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NOTES

Relocalization of Upstream Binding Factor to Viral Replication Compartments Is UL24 Independent and Follows the Onset of Herpes Simplex Virus 1 DNA Synthesis▽†

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Herpes simplex virus 1 (HSV-1) induces relocalization of several nucleolar proteins. We have found that, as for fibrillarin, the HSV-1-induced redistribution of two RNA polymerase I components, upstream binding factor (UBF) and RPA194, was independent of the viral protein UL24, which affects nucleolin localization. Nevertheless, the kinetics and sites of redistribution for fibrillarin and UBF differed. Interestingly, UBF remained associated with RPA194 during infection. Although UBF is redistributed to viral replication compartments during infection, we did not detect foci of UBF at early sites of viral DNA synthesis, suggesting that it may not be directly involved in this process at early times.

Viruses often usurp host cell functions to promote their own replication, and several have been shown to recruit nucleolar proteins (reviewed in references 11 and 12). Examples include the interaction of nucleolin with the internal ribosome entry site (IRES) of poliovirus, where it stimulates IRES-dependent translation (13), or the recruitment of upstream binding factor (UBF) to adenovirus replication centers, where it enhances viral DNA replication (19). Herpes simplex virus 1 (HSV-1) infection induces changes to the morphology and composition of nucleoli; they become elongated (5), and multiple nucleolar proteins are redistributed. Nucleolin is dispersed in a manner dependent on the viral protein UL24 (4, 21, 22), and mutations in the putative endonuclease motif identified in UL24 (17) are important for the dispersal of nucleolin (3). Cells in which nucleolin expression has been knocked down by small interfering RNA (siRNA) treatment for 5 days produced viral yields that were reduced 23-fold and 10-fold at 16 h postinfection (hpi) and 24 hpi, respectively, in a multistep replication assay (8). UL24 is expressed with leaky-late kinetics (29), is important for optimal viral yields (14, 15)—especially in neurons—and transiently localizes to nucleoli during infection (22). In addition, ectopic expression of UL24 and several of its homologs induce a G_2-M cell cycle block (26, 27). Fibrillarin and B23 are also redistributed during HSV-1 infection, redistribution of the former having been shown to be independent of UL24 (8, 22, 25). Nucleolin, B23, and fibrillarin contribute to the formation of mature rRNA (reviewed in references 6 and 9). During infection, fibrillarin is redistributed as small spots within nuclei (22), some of which localize to centromeres (25). The RNA polymerase I (RNAPI) transcription factor UBF stimulates the formation of the RNAPI preinitiation complex at the ribosomal DNA (rDNA) promoter (2) and also binds throughout rDNA (28) to modulate chromatin structure (30). During HSV-1 infection, foci of UBF accumulate in viral replication compartments (VRCs), and UBF has been proposed to be involved in viral DNA replication (32).

The redistribution of nucleolar proteins in HSV-1-infected cells can be grouped into UL24-dependent and UL24-independent events. Because the redistribution pattern of UBF is similar to that of fibrillarin—small spots distributed throughout the nucleus—we hypothesized that it also falls in the UL24-independent category. In this study, we tested this hypothesis and further investigated the subnuclear distribution of UBF during infection and the kinetics of its relocalization to sites of viral DNA synthesis.

UBF and fibrillarin are redistributed independently during HSV-1 infection. Because the redistribution patterns of UBF and fibrillarin are similar in HSV-1-infected cells (22, 32), we asked if they were relocalized together using a time course experiment to compare their spatial and temporal distribution during infection. For all immunostaining described in this report, Vero cells were infected at a multiplicity of infection of 10 and processed as described previously (22) using secondary antibodies coupled to Alexa 488 or 568, unless indicated otherwise. Images were acquired by confocal microscopy, and laser intensities were kept constant within a given experiment. Vero cells grown on coverslips were either mock infected or infected with the wild-type virus KOS. At 3, 6, 12 and 18 hpi, cells were fixed, blocked with human serum (Sigma), and immunostained using antibodies against fibrillarin (Covance) and UBF (Santa Cruz). Cells were visualized by confocal microscopy. As expected, in mock-infected cells both UBF and fibrillarin localized to nucleoli in one or two foci within nuclei (Fig. 1). As early as 3 hpi, we observed small spots of UBF redistributed from nucleoli. As

