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Nuclear Sites of Herpes Simplex Virus Type 1 DNA Replication and Transcription Colocalize at Early Times Postinfection and Are Largely Distinct from RNA Processing Factors

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We have visualized the intracellular localization of herpes simplex virus (HSV) type 1 replication and transcription sites in infected HeLa cells by using direct labelling methods. The number of viral transcription foci increases in a limited way; however, the number of replication sites increases in a near-exponential manner throughout infection, and both replication and transcription sites are found buried throughout the nuclear interior. Simultaneous visualization of viral transcription and replication foci shows that the two processes colocalize at early times, but at later times postinfection, there are additional sites committed solely to replication. This contrasts with the situation in adenovirus-infected cells in which, throughout replication, sites of transcription are adjacent to but do not colocalize with sites of viral DNA replication. The data for an increase in HSV transcription sites suggest an initial phase of replication of input genomes which are then transcribed. Sites of HSV replication colocalize with viral DNA replication and packaging proteins but are largely distinct from the punctate distribution of small nuclear ribonucleoprotein particles. Very high multiplicities of infection have shown an upper limit of some 18 viral transcription foci per nucleus, suggesting cellular constraints on transcription site formation. Use of virus replication mutants confirms that the labelled foci are sites of viral RNA and DNA synthesis; in the absence of viral DNA replication functions, no replication foci and only a limited number of transcription foci were present. Absence of a packaging function had no apparent effect on transcription or replication site formation, illustrating that DNA packaging is not a prerequisite for ongoing DNA synthesis. Further, the essential HSV protein IE63 is required for efficient replication site formation at later times postinfection but is not required for transcription foci formation.

The organization of eukaryotic cells is complex and must ensure that the processes of transcription, RNA processing, transport, and DNA replication are closely regulated both temporally and spatially within the cell nucleus (4). Recently a number of nuclear domains have been identified; some are involved in transcription (49) and RNA processing (52), others are involved in replication (9, 18), and all interact with the underlying nuclear matrix which forms the skeleton and structural basis for the nucleus. However, the dynamics of nuclear compartmentalization and the functional and spatial relationships between the different domains, including those involved in replication, transcription, and RNA metabolism, are largely unknown (45).

Herpes simplex virus type 1 (HSV-1) is a nuclear replicating DNA virus which has lytic and latent phases of replication (40), and virus genes can be divided into three classes based on the kinetics of their expression (5). The immediate-early (IE) proteins, two of which (IE63 and IE175) are essential for virus DNA replication, do not require prior viral protein synthesis for their expression and predominantly act to regulate early and late virus gene expression. Early viral proteins include those involved in DNA metabolism and DNA replication, while the late proteins, encompassing two subclasses, comprise the majority of virus structural components.

Following infection of cells in vitro, HSV-1 DNA replication commences around 3 to 4 h postinfection, reaching maximum efficiency between 8 and 16 h (7). The virus encodes a set of seven proteins required for viral DNA replication (6) which are sufficient for origin-dependent replication in transfected cells (16, 43, 47). These proteins include a DNA polymerase (UL30), a polymerase accessory factor (UL42), helicase-primase activities (UL5, UL8, and UL52), an origin binding protein (UL9), and a single-stranded DNA binding protein (UL29). Early studies demonstrated that viral DNA replication occurs within defined areas of the nucleus, but the spatial organization of these replication structures was poorly understood (36, 39). De Bruyn Kops and Knipe (9) have demonstrated that HSV DNA replication occurs in the interior of the nucleus at sites defined by preexisting host cell nuclear architecture, probably the nuclear matrix. Infected cells in which viral DNA synthesis has been blocked exhibit several of the viral DNA replication proteins in a punctate distribution, termed prereplicative sites (35). The viral DNA replication apparatus (21, 22) and several host cell proteins colocalize with these structures, reflecting the assembly of viral and cellular components required for viral DNA synthesis (9); host cell factors can apparently substitute for some of these viral proteins under certain conditions (21).

