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Assembly of Complete, Functionally Active Herpes Simplex Virus DNA Replication Compartments and Recruitment of Associated Viral and Cellular Proteins in Transient Cotransfection Assays

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Early during the herpes simplex virus (HSV) lytic cycle or in the presence of DNA synthesis inhibitors, core viral replication machinery proteins accumulate in intranuclear speckled punctate prereplicative foci, some of which colocalize with numerous sites of host cellular DNA synthesis initiation known as replisomes. At later times, in the absence of inhibitors, several globular or large irregularly shaped replication compartments are formed; these compartments also contain progeny viral DNA and incorporate the IE175(ICP4) transcription factor together with several cellular proteins involved in DNA replication and repair. In this study, we demonstrate that several forms of both prereplication foci and active viral replication compartments that display an appearance similar to that of the compartments in HSV-infected cells can be successfully assembled in transient assays in DNA-transfected cells receiving genes encoding all seven essential HSV replication fork proteins together with oriS target plasmid DNA. Furthermore, bromodeoxyuridine (BrdU)-pulse-labeled DNA synthesis initiation sites colocalized with the HSV single-stranded DNA-binding protein (SSB) in these replication compartments, implying that active viral DNA replication may be occurring. The assembly of complete HSV replication compartments and incorporation of BrdU were both abolished by treatment with phosphonoacetic acid (PAA) and by omission of any one of the seven viral replication proteins, UL5, UL8, UL9, UL42, UL52, SSB, and Pol, that are essential for viral DNA replication. Consistent with the fact that both HSV IE175 and IE63(ICP27) localize within replication compartments in HSV-infected cells, the assembled HSV replication compartments were also able to recruit both of these essential regulatory proteins. Blocking viral DNA synthesis with PAA, but not omission of oriS, prevented the association of IE175 with prereplication structures. The assembled HSV replication compartments also redistributed cotransfected cellular p53 into the viral replication compartments. However, the other two HSV immediate-early nuclear proteins IE110(ICP0) and IE68(ICP22) did not enter the replication compartments in either infected or transfected cells.

During the productive or lytic cycle of herpes simplex virus type 1 (HSV-1) infection, viral DNA replication begins within 4 h in distinctive nuclear viral DNA replication compartments (RC) or factories containing both viral proteins and host cellular proteins. Genetic analyses have determined that there are seven essential viral proteins required specifically for DNA replication during infection (28, 58). The isolated genes for these same seven proteins when introduced into cultured mammalian cells by transient DNA transfection procedures are also sufficient for the specific amplification of cotransfected bacterial plasmid DNA containing the HSV origin (oriS or oriL) as assayed by *Dpn*I resistance and Southern blot hybridization (8, 60). These seven essential viral replication proteins are the helicase-primase components UL5, UL8, and UL52, the origin DNA-binding protein UL9, the viral DNA polymerase (Pol or UL30), the polymerase accessory protein UL42, and the single-stranded DNA-binding protein (SSB, ICP8, or UL29). UL5, UL8, and UL52 form a stable triplet complex with both helicase and primase activities (12, 63, 64).

Antibody against SSB was initially used to identify and define various prereplicative structures and RC in indirect immunofluorescence assays (IFA) (46). Early during viral infection, a number of small punctate nuclear structures referred to as prereplicative sites or foci are observed. These are defined most clearly by specifically blocking viral DNA synthesis with phosphonoacetic acid (PAA). At least some of the prereplicative foci (pre-RF) colocalize with initiation sites for host cellular DNA synthesis as defined by bromodeoxyuridine (BrdU) pulse-labeling and have been suggested to represent a reorganization of the cellular replisomes (14, 47). Eventually, these numerous punctate foci aggregate into several very large, mostly irregularly shaped globular RC containing progeny viral DNA. All seven essential HSV replication proteins are also found to accumulate in the RC (6, 21, 31, 32, 42) as well as some host cellular proteins, including the tumor suppressors Rb and p53 and several proteins related directly to cellular DNA replication such as DNA polymerase delta and DNA ligase (59). The viral core replication proteins UL5, UL8, UL52, and SSB are all also known to be present in the prereplicative sites in infected cells (6, 7, 21, 31, 32). UL9 may also be required for SSB to localize in prereplicative sites in infected cells (6, 21, 31, 32), but only UL5, UL8, and UL52 are sufficient for the localization of SSB in prereplicative sites in DNA-transfected cells (31).

Both the IE175(ICP4) and IE63(ICP27) nuclear regulatory

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proteins are essential for efficient synthesis of both replication-related proteins and viral DNA in HSV-1 infection, although neither is essential for viral DNA replication per se in transient replication assays. Nevertheless, IE175 is known to redistribute from an early nuclear diffuse pattern into viral replication factories later during infection (30, 49), and we have recently obtained a similar finding of partial redistribution for IE63 also (62). However, IE175 does not localize within the prereplicative sites formed when viral DNA synthesis is blocked (29, 48). IE175 is a DNA-binding protein that behaves as an autoregulatory repressor of immediate-early (IE) and latency promoters and as a transcriptional transactivator of viral delayed-early and late promoters in transient transfection assays (18–20, 40, 41, 47). Furthermore, IE175 deletion or temperature-sensitive mutant viruses are unable to synthesize delayed-early mRNA (16, 43, 56). IE63 is required for normal high levels of viral replication during infection, and some IE63 deletion mutant viruses produce considerably less progeny HSV DNA compared to wild-type virus (35). The efficiency of SSB distribution within viral RC was also found to be greatly altered in cells infected with a IE63 null mutant virus (13). Recently, reduced levels of accumulated mRNA of UL5, UL8, UL9, UL42, UL52, and Pol have also been demonstrated with IE63 mutants (55). However, it is still not clear whether just the assembled HSV RC are sufficient to redistribute IE175 or IE63 during viral infection or whether other factors are involved, nor is it known whether the subnuclear location of either IE175 or IE63 is important for fulfillment of their biological function.

In uninfected mammalian cells, multiprotein replication complexes that contain DNA polymerases alpha and delta, DNA primase, topoisomerases I and II, RNase H, proliferating cell nuclear antigen, a DNA-dependent ATPase, replication factor C, DNA ligase I, DNA helicase, and replication protein A have been characterized (1). Cellular replication initiation sites, sometimes called replisomes, become pulse-labeled by biotin-11-dUTP or BrdU in S-phase cells and associate with the nuclear matrix, where replication occurs as the template moves through them (24). Even though HSV has a relatively large (155-kb) genome and encodes numerous viral proteins, HSV apparently also takes advantage of several host cell proteins, including RNA polymerase II and associated transcription factors, and probably also components of the cellular DNA synthesis and replication machinery as well. The expression and distribution of some cellular replication initiation proteins, such as replication protein A, cdc2, cyclin A, and DNA polymerase alpha, are known to change during the G_1-S phase transition in mammalian cells (5), but little is known about how or whether the cellular DNA replication apparatus changes in response to the formation of functional viral DNA RC. The HSV pre-RF formed in the absence of viral DNA synthesis have been shown to colocalize with BrdU-labeled cellular DNA synthesis initiation sites (14, 15) and are more prone to do so in the presence of PAA in infections with mutant viruses that lack one of the replication proteins (31, 32). Because of these results, it has been presumed that several viral DNA replication proteins (particulately the primase-helicase complex and SSB) may initially target to the preexisting cellular DNA initiation sites before accumulation and assembly of the larger functionally active viral RC. However, it is not clear yet whether such structures are actual direct intermediates in the formation of RC or simply represent storage sites. Indeed, Maul et al. (34) have recently argued that input HSV genomes instead localize at cellular protein PML-containing nuclear bodies (ND10 or PODs) and that initial progeny DNA and transcripts are also associated with PODs.

HSV infection is a complicated process in which many genes are turned on and off in a tightly controlled cascade pattern, and the efficient expression of replication genes is dependent on the presence of several IE regulatory proteins and perhaps virion factors. Furthermore, many other processes, including DNA maturation and capsid assembly, occur simultaneously. To study how HSV-1 replication compartments are formed, together with the impact of assembled HSV RC on the subcellular location of other viral and cellular proteins, we chose to introduce by cotransfection all seven HSV-1 replication fork proteins (Rep mixture) expressed under the control of strong heterologous promoters to avoid complications from other HSV-encoded gene products. In this report, we demonstrate, for the first time, that typical large HSV RC, whose characteristics are remarkably similar to the functionally active structures formed in virus-infected cells, can be successfully assembled in DNA-transfected Vero cells. Our assay involves the introduction of a complete set of expression plasmids containing the UL5, UL8, UL9, UL42, UL52, Pol, and SSB genes driven by constitutive human cytomegalovirus (HCMV) major IE enhancers-promoters, together with the target plasmid containing HSV oriS. In this simplified and easily manipulated system we have (i) identified both the essential and minimal viral replication protein requirements for forming large PAA-sensitive HSV RC; (ii) shown that PAA-sensitive DNA synthesis initiation as defined by pulse-labeled BrdU incorporation occurs within structures formed by the viral HSV replication proteins; (iii) demonstrated that both IE175 and IE63 are efficiently redistributed into assembled RC; and (iv) found that the cellular p53 protein expressed by cotransfection is also recruited into the assembled HSV RC.

MATERIALS AND METHODS

Cells and viruses. Vero cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum in humidified 5% CO_2 incubator. Cells were seeded at 8×10^4 cells per well in two-well slide chambers for transfection and in four-well slide chambers for virus infection studies, and 10^6 cells per well were plated in 100-mm-diameter dishes for the transient *Dpn*I DNA replication assays. Stocks of HSV-1(KOS) were prepared by infecting monolayer Vero cells at 0.01 PFU per cell. Infected cells were incubated in DMEM supplemented with 1% calf serum in humidified 5% CO_2 incubator and were harvested after 2 days. Supernatant virus was collected following three freeze-thaw cycles and centrifuged to pellet cell debris. The clarified viral stocks were titered on monolayer Vero cells by plaque formation in DMEM supplemented with 1% human serum to neutralize cell-free virus and prevent formation of secondary plaques. Infected cells were fixed with methanol for 10 min at room temperature (RT) and stained with crystal violet for 10 min at RT followed by washing with distilled H_2O several times.

For IFA, the titer of the HSV-1(KOS) used was 1.5×10^8 PFU per ml. HSV-1(KOS) was used to infect cells in four-well slides at a multiplicity of infection (MOI) of 0.5 PFU per cell. After absorption in phosphate-buffered saline (PBS)–glucose–inactivated calf serum) at RT for 1 h, the cells were incubated in DMEM supplemented with 1% calf serum and incubated for up to 6 h in a humidified 5% CO_2 incubator. PAA was included at 100 μg/ml in the postabsorption medium when viral DNA synthesis was to be blocked. BrdU (Sigma) was added at final concentration of 10 μM for incorporation into newly synthesized DNA for 30 min just before the cells were fixed.

Expression plasmids. Plasmid pLZ11 DNA (1 μg) carries the IE63 gene driven by its own promoter with four tandemly repeated SNE sites (9) inserted at the *Bam*HI site at −276 upstream of the IE63 promoter (in a fragment from genome map positions 113422 to 115742) to boost basal expression (62). Plasmid pGH114 DNA (0.5 μg) contains the IE175 gene driven by cytomegalovirus enhancer-promoter region (38), plasmid pGH92 DNA (1 μg) contains the IE110 gene driven by its own promoter (38), and plasmid pGR169 DNA (1 μg) contains the IE68 gene driven by its own promoter (40). Plasmid pSVp53 DNA (0.5 μg) carrying the human wild-type p53 gene driven by the simian virus 40 enhancer-promoter region was a gift from Ken Kinsler (Johns Hopkins Oncology Center).

