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Herpes Simplex Virus Type 1 Gene Products Required for DNA Replication: Identification and Overexpression

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Seven herpes simplex virus (HSV) genes have been shown recently to be necessary and sufficient to support the replication of origin-containing plasmids. Two of these genes (*pol* and *dbp*) encode well-known DNA replication proteins (the DNA polymerase and the major single-stranded DNA binding protein), and a third gene (*UL42*) encodes a previously identified infected-cell protein which binds tightly to double-stranded DNA. The products of the four remaining genes have not previously been identified. Using the predicted amino acid sequence data (D. J. McGeoch, M. A. Dalrymple, A. Dolan, D. McNab, L. J. Perry, P. Taylor, and M. D. Challberg, J. Virol. 62:444–453; D. J. McGeoch and J. P. Quinn, Nucleic Acids Res. 13:8143–8163), we have raised rabbit antisera against the products of all seven genes. We report here the use of these reagents to identify these proteins in infected cells. All seven proteins localized to the nucleus and were expressed in a manner consistent with the idea that they are the products of early genes. Various immunological assays suggest that four of these proteins (UL5, UL8, UL9, and UL52) are made in infected cells in very low abundance relative to the other three. To improve our ability to study these proteins, we have expressed UL5, UL8, UL9, and UL52 in insect cells by using the baculovirus expression system. The HSV proteins made in insect cells were immunoprecipitable with the appropriate antisera, and the size of each protein was indistinguishable from the size of the corresponding protein made in HSV-infected Vero cells. Our data offer strong support for the accuracy of open reading frames proposed by McGeoch et al. In addition, the antisera and the overproduced HSV replication proteins should be useful reagents with which to analyze the biochemistry of HSV DNA replication.

A number of features of herpes simplex virus (HSV) make it a useful model system for the study of eucaryotic DNA replication (37). The HSV genome is a 153-kilobase linear double-stranded DNA (dsDNA) molecule which contains three *cis*-acting origins of replication (25, 40, 41, 44, and 47) and at least 72 open reading frames (23). Approximately 50 polypeptides specific to HSV-infected cells have been found, and genetic and biochemical studies have implicated a number of these proteins in viral DNA metabolism and replication (3, 7, 14, 21, 22, 30). These include a thymidine kinase (16), a ribonucleotide reductase (6, 33), an alkaline exonuclease (2, 10, 26), a single-stranded DNA (ssDNA)-binding protein (ICP8) (31, 34, 46), a DNA polymerase (1, 10, 11, 32), an origin-binding protein (8, 28), and, recently, a primase (13) and a helicase (J. Crute and I. R. Lehman, personal communication). It is likely, in fact, that most, if not all, of the proteins involved in HSV DNA replication per se are virally encoded.

Consistent with this idea, recent genetic evidence has shown that seven HSV genes are necessary and sufficient for the replication of HSV origin-containing plasmids (4, 5, 9, 19, 24, 48; S. K. Weller, personal communication). Two of these genes, *dbp* and *pol*, encode proteins which have been studied extensively: ICP8 (a ssDNA-binding protein) and the HSV DNA polymerase (Pol), respectively. A third gene, *UL42*, contains a 51-kilodalton (kDa) open reading frame and encodes a previously identified HSV-2-infected-cell protein (ICSP34/35) with an apparent molecular size of 62 to 65 kDa (29, 34). This protein binds tightly to dsDNA (20; C. A. Wu and M. D. Challberg, unpublished results) and has been reported to copurify with the HSV DNA polymerase (29, 43). The products of the remaining four genes, *UL5*, *UL8*, *UL9*, and *UL52*, have not yet been identified.

As a first step toward a biochemical characterization of HSV DNA replication, we set out to identify the products of all of the genes that are essential for DNA replication. Toward this goal, we raised rabbit antisera against the product of each of the seven genes. The immunogens we used were either synthetic peptides or fusion proteins expressed in *Escherichia coli*. These antisera were then used to analyze the expression of these seven proteins during viral infection. Our antibody reagents directed against previously identified HSV proteins (Pol, ICP8, and UL42) provided us with a useful frame of reference during our analysis of the other four gene products.

We report here the identification of the products of *UL5*, *UL8*, *UL9*, and *UL52* in HSV-infected cells. The apparent molecular weight of each polypeptide is close to the predicted molecular weight (24). As expected for proteins involved in viral DNA synthesis, these proteins all localized to the nucleus, and they were expressed in a manner consistent with that of the products of delayed-early genes. In addition, our data suggest that these four proteins are made in very small amounts relative to the other three proteins (ICP8, Pol, and UL42).

Primarily to overcome problems related to their low abundance, we have expressed *UL5*, *UL8*, *UL9*, and *UL52* in insect cells by using recombinant baculoviruses. The proteins made in insect cells are also approximately of the predicted size and in fact are indistinguishable in size from the corresponding proteins found in HSV-infected Vero cells. Our results, therefore, support the interpretation of the

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HSV DNA sequence proposed by McGeoch et al. (24). In addition, our antibody reagents and recombinant baculoviruses should facilitate further biochemical studies of HSV DNA replication.

MATERIALS AND METHODS

Cells and virus. African green monkey kidney (Vero) cells were propagated in Eagle medium containing 10% (vol/vol) fetal calf serum. The KOS strain of HSV-1 was used, and virus stocks were grown and assayed in Vero cells as described previously (5).

