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Correct Intranuclear Localization of Herpes Simplex Virus DNA Polymerase Requires the Viral ICP8 DNA-Binding Protein

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We used indirect immunofluorescence to examine the factors determining the intranuclear location of herpes simplex virus (HSV) DNA polymerase (Pol) in infected cells. In the absence of viral DNA replication, HSV Pol colocalized with the HSV DNA-binding protein ICP8 in nuclear framework-associated structures called prereplicative sites. In the presence of viral DNA replication, HSV Pol colocalized with ICP8 in globular intranuclear structures called replication compartments. In cells infected with mutant viruses encoding defective ICP8 molecules, Pol localized within the cell nucleus but showed a general diffuse intranuclear distribution. In uninfected cells transfected with a plasmid expressing Pol, Pol similarly showed a diffuse intranuclear distribution. Therefore, Pol can localize to the cell nucleus without other viral proteins, but functional ICP8 is required for Pol to localize to prereplicative sites. In cells infected with mutant viruses encoding defective Pol molecules, ICP8 localized to prereplicative sites. Thus, Pol or the portions of Pol not expressed by the mutant viruses are not essential for the formation of prereplicative sites or the localization of ICP8 to these structures. These results demonstrate that a specific nuclear protein can influence the intranuclear location of another nuclear protein.

Herpes simplex virus (HSV) DNA replication takes place within defined regions of the infected cell nucleus called replication compartments (3, 18). Studies with viral mutants and transfected viral genes have shown that seven viral gene products are required for viral DNA synthesis (reviewed in references 1 and 6). The major DNA-binding protein, or infected-cell protein 8 (ICP8), is one of the HSV proteins required for viral DNA synthesis and was used to define the replication compartment structures (16). Immunofluorescence experiments also showed that viral DNA polymerase (Pol) (15, 17, 22), the UL42 gene product (15), and the UL9 gene product (15) are also localized within structures similar to replication compartments. Nuclear staining was observed for the three other viral proteins required for viral DNA synthesis, UL8, UL5, and UL52 (15), but the staining was too faint to determine precise sites of intranuclear localization.

In the absence of viral DNA replication, ICP8 assumes a punctate distribution within the nuclei of infected cells, at sites called prereplicative sites (16). We have hypothesized that these infected-cell-specific structures are complexes of viral and cellular proteins poised to initiate viral DNA synthesis and that, when viral DNA replication can proceed, progeny viral DNA-protein complexes and additional replication complexes accumulate in one area of the infected-cell nucleus, leading to globular intranuclear structures called replication compartments (3). At least a portion of the host DNA replication apparatus is localized to prereplicative sites, and ICP8 is required for this reorganization of the location of cellular DNA synthesis (3). We have investigated whether viral DNA polymerase is localized to the same intranuclear structures as is ICP8 and, by the use of viral mutants with specific alterations in the ICP8 or *pol* gene products, examined the interrelationship between these two viral gene products for localization within the cell nucleus.

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MATERIALS AND METHODS

Cells and viruses. Monkey CV-1 cells (American Type Culture Collection) were chosen for this study because they gave lower background levels of staining than did Vero cells with the rabbit sera used. These cells were plated onto glass coverslips as described previously for Vero cells (8) and infected the next day. The cultures were mock infected or infected at a multiplicity of infection of 20. The KOS1.1 wild-type (wt) virus; the *pol* gene mutants ΔX17, ΔX14, and ΔS1 (12) (Fig. 1); and the ICP8 gene mutants *d*301, *n*10, and *n*102 (4) (Fig. 2) were described previously. Mutant HP54 (Fig. 1) was constructed as described for mutant HP66 (13) to have 2,253 bp of Pol-coding sequences replaced with the *Escherichia coli lacZ* gene linked to the *tk* promoter. HP54 has an additional deletion of the *pol* locus relative to HP66 (12). The ΔX14 and ΔX17 mutant viruses (Fig. 1) each contain a 1,165-bp deletion that should cause a frameshift in the Pol-coding sequences. On the basis of this deletion, these mutants were predicted to encode a Pol protein of 65 kDa. No Pol protein was detected in cells infected with ΔX17, and ΔX14-infected cells expressed a 96-kDa Pol protein, consistent with an additional frameshift mutation restoring the expression of an additional part of the Pol-coding sequences. The ΔS1 mutant virus (Fig. 1) contains a 2,253-bp deletion within the Pol-coding sequences and expresses a 57-kDa Pol gene product (26). Propagation and determination of titers of the *pol* mutants were done on DP6 cells, which contain the *pol* gene and are capable of complementing the growth of *pol* mutants (13).