Transient DNA transfection. Transient DNA transfection assays for IFA were carried out with 8×10^4 Vero cells in two-well slide chambers. A mixture of seven DNA plasmids (0.3 μg of each) carrying genes encoding UL5, 8, 9, 42, 52, SSB, and Pol driven by the cytomegalovirus enhancer-promoter region, together with plasmid pMC110 (0.3 μg) carrying an HSV oriS origin fragment, was used to represent the complete set of replication plasmids in each well (23). The CsCl-purified plasmid DNAs were cotransfected by the calcium phosphate precipitation procedure in BBS buffer (38). pUC18 DNA was used as a carrier to

normalize the total amount of transfected DNA. Transfected cells were incubated in DMEM supplemented with 10% fetal bovine serum in a humidified 3% CO_2 incubator at 35°C overnight. The medium was changed 18 h after transfection, and the slides were placed into a 5% CO_2 incubator at 37°C. To block viral DNA synthesis, PAA at 400 μg/ml was included in the medium from 18 h after transfection. Cells were fixed 48 h after transfection for IFA. BrdU was added to the culture medium at final concentration of 10 μM for 30 min before fixation when appropriate.

Transient *Dpn*I replication assay. For transient replication assays (8), DNA transfection was carried out as described above for IFA except that 1.8 μg of each plasmid DNA carrying UL5, UL8, UL9, UL42, UL52, SSB, Pol, and oriS was used per 100-mm-diameter dish. Transfected Vero cells were harvested 48 h after transfection. PBS was used to wash the transfected cells twice before the cells were scraped into 2 ml of 150 mM NaCl–40 mM Tris (pH 7.5). Pelleted cells were incubated with 100 μg of RNase A per ml for 1 h at 37°C followed by addition of 2 ml of lysis buffer containing 10 mM Tris (pH 8.0), 10 mM EDTA, 2% sodium dodecyl sulfate (SDS) and 100 μg of proteinase K per ml for 2 h at 37°C. Lysed cells were subsequently extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform-isoamylalcohol (24:1). The upper layer of cellular DNA was precipitated in 70% ethanol containing 0.3 M sodium acetate (pH 5.2) at −20°C overnight. DNA was pelleted by centrifugation and washed with 70% ethanol before being resuspended in 300 μl of distilled H_2O. Each DNA sample (200 μl) was digested with 40 U of *Hin*dIII at 37°C overnight to generate linear monomers of the target oriS DNA from pMC110. Samples (10 μg) of cellular DNA were digested with 30 U of *Dpn*I at 37°C overnight. The digested cellular DNA was resolved by electrophoresis on a 0.8% agarose gel at 1.0 V/cm overnight. The DNA was denatured by incubating the gel in 0.2 M HCl for 10 min at RT followed 0.4 M NaOH and 0.6 M NaCl for 20 min at RT. The DNA was transferred to a NytrAN membrane (Schleicher & Schuell), which had been treated in 10× SSC (1.5 M NaCl, 0.15 M sodium citrate) for 20 min at RT, by vacuum transfer for 1 h and cross-linked by UV radiation onto the membrane after air drying for 1 h at RT. The membrane was preincubated in prehybridization buffer (0.75 M NaCl, 0.05 M Na_2HPO_4, 5 mM Na_2EDTA, 5 mg of Carnation nonfat dried milk per ml, 0.5 mg of heparin per ml, 60 mg of polyethylene glycol 8000, 0.2 mg of denatured salmon sperm DNA per ml, 10% formamide, 1% SDS) at 60°C for 2 h. A gel-purified 230-bp *Sma*I oriS fragment (100 ng) from pMC110 DNA was labeled with [α-^{32}P]ATP and Klenow DNA polymerase by random priming to obtain a specific activity of 10^8 cpm per μg. The membrane was incubated in hybridization buffer containing 10^5 cpm of the probe DNA per ml at 60°C overnight then washed twice with 0.1× SSC–0.1% SDS at 65°C for 45 min before being exposed to Kodak XAR5 film with an intensifying screen at −80°C for 5 days.

IFA. Infected or transfected cells were washed in 1× Tris-saline (100 mM NaCl, 10 mM Tris-HCl [pH 7.5]), fixed with 1% paraformaldehyde in PBS for 10 min at RT, and then permeabilized in 0.2% Triton X-100 in PBS for 20 min on ice. To expose incorporated BrdU residues, pulse-labeled cells were incubated with 4 N HCl for 10 min at RT and then washed for 10 min in PBS. The primary mouse monoclonal antibody (MAb) and rabbit polyclonal antibody (PAb) were diluted together in PBS with 2% goat serum for double labeling or diluted separately for single labeling. Primary antibodies were incubated for 1 h at 37°C and then incubated with the appropriate combination of fluorescein isothiocyanate (FITC)-conjugated and rhodamine-conjugated anti-mouse, anti-rabbit, or anti-human secondary antibodies at 1:100 dilution for 30 min at 37°C for double-labeling. Rhodamine-conjugated anti-mouse secondary antibody was diluted at 1:100 for single labeling. Antibodies used included mouse anti-p53 MAb-1 (Oncogene Science, Inc.), mouse anti-BrdU MAb (Becton Dickinson), and human anti-nucleoli agent (ANA-N) antibody (Sigma). Mouse anti-SSB 39S MAb, anti-IE175 58S MAb, rabbit anti-IE110(N) PAb, and rabbit anti-IE175(N) peptide PAb were described elsewhere (38). Rabbit anti-IE68(N) peptide PAb was generated by immunization with the keyhole limpet hemocyanin-conjugated peptide (14-KARRPALRSPPLGTRK-29) by procedures described previously (45). Rabbit anti-SSB PAb 3-83 was generously provided by David Knipe (Harvard Medical School). Slides were screened and photographed with a 40× oil immersion objective on a Leitz Dialux 20EB epifluorescence microscope, using Kodak T-MAX P3200 and appropriate narrow-band FITC or rhodamine filters.

RESULTS

Characterization of BrdU-labeled replication structures in HSV-infected Vero cells. For an initial examination of the patterns of BrdU incorporation and formation of DNA replication-related structures, we infected Vero cells with HSV-1(KOS) at a relatively low MOI (<1 PFU per cell). In both virus-infected and mock-infected cultures, cellular DNA initiation sites that were pulse-labeled by BrdU for 30 min were randomly distributed as nuclear speckles or networks (replisomes) in 20 to 30% of the cells, presumably representing those in S-phase (Fig. 1b and d). However, in virus-infected cells, some of the BrdU-labeled DNA synthesis initiation sites instead formed distinctive globular or large irregularly shaped nuclear structures at 6 h postinfection; and these usually colocalized with HSV RC, as detected by antibody to the viral SSB in double-label IFA experiments (Fig. 1a and b and c and d).

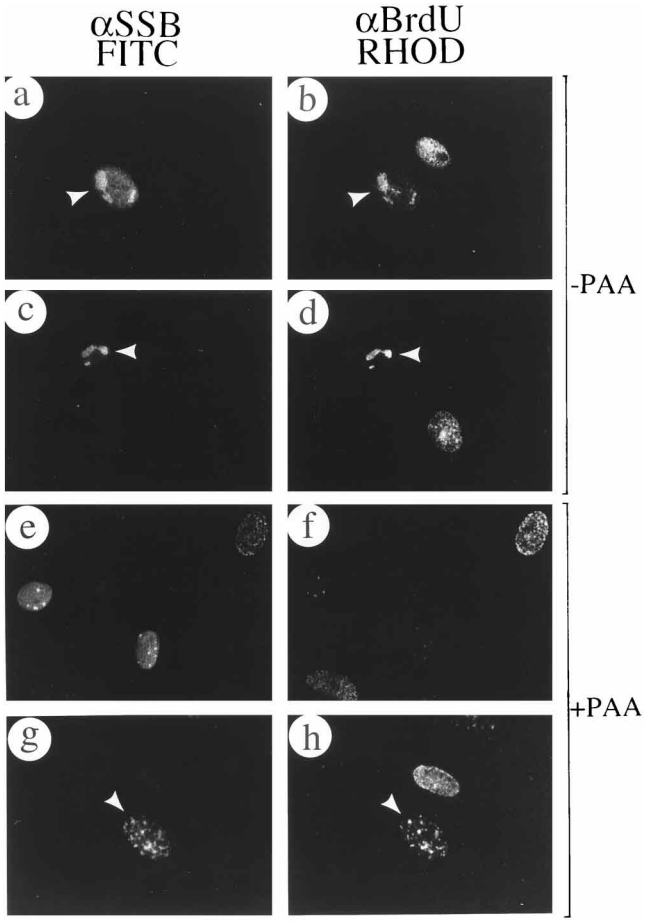

FIG. 1. SSB and BrdU incorporation into viral RC in HSV-infected Vero cells in the presence or absence of PAA. Infected cells were labeled by 10 μM BrdU for 30 min before fixation at 6 h after infection in the absence of PAA (a to d) or in the presence of 400 μM PAA (e to h). SSB (UL30 or ICP8) was detected by IFA with FITC-labeled anti-SSB PAb 3-83 in panels a, c, e, and g; incorporated BrdU was detected by rhodamine-labeled anti-BrdU MAb in panels b, d, f, and h. Panels a and b, c and d, e and f, and g and h show the paired double-label immunofluorescence images of the same fields. In some virus-infected cells (arrowed), BrdU was incorporated into either complete viral DNA RC (a to d) or pre-RF (g and h).

The formation of complete HSV replication compartments was inhibited when PAA was added at a final concentration of either 100 or 400 μg/ml to specifically block viral DNA synthesis. Under these conditions, all of the larger structures disappeared and viral SSB was instead found in relatively small punctate or speckled nuclear structures, which have been shown previously to colocalize with sites of pulse-labeled BrdU incorporation and referred to as prereplication foci (14). The percentages of cells that incorporated BrdU during an infection experiment in Vero cells are shown in Table 1 (experiment A). In uninfected cultures, 29% of the cells incorporated BrdU into replisome speckles at 6 h, whereas 77% of cells infected in the absence of PAA were labeled with BrdU at 6 h, and 89% of the SSB-positive cells and 94% of those containing either globular or large irregular viral replication compartments incorporated BrdU. However, only 53% of cells with large rep-

TABLE 1. Comparison of Percentages of SSB-positive cells that incorporate BrdU during HSV infection or after transient expression in cotransfection assays[a]

IFA pattern	% of cells incorporating BrdU				
	Mock	Infected with:		Transfected with:	
		KOS − PAA	KOS + PAA	Rep − PAA	Rep + PAA
Expt A (infection [6 h])					
SSB^+/total	<0.1	80	78		
$BrdU^+$/total	29	77	48		
$BrdU^+/SBB^+$	NA[b]	89	53		
RC^+/SSB^+	NA	27[c]	1		
Pre-RF^+/SSB^+	NA	47[d]	51[d]		
$BrdU^+/RC^+$	NA	94[c]	NA		
$BrdU^+$/pre-RF^+	NA	87[d]	89[d]		
Expt B (DNA transfection [48 h])					
SSB^+/total	<0.1			6	6
$BrdU^+/SSB^-$	25			24	65
$BrdU^+/SBB^+$	NA			66	7
RC^+/SSB^+	NA			82[c]	37
Pre-RF^+/SSB^+	NA			4	29[e]
$BrdU^+/RC^+$	NA			75[c]	8
$BrdU^+$/pre-RF^+	NA			100	4
$BrdU^+$/uniform $diffuse^+$	NA			8	3

[a] All experiments were carried out in Vero cells in the presence or absence of PAA. DNA transfection involved the complete Rep + oriS plasmid mixture. Mock-infected or mock-transfected cells (Mock) were used as controls. Double-label IFA was performed with rabbit anti-SSB PAb 3-83 and mouse anti-BrdU MAb. A pulse of 10 μM BrdU was incorporated for 30 min before fixation for IFA. $BrdU^+$/total = fraction of total cells incorporating BrdU, etc.