Fusion proteins. Plasmids, each containing one of the seven HSV-1 replication genes (48), were used to obtain restriction fragments that correspond to a portion of the open reading frame of each gene as deduced from the sequence data of McGeoch et al. (24, 36). These restriction fragments were then ligated in frame with the *lacZ* reading frame in an *E. coli* expression vector containing the *lac* operon (pMLB vector series; kindly provided by M. Berman, Bionetics, Inc., Gaithersburg, Md.). The restriction sites used and the region of each open reading frame expressed are shown in Fig. 1. The fusion proteins were generated and analyzed by standard methods (18, 45) and were purified by affinity chromatography by using a monoclonal antibody against β-galactosidase (Promega Biotec, Madison, Wis.).

Peptides. Carboxy or amino octa- or decapeptides were made commercially (Biosearch, San Rafael, Calif.). The locations of the peptides used are shown in Fig. 1. The sequences of the peptides made were as follows: UL5 carboxy terminus, CRDPNVVIVY; UL8 amino terminus, MDTADIVC; UL8 carboxy terminus, CDDKMSFLFA; UL9 carboxy terminus, CQGAVNFSTL; UL42 amino terminus, MTDSPGGVAC; UL42 carboxy terminus, CQTPYG FGFP; and UL52 carboxy terminus, CSTSQPSS. Following purification by gel filtration chromatography, the peptides were coupled to keyhole limpet hemacyanin by standard techniques.

Antisera. New Zealand rabbits were immunized subcutaneously by a standard protocol using 20 to 50 μg of purified fusion protein or 0.5 to 1.0 mg of keyhole limpet hemacyanin-coupled peptide and boosted at least twice at biweekly intervals.

Immunoprecipitation. Cells were infected with 10 to 20 PFU per cell by adsorption for 1 h. At various times after infection, the medium was removed and fresh medium containing [^{35}S]methionine (0.1 mM; specific activity, ~1,000 Ci/mmol; 0.5 mCi/ml; Amersham Corp., Arlington Heights, Ill.) was added, and the cells were incubated for 2 h. After labeling, the cells were disrupted in 20 mM Tris hydrochloride, pH 7.4–150 mM NaCl–0.5% sodium deoxycholate–1% Triton X-100–0.1% sodium dodecyl sulfate (SDS)–1 mM phenylmethylsulfonyl fluoride, and the extract was clarified by centrifugation at ~10,000 × *g*. Immunoprecipitation was performed as previously described (45), and in all cases, the extracts were immunoprecipitated with preimmune sera prior to the use of immune sera. Immunoprecipitated proteins were submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (17). ^{14}C-labeled marker proteins (myosin, phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, and lysozyme) were purchased from Amersham.

Immunofluorescence. Immunofluorescence was performed as previously described (35) by using fluorescein-conjugated goat anti-rabbit immunoglobulin antiserum (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.). Antisera were preabsorbed with methanol-acetone (50:50)-fixed uninfected Vero cells for 2 to 4 h at ambient temperature prior to use in immunofluorescence studies.

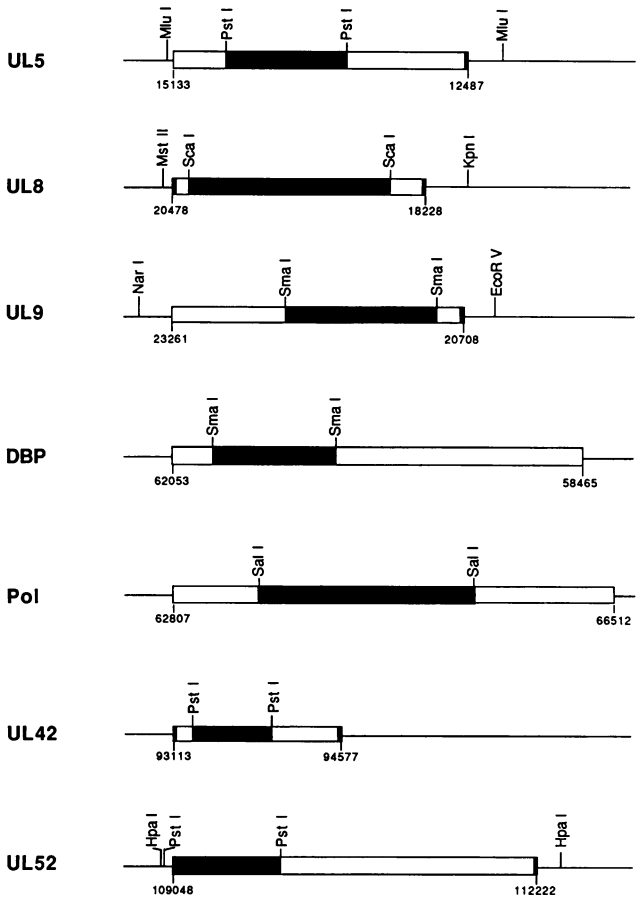

FIG. 1. Schematic representation of the seven essential HSV-1 DNA replication genes. The genes are listed from top to bottom as they occur on the genome from left to right (23). DBP refers to *UL29*, the gene encoding the major ssDNA-binding protein. Pol refers to *UL30*, the gene encoding the DNA polymerase. Each bar indicates the region of the complete open reading frame of each unspliced gene displayed from amino-terminal codon to carboxy-terminal codon. The first residue of the translation initiation codon is given beneath the amino terminus, and the first residue of the stop codon is given beneath the carboxy terminus using the numbering system previously described (23). The solid area of each bar indicates the portion of each open reading frame used to generate fusion proteins as described in Materials and Methods. The restriction sites used to isolate the DNA fragments used to make the fusion protein expression vectors are shown above the limits of the solid bars (note that in the case of UL52, one of the *PstI* sites used in making the fusion protein is upstream of the putative start codon). Also indicated are the restriction sites flanking the open reading frames of *UL5*, *UL8*, *UL9*, and *UL52* that were used to generate the vectors for making recombinant baculoviruses as described in Materials and Methods. The narrow bars at the amino and/or carboxy termini of *UL5*, *UL8*, *UL9*, *UL42*, and *UL52* indicate the terminal peptides used to generate antipeptide antisera as described in Materials and Methods, where the sequence of each peptide can be found.

