts not treated with antibody (lanes 1 to 5) represent one-tenth of the sample immunoprecipitated.

UL42 was of interest because there has been some debate on the exact C-terminal residues of pol which are required for physical association with UL42 (12, 49). Some possible interpretations for the failure of the pol antibody to coprecipitate UL42 are (i) that the antibodies to pol sterically interfered with the ability of pol and UL42 to associate and (ii) that the epitope recognized by 771 was masked when pol and UL42 are physically associated, but not when pol is free. Since we could coimmunoprecipitate only small amounts of pol and UL42 from in vitro translation products with the UL42 antibody under highly stringent wash conditions (500 mM NaCl), it appeared that only a small percentage of UL42 and pol formed stable complexes in rabbit reticulocyte lysates. Furthermore, because the pol-specific antibody 771 is less avid than the 834 UL42-specific antibody (unpublished results), our failure to detect coprecipitation of UL42 with the pol antiserum may have reflected the lack of sensitivity of this assay system. Therefore, we tested the ability of the pol antibody 771 to coprecipitate the two proteins coexpressed at high levels in insect cells coinfected with pol and UL42 baculovirus recombinants (Fig. 5). In this experiment, MAb 6898, which recognizes essentially the same region as 834, was employed as the UL42 antibody. The specificity of each antibody was confirmed by the ability of each to precipitate protein only from cells singly infected by the recombinant expressing the cognate protein despite the presence of large amounts of other labelled proteins in the extracts. However, large amounts of pol were coimmunoprecipitated with MAb 6898 (α-UL42) from extracts of cells coinfected with both the UL42 and the pol recombinants. It is also apparent that a small amount of UL42 could be coimmunoprecipitated with pol by using the 771 serum (α-pol). These results demonstrate that the residues recognized by the pol antiserum (1216 to 1224) are accessible in at least a subpopulation of complexes of pol and UL42 and suggest that they may not be essential for the physical interaction of pol with UL42.

DISCUSSION

Analogously to many prokaryotic and eukaryotic replication systems, the HSV-1 DNA polymerase is a multiprotein complex which contains a 1:1 ratio of pol and UL42 (9, 24). Both of these viral proteins are essential for viral DNA replication in vivo (6, 27–29, 31, 42, 53). Although pol has inherent enzymatic activity (15, 18, 24, 25, 30), UL42 stimulates the activity of pol (18, 49), acting as a processivity factor in vitro (24, 26) perhaps by increasing the affinity by which pol binds to the DNA template. In heterologous expression systems, a pol-UL42 complex forms which is detectable by immunoprecipitation with antibodies to pol and UL42 (12, 13, 21, 49) (Fig. 4 and 5). We have examined the physical and functional interactions between UL30 (pol) and UL42 in an in vitro expression system. By coexpressing pol and mutant forms of UL42 in vitro in rabbit reticulocyte lysates, we have identified two distinct domains of UL42 which are critical for stimulation of pol activity.

To ensure that mutant forms of UL42 which failed to stimulate pol lacked critical domains and were not simply degraded rapidly, all UL42 mutants were incubated under polymerase reaction conditions (Fig. 2) to assess stability, since proteins which do not fold properly are often susceptible to increased proteolysis (40). We found that with the exception of three C-terminal deletion mutants generated from linearized template, all mutant forms of UL42 were stable (Fig. 2). However, because the same three deletion mutants created by linker mutagenesis were stable, the addition of a nonsense peptide at the C-terminal end of these deletion mutants was sufficient for stability. The fact that none of the other deletion or insertion mutations in UL42 resulted in unstable polypeptides suggests that these mutations did not result in large perturbations in global folding of the protein.

We have found that two domains of UL42 critical for its ability to stimulate pol activity are located within the amino two-thirds of the protein. The C-terminal 149 amino acids of UL42 can be deleted without affecting its stability or its ability to stimulate pol activity (Table 2). By coexpressing in in vitro transcription-translation reactions internal insertion and deletion mutants of UL42 with pol (Fig. 3), we found two physically separable regions which are critical for pol stimulation. Region I is located between amino acids 129 and 163, while region II is located between amino acids 202 and 337. Because of the relatively large number of amino acid residues encompassing region II, we cannot exclude the possibility that it contains more than one functional domain. The information contained in the amino-terminal 157 residues is essential for UL42 function, as indicated by the failure of initiation products from the third AUG codon to stimulate pol activity (Fig. 2; Table 2).