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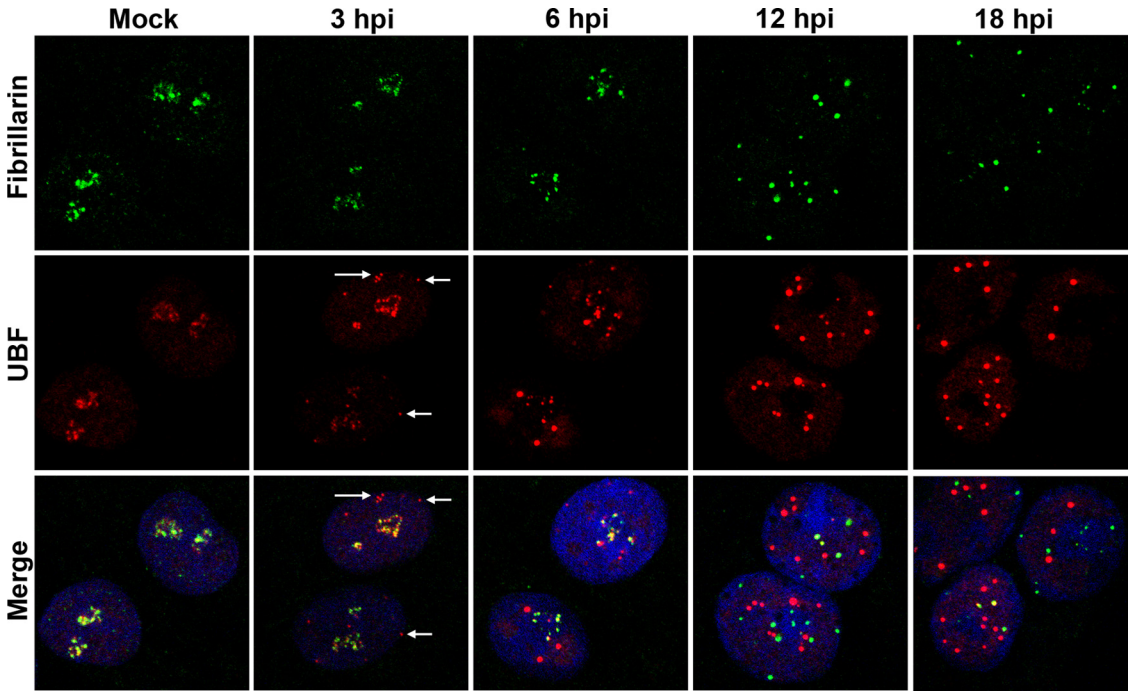

FIG. 1. UBF and fibrillarin are redistributed separately during HSV-1 infection. Confocal images show a time course of fibrillarin (green) and UBF (red) redistribution during infection. Arrows indicate relocalized spots of UBF prior to the redistribution of fibrillarin. Merged images are shown in the bottom panels. Nuclei were stained with Draq 5 (blue; Biostatus Limited).

infection progressed, both UBF and fibrillarin were relocalized as spots throughout the nucleoplasm but mainly remained separate. Thus, the localization and kinetics of fibrillarin and UBF redistribution differed during HSV-1 infection, suggesting that different mechanisms are involved in their relocalization.

Redistribution of UBF and a catalytic subunit of RNAPI is UL24 independent. Previously we found that HSV-1-induced nucleolar modifications could be classified according to their dependence on the viral protein UL24 (22). Here we asked whether the relocalization of UBF was dependent on UL24 and how the association of UBF with a catalytic subunit of RNAPI, RPA194, was affected during infection. Cells were either mock infected or infected with KOS or the UL24-deficient virus UL24X (14), fixed at 18 hpi, and stained for UBF or RPA194 (Santa Cruz). In mock-infected cells, UBF and RPA194 were found in foci corresponding to nucleoli (Fig. 2A, left panels). We found that, as for UBF, HSV-1 caused the redistribution of RPA194 into small spots throughout the nucleus (Fig. 2A, middle panels). Similar staining patterns were observed for both proteins in UL24X-infected cells, indicating that UL24 is not required for these effects (Fig. 2A, right panels). To determine if the redistribution of UBF and RPA194 was dependent on viral DNA replication, cells were infected in the presence of 300 μg/ml phosphonoacetic acid (PAA) (Sigma). We continued to detect some redistribution of UBF in the presence of PAA (Fig. 2B), suggesting that the trigger for the relocalization of these factors occurs independently of viral DNA replication.