Construction of recombinant baculoviruses. Recombinant and wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV) stocks were propagated in *Spodoptera frug-*

iperda cells (Sf9) as described by Summers and colleagues (38, 39, 42). Restriction fragments containing the entire open reading frame of *UL5*, *UL8*, *UL9*, or *UL52* were ligated into the *Bam*HI site of the plasmid pAc373 by either blunt-end ligation or by using *Bam*HI linkers. The restriction sites flanking each open reading frame which were used in the construction of each recombinant plasmid are shown in Fig. 1. Recombinant baculoviruses (AcNPV/UL5, AcNPV/UL8, AcNPV/UL9, and AcNPV/UL52) were generated as previously described (42).

RESULTS

As mentioned above, the products of three of the genes required for DNA replication (ICP8, Pol, and UL42) are well-known infected-cell proteins; the products of the other four genes (*UL5*, *UL8*, *UL9*, and *UL52*) have not hitherto been identified. To identify the products of these genes present in HSV-infected cells, we raised polyclonal rabbit antisera against portions of the open reading frames predicted from the DNA sequences of all seven genes. We anticipated that the interpretation of our results regarding the unidentified proteins would be strengthened by using the same methodology with the three known proteins. The immunogens used in this analysis were either fusion proteins expressed in *E. coli* or synthetic peptides. Figure 1 shows the regions of the open reading frames which were expressed as fusion proteins and the locations of the carboxy- and/or amino-terminal peptides against which antisera were raised.

Immunoassay of infected-cell proteins. The complete set of antisera was used to test for the presence of proteins unique to HSV-1-infected Vero cells by immunoprecipitation of [^{35}S]methionine-labeled proteins and immunoblot analysis. The results of the immunoprecipitation analysis are shown in Fig. 2 and, along with the results of immunoblot experiments, are described in detail below. In summary, one or more antisera against each of the seven gene products reacted with an infected-cell-specific protein whose size was close to that predicted from DNA sequence analysis (24, 36).

Antiserum against the polymerase fusion protein immunoprecipitated an HSV-infected cell-specific protein of 140 kDa (Fig. 2A, lane 2). This antiserum also detected a 140-kDa polypeptide on immunoblots of infected-cell extracts, and this immunoreactivity cochromatographed with HSV DNA polymerase activity during purification (27; data not shown). An abundant infected-cell protein of 130 kDa was immunoprecipitated by antiserum against the ICP8 fusion protein (Fig. 2A, lane 4). (The multiple smaller polypeptides specific to infected cells which can be seen in lane 4 are likely degradation products of ICP8 on the basis of other data not shown). A 130-kDa protein was also detected by immunoblot analysis, and again, the antiserum reacted with purified ICP8 (27; data not shown). Antiserum against the carboxy-terminal peptide of UL42 immunoprecipitated an abundant 62-kDa polypeptide from infected cells (Fig. 2A, lane 14). An HSV protein of the same size was also seen on immunoblots using antisera made against a UL42 amino-terminal peptide and the UL42 fusion protein (data not shown), suggesting that the 62-kDa polypeptide represents the undegraded product of this gene. This 62-kDa protein was purified to homogeneity, and the purified protein was shown to bind tightly, but nonspecifically, to dsDNA (C. A. Wu and M. D. Challberg, unpublished results), in agreement with reported properties of the product of the *UL42* gene (20, 29). Thus, our results using antisera directed against these three previously identified HSV proteins are in agreement with previously reported results regarding their molecular weights, and our results offer independent confirmation of the accuracy of the reported DNA sequence interpretation (24, 36).