Small alterations have been introduced within region I of UL42 to further define residues which are critical for the functional interaction with pol. Deletion of residue 140 (isoleucine) decreased pol stimulation almost twofold. Furthermore, an insertion mutation after amino acid 140 failed to stimulate pol. Thus, the region at or close to amino acid 140 is particularly sensitive to perturbations. We are currently introducing other small deletions around amino acid 140 as well as point mutations in this region to further define which residues are critical for functional interaction with pol.

To determine whether region I or II is involved in the physical association of pol and UL42, mutant and wild-type UL42 were cotranslated with pol in vitro and immunoprecipitated with antibody specific to UL42 (Fig. 4). pol was coimmunoprecipitated with wild-type UL42 and the region I mutant, *d*129–163, indicating that region I is not required for physical association with pol. Although pol was not detected

above background levels when coexpressed with the region II mutant, the nonspecific adherence of pol and the poor sensitivity of the assay make it difficult to assign whether region II is required for complex formation. Indeed, the low level of coimmunoprecipitation of pol and UL42 observed in cotranslations of even the wild-type UL42 with pol may be specific to the in vitro translation system used. It should be noted that the wash conditions which we utilized in immunoprecipitations were more stringent (containing 500 mM NaCl) than those used by other investigators (12, 13, 21, 49). However, even under these stringent conditions, we observed a much more quantitative coimmunoprecipitation of pol and UL42 with antibody to UL42 in extracts of cells which coexpressed pol and UL42 from recombinant baculoviruses (Fig. 5). The differences observed in the ability of the two proteins to be coimmunoprecipitated from the different expression systems is not due to the differences in the antibodies used, since we also observed low-level coprecipitation of pol with UL42 from in vitro translations with MAb 6898 (results not shown). It is possible that the differences observed may reflect a poor ability of proteins expressed in the rabbit reticulocyte lysates to assume a conformation necessary for the two proteins to form a stable physical association. It is also possible that the K_d for the pol-UL42 heterodimer is such that the low level of protein expressed in rabbit reticulocyte lysates prevents the formation of significant amounts of stable pol-UL42. Nevertheless, the association of the two proteins translated in vitro is sufficient to enable the UL42 to stimulate pol activity.

Another possible explanation for the rather poor ability of pol and UL42 to be coimmunoprecipitated from in vitro translation products may be the presence of truncated polypeptides produced in rabbit reticulocyte lysates as a result of initiation of translation from internal AUG codons. Such products are not present at high levels in nuclear extracts of cells infected with the recombinant baculoviruses. It is possible that although such polypeptides are not sufficient to stimulate pol activity, they could compete to some extent with the wild-type UL42 protein for physical association with pol. Although we have not been able to observe any such dominant negative effects of competing polypeptides on the ability of the wild-type UL42 to stimulate pol activity (unpublished results), we cannot exclude the possibility that these polypeptides destabilize the interaction between the wild-type UL42 and pol.

Our ability to detect coimmunoprecipitation of UL42 with pol antiserum to residues 1216 to 1224 from nuclear extracts of cells which coexpress the proteins from recombinant baculoviruses, and not from in vitro translation products, indicates that caution must be exercised when interpreting negative immunoprecipitation results from experiments involving in vitro translation products. Thus, the failure of a pol polypeptide lacking the 19 C-terminal residues to coprecipitate with UL42 from translation reactions (12) and the success of such mutant proteins to be immunoprecipitated with UL42 when expressed in *Saccharomyces cerevisiae* (49) may be accounted for by the relatively unstable interaction between the proteins present in the rabbit reticulocyte lysates, the amount of protein made, or differences in posttranslational modifications among expression systems. Therefore, although we have not observed significant levels of coimmunoprecipitation of the region II UL42 mutant protein *d*202–337 in cotranslations with pol (Fig. 4), we must await the construction of a baculovirus recombinant expressing mutations in this region for more definitive coimmunoprecipitation analysis.