The ICP8 gene mutant *d*301 (Fig. 2) contains a 2,001-bp deletion within the ICP8-coding sequences and expresses a

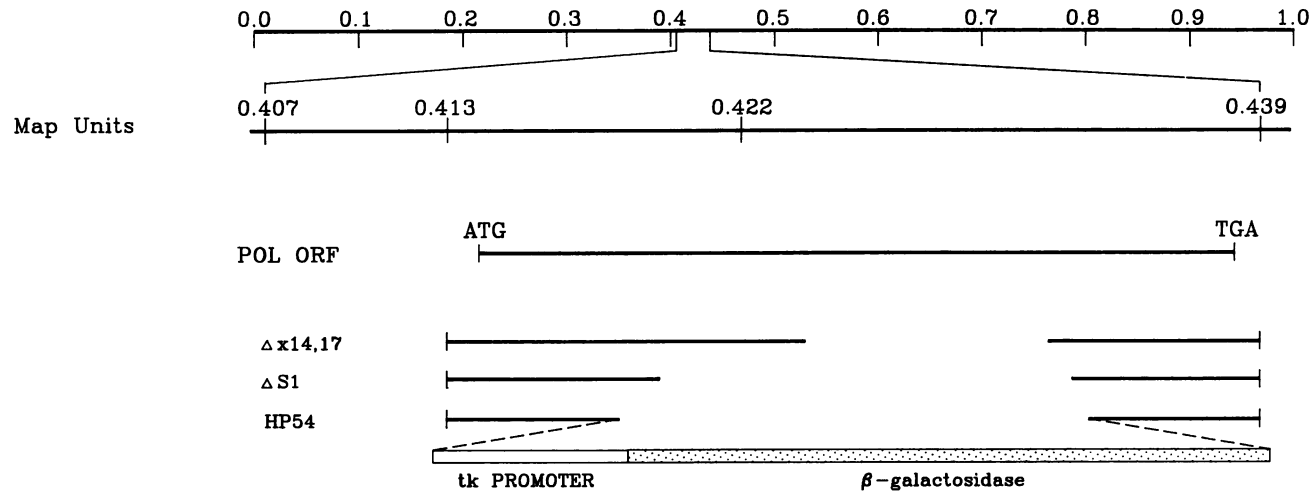


FIG. 1. Structures of viral DNA Pol genes in viral mutants used in this study. The location of the Pol open reading frame (ORF) in the viral genome is shown in the top half of the figure. The bottom lines show the locations of the deletions in ΔX14, ΔX17, and ΔS1 and the location of the *tk-lacZ* insertion in HP54.

53-kDa ICP8 gene product which localizes to the cell nucleus (4). The mutant *n*10 genome (Fig. 2) contains a nonsense codon after codon 1160 of the ICP8-coding sequences, and this mutant virus expresses an ICP8 gene product of approximately 120,000 kDa which fails to localize to the cell nucleus (4). The ICP8 gene mutant *d*102 (Fig. 2) contains a 1,188-bp deletion in the ICP8-coding sequences and expresses an ICP8 gene product of 80,000 kDa which fails to localize to the cell nucleus (4). Propagation and determination of titers of the ICP8 mutant viruses were done on S-2 cells, which are derived by introduction of the ICP8 gene and are capable of complementing the growth of ICP8 mutants (4).

To block viral DNA synthesis in wt virus-infected cells, we added 400 μg of sodium phosphonoacetate (PAA) per ml of culture medium. Under these conditions, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer (pH 7.6) was added to the medium.

Plasmids. pCIE-Pol contains the HSV Pol major open reading frame under the control of the human cytomegalovirus immediate-early enhancer-promoter and was constructed as follows. A 1.1-kbp *Xba*I fragment containing the cytomegalovirus control region was isolated from pEQ176 (a generous gift from A. Geballe) and treated with the Klenow fragment and deoxynucleoside triphosphates to fill in the ends. The treated fragment was inserted into similarly treated *Hin*dIII-cleaved pT7Pol-01. pT7Pol-01 (24) consists of a pGEM4 backbone and HSV sequences starting 6 bp upstream of the start site of transcription of the major Pol