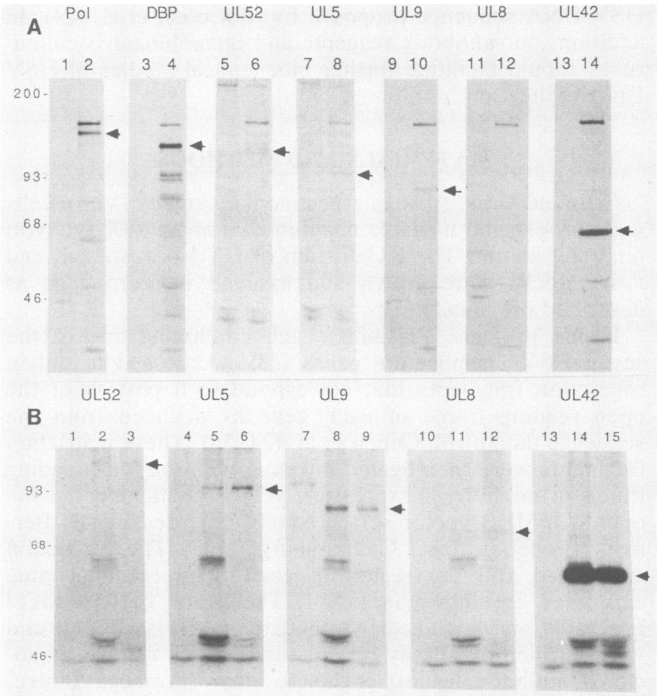

FIG. 2. Immunoprecipitation of the HSV replication proteins from infected cells. The HSV-infected-cell-specific protein immunoprecipitated by each serum is indicated by an arrow. Antipolymerase and anti-DBP sera were made against fusion proteins; the other antisera used were against carboxy-terminal peptides (see text for details). The numbers at the left indicate the positions and sizes (in kilodaltons) of ^{14}C-labeled marker proteins run on the same gel. (A) Antibodies to the indicated gene products were reacted with extracts of mock-infected (lanes 1, 3, 5, 7, 9, 11, and 13) or HSV-infected (lanes 2, 4, 6, 8, 10, 12, and 14) Vero cells which had been labeled with [^{35}S]methionine from 8 to 10 h after infection. (B) Antisera to the indicated gene products were reacted with [^{35}S]methionine-labeled extracts made from mock-infected (lanes 1, 4, 7, 10, and 13), HSV-infected (lanes 2, 5, 8, 11, and 14), or phosphonoacetic acid (100 mg/ml)-treated HSV-infected (lanes 3, 6, 9, 12, and 15) Vero cells.

We were also able to identify an infected-cell-specific protein with our antisera against the four previously unidentified gene products. The UL5 carboxy-terminal peptide antiserum immunoprecipitated a 95-kDa HSV-infected-cell-specific protein (Fig. 2A, lane 8). A 95-kDa protein was also seen on immunoblots using this same antiserum as well as antiserum against the UL5 fusion protein (27; data not shown). Antiserum against a UL9 carboxy-terminal peptide immunoprecipitated an 82-kDa polypeptide from HSV-infected cells (Fig. 2A, lane 10). A polypeptide of the same mobility was seen on immunoblots using both the antipeptide antiserum and antiserum raised against the UL9 fusion protein (27; data not shown). A barely detectable band migrating at 115 kDa was seen when infected-cell extracts were immunoprecipitated with an anti-UL52 carboxy-terminal peptide antiserum (Fig. 2A, lane 6). Finally, antiserum against the UL8 carboxy-terminal peptide immunoprecipitated a 75-kDa polypeptide from infected-cell extracts. This protein was also of very low abundance and was not seen on some autoradiographs (e.g., Fig. 2A) but was seen clearly on others (Fig. 2B, lanes 11 and 12, and Fig. 5, lane 2). All of

TABLE 1. Summary of the HSV proteins required for DNA replication

Gene[a]	Predicted mol wt[b]	Observed mol wt[c]	Relative abundance	Activity	Reference
UL5	99	95	++		24, 48, this work
UL8	80	75	+		24, 48, this work
UL9	94	82	++	Binds origin	8, 24, 28, 48, this work
dbp	130	130	++++	Binds ssDNA	31, 36, this work
pol	136	140	+++	DNA polymerase	32, 36, this work
UL42	51	62	+++++	Binds dsDNA	20, 24, 29, 48, this work
UL52	114	115	+		24, 48, this work

[a] Genes are listed in order of occurrence on the genome from left to right (see reference 23 for map coordinates).
[b] Size in kilodaltons as predicted by the nucleotide sequence of the open reading frame (23, 24, 36).
[c] Size in kilodaltons as determined by mobility in SDS-polyacrylamide gels.

our antisera against UL8 and UL52 were unable to detect HSV-infected-cell-specific proteins when tested by immunoblot analysis, despite the fact that these sera did detect the appropriate protein on immunoblots when UL8 and UL9 were overexpressed in insect cells (see below). We suspect that the failure to detect the UL8 and UL9 proteins by immunoblot of HSV-infected cells is a reflection of low abundance of the proteins, although other explanations are possible.

Overall our immunoprecipitation and immunoblot data using antisera made against immunogens derived from the DNA sequence information of McGeoch et al. confirm the open reading frame interpretation published for each of these genes (24, 36). In addition, our results suggest that these proteins are not subject to extensive posttranslational processing. These results are summarized in Table 1.

The kinetics of synthesis of these proteins was examined by pulse-labeling infected cells with [^{35}S]methionine for 2 h at various times following infection with HSV. Extracts of labeled proteins were then subjected to immunoprecipitation with the appropriate antisera. Polymerase, UL42, and UL9 were first detected at 2 to 4 h after infection; their synthesis peaked between 6 and 10 h after infection and declined thereafter (Fig. 3). Immunoblot analysis of infected-cell extracts made at various times after infection showed that the amounts of these proteins increased until about 12 h after infection and did not appreciably decline over the next 12 h (25). These data suggest that these proteins are not rapidly turned over. All seven proteins exhibited roughly the same kinetics of appearance, but it was difficult to demonstrate this reproducibly for UL8 and UL52 owing to their apparently extremely low levels of synthesis. The effect of inhibition of viral DNA replication on the rates of synthesis of these proteins was examined by using the drug phosphonacetic acid. This drug specifically blocks viral DNA synthesis, which in turn results in greatly diminished levels of expression of late genes, but not early genes or immediate-early genes (15). The data in Fig. 2B show that the rates of synthesis of the products of *UL5*, *UL8*, *UL9*, *UL42*, and *UL52* were not appreciably altered in the presence of phosphonacetic acid at a level which demonstrably blocked late-protein synthesis (data not shown). These data are consistent with the view that the genes *UL5*, *UL8*, *UL9*, *UL42*, and *UL52*, like the genes encoding ICP8 and polymerase (12, 15), are all delayed-early genes, as expected for