Although region I is not essential for physical interactions with pol, it is required for stimulation of pol activity in vitro. Disruption of functional domains without affecting protein-protein interactions has been reported for a component of the HSV-1 helicase-primase complex. Single amino acid substitutions introduced into conserved motifs of UL5 disrupted its function in viral replication but did not affect UL5-UL8-UL52 complex formation (56). It is possible that region I mediates the binding of the UL42 and/or the pol-UL42 complex to the DNA template. Chou-Fasman (7) and Robson-Garnier (20) algorithms predict that residues 130 to 139, 140 to 144, and 145 to 162 form a helix-loop-helix motif. A similar motif is directly involved in the DNA binding of many prokaryotic regulatory proteins and eukaryotic transcription factors (39). It is also possible that region I is required to maintain the conformational integrity of UL42, although any perturbations which result from the mutations tested are not sufficient to alter the global folding enough to lead to increased proteolysis. Because of the ability of UL42 to bind to double-stranded DNA with no apparent sequence specificity (19, 19a, 33), it will be necessary to purify the mutant UL42 proteins to determine whether their abilities to bind to DNA have been altered.

We have shown that UL42 has multiple, separable domains required for pol-accessory functions in vitro. However, it will be necessary to further define critical residues within both region I and region II of UL42. Because many synthetic peptides corresponding to linear arrays of residues from UL42 were found to inhibit pol activity by their abilities to interact with DNA rather than their abilities to inhibit the interaction between pol and UL42 (38), care must be exercised in defining peptide domains which interfere only with the ability of pol and UL42 to functionally and physically interact. Thus, it will be important to gain a more complete understanding of the function of these residues in the overall process of viral DNA replication to provide a basis from which to design peptidomimetic antiviral drugs.

ACKNOWLEDGMENTS

We thank Mark Challberg and Bob Lehman for the recombinant baculovirus constructs, Howard Marsden and Ann Cross for MAb 6898, Fred Hink for advice on the propagation of baculoviruses and insect cells, and Pravin Kaumaya for advice and help in designing immunogenic peptides.

This work was supported in part by grant GM34930 from the National Institutes of Health (D.S.P.) and by OSU Comprehensive Cancer Center core grant CA16958 from the National Cancer Institute. S.J.M. was supported in part by National Research Service award T32 CA09498-06 from the National Cancer Institute and by fellowship F32 AI08566-01 from the National Institutes of Health. T.F.B. was supported by National Institute of Allergy and Infectious Diseases training grant 5T32 AI07387-03 for AIDS research.

REFERENCES

1. **Bruckner, R. C., J. J. Crute, M. S. Dodson, and I. R. Lehman.** 1991. The herpes simplex virus 1 origin binding protein: a DNA helicase. J. Biol. Chem. **266**:2669–2674.
2. **Bush, M., D. R. Yager, M. Gao, K. Weisshart, A. I. Marcy, D. M. Coen, and D. M. Knipe.** 1991. Correct intranuclear localization of herpes simplex virus DNA polymerase requires the viral ICP8 DNA-binding protein. J. Virol. **65**:1082–1089.
3. **Chadler, J. M., and N. D. Stow.** 1990. Herpes simplex virus helicase-primase: the UL8 protein is not required for DNA-dependent ATPase and DNA helicase activities. Nucleic Acids Res. **18**:3573–3578.
4. **Challberg, M. D.** 1986. A method for identifying the viral genes required for herpesvirus DNA replication. Proc. Natl. Acad.