UBF associates with the RNAPI transcription initiation machinery through interactions with RNAPI-associated factor 53 (PAF53) (10) and SL1 subunits (1, 18). We next investigated whether UBF remained associated with RNAPI during infection. KOS-infected cells were fixed at either 9 or 18 hpi and then blocked with human serum and costained for UBF and the largest subunit of RNAPI, RPA194 (Santa Cruz). As expected, they colocalized within nucleoli in mock-infected cells (Fig. 2C, left panels). Interestingly, in contrast to what we observed for fibrillarin, UBF and RPA194 remained colocalized during HSV-1 infection (Fig. 2C, middle and right panels). This is also in contrast to what was seen by Stow et al. (32); however, their RNAPI antibody likely recognized the RPA43 subunit (23), while we have looked at the largest RNAPI subunit, which also forms part of the catalytic site of the enzyme.

Because much of the UBF and RNAPI appeared to be released from nucleoli during HSV-1 infection, we investigated whether UBF could still be detected bound to ribosomal genes in infected cells. We carried out chromatin immunoprecipitations (ChIP) with mock- and KOS-infected cells harvested at 10 hpi. ChIPs were performed using the Magna ChIP G kit (Millipore) and antibodies against either UBF or ERp57 (Santa Cruz), a negative control for rDNA binding. Immunoprecipitated DNA was amplified by PCR using primers that detect rDNA promoter sequences. As expected, in mock-infected cells, we detected UBF bound to rDNA (Fig. 2D). Similarly, even though it appeared that large amounts of RNAPI-associated UBF were no longer in nucleoli, we found that following HSV-1 infection, some UBF remained bound to rDNA. This result is consistent with findings suggesting that despite a decrease in mature rRNA synthesis following HSV-1 infection, rDNA continues to be transcribed (31, 33).

Subnuclear distribution of UBF during infection. To investigate the early kinetics of UBF relocalization to VRCs, in-