HSV DNA is transported to the nucleus together with certain structural proteins such as Vmw65 (2) which interact with cellular transcription factors to initiate gene expression from the circularized viral genome (27; reviewed in reference 28). Viral transcripts can be detected between 30 and 60 min postinfection, and throughout infection, viral mRNAs are synthesized by a modified cellular RNA polymerase II (37, 38). Recent evidence suggests that up to 90% of transcription of some cellular genes can be inhibited as early as 3 h postinfection and that virus proteins IE110, IE175, and IE63 are involved in this inhibition (46). All HSV-1 transcripts (except certain latency-associated transcripts [11]) are processed by

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polyadenylation, but as few HSV genes contain introns (12), there is a limited requirement for the cell's splicing machinery (31). Virus infection initiates important structural rearrangements in the nucleus as HSV subverts host cellular mechanisms. Not least of these is the redistribution of the splicing small nuclear ribonucleoprotein particles (snRNPs) away from a widespread diffuse pattern to a highly punctate nuclear organization (23, 31), while the distribution of polyadenylation factors remains largely unaltered (26). The essential (41) product of IE gene 2, IE63 (also known as ICP27), has been shown to be both necessary and sufficient to cause this effect and colocalizes with the redistributed snRNPs (31, 42); IE63 causes an inhibition of cellular splicing both in vitro and in vivo (14) and binds RNA directly (19). Mutant viruses which lack IE63 exhibit reduced levels of DNA replication (25); IE63 promotes viral DNA replication by stimulating expression of certain early genes (26, 48) and affects the nuclear localization of UL29 and thus replication complex formation (8).

In this study, we used direct procedures for labelling DNA and RNA synthesis in vivo (17, 20) to determine how the sites of transcription and replication are spatially related. Infections with viruses mutated in the IE63 gene and virus replication and DNA packaging functions together with use of viral and cellular antibodies facilitated our analysis. We demonstrate that the IE63 protein is essential for efficient DNA replication focus formation and that these foci colocalize with viral DNA replication and packaging proteins.

Our data demonstrate that the organization of the HSV-infected nucleus differs significantly from that of cells infected with adenovirus (3, 33). Like HSV-1, adenovirus is a nuclear replicating virus, but it makes extensive use of the host cell's splicing machinery for gene expression and utilizes a different DNA replication mechanism.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were grown as monolayers in Dulbecco's modified minimal essential medium supplemented with 5% newborn calf serum and 5% fetal calf serum (FCS). Vero 2-2 cells were maintained in Glasgow minimal essential medium supplemented with 5% newborn calf serum and 5% FCS. A26 cells (30) were maintained in Dulbecco's modified minimal essential medium supplemented with 5% FCS and containing G418 (300 μm/ml) to maintain selection for integrated sequences which include the UL6, UL8, and UL9 genes. All cells were grown at 37°C in an atmosphere of 5% CO_2.

Stocks of wild-type HSV-1 (strain 17+) were grown on HeLa cells. Stocks of the IE63 insertion mutant virus 27-lacZ (44) (a gift from R. Sandri-Goldin) were grown on the complementing IE63-expressing Vero 2-2 cell line. Stocks of the UL6 mutant (lacZ-UL6-1), UL8 mutant (ambUL8), and UL9 mutant (7-2) viruses (29, 30) were grown on a complementing A26 cell line which expresses all three functions. An aphidicolin-resistant HSV-1 mutant (aph^R) capable of replicating in aphidicolin concentrations of 1 to 2 μg/ml was grown on HeLa cells (kind gift from N. Stow, Institute of Virology, Glasgow, Scotland).

HSV-1 infection of cell monolayers. All cells were grown as monolayers on sterile glass coverslips previously treated with 10% poly-L-lysine for 10 min after sterilization. Subconfluent cell monolayers were infected with either wild-type or mutant HSV-1 at a multiplicity of 10 PFU per cell. After 1 h at 37°C, the infected medium was removed and replaced with fresh prewarmed medium until the time of harvesting. Coverslips were harvested at 2-h intervals until 16 h postinfection. Mock-infected (uninfected) cells were treated in identically, with the omission of virus.

Immunofluorescence and antibodies. Indirect immunofluorescence experiments were performed as previously described (31). All antibodies were diluted in phosphate-buffered saline before use. The U2 splicing snRNPs were labelled with the B″ monoclonal antibody 4G3 (13) at a dilution of 1:5. The HSV-1 DNA polymerase UL30 protein was detected by using monoclonal antibody 13429 (kind gift from A. Cross, Institute of Virology) at a dilution of 1:2. The HSV-1 packaging protein UL19 was detected by using monoclonal antibody LP12 (kind gift from A. C. Minson, University of Cambridge) at a dilution of 1:100. The intracellular localization of individual antigens was established prior to double-labelling experiments; antibodies used (both primary and secondary) were titered to obtain the optimum working concentrations. The filter set used did not give bleed-through of the two fluorochromes.