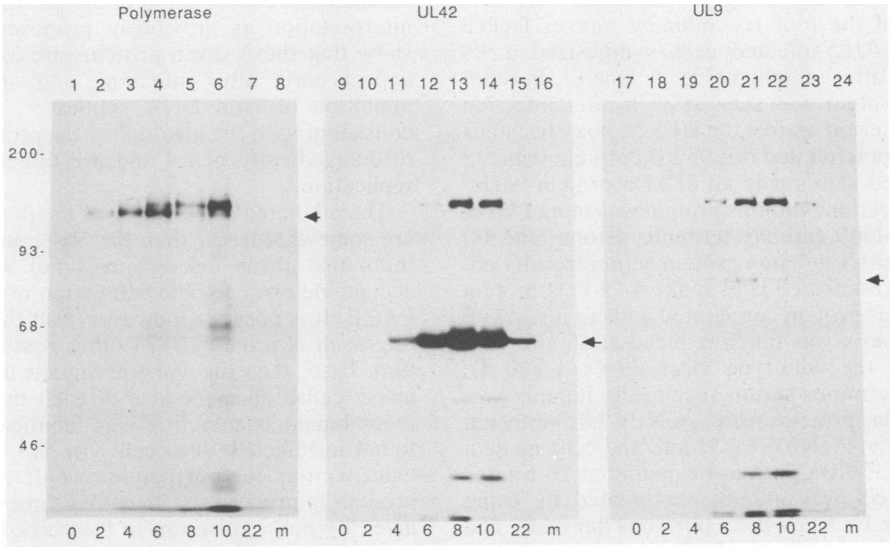

FIG. 3. Immunoprecipitation of HSV replication gene products from [^{35}S]methionine-labeled extracts made at various times after infection. The antisera were the same as those used in Fig. 2. The numbers at the left indicate the positions and sizes (in kilodaltons) of ^{14}C-labeled marker proteins (lanes m) run on the same gel. The numbers below indicate the time (in hours) after infection at which the 2-h labeling was begun. The extracts were made immediately following the labeling period. Arrows indicate the specifically immunoprecipitated bands. In lanes 8, 16, and 24, mock-infected extracts were used.

genes encoding proteins that are involved directly in viral DNA replication.

Immunofluorescence analysis. The intracellular localization of the seven proteins was examined by indirect immunofluorescence analysis of Vero cells at various times after infection with HSV (Fig. 4). Several of the antisera displayed cytoplasmic staining at time zero, but the different antisera against the seven replication proteins showed distinct nuclear staining beginning at 3 to 6 h postinfection. The intensity of fluorescence observed with each antiserum correlated well with the abundance of the corresponding protein noted by immunoblot and immunoprecipitation analyses. Two basic staining patterns were seen: intense globular nuclear staining with sparing of the nucleolus (Pol, ICP8, UL9, and UL42) and faint diffuse nuclear staining (UL5, UL8, and UL52). We were unable to do colocalization experiments since all our antisera were made in rabbits. We conclude that the predominant site of localization of all seven proteins is the nucleus.

Expression of *UL5*, *UL8*, *UL9*, and *UL52* in insect cells. All the results presented above are consistent with the view that the products of the *UL5*, *UL8*, *UL9*, and *UL52* genes are present in infected cells at very low levels. Owing to these low levels, our initial attempts at purification of these proteins from extracts of infected cells were very much hampered despite the use of large numbers of infected cells. To increase the amounts of these proteins available to us for biochemical studies, we have overexpressed each of these four genes in insect cells by using the baculovirus expression system. The restriction sites that were used to construct the vectors for making recombinant baculoviruses expressing *UL5*, *UL8*, *UL9*, and *UL52* are shown in Fig. 1. The sizes of the nontranslated leader sequences for the genes differed considerably and were dictated by the available restriction enzyme sites.

Figure 5 shows an SDS-PAGE analysis of the [^{35}S]methionine-labeled proteins made in insect cells at various times after infection with wild-type AcNPV or one of the recombinant baculoviruses. Wild-type AcNPV-infected cells synthesized large amounts of the 30-kDa polyhedrin protein beginning 36 h after infection, and as expected (38), cells infected with any of the four recombinant viruses lacked polyhedrin. AcNPV/UL5-infected cells synthesized a 95-kDa protein at 48 h after infection (Fig. 5, lane 8). In other experiments, this protein was seen at 36 h after infection with this virus. Antiserum against the UL5 carboxy terminus specifically immunoprecipitated this 95-kDa protein (lane 9). AcNPV/UL9-infected cells made an 82-kDa protein beginning at 36 h after infection, and this protein was immunoprecipitated by an anti-UL9 carboxy terminus serum (lane 14) as well as by the anti-UL9 fusion protein serum (results not shown). AcNPV/UL8-infected cells make a 75-kDa protein (lanes 17 and 18). This protein comigrated with an insect cell protein whose synthesis was much reduced at 36 and 48 h after infection with the wild-type virus (lanes 3 and 4). Anti-UL8 carboxy terminus serum specifically immunoprecipitated the 75-kDa protein from AcNPV/UL8-infected cells (lane 19). Finally, AcNPV/UL52-infected cells made a small amount of a 115-kDa protein beginning at 36 h after infection. This protein was immunoprecipitated by using antiserum against the UL52 carboxy terminus (lane 24). The apparent molecular weight of each HSV protein produced by insect cells is virtually identical to that of the corresponding protein immunoprecipitated from HSV-infected Vero cells (Fig. 6).