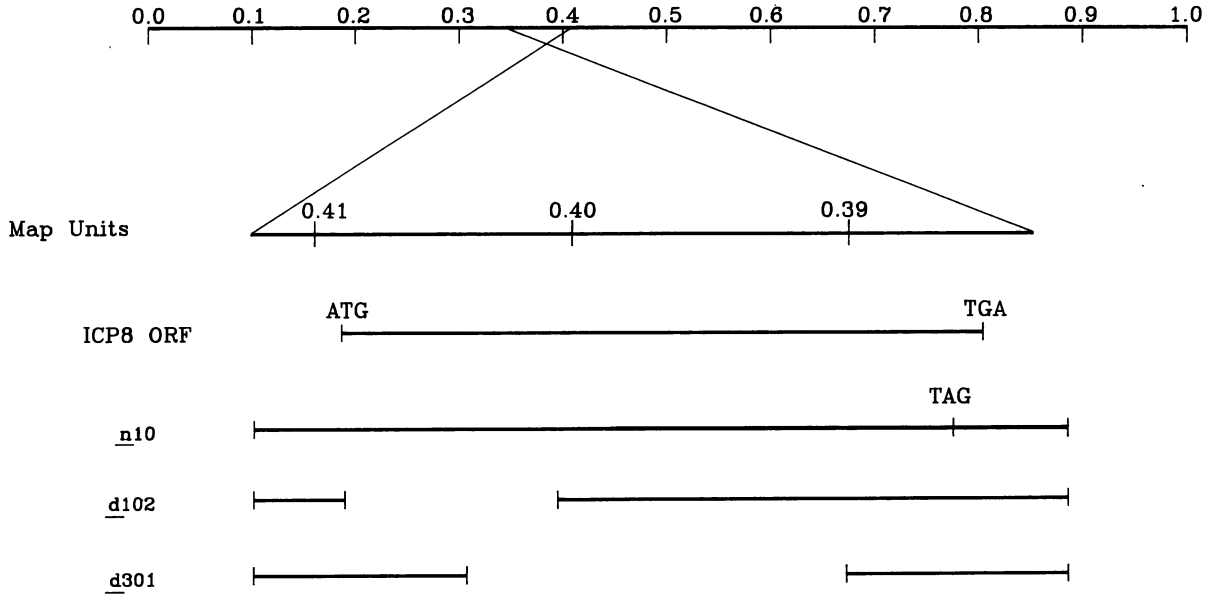


FIG. 2. Structures of ICP8 genes in viral mutants used in this study. The location of the ICP8 open reading frame (ORF) in the viral genome is shown in the top half of the figure. The bottom lines show the location of the nonsense codon in *n*10 and the locations of the deletions in *d*102 and *d*301.

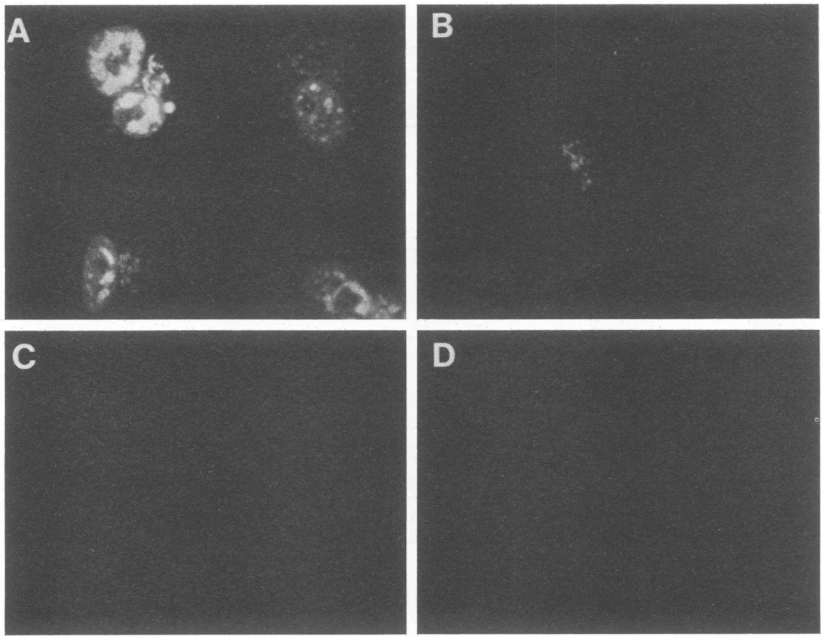


FIG. 3. Specificity of anti-Pol serum PP5 for immunofluorescence. CV-1 cells were infected with KOS1.1 wt virus or mock infected. At 5 h postinfection, the cells were fixed and reacted with anti-Pol serum PP5 or preimmune serum and then with fluorescein-conjugated goat antirabbit immunoglobulin antibody. (A) Infected cells reacted with anti-Pol serum. (B) Infected cells reacted with preimmune serum. (C) Mock-infected cells reacted with anti-Pol serum. (D) Mock-infected cells reacted with preimmune serum.

transcript (the location of the *Hin*dIII site into which the cytomegalovirus control region was inserted) and continuing to a *Kpn*I site just downstream of the Pol adenylation site (25).

Indirect immunofluorescence. Mock-infected or infected cells were fixed and permeabilized as described previously (16) at 5 h postinfection. The cells were incubated with a 1:10 dilution of anti-Pol serum PP5 (26) and/or a 1:10 dilution of mouse monoclonal antibody 39S (20), with a 1:50 dilution of fluorescein-conjugated goat antirabbit immunoglobulin antibody, and with a 1:30 dilution of rhodamine-conjugated goat antimouse immunoglobulin antibody. The last two antibodies were diluted in phosphate-buffered saline (PBS) containing 10% normal goat serum to lower nonspecific staining by the fluorochrome-conjugated antibodies. For double-label immunofluorescence, the cells were photographed as described previously (7, 8).