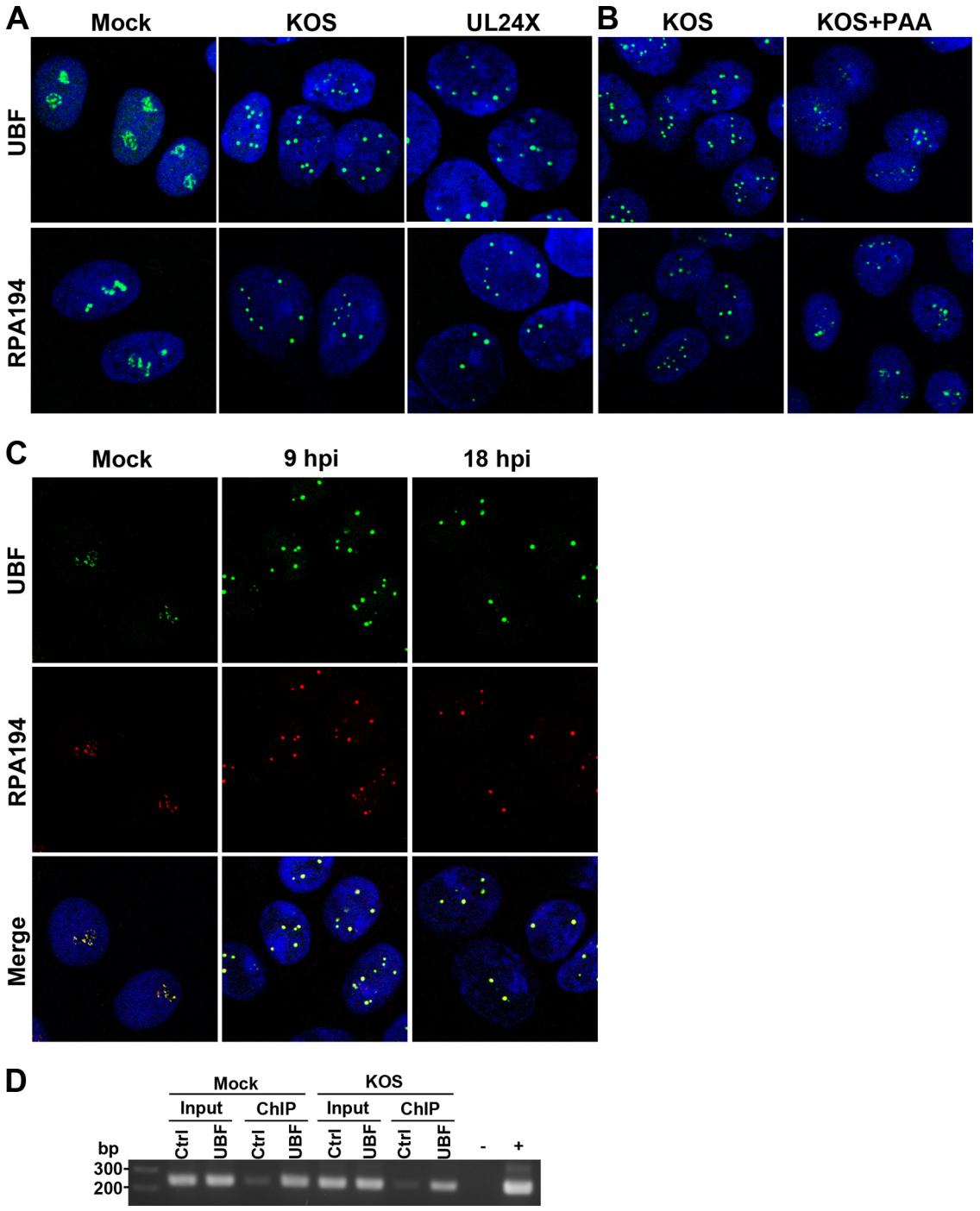

FIG. 2. UL24-independent relocalization of components of the RNAPI machinery upon infection with HSV-1. (A) Confocal images of mock-, KOS-, and UL24X-infected cells stained for either UBF (top panels) or RPA194 (bottom panels) at 18 hpi. (B) Shown are KOS-infected cells that remained untreated (left panels) or that were treated with PAA (right panels) and stained for either UBF or RPA194 at 18 hpi. Nuclei were stained with Draq 5 (blue). (C) Mock- or KOS-infected cells were costained for UBF (green) and RPA194 (red). Merged confocal images are shown in the bottom panels. Nuclei were stained with Draq 5 (blue). (D) ChIP assays on lysates from mock- or KOS-infected cells at 10 hpi using UBF or control antibodies. Input represents 1/100 of sample analyzed in ChIPs. Shown is an agarose gel of the PCR products obtained using primers detecting rDNA promoter sequences. Minus and plus signs represent negative (water) and positive (Vero cell DNA) PCR controls, respectively.

fected cells were fixed at 4, 5, 6, and 9 hpi, blocked with human serum, and costained using antibodies against UBF and ICP8 (Santa Cruz), a marker for VRCs. At 4 hpi, negligible amounts of UBF colocalized with early replication compartments (Fig. 3A). At 5 hpi, we began to detect spots of UBF colocalizing with ICP8. As infection progressed and VRCs coalesced, increasing amounts of UBF appeared to be relocalized to these compartments. At both 6 and 9 hpi, most UBF was detected in