The levels of expression of these four HSV genes in insect cells varied widely despite the fact that they are all presumably expressed from the same polyhedrin promoter; the factors which lead to these different levels are not yet clear. There does not appear to be a correlation between the level of protein made in insect cells and the size of the HSV sequence in the mRNA leader, although this was not tested systematically. There is also no correlation between the amount of each protein made in HSV-infected Vero cells and the amount made in recombinant baculovirus-infected insect cells. We did test the stabilities of these four proteins in insect cells by pulse-chase experiments (data not shown). UL8 and UL9, which displayed the highest levels of expression, were found to have half-lives of at least 8 h. UL5 had a half-life of between 2 and 8 h. Finally, UL52, which showed very low levels of expression, was very unstable, with a half-life of less than 2 h. Thus, it appears that the levels of expression of these proteins in insect cells are at least partially related to protein stability. It is unclear, however, whether this represents an intrinsic property of each protein of a phenomenon unique to its occurrence in insect cells. We were unable to do pulse-chase experiments in HSV-infected Vero cells due to the low levels of incorporation of label into these four proteins.

DISCUSSION

In this report, we describe the characterization of a complete set of antisera against the products of the seven HSV genes that have been shown previously to be required for the replication of HSV DNA (48). All of our antisera were raised against reagents (fusion proteins or peptides) which were made on the basis of DNA sequence data. Our antisera against the three known proteins (Pol, ICP8, and UL42) are specific for the appropriate infected-cell proteins and have provided a useful reference point from which to evaluate results obtained using antisera against the other four gene products. We have identified the products of *UL5*, *UL8*, *UL9*, and *UL52* in infected cells and have shown that the apparent molecular weight of each protein is similar to the predicted molecular weight. These data thus provide independent confirmation of the accuracy of DNA sequence interpretation as previously proposed (24, 36). Our data show that these seven proteins are located in the nucleus, appear early after infection, and are expressed despite inhibition of viral DNA synthesis. These results are all consistent with the idea that these proteins are the products of delayed-early genes and are directly involved in DNA replication.

The predicted sizes of two of the proteins (UL8 and UL9) are somewhat larger than the observed sizes (Table 1). We think that these discrepancies fall within the bounds of acceptable error for the estimation of protein size by SDS-PAGE. It is possible, however, that these discrepancies are the result of proteolysis or other posttranslational modification. If so, then the same or similar modifications occur in insect cells, since the size of each protein expressed by a recombinant baculovirus was identical to the size of that found in infected Vero cells. In one case (UL8), we have evidence that the polypeptide we detect is unlikely to be a product of proteolysis, since the same-size protein is recognized by both antiserum to the carboxy terminus and antiserum to the amino terminus. In the case of UL9, we know that the protein has an intact carboxy terminus, since it is recognized by antiserum made against a carboxy-terminal peptide; we do not yet have available anti-amino-terminal peptide serum. We have shown elsewhere that the 82-kDa