[b] NA, not applicable.

[c] Includes both globules (pre-RC) and irregular bodies (full RC).

[d] Predominantly speckled or micropunctate structures that colocalize with BrdU-labeled replisomes (see Table 2).

[e] Includes primarily punctate plus a few speckled or micropunctate forms (see Table 4).

lication structures were labeled with BrdU when PAA was used to inhibit viral DNA replication. The higher percentage of cells with replication structures that labeled with BrdU in the absence of PAA indicates that ongoing viral DNA synthesis was probably occurring in these structures. Curiously, the percentage of cells with SSB in small punctate or speckled pre-RF that incorporated BrdU in a similar pattern remained at just under 90% both before and after PAA treatment. The latter result appears to indicate that these cells may have been undergoing cellular but not viral polymerase-driven DNA synthesis.

Recognition of two distinct types of viral pre-RF. To further evaluate the effects of PAA on SSB-associated structures observed in infected cells, we compared and tabulated the several different categories of IFA patterns observed in parallel sample cultures at 6 h after infection (Table 2). Five distinct SSB patterns were recognized, four in the presence of PAA and two in the absence of PAA. First, nearly 40% of the SSB-positive cells contained between 50 and 200 speckled or micropunctate SSB structures that closely resembled the replisomes seen in uninfected S-phase cells (Fig. 1g). Indeed, these structures were predominately colocalized with strongly BrdU-incorporating speckled patterns in the absence of PAA, and both the SSB and BrdU patterns were totally unaffected by the presence of PAA (Fig. 1h). Another 21% of the SSB-positive cells contained a mixture of several larger globules together with the speckled pattern, although only the speckled structures labeled strongly with BrdU and the globules disappeared in the presence of PAA. Approximately 27% of the infected cells contained between 2 and 10 spherical globular SSB-positive structures of various sizes without the speckled background. These structures, referred to as prereplication compartments (pre-RC), usually labeled weakly with BrdU in the absence of PAA but disappeared almost completely in the presence of PAA. Finally, 13% of the SSB-positive cells at this stage of infection displayed large irregular bodies or multilobed structures that sometimes nearly filled all of the nucleoplasm surrounding the nucleolus (similar to the structures shown in Fig. 2d to f or 4a and c). These forms, which we refer to as true fully active virus RC, all labeled strongly with BrdU, and their formation was abolished in the presence of PAA. At later stages of infection, cells with these complete RC became much more abundant.

In contrast, in the presence of PAA, essentially only two types of patterns were observed, either or both of which represent pre-RF. Approximately 57% of the SSB-positive cells contained exactly the same patterns of small numerous BrdU-labeled SSB speckles described above that were unaffected by PAA and which we have interpreted to be associated with cellular S-phase replisomes (Table 2). However, 40% displayed a novel SSB pattern not seen in the absence of PAA, in which between two and five small punctate spots were accompanied by a uniform diffuse background nuclear staining (Fig. 1e). Most of these cells failed to incorporate any BrdU either into the punctate spots or into any speckled patterns and were therefore interpreted to represent non-S-phase cells.

Assembly of HSV RC in transient DNA transfection assays. We next asked whether similar viral DNA replication-related structures can be formed in transient expression assays in DNA cotransfected cells receiving just the seven essential HSV replication genes UL5, UL8, UL9, UL42, UL52, SSB, and Pol under the control of the strong constitutive HCMV enhancer-promoter region. Various combinations of these genes were cotransfected into Vero cells together with the HSV oriS target plasmid DNA (pMC110). Initially, a plasmid encoding the viral SSB gene (pSSB) was transfected alone, and the expressed SSB protein (detected with anti-SSB 39S MAb) was found to be distributed in a typical uniform nuclear diffuse pattern at 48 h after transfection (Fig. 2a to c). In contrast, when the

TABLE 2. Alteration in the patterns of SSB- and BrdU-associated replication structures in HSV-infected cells in the presence and absence of PAA

SSB IFA pattern[a]	Level of BrdU incorporation[b] (%)					
	Strong		Weak		Negative	
PAA added	−	+	−	+	−	+
Speckled or micropunctate[c]	30	50	2	1	7	6
Globules[d] plus speckled[c]	17	<1	4	<1	<1	<1
Spherical globules[c] only (=pre-RC)	3	<1	20	1	4	1
Large irregular bodies (=RC)	13	<1	<1	<1	<1	<1
Few punctate plus diffuse (=pre-RF)	<1	<1	<1	1	<1	40
Total	63	50	26	3	11	47

[a] Vero cells at 6 h after infection with HSV-1(KOS) at an MOI of 5; 80% of the cells were positive for SSB; the total numbers of cells scored were 102 in the absence of PAA (−) and 82 in the presence of PAA (+).

[b] Strong BrdU incorporation represented FITC and rhodamine IFA signals of approximately equal intensity, whereas cells with weak BrdU incorporation had much stronger SSB IFA signals than BrdU signals.

[c] The SSB pattern was predominantly colocalized with a speckled replisome-like BrdU pulse-label pattern both before and after PAA.

[d] Globules show a large range of different sizes.

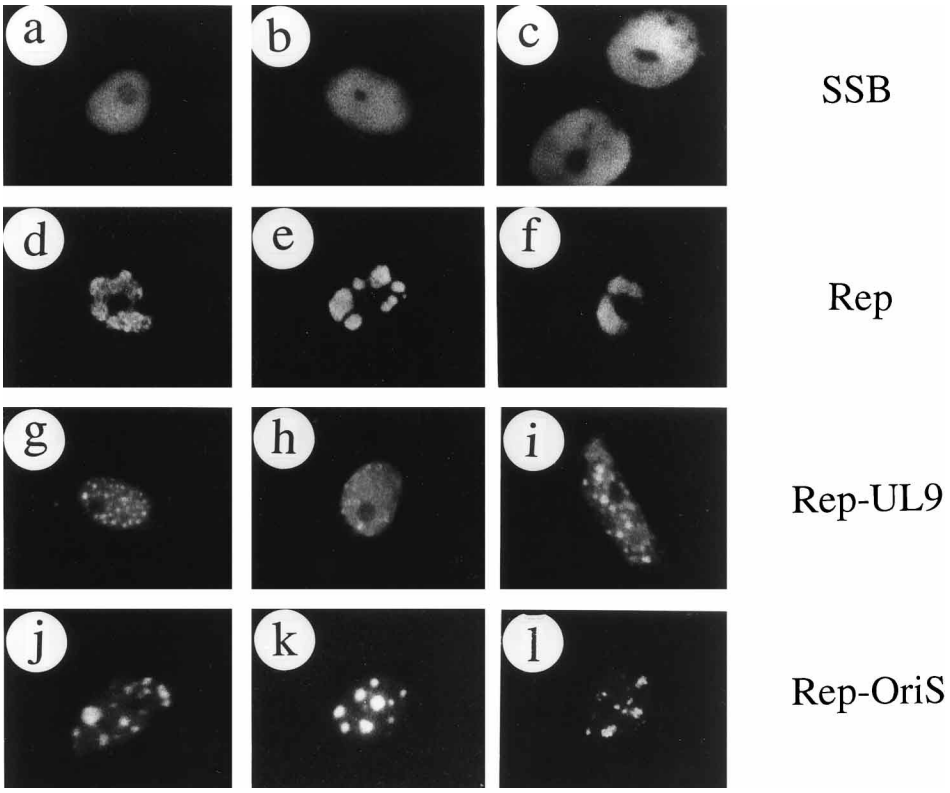

FIG. 2. Assembly of HSV RC in cotransfected cells. All panels show three examples of single-label IFA patterns for SSB detected with rhodamine-labeled anti-SSB 39S MAb in transfected Vero cells. (a to c) Typical nuclear diffuse pattern in cells receiving only the plasmid carrying the SSB gene; (d to f) assembled replication compartments in cells cotransfected with the oriS plasmid pMC110 and expression plasmids encoding UL5, UL8, UL9, UL42, UL52, SSB, and Pol; (g to i) diffuse plus pre-RF patterns in cells cotransfected with oriS and plasmids encoding UL5, UL8, UL42, UL52, SSB, and Pol without UL9; (j to l) pre-RC in cells cotransfected with plasmids encoding UL5, UL8, UL9, UL42, UL52, SSB, and Pol without oriS.

whole set of seven replication genes and oriS were cotransfected into Vero cells, most of the SSB-positive cells formed either globular structures (pre-RC [not shown]) or large irregularly shaped bodies that closely resembled the complete HSV RC obtained in virus-infected cells (Fig. 2d to f). Cotransfected cells receiving plasmids carrying the other six essential HSV core replication genes only, but omitting the UL9 origin DNA-binding protein gene, failed to assemble any large replication-associated structures. Instead, SSB remained in numerous punctate or small globular pre-RF within a diffuse nuclear background (Fig. 2g to i). However, in the absence of oriS, SSB still formed several mid-sized spherical globules in most DNA-transfected cells (Fig. 2j to l), and there were even a few cells that contained large irregular bodies similar to the complete RC assembled in the presence of oriS. Therefore, oriS was not required for assembly of the large globular replication structures formed in transiently transfected cells, but it did appear to increase the efficiency of formation of complete RC. Most of the large globular structures formed in the absence of HSV oriS probably represent intermediates between the pre-RF and active viral RC, and therefore we will also refer to them as pre-RC on the presumption that they do not synthesize viral DNA.

Functional characterization of the HSV replication plasmids. A transient *Dpn*I resistance replication assay was also carried out by using a ^{32}P-labeled oriS-containing DNA fragment as a probe on a Southern blot to confirm that our plasmid DNA containing HSV oriS was replicated in the same cotransfected cultures and under the same transfection conditions used for the assembly of the HSV RC (Fig. 3). The full set of plasmids carrying genes encoding all seven replication proteins and the oriS target plasmid was cotransfected into Vero cells. Cells receiving the same complete Rep plasmid mixture but omitting the plasmid carrying the Pol gene (Rep − Pol) or the oriS-containing plasmid, (Rep − oriS) were included in the same replication assay as negative controls. Input oriS-containing DNA plasmid and amplified DNA were cleaved to give a 3.3-kb linear monomer band by digestion with *Hin*dIII in cells transfected with Rep (lane 1) or Rep − Pol (lane 2). Since cells transfected with the Rep − oriS mixture did not have oriS-containing DNA, no input DNA was detected in that sample (lane 3). Somewhat more monomer linearized oriS-containing DNA was recovered from cells transfected with Rep than that with Rep − Pol, although each received the same amount of input transfected oriS DNA. By double digestion with *Dpn*I and *Hin*dIII, amplified DNA that was resistant to methylation-specific digestion by *Dpn*I was also detected as a linear band of 3.3 kb. As expected, a significant amount of oriS-containing DNA was found to be resistant to *Dpn*I digestion from cells transfected with the complete Rep mixture (lane 4), whereas there was no *Dpn*I-resistant replicated DNA detected from cells transfected with Rep − Pol (lane 5) or Rep − oriS (lane 6). This result confirms that the oriS-containing DNA was replicated in those transfected Vero cells receiving our complete set of Rep plasmids but not when Pol was absent.