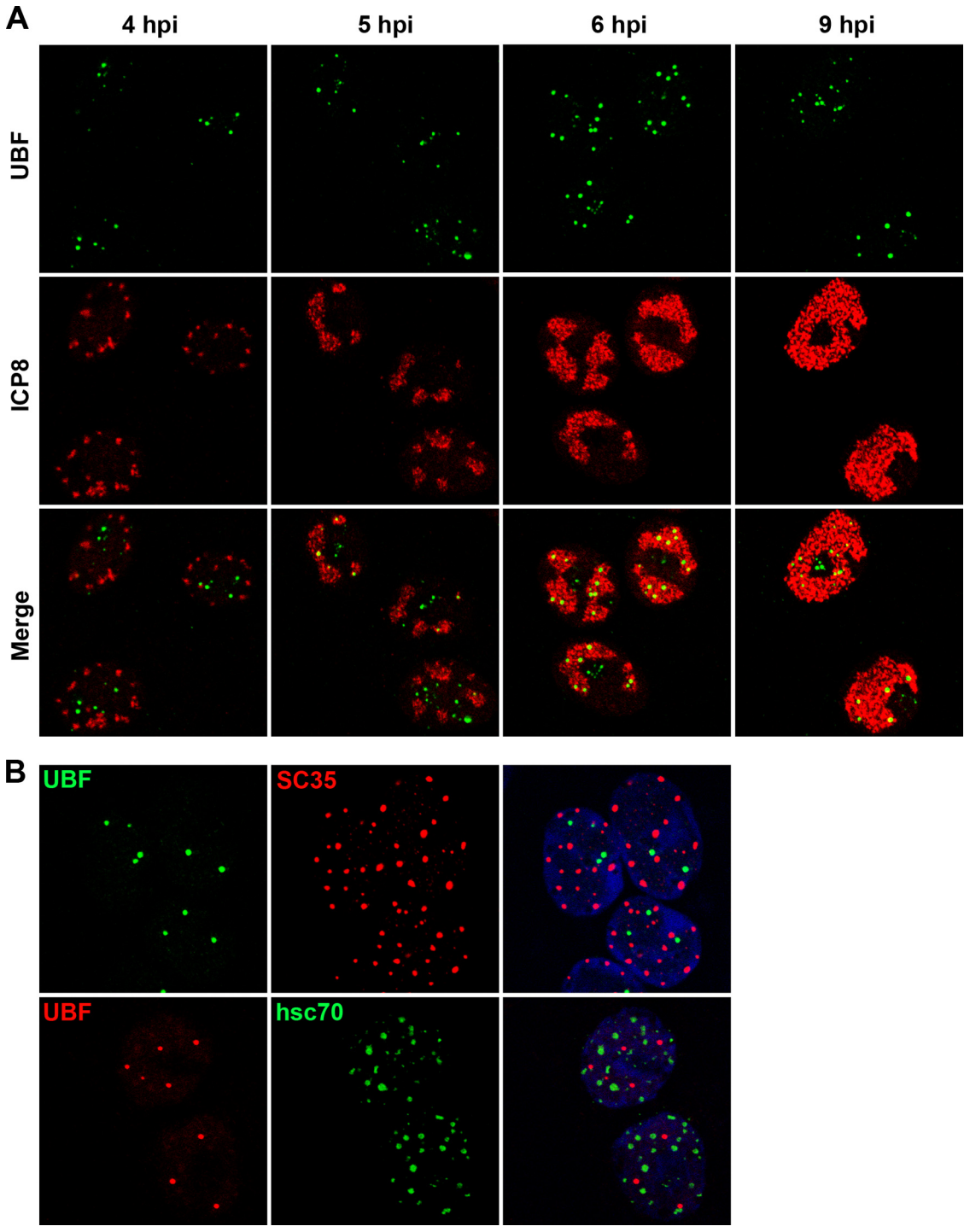

FIG. 3. Subnuclear localization of UBF in HSV-1-infected cells. (A) Time course of UBF relocalization to VRCs. Infected cells were costained for UBF (green) and ICP8 (red). Merged confocal images are presented in the bottom panels. (B) Vero cells were costained at 18 hpi for either UBF (green) and SC35, a marker for nuclear speckles (red) (top panels), or UBF (red) and hsc70, a marker for VICE domains (green) (bottom panels). Merged confocal images are shown in the right panels. Nuclei were stained with Draq 5 (blue).

spots within VRCs (Fig. 3A), while a portion of UBF staining remained outside VRCs, which is similar to what has been reported previously (32). The UBF outside VRCs likely represents the portion that remains associated with rDNA promoters (Fig. 2D) and continues to function in rRNA synthesis. VRCs are not homogeneous structures, and several subcompartments have been identified, such as splicing speckles and virus-induced chaperone-enriched (VICE) domains (7, 20). To determine whether UBF was recruited to either of these subcompartments, fixed cells were blocked using human serum