Sci. USA **83**:9094–9098.
5. **Challberg, M. D., and T. J. Kelly.** 1989. Animal virus DNA replication. Annu. Rev. Biochem. **58**:671–717.
6. **Chartrand, P., N. D. Stow, M. C. Timbury, and N. M. Wilkie.** 1979. Physical mapping of *paa*^{r} mutations of herpes simplex virus type 1 and type 2 by intertypic marker rescue. J. Virol. **31**:265–276.
7. **Chou, P. Y., and G. D. Fasman.** 1978. Empirical predictions of protein conformation. Annu. Rev. Biochem. **47**:251–276.
8. **Conley, A. J., D. M. Knipe, P. C. Jones, and B. Roizman.** 1981. Molecular genetics of herpes simplex virus. VII. Characterization of a temperature-sensitive mutant produced by in vitro mutagenesis and defective in DNA synthesis and accumulation of γ polypeptides. J. Virol. **37**:191–206.
9. **Crute, J. J., and I. R. Lehman.** 1989. Herpes simplex-1 DNA polymerase. Identification of an intrinsic 5′-3′ exonuclease with ribonuclease H activity. J. Biol. Chem. **264**:19266–19270.
10. **Crute, J. J., T. Tsurumi, L. Zhu, S. K. Weller, P. D. Olivo, M. D. Challberg, E. S. Mocarski, and I. R. Lehman.** 1989. Herpes simplex virus 1 helicase-primase: a complex of three herpes-encoded gene products. Proc. Natl. Acad. Sci. USA **86**:2186–2189.
11. **de Bruyn Kops, A., and D. M. Knipe.** 1988. Formation of DNA replication structures in herpes virus-infected cells requires a viral DNA binding protein. Cell **55**:857–868.
12. **Digard, P., C. S. Chow, L. Pirrit, and D. M. Coen.** 1993. Functional analysis of the herpes simplex virus UL42 protein. J. Virol. **67**:1159–1168.
13. **Digard, P., and D. M. Coen.** 1990. A novel functional domain of an alpha-like DNA polymerase. The binding site on the herpes simplex virus polymerase for the viral UL42 protein. J. Biol. Chem. **265**:17393–17396.
14. **Dodson, M. S., and I. R. Lehman.** 1991. Association of DNA helicase and primase activities with a subassembly of the herpes simplex virus 1 helicase-primase composed of the UL5 and UL52 gene products. Proc. Natl. Acad. Sci. USA **88**:1105–1109.
15. **Dorsky, D. I., and C. S. Crumpacker.** 1988. Expression of herpes simplex virus type 1 DNA polymerase gene by in vitro translation and effects of gene deletions on activity. J. Virol. **62**:3224–3232.
16. **Elias, P., M. E. O'Donnell, E. S. Mocarski, and I. R. Lehman.** 1986. A DNA binding protein specific for an origin of replication of herpes simplex virus type 1. Proc. Natl. Acad. Sci. USA **83**:6322–6326.
17. **Fierer, D. S., and M. D. Challberg.** 1992. Purification and characterization of UL9, the herpes simplex virus type 1 origin-binding protein. J. Virol. **66**:3986–3995.
18. **Gallo, M. L., D. I. Dorsky, C. S. Crumpacker, and D. S. Parris.** 1989. The essential 65-kilodalton DNA-binding protein of herpes simplex virus stimulates the virus-encoded DNA polymerase. J. Virol. **63**:5023–5029.
19. **Gallo, M. L., D. H. Jackwood, M. Murphy, H. S. Marsden, and D. S. Parris.** 1988. Purification of the herpes simplex virus type 1 65-kilodalton DNA-binding protein: properties of the protein and evidence of its association with the virus-encoded DNA polymerase. J. Virol. **62**:2874–2883.
19a. **Gallo, M. L., and D. S. Parris.** Unpublished observations.
20. **Garnier, J., D. J. Osguthorpe, and B. Robson.** 1978. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. J. Mol. Biol. **120**:97–120.
21. **Gibbs, J. S., K. Weisshart, P. Digard, A. deBruyn Kops, D. M. Knipe, and D. M. Coen.** 1991. Polymerization activity of an alpha-like DNA polymerase requires a conserved 3′-5′ exonuclease active site. Mol. Cell. Biol. **11**:4786–4795.
22. **Goodrich, L. D., F. J. Rixon, and D. S. Parris.** 1989. Kinetics of expression of the gene encoding the 65-kilodalton DNA-binding protein of herpes simplex virus type 1. J. Virol. **63**:137–147.
23. **Goodrich, L. D., P. A. Schaffer, D. I. Dorsky, C. S. Crumpacker, and D. S. Parris.** 1990. Localization of the herpes simplex virus type 1 65-kilodalton DNA-binding protein and DNA polymerase in the presence and absence of viral DNA synthesis. J. Virol. **64**:5738–5749.