Inhibition of viral DNA synthesis with PAA blocks the assembly of HSV RC in cotransfected cells. Since the formation of complete RC in HSV-infected cells was inhibited by PAA,

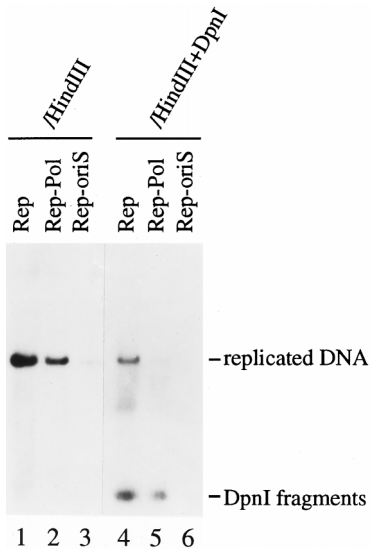

FIG. 3. HSV oriS-dependent transient DNA replication assay in cotransfected Vero cells receiving all seven essential viral replication proteins. Southern blotting to detect oriS DNA was performed with size-fractionated cellular DNA from transfected Vero cells receiving various sets of plasmid DNAs. Lane 1, UL5, UL8, UL9, UL42, UL52, Pol, and SSB genes plus oriS; lane 2, complete sets of plasmids except that the plasmid encoding the Pol gene was omitted; lane 3, complete set of plasmids except that the oriS-containing plasmid was omitted. Each DNA sample (10 μg) was digested with *Hin*dIII to linearize the input oriS-containing plasmid or digested with *Hin*dIII and *Dpn*I to detect amplified unmethylated oriS-containing DNA in transfected cells. A 230-bp *Sma*I-*Sma*I fragment containing oriS isolated from pMC110 was used as the hybridization probe.

the effect of PAA on the assembly of RC in transfected cells was also studied. Compared with the SSB distribution in replication compartments in the absence of PAA (Fig. 4a and c), the addition of PAA proved to efficiently inhibit the assembly of virtually all large RC and SSB remained predominantly as small nuclear globules or punctate structures in the presence of PAA (Fig. 4e and g). Quantitatively, the proportion of SSB-positive cells with globules or large irregular bodies decreased from 82 to 37%, and those with small punctate structures increased from 4 to 29% in one typical experiment after addition of PAA (Table 1, experiment B).

Because of possible morphological similarities between the assembled pre-RC or RC with large globular nucleoli, anti-SSB 39S MAb and an ANA-N antibody were also used in double-label IFA experiments to compare the two structures in DNA-transfected cells. The results confirmed that the assembled globular replication compartments were clearly not associated with nucleolar domains labeled by the ANA-N antibody (Fig. 4a to d). Similarly, the SSB-positive punctate structures formed in the presence of PAA were quite distinct from nucleoli (Fig. 4e to h).

Assembly of HSV RC requires each of the seven HSV essential replication proteins. In transient *Dpn*I replication assays, all seven essential HSV replication gene products, including UL5, UL8, UL9, UL42, UL52, SSB, and Pol, are required for the replication of plasmid DNA containing HSV oriS. Elimination of each of these gene products one at a time was tested to identify the essential protein requirements for assembling HSV replication-associated structures in transient cotransfected cells. In the presence of the whole set of replication gene products and oriS, complete HSV replication compartments were frequently detected by the anti-SSB 39S MAb (Fig. 5a and b). In the absence of UL5, UL8, or UL52, SSB gave a somewhat uneven nuclear diffuse distribution only (Fig. 5c to h). However, consistent with previous mutant HSV infection data (31, 33), SSB formed numerous small nuclear micropunctate structures in the absence of Pol or UL42 (Fig. 5i to l). Furthermore, SSB gave a similar nuclear punctate pattern in the presence of UL5, UL8, and UL52 only (Fig. 5m and n). Therefore, the helicase-primase complex of UL5, UL8, and UL52 was all that was required for SSB to localize into nuclear micropunctate structures that are similar to some of the pre-RF seen in infected cells in the absence of viral DNA synthesis. However, because each of the seven essential HSV replication gene products was required for SSB to locate in complete assembled RC, the results are fully consistent with the requirements for the amplification of HSV oriS DNA plasmids in the *Dpn*I replication assay in transfected cells. Therefore, based on the similarities between the assembled compartments and those found in HSV-infected cells, it is reasonable to claim that even after transient expression in DNA-cotrans-

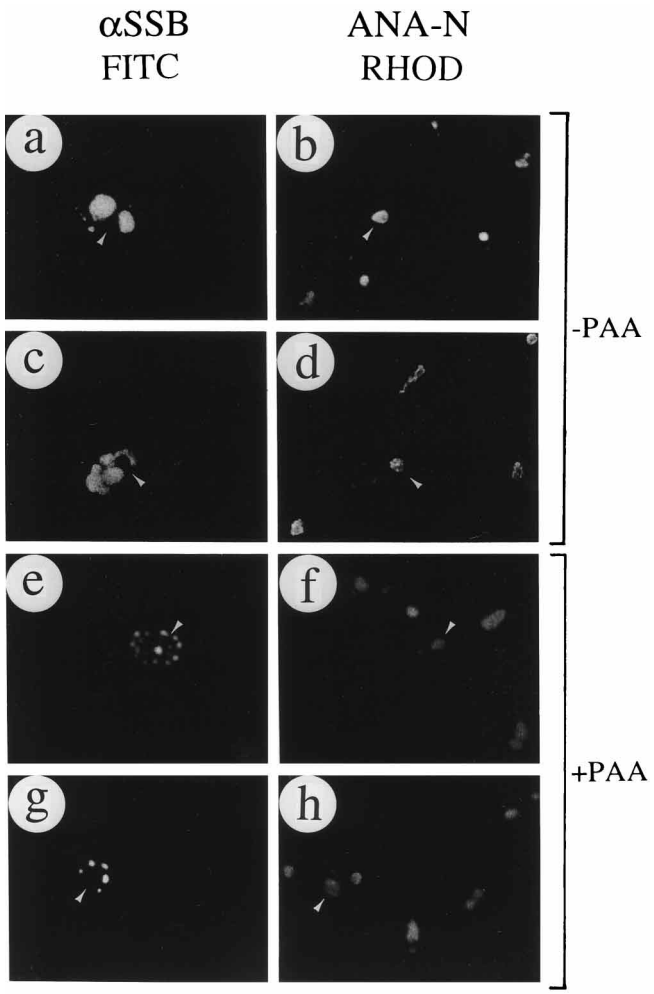

FIG. 4. Neither complete RC nor pre-RF assembled in the presence of PAA are associated with nucleoli. oriS DNA and the whole set of plasmids encoding UL5, UL8, UL9, UL42, UL52, SSB, and Pol were cotransfected in the absence of PAA (a to d) or in the presence of 400 μM PAA (e to h). SSB was detected by rhodamine-labeled anti-SSB MAb 39S in panels a, c, e, and g; nucleoli were detected by FITC-labeled ANA-N antibody in panels b, d, f, and h. Panels a and b, c and d, e and f, and g and h are paired double-label frames for the same fields. Arrowed cells show that nucleoli are localized outside SSB RC in the absence of PAA (a to d) and outside pre-RF in the presence of PAA (e to h).

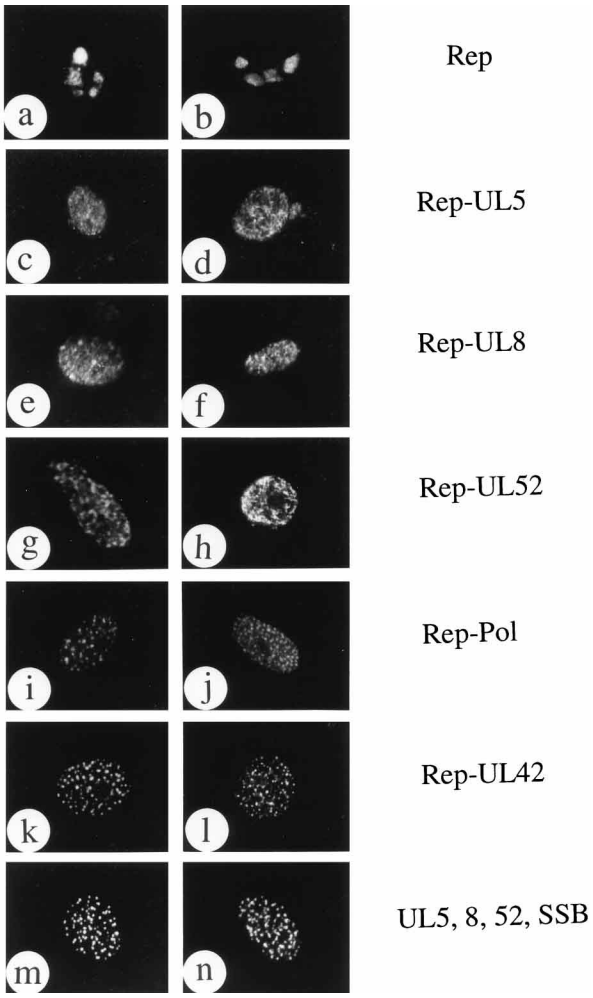

FIG. 5. All seven essential viral replication gene products are required for the assembly of complete HSV RC in cotransfected cells. SSB was detected by rhodamine-labeled anti-SSB 39S MAb. (a and b) Two separate single-label frames showing cells cotransfected with oriS DNA and the whole set of plasmids encoding UL5, UL8, UL9, UL42, UL52, SSB, and Pol; (c to l) omission experiments showing cotransfection of all plasmids except the one encoding UL5 (c and d), UL8 (e and f), UL52 (g and h), Pol (i and j), or UL42 (k and l); (m and n) cotransfection of plasmids encoding UL5, UL8, UL52, and SSB only.

fected cells, SSB can be localized into structures that represent assembled fully active HSV RC and that the presence of UL5, UL8, UL9, UL42, UL52, Pol, and SSB is both necessary and sufficient for the process.

Incorporation of BrdU into assembled HSV replication structures. Because the assembled HSV RC produced by DNA transfection are morphologically similar to those formed in virus-infected cells, we asked whether DNA synthesis initiation as defined by BrdU incorporation also occurred at these sites. The results revealed that in the presence of all seven replication proteins and the oriS target plasmid DNA (Rep), a 30-min pulse with BrdU was incorporated into newly synthesized DNA in 66% of the SSB-positive cells and as many as 75% of the assembled RC (Table 1, experiment B), where it often colocalized with SSB (Fig. 6a and b). However, with the same DNA plasmid mixture in the presence of PAA, BrdU was incorporated into only 7% of the SSB-positive cells (Table 1, experiment B). In the absence of HSV polymerase, a few SSB-positive structures colocalized with DNA synthesis initiation sites, and these resembled micropunctate pre-RF whose distribution was similar to the speckled BrdU patterns obtained in untransfected S-phase cells (Fig. 6c and d). Globular pre-RC that still incorporated BrdU were also formed relatively frequently in the Rep − oriS mixture (Fig. 6e and f).