Transfected cells were fixed and permeabilized 48 h posttransfection by incubation for 10 min in methanol and for 5 min in acetone at −20°C. The coverslips were air dried and stored at −20°C. Prior to reaction with antibodies, the coverslips were thawed and washed twice in PBS, incubated for 20 min at 37°C in 10% fetal calf serum in PBS, and reacted for 20 min at 37°C with a 1:20 dilution in PBS of either preimmune serum or EX6 anti-Pol rabbit antiserum (23). The coverslips were washed and incubated for 20 min with fluorescein-conjugated goat antirabbit immunoglobulin antibody diluted 1:50 in 2% calf serum in PBS.

RESULTS

We used indirect immunofluorescence to examine the localization of HSV DNA Pol under various conditions. For analysis of infected cells, we used anti-Pol serum PP5, prepared by immunizing rabbits with a *lacZ-pol* fusion protein and previously shown to be specific for Pol (26). On HSV-infected cells, the antiserum stained intranuclear globular structures (Fig. 3A), as reported for other Pol antisera (15, 17, 22). No staining was seen with the PP5 antiserum (Fig. 3C) or preimmune serum (Fig. 3D) on mock-infected cells. Preimmune serum did react with some perinuclear structures in infected cells (Fig. 3B). This reaction appeared to be due to nonspecific binding of rabbit immunoglobulin G to some component of the Golgi apparatus in infected cells (results not shown), possibly the virus-encoded Fc receptor (21). We have not been able to block this apparent nonspecific binding but have focused on the specific nuclear reactivity of the Pol antiserum.

Localization of Pol to prereplicative sites and replication compartments. The intranuclear distribution of Pol shown in Fig. 3 was very similar to that previously observed for the virus-encoded major DNA-binding protein ICP8 (16) and for the viral transactivator ICP4 (7, 17). We performed double-label immunofluorescence with rabbit anti-Pol serum PP5 (26) and mouse anti-ICP8 monoclonal antibody 39S (20). A major portion of Pol was codistributed with ICP8 in replication compartments (Fig. 4A and B). Single-antibody staining did not reveal a spillover of the two fluorescent images between the two filters (7; results not shown).

We also examined the distribution of Pol in cells infected in the presence of PAA, a specific inhibitor of viral DNA polymerase (9, 11), to determine the site of localization of Pol in the absence of viral DNA synthesis. Under these conditions, a major portion of Pol showed an intranuclear punctate distribution that corresponded to the prereplicative sites at which ICP8 was localized (Fig. 4C and D). Thus, Pol was codistributed with ICP8 in prereplicative sites in the absence of viral DNA replication and in replication compartments in the presence of viral DNA replication.

Requirement for functional ICP8 for correct nuclear localization of Pol. We had previously shown that functional ICP8