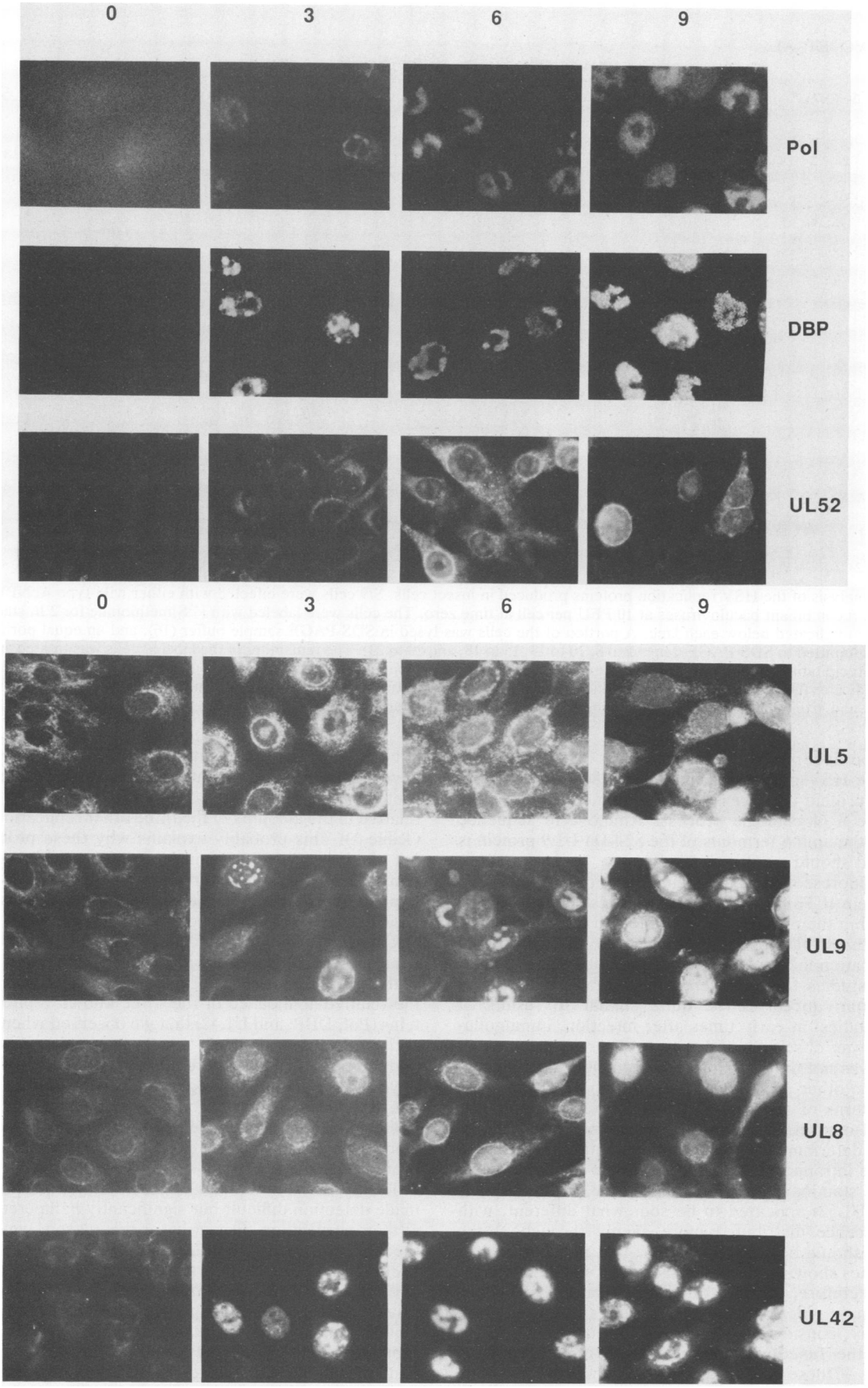

FIG. 4. Immunofluorescence of HSV-infected cells at various times after infection by using antisera against the indicated HSV proteins. Above the panels, the time after infection (in hours) is indicated. On the right of each row, the specifity of each antiserum is indicated. The antisera were the same as those used in Fig. 2 (see text for details).

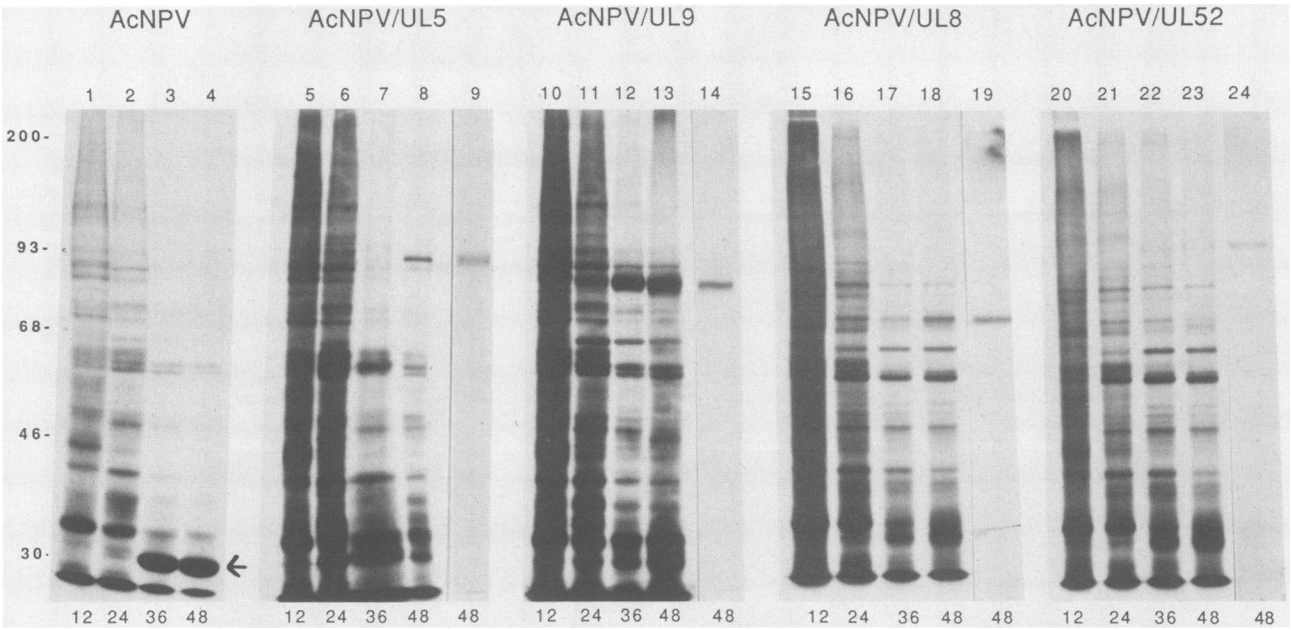

FIG. 5. Analysis of the HSV replication proteins produced in insect cells. Sf9 cells were infected with either wild-type AcNPV or one of the indicated recombinant baculoviruses at 10 PFU per cell at time zero. The cells were labeled with [^{35}S]methionine for 2 h, starting at the time (in hours) indicated below each lane. A portion of the cells was lysed in SDS-PAGE sample buffer (16), and an equal portion of each extract was submitted to SDS-PAGE (lanes 1 to 8, 10 to 13, 15 to 18, and 20 to 23). The remainder of the labeled cells was treated as described for immunoprecipitation of labeled Vero cells. Immunoprecipitation was done using the same antisera as for Fig. 1 (lanes 9, 14, 19, and 24). The arrow indicates the position of the polyhedrin protein band seen in wild-type infected cells (lanes 3 and 4). The numbers at the left indicate the positions and sizes (in kilodaltons) of ^{14}C-labeled marker proteins run on the same gel.