To assess the effect of omission of core protein components on the BrdU incorporation patterns of replication structures formed in transient assays, we tabulated the results from a set of experiments similar to these described above (Table 3). Approximately 50 SSB-positive cells were scored in each sample. In the complete Rep control, 64% of the SSB-positive cells contained either globules or irregular bodies with strong colocalized BrdU patterns, whereas there were very few colocalized speckles or punctate structures (6% in Table 1, experiment B). In contrast, the Rep − UL42, Rep − Pol, and Rep − UL9 samples all gave nearly 70% uniform diffuse SSB patterns without any BrdU incorporation (Table 3). However, most of the remaining 30% gave micropunctate SSB, and approximately half of these occurred in cells with S-phase-like speckled BrdU patterns of which at least some were colocalized (Fig. 6c and d). In the case of the sample receiving UL5, UL8, UL52, and SSB only, 67% of the SSB-positive cells were speckled or micropunctate and almost 25% colocalized with

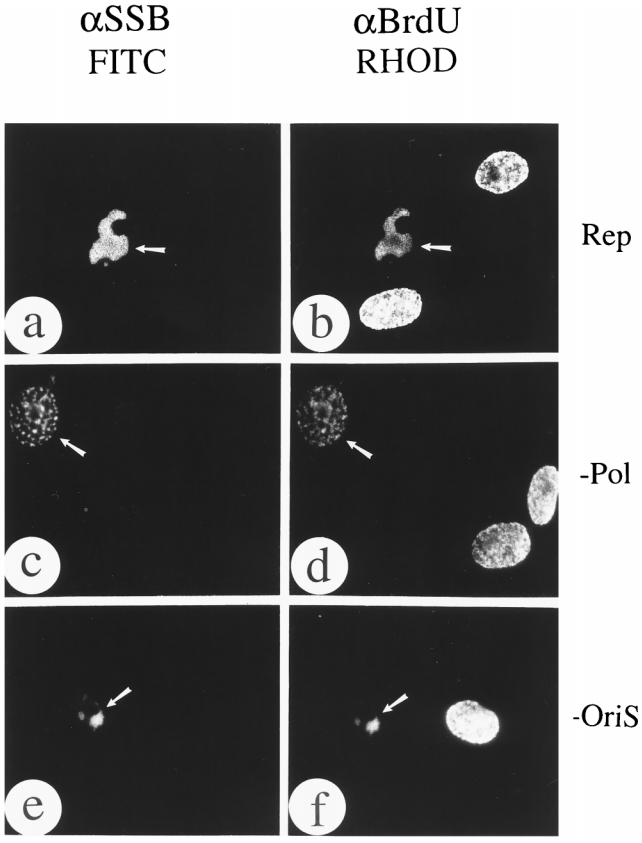

FIG. 6. Colocalization of assembled RC and BrdU-labeled DNA initiation sites. SSB was detected with FITC-labeled rabbit anti-SSB 3-83 PAb in panels a, c, and e; incorporated BrdU was detected with rhodamine-labeled anti-BrdU MAb in panels b, d, and f. (a and b) oriS DNA and the whole set of plasmids encoding UL5, UL8, UL9, UL42, UL52, SSB and Pol cotransfected together; (c to f) cotransfection of all plasmids except the one encoding Pol (c and d) or the one encoding oriS (e and f). Paired panels show the immunofluorescence images of the same fields labeled with anti-SSB (a, c, and e) and anti-BrdU (b, d, and f) from each transfection. In some cotransfected cells (arrowed), incorporated BrdU colocalized with the SSB protein in either RC (a and b), pre-RF (c and d), or pre-RC (e and f).

TABLE 3. Effect of removing UL42, Pol, or UL9 from the complete transfection plasmid mixture[a]

SSB IFA pattern	No. of SSB-positive cells											
	Complete Rep		Rep − UL42		Rep − Pol		Rep − UL9		Rep − all 3		SSB alone	
BrdU incorporation	+	−	+	−	+	−	+	−	+	−	+	−
Uniform diffuse	0	5	0	34	0	32	0	34	0	9	2	47
Speckled micropunctate (=pre-RF)	1	0	8	11	11	4	2	7	10	18	0	0
Few punctate (=pre-RF)	2	4	0	2	1	1	0	1	0	2	0	1
Globular (=pre-RC)	16	6	0	1	0	4	0	7	0	4	0	0
Irregular bodies (=RC)	13	3	0	0	0	0	0	0	0	0	0	0
Total (%)	64	36	17	83	22	78	4	96	30	70	4	96

[a] Vero cells received either the complete Rep + oriS mixture of plasmid DNAs or mixtures lacking the components indicated. A total of between 43 and 56 SSB-positive cells were scored in each sample. All cell cultures were pulse-labeled with BrdU for 30 min at 48 h after transfection and then scored for the presence (+) or absence (−) of BrdU incorporation and SSB patterns by double-label IFA.

S-phase-like BrdU patterns (Table 3). Surprisingly, even when SSB was transfected alone, 98% of the cells gave a uniform diffuse SSB-positive pattern but only 5% of these displayed S-phase BrdU speckles (Table 3), whereas the normal 25% of nonexpressing cells still did so. Evidently expression of SSB in a uniform diffuse pattern occurs only in non-S-phase cells under the conditions of our transient assays, whereas assembly into micropunctate or replisome-like patterns together with UL5, UL8, and UL52 shows some preference for S-phase cells.

Inhibitory effect of PAA on BrdU incorporation into RC in transient assays. A quantitative comparison of the incorporation of BrdU into the various different SSB-associated replication-related structures in a transient expression assay in the presence and absence of PAA is shown in Table 4. In DNA-transfected Vero cells receiving the full Rep plasmid mixture plus oriS in the absence of PAA, the predominant patterns observed in SSB-positive cells were the typical spherical globules (pre-RC) and large irregular bodies (RC) similar to those found in virus-infected cells. In this experiment, up to 53% of the cells contained globules, with 18% showing strong colocalized BrdU staining and another 20% giving a weak BrdU-positive pattern. Among another 29% of the SSB-positive cells that we categorized as full replication compartments, 13% had strong colocalized BrdU staining and 9% incorporated BrdU relatively weakly.

Virtually all of the full RC and many of the large prereplication globules disappeared in the presence of PAA (400 μg/μl), leaving a new distribution of SSB-positive nuclei consisting of 34% uniform diffuse, 34% globular, and 24% punctate-plus-diffuse patterns (Table 4). Very few of the SSB-positive cells in the presence of PAA (7% overall [Table 1, experiment B]) incorporated any BrdU at all, and only 5% showed the numerous micropunctate foci pattern, of which only a small subset colocalized with a strong BrdU-labeled speckled pattern (1%). In fact, the very rare presence (2% only) of either SSB or BrdU-labeled typical S-phase speckled patterns (even in the absence of PAA) represented the major difference between the IFA results observed in transfected cells compared to virus-infected cells (compare Tables 2 and 4). On the other hand, the 24% of SSB-positive cells in PAA that showed a small number of punctate spots within a uniform diffuse background closely resembled the type of pre-RF seen in infected non-S-phase cells. Furthermore, in the Rep + oriS experiment described above, where only 1% of the SSB-expressing cells grown in PAA for 48 h gave typical speckled BrdU incorporation patterns, the percentage of non-SSB-expressing cells in the same culture that gave strong speckled BrdU-positive patterns increased from 25 to 65% (Table 1, experiment B). We conclude that virtually all of the SSB-associated replicating structures formed in transfected cells are PAA sensitive and that the process of transfection led in some way to synchronization or selection against S-phase characteristics only in those cells that expressed viral proteins.

Role of oriS in formation of replication structures obtained in DNA-transfected cells. To examine further the rather surprising observation that many prereplication globules and even some full RC-like structures were still generated in the Rep − oriS mixtures in the transient transfection assay (Fig. 2j to l; Fig. 6e and f), we tabulated the BrdU incorporation results from two separate experiments carried out in the presence and absence of oriS in which the levels of SSB expression were widely different (Table 5). In experiment A, the transfection efficiency was very high, giving globular or irregular bodies in 93% of the SSB-positive cells and uniform diffuse patterns in only 5% or less, whereas in experiment B, with a much lower transfection efficiency, approximately 55% of the SSB-positive cells displayed uniform diffuse patterns only or a mixture of uniform diffuse plus globules. Again, no more than 1 to 2% of the SSB-positive cells in experiment A gave S-phase-like BrdU-pulse-labeled speckles, whereas in both experiments, 20 to 23% of the cells containing viral SSB structures showed strong BrdU incorporation in the presence of oriS, and 11 to 12% did so in the absence of oriS. Only the number of fully active irregular bodies (RCs) incorporating high levels of BrdU appeared to be significantly affected by the absence of oriS (from 12 to 4% or 11 to 5%), whereas the proportion of cells with globular pre-RC incorporating low levels of BrdU were essentially unaffected. The proportion of such structures that

TABLE 4. Alteration in the patterns of SSB- and BrdU-associated replication structures in transient expression assays in the presence and absence of PAA[a]

SSB IFA pattern	Level of BrdU incorporation (%)					
	High		Low		Negative	
PAA added	−	+	−	+	−	+
Uniform diffuse	1	<1	1	1	12	33
Speckled micropunctate (=pre-RF)	2	1	<1	1	<1	3
Globules[b] only (=pre-RC)	18	1	20	3	15	30
Large irregular bodies (=RC)	13	<1	9	<1	7	3
Few punctate plus diffuse (=pre-RF)	2	<1	<1	<1	<1	24
Total	36	2	30	5	34	93

[a] Vero cells received the complete Rep + oriS mixture of plasmid DNAs; 6% of the cells were positive for SSB; the total numbers of cells scored were 259 in the absence of PAA (−) and 134 in the presence of PAA (400 μg/ml) (+).

[b] Includes mixed globules plus diffuse in some cases. Globules show a large range of different sizes.

TABLE 5. Relatively small effects of the presence or absence of oriS on BrdU incorporation into various replication structures generated in transient expression assays[a]

SSB IFA pattern	Level of BrdU incorporation (%)					
	High		Low		Negative	
OriS DNA added	+	−	+	−	+	−
Expt A (high efficiency)						
Uniform diffuse	<1	<1	<1	<1	5	1
Speckled micropunctate (=pre-RF)	2	1	<1	<1	<1	<1
Globules only (=pre-RC)	11	7	16	15	26	30
Large irregular bodies (=RC)	12	4	11	12	17	30
Total	25	12	27	27	48	61
Expt B (low efficiency)						
Uniform diffuse	<1	<1	<1	<1	29	31
Diffuse plus globules (=pre-RC)	3	1	13	13	8	13
Globules (=pre-RC)	6	6	13	20	8	11
Large irregular bodies (=RC)	11	5	5	11	3	1
Total	20	12	31	34	48	56

[a] Vero cells received the complete Rep mixture of plasmid DNAs with (+) or without (−) the oriS plasmid; 8 and 1.5% of the cells were positive for SSB in experiments A and B, respectively. The total numbers of SSB-positive cells scored were 122 and 126 in experiment A and 82 and 83 in experiment B.

lacked any BrdU incorporation also increased somewhat (from 43 to 60% or 19 to 25%) in the absence of oriS. Although we have not shown directly that BrdU incorporation in this situation is PAA sensitive, we conclude that even in the absence of the specific oriS plasmid, not only was extensive formation of viral replication associated structures occurring, but some DNA synthesis associated with the viral biochemical machinery was probably ongoing also.