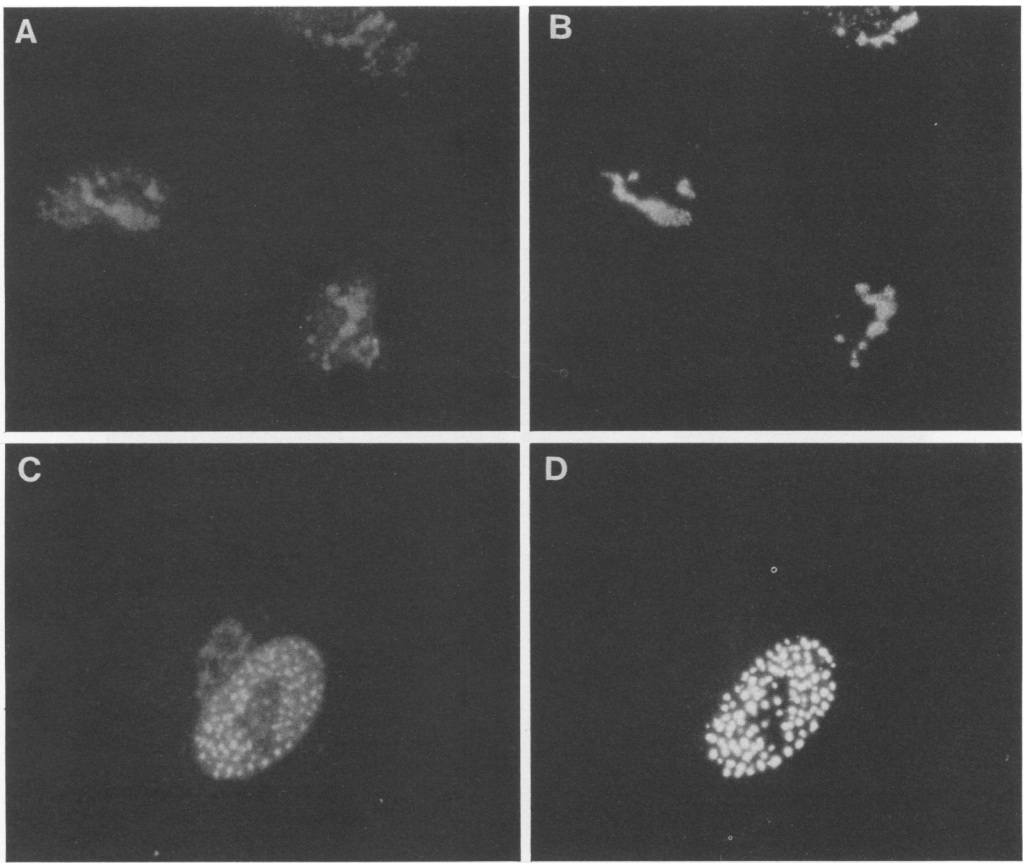
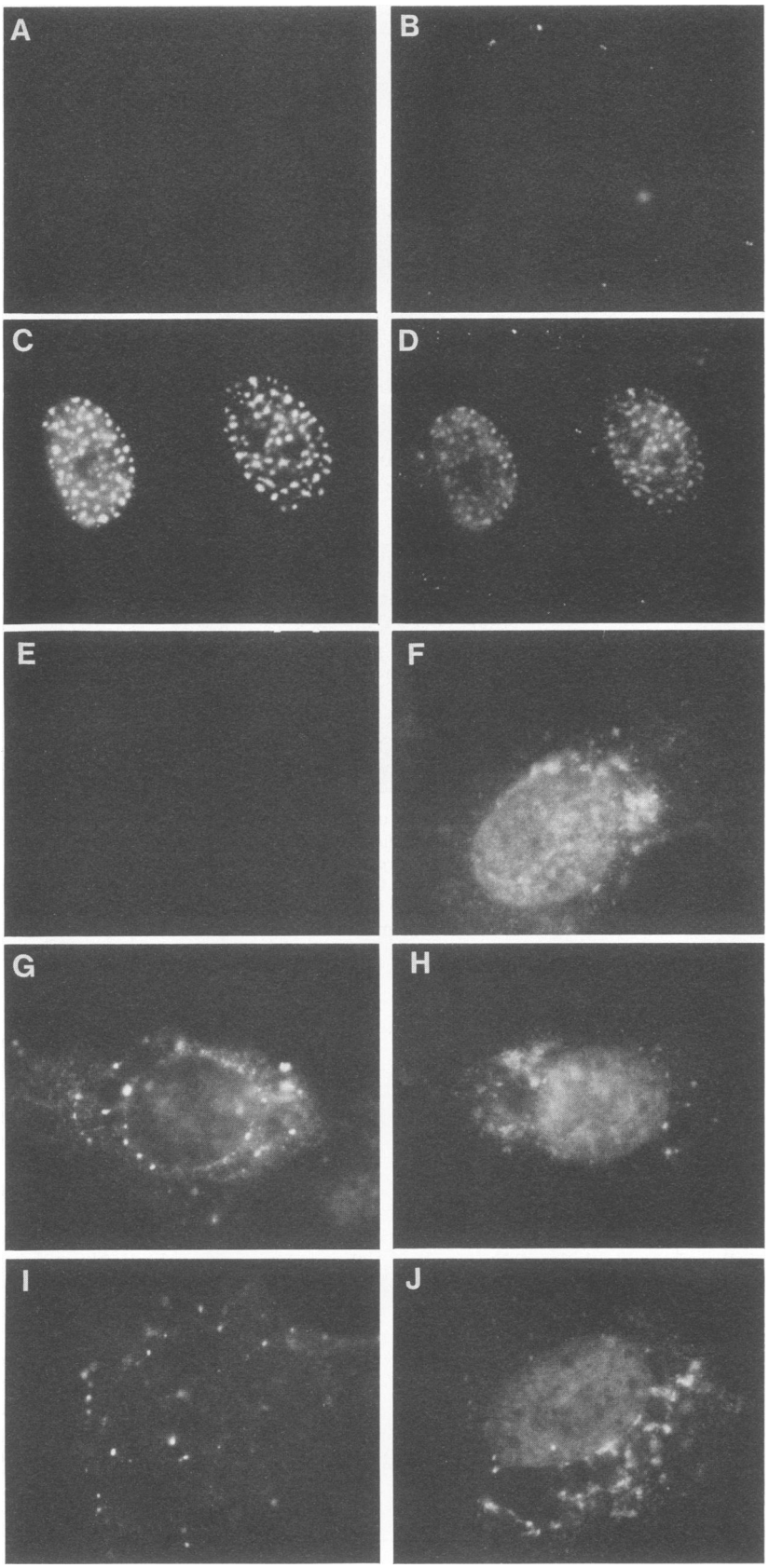


FIG. 4. Codistribution of ICP8 and Pol in the presence or absence of viral DNA replication. CV-1 cells were infected with wt virus in the absence or presence of 400 μg of PAA per ml to inhibit viral DNA replication. At 5 h postinfection, the cells were fixed and reacted with anti-Pol serum PP5 and/or anti-ICP8 monoclonal antibody 39S, with fluorescein-conjugated goat antirabbit immunoglobulin antibody, and with rhodamine-conjugated goat antimouse immunoglobulin antibody. (A) Cells infected in the absence of PAA and reacted with Pol antiserum. (B) Same field of cells as in panel A; the fluorescence image is due to the reaction with ICP8 antibody. (C) Cells infected in the presence of PAA; the fluorescence image is due to the reaction with Pol antiserum. (D) Same field of cells as in panel C; the fluorescence image is due to the reaction with ICP8 antibody.