24. **Gottlieb, J., A. I. Marcy, D. M. Coen, and M. D. Challberg.** 1990. The herpes simplex virus type 1 UL42 gene product: a subunit of DNA polymerase that functions to increase processivity. J. Virol. **64**:5976–5987.
25. **Haffey, M. L., J. T. Stevens, B. J. Terry, D. I. Dorsky, C. S. Crumpacker, S. M. Wietstock, W. T. Ruyechan, and A. K. Field.** 1988. Expression of herpes simplex virus type 1 DNA polymerase in *Saccharomyces cerevisiae* and detection of virus-specific enzyme activity in cell-free lysates. J. Virol. **62**:4493–4498.
26. **Hernandez, T. R., and I. R. Lehman.** 1990. Functional interaction between the herpes simplex-1 DNA polymerase and UL42 protein. J. Biol. Chem. **265**:11227–11232.
27. **Jofre, J. T., P. A. Schaffer, and D. S. Parris.** 1977. Genetics of resistance to phosphonoacetic acid in strain KOS of herpes simplex virus type 1. J. Virol. **23**:833–836.
28. **Johnson, P. A., M. G. Best, T. Friedmann, and D. S. Parris.** 1991. Isolation of a herpes simplex virus type 1 mutant deleted for the essential UL42 gene and characterization of its null phenotype. J. Virol. **65**:700–710.
29. **Marchetti, M. M., C. A. Smith, and P. A. Schaffer.** 1988. A temperature-sensitive mutation in a herpes simplex virus type 1 gene required for viral DNA synthesis maps to coordinates 0.609 through 0.614 in U_L. J. Virol. **62**:715–721.
30. **Marcy, A. I., P. D. Olivo, M. D. Challberg, and D. M. Coen.** 1990. Enzymatic activities of overexpressed herpes simplex virus DNA polymerase purified from recombinant baculovirus-infected insect cells. Nucleic Acids Res. **18**:1207–1215.
31. **Marcy, A. I., D. R. Yager, and D. M. Coen.** 1990. Isolation and characterization of herpes simplex virus mutants containing engineered mutations at the DNA polymerase locus. J. Virol. **64**:2208–2216.
32. **Marians, K. J.** 1992. Prokaryotic DNA replication. Annu. Rev. Biochem. **61**:673–719.
33. **Marsden, H. S., M. E. M. Campbell, L. Haarr, M. C. Frame, D. S. Parris, M. Murphy, R. G. Hope, M. T. Muller, and C. M. Preston.** 1987. The 65,000-M_r DNA-binding and virion *trans*-inducing proteins of herpes simplex virus type 1. J. Virol. **61**:2428–2437.
34. **McGeoch, D. J., M. A. Dalrymple, A. J. Davison, A. Dolan, M. C. Frame, D. McNab, L. J. Perry, J. E. Scott, and P. Taylor.** 1988. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. **69**:1531–1574.
35. **Murphy, M., P. Schenk, H. M. Lankinen, A. M. Cross, P. Taylor, A. Owsianka, R. G. Hope, H. Ludwig, and H. S. Marsden.** 1989. Mapping the epitopes on the 65K DNA-binding protein of herpes simplex virus type 1. J. Gen. Virol. **70**:2357–2364.
36. **O'Donnell, M. E., P. Elias, B. E. Funnell, and I. R. Lehman.** 1987. Interaction between the DNA polymerase and single-stranded DNA-binding protein (infected cell protein 8) of herpes simplex virus 1. J. Biol. Chem. **262**:4260–4266.
37. **Olivo, P. D., N. J. Nelson, and M. D. Challberg.** 1988. Herpes simplex virus DNA replication: the UL9 gene encodes an origin binding protein. Proc. Natl. Acad. Sci. USA **85**:5414–5418.
38. **Owsianka, A. M., G. Hart, M. Murphy, J. Gottlieb, R. Boehme, M. Challberg, and H. S. Marsden.** 1993. Inhibition of herpes simplex virus type 1 DNA polymerase activity by peptides from the UL42 accessory protein is largely nonspecific. J. Virol. **67**:258–264.
39. **Pabo, C. O., and R. T. Sauer.** 1992. Transcription factors: structural families and principles of DNA recognition. Annu. Rev. Biochem. **61**:1053–1095.
40. **Pakula, A. A., and R. T. Sauer.** 1989. Genetic analysis of protein stability and function. Annu. Rev. Biochem. **23**:289–310.
41. **Parris, D. S., A. Cross, L. Haarr, A. Orr, M. C. Frame, M. Murphy, D. J. McGeoch, and H. S. Marsden.** 1988. Identification of the gene encoding the 65-kilodalton DNA-binding protein of herpes simplex virus type 1. J. Virol. **62**:818–825.
42. **Purifoy, D. J. M., R. B. Lewis, and K. L. Powell.** 1977. Identification of the herpes simplex virus DNA polymerase gene. Nature (London) **269**:621–623.