Recruitment of the viral IE175 and IE63 proteins into assembled HSV RC. During HSV infection, the viral DNA RC that are formed in many Vero cells by 6 h after infection at low MOI appear to incorporate most of the HSV IE175 protein present at that time into colocalized structures (30, 49). We have also found that some but not all of the IE63 protein present at that time is also incorporated into the RC (62). Since both IE175 and IE63 are essential for viral reproduction, the subnuclear location of both IE175 and IE63 proteins during viral infection is probably important for their biological function. To test whether the assembled HSV RC in cotransfected cells were also able to recruit the IE175 or IE63 proteins, double-label IFA of IE175 or IE63 together with SSB and the assembled RC components was performed in Vero cells.

A typical nuclear diffuse distribution was obtained with anti-IE175 antibody when a plasmid expressing the IE175 gene (pGH114) was transfected alone into Vero cells (IE175) (Fig. 7a and b). However, the IE175 protein was found in either globules or irregularly shaped bodies within the nucleus when the IE175 plasmid was cotransfected with the complete set of seven replication gene plasmids encoding the UL5, UL8, UL9, UL42, UL52, SSB, and Pol proteins and the oriS plasmid. These structures were colocalized with the SSB protein in all cells that contained them (Fig. 7c and d). In the presence of PAA, SSB was found only in relatively small punctate structures (Fig. 7e), but IE175 always remained totally nuclear diffuse in the same cells (Fig. 7e and f). Therefore, in cotransfected cells, the assembled HSV replication compartments were able to recruit the essential IE175 gene product, whereas IE175 did not enter pre-RF. Surprisingly, IE175 relocalization still occurred within the nucleus in assembled replication structures (pre-RC and RC-like) in the absence of oriS. However, only 20% of the cells gave complete colocalization (Fig. 7g and h), whereas the majority of the cells gave a mixed pattern of partial colocalization together with a uniform diffuse background.

IE63 expressed on its own from plasmid pLZ11 in transfected Vero cells is distributed partially in a nuclear diffuse pattern and partly in a punctate colocalized pattern with the SC-35 spliceosome-associated antigen SC35 (44, 51, 52, 62). However, IE63 was also recruited into assembled RC in Vero cells cotransfected with the full set of replication plasmids carrying UL5, UL8, UL9, UL42, UL52, SSB, Pol, and oriS (Fig. 8a and b). When both viral transactivators were cotransfected into Vero cells with the full set of replication plasmids, the assembled replication compartments recruited both IE63 (Fig. 8e and f) and IE175 (Fig. 8g and h) into similar structures. Furthermore, the recruitment of either IE63 or IE175 was independent of the presence of oriS (Fig. 8i to l). IE175 always remained in a nuclear diffuse pattern when SSB was not detected in the same cells (Fig. 8l). Interestingly, in the absence of UL42, some IE63 was still redistributed into nuclear punctate pre-RF containing SSB, although some remained in a

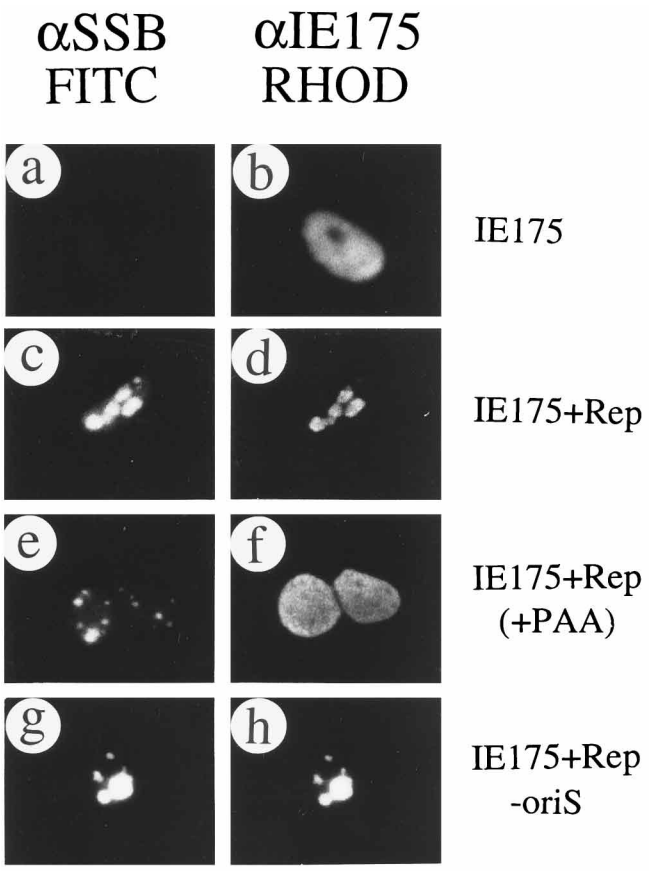

FIG. 7. Recruitment of the HSV IE175 protein by assembled RC. SSB was detected by FITC-labeled anti-SSB 39S MAb in panels a, c, e, and f; IE175 was detected by rhodamine-labeled anti-IE175(N) PAb in panels b, d, f, and h. (a and b) Two immunofluorescence images of the same field when IE175-encoding plasmid pGH114 was transfected alone; (c and d) cotransfection of IE175 (pGH114), oriS, and the whole set of plasmids encoding UL5, UL8, UL9, UL42, UL52, Pol, and SSB; (e and f) cotransfection of IE175 (pGH114), oriS, and the whole set of plasmids encoding UL5, UL8, UL9, UL42, UL52, SSB, and Pol in the presence of PAA; (g and h) cotransfection of IE175 (pGH114) and the whole set of plasmids encoding UL5, UL8, UL9, UL42, UL52, SSB, and Pol without oriS.

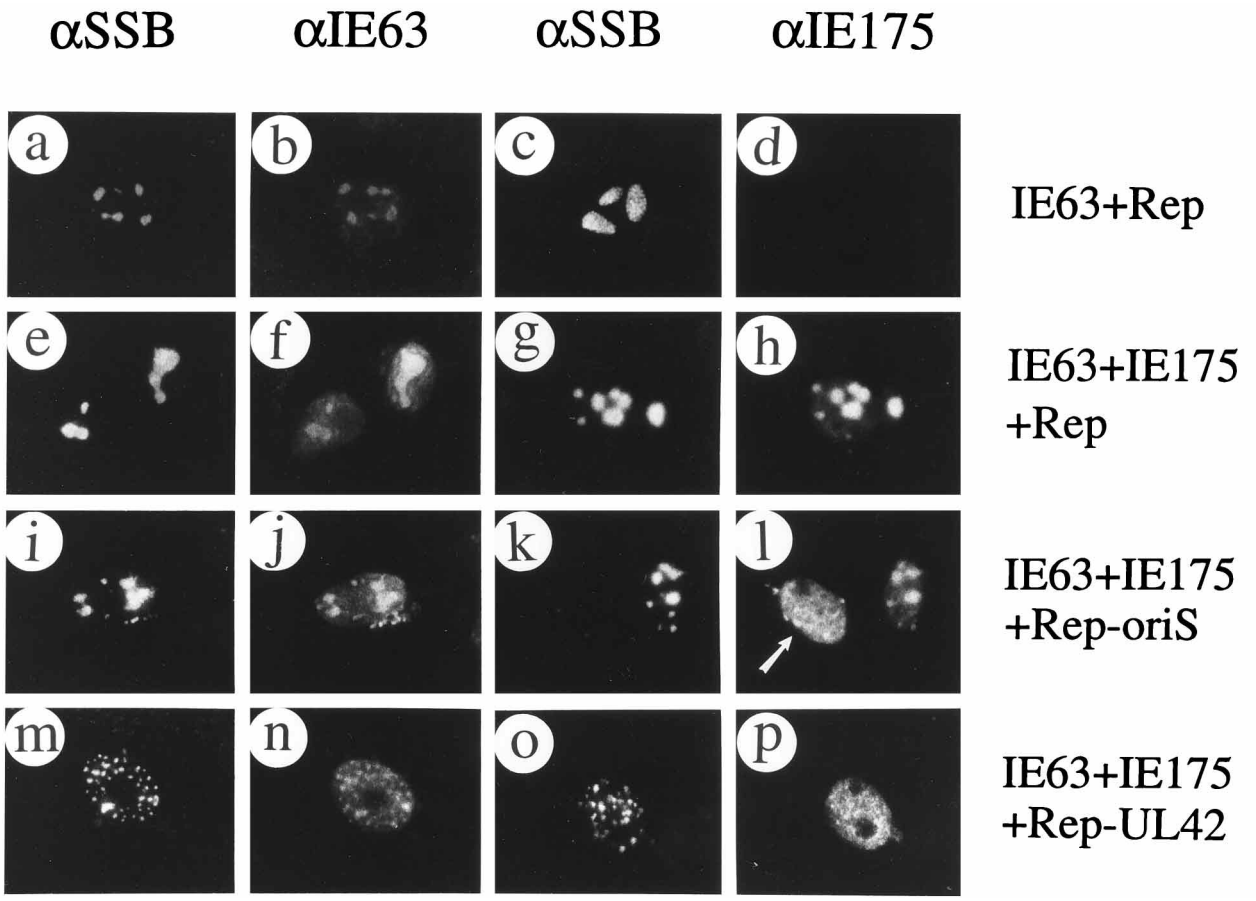

FIG. 8. Recruitment of the HSV IE63 protein in the presence and absence of IE175 by assembled RC. SSB was detected with FITC-labeled anti-SSB 39S MAb (a, c, e, g, i, k, m, and o); IE63 was detected with rhodamine-labeled anti-IE63(N) PAb (b, f, j, and n); IE175 was detected with rhodamine-labeled anti-IE175(N) PAb (d, h, l, and p). Paired double-label immunofluorescence images of the same fields are shown in panels a and b, c and d, e and f, g and h, i and j, k and l, m and n, and o and p. (a to d) Cotransfection of IE63 (pLZ11), oriS, and plasmids encoding UL5, UL8, UL9, UL42, UL52, SSB and Pol; (e to h) cotransfection of IE63 (pLZ11), IE175 (pGH114), oriS, and plasmids encoding UL5, UL8, UL9, UL42, UL52, SSB, and Pol; (i to l) cotransfection of IE63 (pLZ11), IE175 (pGH114), and plasmids encoding UL5, UL8, UL9, UL42, UL52, SSB and Pol (the IE175 protein remained in a nuclear diffuse pattern when SSB RC were not assembled in the same transfected cells [arrowed cells in panel l]); (m to p) cotransfection of IE63 (pLZ11), IE175 (pGH114), oriS, and plasmids encoding UL5, UL8, UL9, UL52, SSB and Pol.

nuclear diffuse background as well (Fig. 8m and n). In contrast, in the same cells, IE175 stayed in a nuclear diffuse pattern, even in those cells displaying SSB as a pattern of nuclear punctate pre-RF (Fig. 8o and p).