is needed for the localization of at least a portion of the host DNA replication apparatus to prereplicative sites (3). To determine whether functional ICP8 was needed for the localization of Pol to prereplicative sites, we examined the localization of Pol in cells infected with various viral mutants (Fig. 2) expressing defective ICP8 molecules (4). All of these ICP8 mutants are defective for DNA replication (4), so wt virus infection was carried out in the presence of PAA to inhibit viral DNA replication. Under these infection conditions, wt Pol and ICP8 localized to prereplicative site structures (Fig. 5C and D). In cells infected with the *d*301 mutant, monoclonal antibody 39S did not recognize the truncated ICP8 molecule expressed (Fig. 5E), but the Pol antiserum showed diffuse nuclear staining (Fig. 5F). In cells infected with the *n*10 mutant, ICP8 showed a cytoplasmic distribution with some perinuclear accumulation (Fig. 5G), while Pol showed a diffuse distribution throughout the nucleus (Fig. 5H). In cells infected with the *n*102 mutant, ICP8 reacted poorly with monoclonal antibody 39S but appeared to be distributed throughout the cell (Fig. 5I), and Pol exhibited a diffuse distribution throughout the nucleus (Fig. 5J). On the basis of these results, we conclude that the nuclear localization of Pol is independent of the nuclear uptake of ICP8 but that functional ICP8 is needed for the correct intranuclear localization of Pol to prereplicative sites.

Localization of Pol in transfected cells. To determine the intracellular distribution of Pol in the absence of any other viral proteins, we transfected cells with pCIE-Pol, a plasmid that contains the *pol* gene under the control of the strong immediate-early enhancer-promoter of cytomegalovirus, and analyzed the cells for Pol expression and intracellular distribution by immunofluorescence. Approximately 5% of the cells exhibited fluorescence staining, which was almost exclusively nuclear (Fig. 6). The intranuclear fluorescence showed a diffuse, slightly granular nuclear distribution, with the exclusion of nucleoli. No staining above the background was observed with preimmune sera against pCIE-Pol plasmid-transfected cells or with anti-Pol serum or preimmune serum against cells transfected with a control plasmid (pGEM4) lacking the *pol* open reading frame (results not shown). We conclude from these results that Pol can localize to the nucleus in the absence of other viral proteins but can not localize to prereplicative sites by itself.

Localization of ICP8 in cells infected with *pol* mutants. To determine whether there was a role for Pol in the assembly of prereplicative sites and in the localization of ICP8 to these structures, we examined the localization of ICP8 in cells infected with viral mutants containing lethal mutations in the *pol* gene (13). Previous work with *pol ts* mutants and Pol inhibitors had indicated that Pol might not be needed for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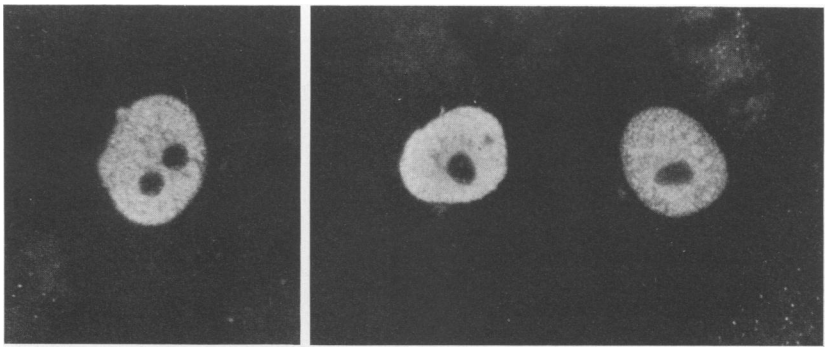

FIG. 6. Nuclear localization of Pol expressed in transfected cells. Vero cells transfected with pCIE-Pol were fixed at 48 h posttransfection and processed as described in Materials and Methods to detect Pol by immunofluorescence. Immunofluorescence images of fields are shown.

these assembly processes (3, 16, 17), but the possibility remained that some Pol function was still expressed by Pol proteins to promote assembly of the prereplicative sites. We used various replication-defective Pol mutants (Fig. 1) to determine whether there was any role for Pol in the assembly of prereplicative sites. In cells infected with mutant HP54 containing a deletion in the *pol* coding sequences and a *lacZ* insertion, ICP8 localized to prereplicative sites (Fig. 7A), but no Pol was detected by immunofluorescence (Fig. 7B). Similarly, in cells infected with ΔX17 or ΔX14, viruses containing 1.2-kb deletions in the *pol* gene, ICP8 localized to prereplicative sites (Fig. 7C and E), but little Pol could be detected (Fig. 7D and F). In cells infected with mutant ΔS1, containing a 2-kb deletion in the Pol-coding sequences, ICP8 localized to prereplicative sites (Fig. 7G). Pol staining was faint but appeared to coincide with prereplicative sites containing ICP8 (Fig. 7H). Thus, Pol or at least the regions missing from these mutant proteins are not essential for the assembly of prereplicative sites or the localization of ICP8 to these structures.