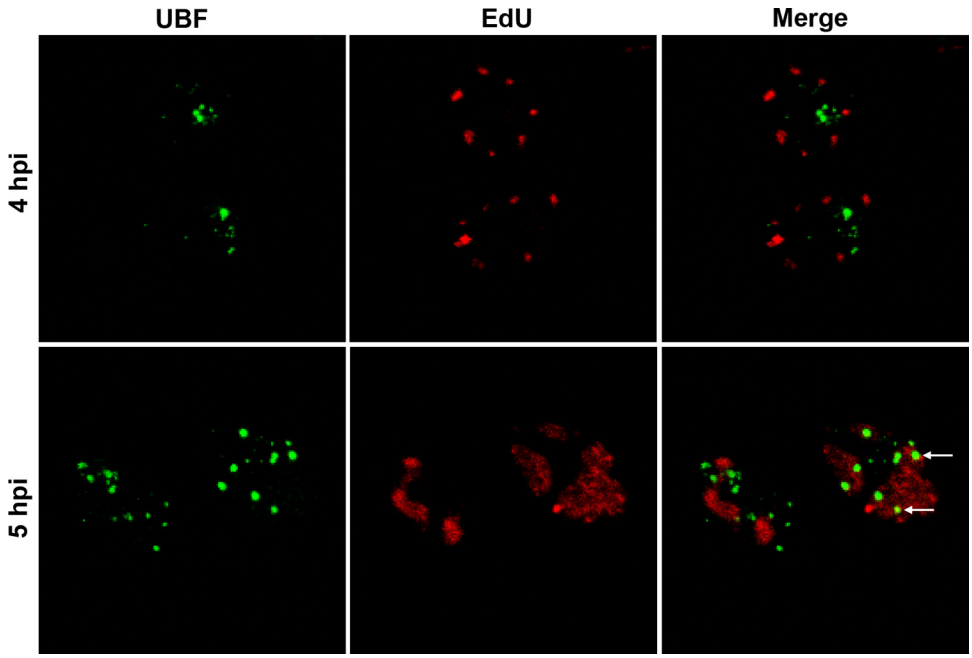

FIG. 4. HSV-1 DNA synthesis begins prior to the relocalization of UBF to VRCs. Vero cells were infected for 4 (top panels) or 5 h (bottom panels) and costained for UBF (green) and EdU, a marker for active DNA synthesis (red). Images are Z-stacks taken using a confocal microscope. Merged images are shown in the right panels. Arrows indicate colocalization of redistributed spots of UBF and sites of DNA synthesis at 5 hpi. A video of the Z-stack at 4 hpi is provided in the supplemental material.

and costained for UBF and either SC35 (Abcam) or hsc70 (Stressgen), respectively. For the most part, UBF did not colocalize with either splicing speckles or VICE domains (Fig. 3B) in infected cells and thus appeared to represent a distinct subcompartment found in VRCs; however, we cannot rule out the possibility that the presence of UBF in VRCs is an indirect consequence of the large space occupied by these compartments in the infected cell nucleus.

Absence of UBF from sites of HSV-1 DNA replication at early times in infection. It has been proposed that UBF recruited to VRCs is involved in HSV-1 DNA replication (32); however, we did not detect spots of UBF in early VRCs (Fig. 3A). To determine if relocalized spots of UBF were present at sites of HSV-1 DNA replication at these early times, cells were stained with Click-iT EdU (Invitrogen), which marks active sites of DNA synthesis. Cells were incubated with EdU for 30 min prior to fixation at either 4 or 5 hpi and costained for EdU and UBF, using secondary antibodies coupled to Alexa 647 and Alexa 488, respectively. Z-stack analyses were performed using a confocal microscope; EdU staining is shown in red to facilitate visualization (Fig. 4). At 4 hpi, we observed many sites of viral DNA synthesis in which we were unable to detect any spots of UBF, while by 5 hpi, UBF began to associate with the growing VRCs. A three-dimensional (3D) video of the Z-stack image at 4 hpi is included in the supplemental material. Therefore, we found that early viral DNA synthesis occurred in the apparent absence of UBF.

Impact of HSV-1 infection on RNAPI transcription machinery. Our results suggest that UL24-independent nucleolar modifications can be subdivided into at least two classes—those represented by the redistribution of fibrillarin and those comprising the redistribution of UBF and RPA194. The redistribution of spots of UBF to VRCs has led to the hypothesis that this factor functions in viral DNA replication. This possibility was supported by the observation of Stow et al. that in a cotransfection assay, expression of UBF-EGFP fusion proteins inhibited origin-dependent HSV DNA replication (32). Our results showing that the redistribution of UBF to VRCs follows the onset of viral DNA replication suggest that UBF may not be required for viral DNA synthesis, at least at early times in infection; however, we cannot rule out a possible indirect role for UBF in this process. Nucleoli form around nucleolar organizing regions (NORs), which are clusters of domains on chromosomes that contain the rDNA repeats (16, 24). The virus-induced structures containing UBF and RNAPI that we have observed are reminiscent of pseudo-NORs (23). Pseudo-NORs are RNAPI transcription complexes that assemble on arrays of ribosomal genes introduced at ectopic sites within the human genome. Pseudo-NORs are transcriptionally silent and, unlike nucleoli, do not appear to contain nucleolin, B23, or fibrillarin. Although we recognize that this is an artificial system, the formation of these structures in infected cells suggests that they may serve a particular function in this context. Because the formation of pseudo-NORs is based on the interaction of UBF with a specific DNA target, it suggests that the UBF/RNAPI complexes also form on specific DNA sequences within VRCs; however, to date we have not detected any specific interaction between UBF and viral promoter DNA in infected cells. Given the role of UBF in altering the chromatin structure of rDNA, one possible function of the UBF/RNAPI complexes in VRCs may be to modulate the structure of particular loci in the viral genome to promote a particular stage in viral replication.

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