Recruitment of p53 by assembled HSV RC in cotransfected cells. Several cellular proteins, including p53, have been suggested to colocalize with HSV RC in HSV-infected cells (59). Since endogenous p53 in Vero cells gives no detectable IFA signal with anti-p53 MAb-1, plasmid pSVp53 expressing the wild-type p53 protein was transfected into Vero cells in the presence or absence of complete set of plasmids carrying UL5, UL8, UL9, UL42, UL52, SSB, Pol, and oriS. Expression of p53 alone produced a typical nuclear diffuse pattern (p53) (Fig. 9a and b), but double-label IFA of cotransfected cells revealed that p53 was efficiently redistributed into the HSV replication compartments together with SSB (Fig. 9c to h). p53 is known to be involved in DNA repair and the G_1/S cell cycle control checkpoint (22) and has been suggested to colocalize with viral DNA replication compartments in HSV-infected cells (59). Therefore, the colocalization between p53 and the assembled HSV RC in transiently cotransfected cells strengthens the idea that these assembled RC are biologically functional and that this model may provide a simplified system to study how host cellular factors contribute to viral DNA replication.

Specificity of the recruitment by assembled HSV RC. The assembled HSV RC have many similarities to those found in HSV-infected cells based on the requirement for all seven essential replication gene products, colocalization with cellular DNA synthesis initiation sites, the inhibition effect by PAA, and the ability to recruit both the viral IE175 and IE63 proteins and p53. To test the specificity of the recruitment of HSV nuclear proteins by the assembled HSV RC, the other two HSV IE nuclear proteins, IE110 and IE68, were also tested by cotransfection in the presence of UL5, UL8, UL9, UL42, UL52, SSB, Pol, and oriS. In infected Vero cells, both IE110 and IE68 gave a nuclear punctate distribution, which is unrelated to the viral RC (61). Similarly in DNA-transfected cells, both IE110 and IE68 remained in typical nuclear punctate patterns despite the presence of the assembled SSB-positive RC in the same cells (Fig. 10). Even though IE110 has been demonstrated to be able to associate with many other HSV proteins in punctate structures in cotransfected cells, including IE175 (38), IE63 (62), IE68 (62), and UL5, UL8, and UL52 (33), as well as with cellular proteins such as p53 and RAG-1 (62), IE110 evidently does not associate with assembled RC in DNA-transfected cells. This result demonstrates both the specificity of recruitment by assembled RC and the selectivity of

colocalization between IE110 and other viral or cellular proteins.

DISCUSSION

Efficient assembly of functionally active HSV RC in transient expression assays. Since HSV origin-specific DNA replication can be reproduced in *Dpn*I resistance cotransfection assays, and the requirements for viral replication proteins are the same as those in infected cells, we investigated whether the transient transfection system could also be extended visually at the level of single cells to understand how HSV RC are assembled. By cotransfection of constitutive expression plasmids encoding only UL5, UL8, UL52, and SSB into Vero cells, we were able to form numerous SSB-containing micropunctate structures, which are similar to the pre-RF described recently by others (31). Importantly, as summarized in the model in Fig. 11, we took this a step further to demonstrate that large globular structures or irregularly shaped bodies containing SSB were also observed in the nucleus when the complete set of Rep plasmids carrying each of the seven HSV essential replication genes were cotransfected into Vero cells together with the HSV oriS-containing target plasmid. This is the first demonstration of the apparently complete assembly of functional HSV RC in DNA-transfected cells.

These SSB RC-like structures have been examined in several different ways to determine whether they are biologically functional. The viral protein requirements for assembly were demonstrated to be the same as for positive signals in the *Dpn*I DNA replication assay in transfected cells, as well as for viral DNA replication in virus-infected cells (28, 58). Importantly, omission of any one of the seven essential HSV replication proteins abolished assembly of the largest forms of the RC, although much smaller micropunctate structures resembling pre-RF remained, especially in the absence of UL42, Pol, or UL9. Of even greater significance, the largest globular structures and irregular bodies assembled, which were most morphologically similar to active viral RC, frequently incorporated high levels of pulse-labeled BrdU, and addition of the HSV DNA polymerase-specific inhibitor PAA abolished both assembly of the largest forms and BrdU incorporation into the small globular or punctate structures that remained. Therefore, it appears entirely reasonable to claim that specific viral Pol-dependent DNA synthesis was occurring in these structures and that they are functionally equivalent to the active viral DNA RC generated in HSV-infected cells.

As expected, the *Dpn*I replication assay carried out with the same input plasmids under the same conditions showed that they were competent to carry out replication of the viral oriS target plasmid DNA in an HSV Pol-dependent fashion. Others have previously confirmed the specificity of such assays by showing that similar target plasmids lacking key oriS motifs failed to give detectable replication both in transient cotransfection assays and after coinfection with baculovirus vectors expressing the seven HSV replication proteins (54, 57). All of these pieces of evidence suggest that assembled HSV RC are probably biologically functional and active in synthesizing viral DNA in cotransfected cells.

Do the assembled HSV replication structures initiate at or incorporate cellular replisome sites? Based on the previous observations that PAA-resistant micropunctate structures in infected cells colocalize with cellular BrdU-pulse-labeled speckles or replisomes, and that both complete viral RC and pre-RF apparently contain several cellular replication-related proteins (58), the simple model that cellular S-phase matrix-associated replisomes represent initial sites of formation of

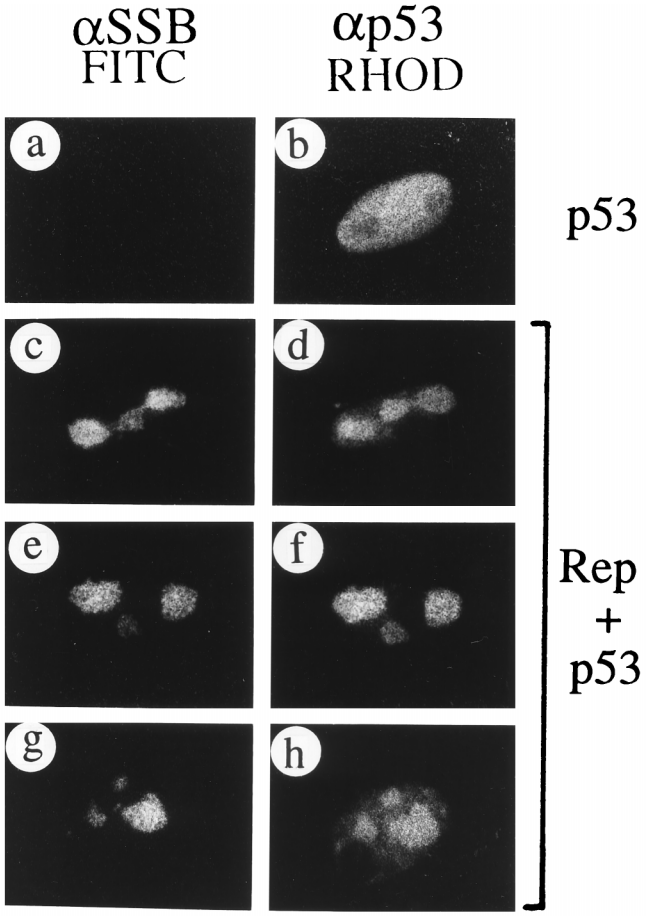

FIG. 9. Redistribution of cotransfected p53 protein by assembled RC. (a and b) Plasmid pSVp53 transfected alone; (c to h) cotransfection of pSVp53 and the whole set of plasmids encoding UL5, UL8, UL9, UL42, UL52, SSB, and Pol plus oriS. Paired double-label IFA panels show SSB detected with FITC-labeled anti-SSB PAb 3-83 (a, c, e, and g) and p53 detected with rhodamine-labeled anti-p53 MAb in the same field (b, d, f, and h).

viral pre-RF, which then coalesce into a smaller number of larger bodies, appears both plausible and attractive. However, there is no direct evidence that the micropunctate SSB pre-RF observed both before and after PAA treatment in S-phase cells are actual intermediates in the process. Furthermore, Maul et al. (34) have recently suggested that input HSV genomes are targeted to a small number of punctate matrix-associated intranuclear loci referred to as ND10 or PODs that contain the cellular protein PML.

Our observations that infected cells that are not in S phase form a second type of PAA-resistant punctate pattern containing only a small number of pre-RF that do not incorporate BrdU (Table 2), together with our evidence that fully active viral RC form efficiently in DNA-transfected cells that apparently lack S-phase characteristics, also suggest that alternative pathways might occur. Indeed, the similarity in number of the punctate SSB foci seen in non-S-phase infected cells in the presence of PAA to the number of complete RC in infected cells (an average of four to five per cell) might make these more likely to be functional intermediates than the far more numerous and smaller replisome-associated structures. However, we do not know whether these few punctate foci are derived from ND10 or PODs, nor do we know whether they contain viral DNA or cellular replication proteins. Further-

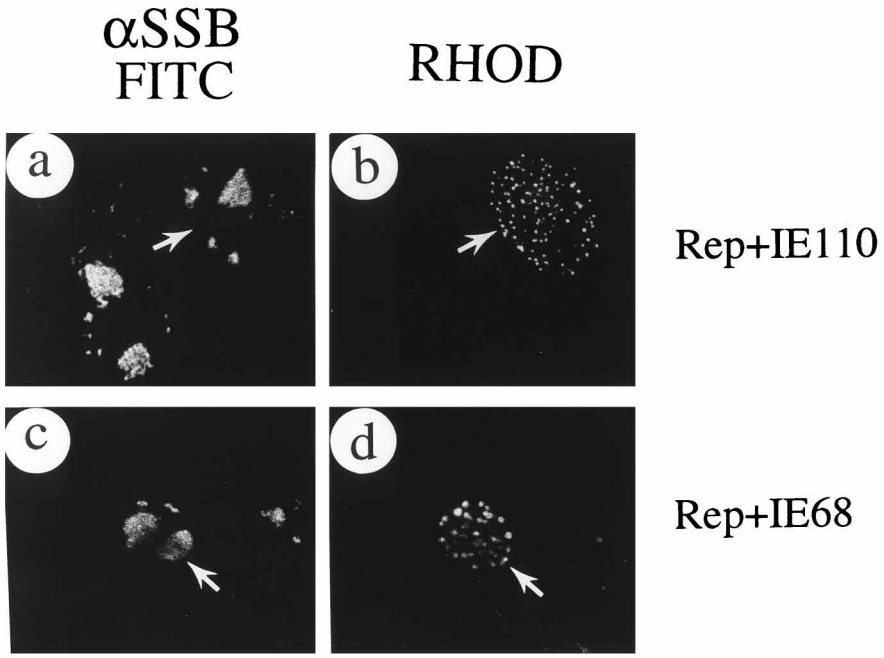

FIG. 10. Failure to recruit IE110 or IE68 protein by assembled RC. (a and b) Paired double-label IFA panels showing IE110 (pGH92) cotransfected with oriS and the whole set of plasmids encoding UL5, UL8, UL9, UL42, UL52, SSB, and Pol; (c and d) IE68 (pGR169) cotransfected with oriS and the whole set of plasmids encoding UL5, UL8, UL9, UL42, UL52, SSB, and Pol. SSB was labeled with rhodamine-labeled anti-SSB 39S MAb in panels a and c; IE110 was detected with FITC-labeled anti-IE110(N) PAb in panel b; IE68 was detected with FITC-labeled anti-IE68(N) PAb in panel d. Both the IE110 and IE68 proteins remained in nuclear punctate structures despite the presence of assembled SSB-positive RC in the same cotransfected cells (arrowed).

more, despite their close resemblance, fewer than 30% of the micropunctate structures formed by transfection of the helicase-primase (UL5, UL8, and UL52) and SSB components alone or by omission of UL42, UL9, or Pol colocalized with BrdU (Table 3), and therefore they may not all correlate with the predominantly replisome-like speckled pre-RF seen in infected S-phase cells in the presence and absence of PAA.