DISCUSSION

The eucaryotic cell nucleus is organized structurally so that at least certain functions are compartmentalized within specific regions of the organelle. In a similar fashion, viral DNA synthesis in cells infected with HSV is organized within globular structures called replication compartments. We have hypothesized that viral DNA replication is initiated at complexes of viral DNA replication proteins similar to the prereplicative sites at which viral proteins accumulate in the absence of viral DNA synthesis (3). The accumulation of viral progeny DNA and additional replication complexes would then lead to the formation of larger intranuclear structures called replication compartments. In this report, we show that HSV Pol is localized to both prereplicative sites and replication compartments and is colocalized with ICP8 in these structures. The localization of Pol to the cell nucleus is independent of ICP8 but, in the absence of ICP8, Pol remains diffusely distributed throughout the cell nucleus. Thus, Pol is dependent on ICP8 to promote the formation of prereplicative site structures (3) or to directly interact with it in these structures.

Previously reported results support a possible direct interaction between Pol and ICP8. First, ICP8 was reported to stimulate Pol activity in vitro, although one report described the stimulation of Pol activity on an activated DNA template (19), a second report described inhibition by ICP8 on a primed single-stranded DNA template but stimulation on a double-stranded DNA template and stimulation of 3′-5′ exonuclease (14), and a third report described stimulation by ICP8 on a primed single-stranded DNA template (5). Second, Pol activity was reduced in extracts from cells infected with HSV type 2 ICP8 *ts* mutants at the nonpermissive temperature (10). Finally, some viral mutations altering sensitivity to Pol inhibitors mapped within the ICP8 gene (2). However, no direct biochemical evidence for an interaction between Pol and ICP8 has been reported.

Although it is known that certain molecules are located at specific sites within the cell nucleus, little is known about the mechanisms localizing them to these sites. This report provides one of the first demonstrations that a specific protein molecule can influence the intranuclear location of another protein molecule. The HSV DNA replication system provides a unique situation for studying the assembly of nuclear structures because it encodes seven gene products involved in DNA synthesis, and it is likely that several of these molecules interact within the nucleus. Furthermore, the viral genes can be individually mutated, and the mutations can be incorporated easily into the viral genome to investigate the result of altering or eliminating a specific gene product. Thus, this system provides the ability to perform a genetic analysis of the role of individual gene products in

FIG. 5. Requirement for functional ICP8 for intranuclear localization of Pol. CV-1 cells were infected with wt or ICP8 mutant viruses, fixed for immunofluorescence at 5 h postinfection, and reacted with antibodies as described in the legend to Fig. 3. (A) Mock-infected cells; the fluorescence image is due to the reaction with ICP8 antibody. (B) Mock-infected cells; the fluorescence image is due to the reaction with anti-Pol serum. (C) Cells infected with wt virus in the presence of PAA; the fluorescence image is due to the reaction with ICP8 antibody. (D) Same field of cells as in panel C; the fluorescence image is due to the reaction with anti-Pol serum. (E) Cells infected with *d*301 mutant virus; the fluorescence image is due to the reaction with ICP8 antibody. (F) Same field of cells as in panel E; the fluorescence image is due to the reaction with anti-Pol serum. (G) Cells infected with mutant *n*10; the fluorescence image is due to the reaction with ICP8 antibody. (H) Same field of cells as in panel G; the fluorescence image is due to the reaction with anti-Pol serum. (I) Cells infected with *d*102; the fluorescence image is due to the reaction with ICP8 antibody. (J) Same field of cells as in panel I; the fluorescence image is due to the reaction with anti-Pol serum.