UL9 gene product purified from insect cells is functional and binds to the HSV origins of replication (28). This suggests that if this protein is processed at the amino terminus, it is likely to be a physiologic process. Amino acid sequence analysis of the amino terminus of the 82-kDa UL9 protein is ongoing and should resolve this issue.

Immunofluorescence studies using a monoclonal antibody to the protein ICP8 have previously suggested that ICP8 is located within the nucleus of HSV-infected cells in discrete focal structures that correspond to the sites at which viral DNA replication takes place (35). The results of immunofluorescence studies reported here using an anti-ICP8 fusion protein serum appear to be quite similar to results of previous studies: at early times after infection, immunofluorescence was observed primarily in discrete focal structures and was not diffusely located throughout the nucleus. Three of the other proteins (Pol, UL42, and UL9) showed similar patterns of staining, although as mentioned above, since all of our sera were produced in rabbits, it was not possible to determine whether different proteins colocalized to the same intranuclear structures. On the other hand, the patterns of staining of the remaining three proteins (UL5, UL8, and UL52) appeared to be somewhat different, with the fluorescence distributed more evenly throughout the nucleus. It should be noted, however, that these three sera were the ones showing the weakest fluorescent staining; it is possible, therefore, that the different apparent patterns of staining simply reflect different sensitivities of the different sera. Clearly, considerably more work needs to be done to determine the functional significance of the intranuclear localization of these seven proteins, all of which have been implicated by genetic experiments as playing a direct role in DNA replication.

Our results using several different immunoassays (immunoblots, immunoprecipitation, and immunofluorescence) have led us to conclude that the UL5, UL8, UL9, and UL52 proteins occur in infected cells in very small amounts relative to the amounts of the three other replication proteins (Table 1). This probably explains why these proteins have not previously been recognized as infected-cell-specific proteins. A number of factors, however, could give a false impression of low abundance, such as low avidity of the antisera, poor incorporation of [^{35}S]methionine, or poor electrophoretic transfer of the proteins during immunoblot analysis. Several lines of evidence suggest that our estimates of the relative abundance of these proteins is accurate. First, the relative abundance of the three known replication proteins (Pol, DBP, and UL42) that we observed when using our antisera is consistent with results from other reports. Second, our results for any given protein are generally the same regardless of the immunogen or rabbit used to raise the antiserum. Finally, the amount of each protein immunoprecipitable from insect cells reflects the amount of each protein made in insect cells as revealed by analysis of total labeled proteins.

The apparent low levels of these HSV proteins not only made detection difficult but significantly hampered attempts at their purification. Primarily for this reason, we expressed the UL5, *UL8*, *UL9*, and *UL52* genes in insect cells using the baculovirus expression system. In each case, the protein made in insect cells was immunoprecipitable with the appropriate antiserum and displayed the same electophoretic mobility on SDS-polyacrylamide gels as the corresponding protein made in HSV-infected Vero cells. This reassured us that the proteins we detected in HSV-infected cells with our antisera are likely to be the authentic and intact gene products of these genes.

The identification, purification, and functional characteri-

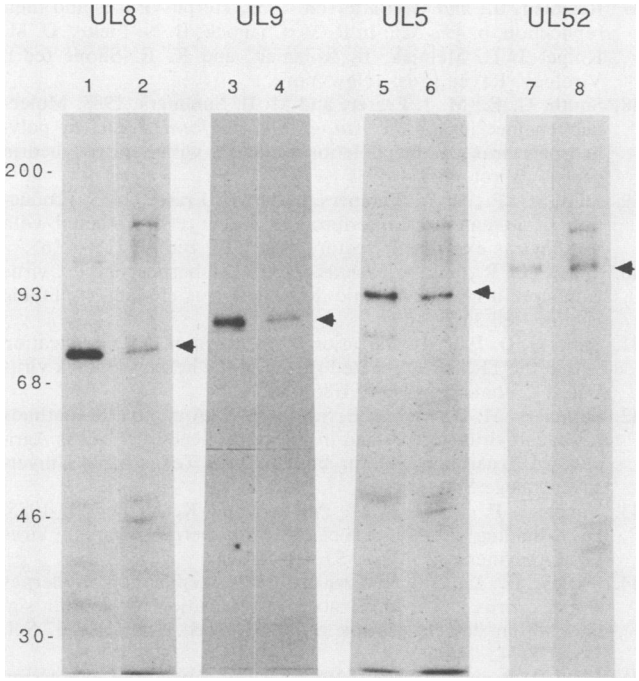

FIG. 6. Immunoprecipitation of HSV-infected Vero cell proteins and recombinant-baculovirus-infected SF9 cell proteins using antisera against HSV replication proteins. Immunoprecipitation of the labeled SF9 cells infected with the indicated recombinant baculovirus (lanes 1, 3, 5, and 7) was done as described in the legend to Fig. 5. Immunoprecipitation of the HSV-infected Vero cell extracts (lanes 2, 4, 6, and 8) was done as described in the legend to Fig. 2. The arrows indicate the bands that were specifically immunoprecipitated. The numbers at the left indicate the positions and sizes (in kilodaltons) of ^{14}C-labeled marker proteins run on the same gel. The antisera were the same as used for Fig. 2.

zation of all the HSV-encoded proteins that have a direct role in viral DNA replication are prerequisites to an analysis of the biochemical events which occur during HSV DNA replication. The products of all seven essential HSV replication genes have been identified. The production of a complete set of antisera against these seven HSV proteins should facilitate further work on their characterization. In addition, the ability to produce four of these proteins in insect cells free from contamination by other HSV proteins should aid the determination of their roles in the replication process.

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