Cellular DNA replication sites in mammalian cells have been visualized by biotin-dUTP pulse-labeling to be able to fuse with each other (25). Therefore, it is possible that viral pre-RF and cellular DNA replication initiation sites also fuse and coalesce into larger globular viral pre-RC containing both viral and cellular proteins. Since viral oriS was not required for targeting of SSB into the globular pre-RC, oriS is probably recruited into the complete RC later, either with or without UL9, to stimulate efficient assembly of viral RC followed by specific replication of viral DNA within these structures.

Diminished role of oriS in the transient replication assay. In experiments using DNA transfection mixtures containing all seven protein components (UL5, UL8, UL9, UL42, UL52, SSB, and Pol) but in the absence of the added oriS plasmid, there were still many large globular and irregularly shaped structures formed, although strong BrdU pulse-labeling of the full RC-like forms was reduced two- to threefold compared to parallel samples in the presence of oriS (Table 5). Clearly, the role of oriS sequences was much less than expected in these assays for reasons that we do not yet understand. Interestingly, in a similar transient cotransfection assembly assay that we have recently described for formation of functionally active HCMV RC (53), the formation of the complete large irregular bodies was much more dependent on the inclusion of ori-lyt plasmid DNA than was the case here with the HSV system.

In both the presence and absence of oriS, low-level pulse-labeled BrdU incorporation still occurred in many of the assembled viral structures, especially the medium-sized globules (pre-RC), implying that they were active at some level in DNA synthesis. Based on the PAA sensitivity of most of these globules in the presence of oriS (Table 4), we presume that this residual DNA synthesis was driven by the viral DNA polymerase. Because bacterial plasmids lacking core oriS motifs are not replicated in the transient *Dpn*I assay (54, 57), it is clear that HSV origin-specific DNA replication could not be occurring. Therefore, the question arises as to whether this type of DNA synthesis involves cellular DNA or some unknown cryptic origins in our input viral replication gene sequences or perhaps represents some form of repair synthesis. Obviously, we also cannot exclude the possibility that the viral replication machinery is also capable of amplifying cellular DNA even in the full RC, as well as in the pre-RC in the presence or absence of oriS, although host DNA synthesis is generally thought to be shut off in wild-type HSV-infected cells at high MOI. We are currently attempting to resolve some of these issues by using combined fluorescence in situ hybridization and antibody IFA procedures.

Specific recruitment of viral IE175 and IE63 by assembled HSV RC. Two of the four HSV IE gene products IE175 and IE63 are essential for productive HSV-1 replication in virus-infected cells. Although neither of them is involved in viral DNA replication directly, they both relocate into the RC at later times during infection. By using assembled HSV RC, we showed that both IE175 and IE63 could be recruited either independently or simultaneously by the assembled RC (into both globular pre-RC and full-sized RC). Curiously, oriS was found to be dispensable for the redistribution of IE175 and IE63 in transfected cells. This finding implies that they are able to recognize the assembled pre-RC, probably through protein-protein interactions. However, consistent with previous reports of studies using virus-infected cells (29, 48), IE175 was unable

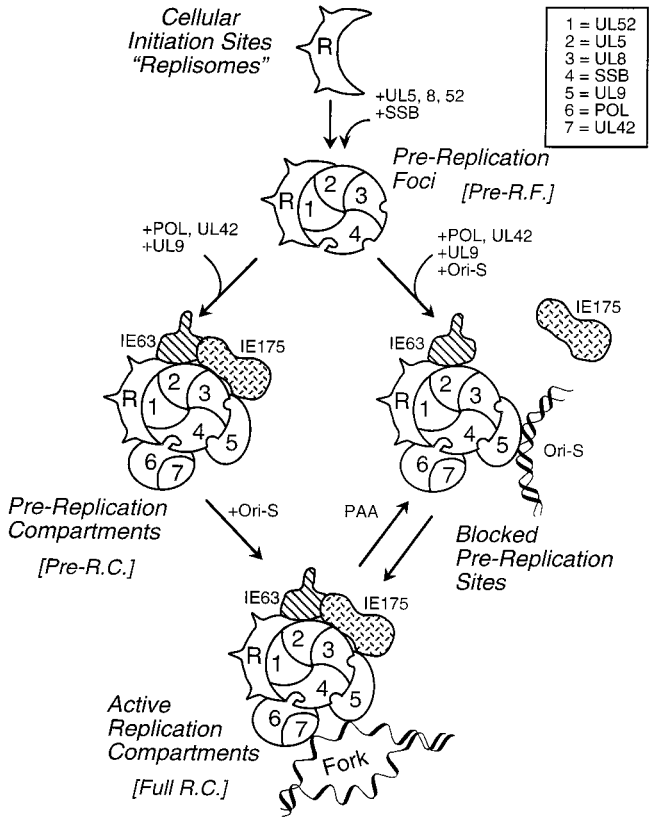

FIG. 11. Model for the assembly of functionally active HSV RC and several intermediate structures in cotransfected cells. Initially, SSB joins the tripartite helicase-primase complex consisting of UL5, UL8, and UL52 and forms numerous small punctate prereplicative foci, which colocalize with cellular replisomes in S-phase cells. Larger pre-RC are formed upon the arrival of UL9, Pol, and UL42 but not oriS. These large pre-RC formed in the absence of oriS are able to recruit both the IE175 and IE63 proteins. Complete viral RC are assembled only in the presence of oriS and incorporate BrdU at higher rates than those formed in the absence of oriS. In the presence of the complete set of seven replication fork proteins plus oriS and the HSV Pol-specific inhibitor PAA, relatively small structures similar to those referred to as prereplication sites in PAA-treated infected cells are formed. The exact composition of the latter in cotransfected cells is not yet known, but they differ from the pre-RC formed in the absence of oriS by being able to recruit IE63 but not IE175.

to move into assembled SSB-positive pre-RF, either when PAA was used to block viral DNA synthesis or when UL42 was omitted. Nevertheless, IE63, unlike IE175, was still able to redistribute with SSB into punctate pre-RF in the absence of UL42. Efficient recruitment of both IE175 and IE63, but not IE110 or IE68, tends to further confirm that the assembled RC in DNA-transfected cells behave similarly to those in virus-infected cells. Both IE175 and IE63 are thought to be involved directly in control of the late phase of viral gene expression, and late transcription might be expected to be localized within the RC.

Recruitment of cellular proteins into the assembled viral RC. Evidently, some cellular DNA replication or repair factors and associated proteins, such as p53, can be incorporated into viral RC in the DNA transfection system, and these may play important modulatory roles in viral DNA replication. The specificity of this interaction has yet to be resolved, but the result is consistent with an earlier report about several replication-related cellular proteins, including p53, colocalizing with HSV-1 viral pre-RF and RC in virus-infected cells (59). Among the cellular proteins found colocalizing with SSB in the viral RC, most are cellular replication or repair proteins and tumor suppressors (e.g., Rb and p53). p53 participates in both DNA replication, cell cycle control, and DNA repair (17, 27) and has been suggested to have modulatory effects on the DNA replication initiation process in both simian virus 40 and polyomavirus (2, 11, 26, 37). We expect that many other cellular replication-related proteins might also be found to be incorporated within these assembled viral DNA RC.

Proposed assembly model of functionally active HSV RC in transient assays. The successful demonstration of assembled functionally active HSV replication compartments detected by IFA in DNA-transfected cells will provide new opportunities to study both the viral and cellular proteins involved, the order of assembly events, and the nature of the cellular processes involved at the single-cell level. Our current model for the assembly of functionally active HSV RC and recruitment of transcription factors is shown in the schematic diagram in Fig. 11. Initially, the UL5-UL8-UL52 tripartite helicase-primase complex and SSB associate together within numerous small speckled or micropunctate structures in the nucleus. In S-phase cells, these structures clearly colocalize with sites of cellular DNA synthesis detected by BrdU incorporation and are unaffected by PAA. Presumably these foci contain cellular helicases, polymerases, initiator proteins, etc., plus other components of the cellular replication fork complexes that may be required subsequently for viral DNA synthesis, such as DNA ligase and topoisomerase. Therefore, targeting might be accomplished by interacting with a cellular DNA origin-binding protein or with its associated helicases, etc.

Subsequently, UL9 and Pol are added through the interactions with SSB and UL8, and UL42 probably also enters into the complex as the accessory processivity factor for the viral Pol protein. Although it is apparently required for this association in HSV-infected cells (31), UL9 (origin-binding protein) is not required for the colocalization with replisomes in transient expression assays in DNA-transfected cells. In virus-infected cells, the targeting of UL5, UL8, UL52, and SSB into cellular replisomes is probably more strictly controlled through UL9 or by other viral proteins. Direct protein-protein interactions between SSB and Pol, between SSB and UL9, and between UL8 and UL9, which have been documented previously (3, 4, 10, 36, 39, 50), probably help to recruit Pol and UL9 into pre-RF where cellular DNA synthesis initiation may or may not be occurring, depending on the stage of the cell cycle. The UL42 processivity factor, which interacts directly with Pol, was also demonstrated here to be required to complete the assembly of the larger active HSV RC, although its presence there has not yet been confirmed directly.

In both virus-infected and transfected cells treated with PAA, we recognized a second class of viral pre-RF consisting of a small number of punctate bodies (between two and eight per cell) that were formed in many cells that did not incorporate BrdU and therefore may not be in S phase. It remains to be determined whether these latter foci also colocalize with cellular replication machinery proteins, or indeed whether inactive cellular replisomes even exist in non-S-phase cells. Although we recognize that this second class of pre-RF may be more valid intermediates in the assembly process than the speckled replisome-associated foci, and that the input viral genomes may be recruited through these or yet another POD-derived punctate structure (34), too little is known about these forms as yet to incorporate them into our assembly and recruitment model.

Upon the arrival of all seven replication gene products, either or both of the small speckled (S-phase) or punctate (non-S-phase) pre-RF are further reorganized into large as-

sembled pre-RC, even in the absence of oriS, some of which also incorporate low levels of BrdU. The transient transfection assay in the absence of oriS allowed us to define some properties of these proposed pre-RC (Fig. 11), which cannot easily be studied in virus-infected cells. The globular pre-RC resemble complete RC to the extent of being relatively large and being able to recruit both IE175 and IE63, but they differed from them by not incorporating BrdU at high rates and by being unable to synthesize viral DNA because of the absence of oriS. In contrast, the punctate pre-RF formed in the presence of PAA failed to recruit IE175 and were smaller than the pre-RC. Cells with the complete irregular body type of RC incorporated BrdU at much higher rates in both transfected cells and in infected cells than did those with globular pre-RC. Since similar globular forms were produced in the absence of oriS, this difference presumably represents new oriS-directed synthesis of viral DNA. Although it is still unclear whether HSV oriS enters the compartments with or without being associated with the bound UL9 origin-binding protein, oriS did stimulate the efficiency of assembly of the complete viral replication compartments. Although the viral origin sequences might be essential for the formation of RC in virus infection, it is still unknown whether cellular DNA synthesis is totally shut down within cells undergoing viral DNA replication, whether HSV DNA synthesis can be initiated at any stage of the cell cycle, and whether other viral functions besides those used here in the transient assays may be needed to bypass cell cycle controls. The presence of IE175 and IE63 in the assembled complete RC hints that late viral transcription might be occurring here too, but whether the two processes (DNA synthesis and late class transcription) are actually coincident is not yet known.

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