43. **Quinlan, M. P., L. B. Chen, and D. M. Knipe.** 1984. The intranuclear location of a herpes simplex virus DNA-binding protein is determined by the status of viral DNA replication. Cell **36**:857–868.
44. **Roizman, B., and A. E. Sears.** 1990. Herpes simplex viruses and their replication, p. 1795–1841. *In* B. N. Fields and D. M. Knipe (ed.), Virology. Raven Press, New York.
45. **Ruyechan, W. T., and A. C. Weir.** 1984. Interaction with nucleic acids and stimulation of the viral DNA polymerase by the herpes simplex virus type 1 major DNA-binding protein. J. Virol. **52**:727–737.
46. **Schaffer, P. A., E. K. Wagner, G. B. Devi-Rao, and V. G. Preston.** 1987. Herpes simplex virus, p. 93–98. *In* S. J. O'Brien (ed.), Genetic maps, vol. 4. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
47. **Seo, Y. H., S. Kobs-Conrad, and P. T. P. Kaumaya.** 1993. Promiscuous T cell epitopes of tetanus toxoid and measles virus enhances immune response to B cell epitopes of the protein antigen LDH-C_4, p. 866–868. *In* C. H. Schneider and A. N. Eberle (ed.), Peptides 1992. Escom Science Publishers, Leiden, the Netherlands.
48. **Sherman, G., J. Gottlieb, and M. D. Challberg.** 1992. The UL8 subunit of the herpes simplex virus helicase-primase complex is required for efficient primer utilization. J. Virol. **66**:4884–4892.
49. **Tenney, D. J., P. A. Micheletti, J. T. Stevens, R. K. Hamatake, J. T. Matthews, A. R. Sanchez, W. W. Hurlburt, M. Bifano, and M. G. Cordingley.** 1993. Mutations in the C terminus of herpes simplex virus type 1 DNA polymerase can affect binding and stimulation by its accessory protein UL42 without affecting basal polymerase activity. J. Virol. **67**:543–547.
50. **Vaughan, P. J., M. Banks, D. J. M. Purifoy, and K. L. Powell.** 1984. Interactions between herpes simplex virus DNA-binding proteins. J. Gen. Virol. **65**:2033–2041.
51. **Vaughan, P. J., D. J. M. Purifoy, and K. L. Powell.** 1985. DNA-binding protein associated with herpes simplex virus DNA polymerase. J. Virol. **53**:501–508.
52. **Weller, S. K., K. J. Lee, D. J. Sabourin, and P. A. Schaffer.** 1983. Genetic analysis of temperature-sensitive mutants which define the gene for the major herpes simplex virus type 1 DNA-binding protein. J. Virol. **45**:354–366.
53. **Wu, C. A., N. J. Nelson, D. J. McGeoch, and M. D. Challberg.** 1988. Identification of herpes simplex virus type 1 genes required for origin-dependent DNA synthesis. J. Virol. **62**:435–443.
54. **Yager, D. R., A. I. Marcy, and D. M. Coen.** 1990. Translational regulation of herpes simplex virus DNA polymerase. J. Virol. **64**:2217–2225.
55. **Zhu, L., and S. K. Weller.** 1992. The UL5 gene of herpes simplex virus type 1: isolation of a *lacZ* insertion mutant and association of the UL5 gene product with other members of the helicase-primase complex. J. Virol. **66**:458–468.
56. **Zhu, L., and S. K. Weller.** 1992. The six conserved helicase motifs of the UL5 gene product, a component of the herpes simplex virus type 1 helicase-primase, are essential for its function. J. Virol. **66**:469–479.