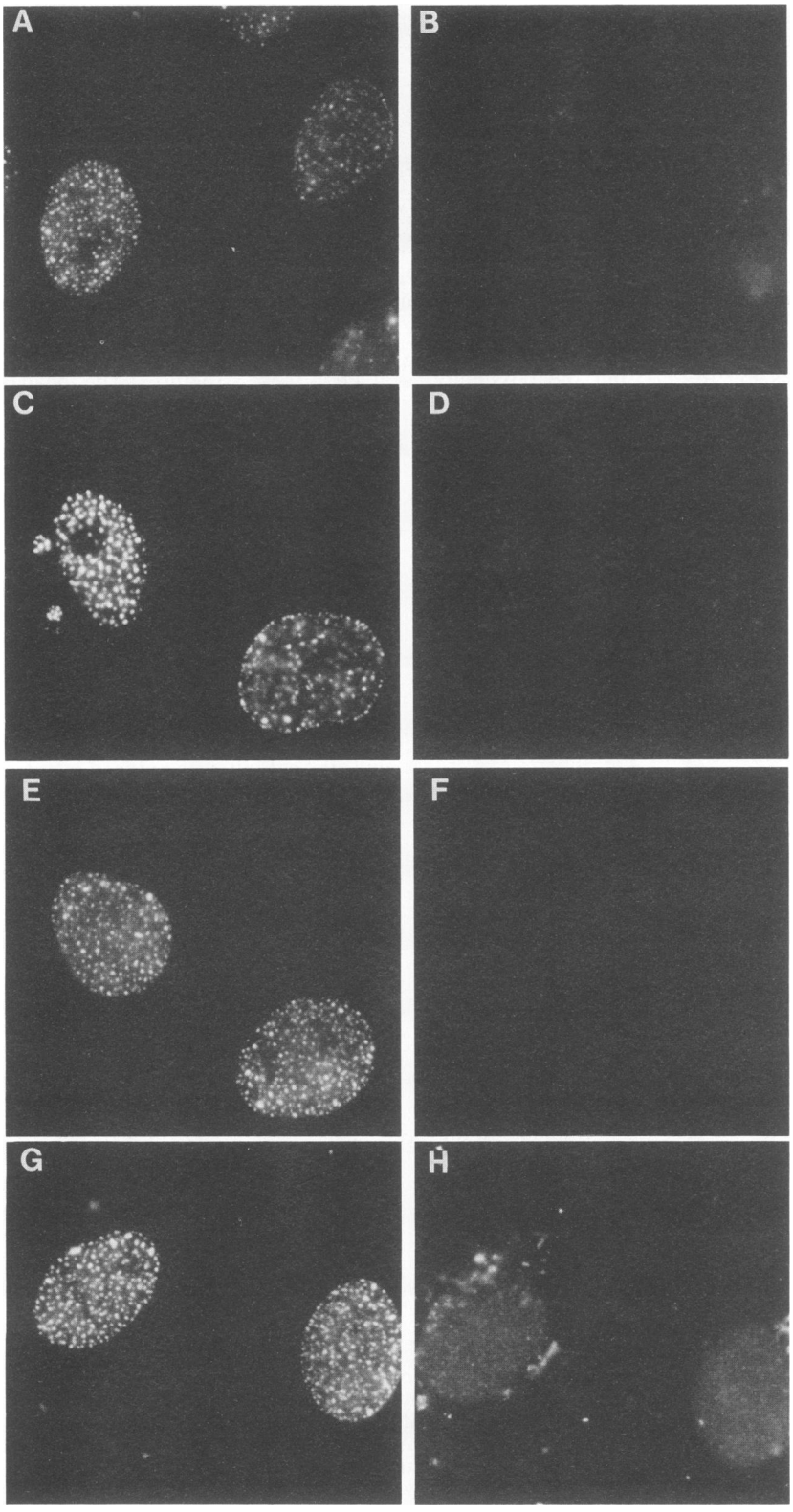
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FIG. 7. Formation of prereplicative site structures in cells infected with *pol* mutants. Cells were infected with mutant viruses encoding defective Pol proteins and fixed for immunofluorescence as described in the legend to Fig. 3. (A) Cells infected with mutant HP54; the fluorescence image is due to the reaction with ICP8 antibody. (B) Same field of cells as in panel A; the fluorescence image is due to the reaction with anti-Pol serum. (C) Cells infected with ΔX17; the fluorescence image is due to the reaction with ICP8 antibody. (D) Same field of cells as in panel C; the fluorescence image is due to the reaction with anti-Pol serum. (E) Cells infected with ΔX14; the fluorescence image is due to the reaction with ICP8 antibody. (F) Same field of cells as in panel E; the fluorescence image is due to the reaction with anti-Pol serum. (G) Cells infected with ΔS1; the fluorescence image is due to the reaction with ICP8 antibody. (H) Same field of cells as in panel G; the fluorescence image is due to the reaction with anti-Pol serum.

parallel with the ability to analyze the intranuclear distribution of the viral gene products within a mammalian cell nucleus.

It is not known whether ICP8 acts as a carrier protein to direct Pol to the prereplicative sites or whether ICP8 promotes the assembly of prereplicative sites and Pol then binds to or interacts with these structures. The results presented in this report indicate that Pol can localize into the nucleus even when ICP8 remains within the cytoplasm or in cells transfected with only the *pol* gene. Therefore, Pol has its own nuclear localization signal and can localize into the nucleus independently of other viral proteins. Elucidation of the events involved in the localization of Pol to the prereplicative sites is likely to provide basic information about the mechanisms controlling the intranuclear location of proteins.

It will be of interest to map the portion(s) of Pol needed for its localization into the nucleus and for its association with prereplicative site structures. Additional antibodies specific for Pol will be necessary, because several of the mutant proteins react poorly with the PP5 antiserum used in this study. Identification of the site on Pol involved in the interaction with other viral or cellular proteins may allow the design of antiviral agents that block this interaction, which is likely to be essential for productive infection by HSV.

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