In vivo transcription label. Visualization of transcription sites was performed essentially as described by Jackson et al. (20) and Wansink et al. (49). Briefly, cells grown as monolayers on coverslips were permeabilized with 0.05% Triton X-100 in physiological buffer for 1 to 2 min at 4°C and incubated with 100 mM bromodeoxyuridine (Br)-UTP (Sigma) for 10 to 20 min at 33°C. Incorporated Br-UTP was detected by using a primary monoclonal anti-Br antibody (Boehringer Mannheim) at a dilution of 1:50 and a secondary anti-mouse fluorescein isothiocyanate (FITC)- or Texas red-conjugated antibody (Amersham) at a dilution of 1:60. Alternatively, to enhance the signal, a biotinylated anti-mouse antibody step was included followed by avidin-FITC detection. As a control to check the specificity of the mouse anti-Br antibody, 100 μM UTP replaced Br-UTP; no signal was detected under these circumstances. If added, α-amanitin (100 μm/ml) was present for 10 to 30 min at 4°C prior to and during transcription. RNase A (200 μm/ml) treatment for 1 h at 37°C prior to hybridization removed all signal.

In vivo replication label. Sites of viral and cellular DNA replication were labelled by using a technique described by Hozak et al. (17), which is very similar to the transcription label method. Cells were permeabilized as described by Jackson et al. (20), and then sites of DNA replication were labelled by the incorporation of 100 mM biotin-11-dUTP (Boehringer Mannheim) for 15 min at 33°C. Incorporated biotin was detected with avidin-FITC or avidin-rhodamine at a dilution of 1:100. After replication label, samples were either mounted directly or double immunolabelled by using a monoclonal antibody against B″, UL19, or UL30, with subsequent detection using anti-mouse FITC- or Texas red (Amersham)-conjugated antibodies at a dilution of 1:60. If added, aphidicolin was present for between 30 min to 5 h prior to and during the replication label. An aphidicolin concentration of 1 μg/ml allowed replication of the resistant mutant aph^R; treatments with 10 and 100 μg aphidicolin per ml prevented DNA replication of this mutant.

Transcription-replication double label. For the simultaneous visualization of sites of transcription and replication, cells were permeabilized in 0.05% Triton X-100 in physiological buffer for 1.5 min and then incubated with 0.2 mM Br-UTP and 0.1 mM biotin-11-dUTP for 15 min at 33°C (33). The incorporated nucleotides were then detected by using mouse anti-Br and then FITC-conjugated anti-mouse (1:60) antibodies and rhodamine-conjugated avidin (1:100). To assess relative sensitivity in the detection of sites of transcription and replication, the concentration of Br-UTP was increased to 1 mM or the concentration of biotin-11-dUTP was increased to 0.5 mM.

In situ hybridization. HSV-1 genomic DNA was labelled by random priming in the presence of fluorescein-dUTP (Amersham); the probe was hydrolyzed until fragments of 200 to 400 bp were obtained. In situ hybridization was performed as described in the Amersham DNA color kit protocol, with omission of the proteinase K step. The hybridized probe was detected with an anti-fluorescein alkaline phosphatase-conjugated antibody and alkaline phosphatase color development. DNase, RNase A, and RNase H (200 U/ml) treatments were performed for 1 h at 37°C to demonstrate signal specificity; effectiveness of the DNase treatment was judged by 4′,6-diamidino-2-phenylindole (DAPI) staining.

Microscopy. Cells were examined by using a Zeiss fluorescence microscope, and images were captured either by using a Noran Instruments Odyssey laser confocal microscope or by photography with Fujicolor ASA 400 film. For every experiment, approximately 2,500 nuclei were examined at each time point, and each experiment was performed on at least eight separate occasions; thus, the average numerical data for foci per nucleus were generated from more than 20,000 cells. A focus was defined as a site of label clearly distinguishable from its surrounding area under the fluorescence microscope as an intensely labelled spot.

RESULTS

Visualization of viral transcription sites. Sites of HSV-1 transcription in infected HeLa cells were labelled at 2-h intervals by incorporation of Br-UTP into nascent transcripts followed by fluorescence immunodetection until 16 h postinfection; representative cells are shown in Fig. 1a to f. In mock-infected cells, a large number of cellular transcription sites which labeled the nucleus in a diffuse pattern of tiny foci could be seen (Fig. 1a); this corresponds well with the 300 to 500 cellular transcription foci described by Jackson et al. (20) and Wansink et al. (49). At 1 h postinfection, nuclei labelled similarly to uninfected cells, with either no or one intense spot against a brightly labelled background (not shown). At 2 h, between two and four very bright foci (average, 2.55) were observed in 95% of cells (Fig. 1b). The number of transcription foci remained constant until 4 h, increasing to an average of 3.17 by 6 h (Fig. 1c). By 8 h, the number of viral transcription sites had doubled, with an average of six foci per cell set against a fainter overall background label (Fig. 1d). At 16 h postinfection, toward the end of the replication cycle, a maximum of 9

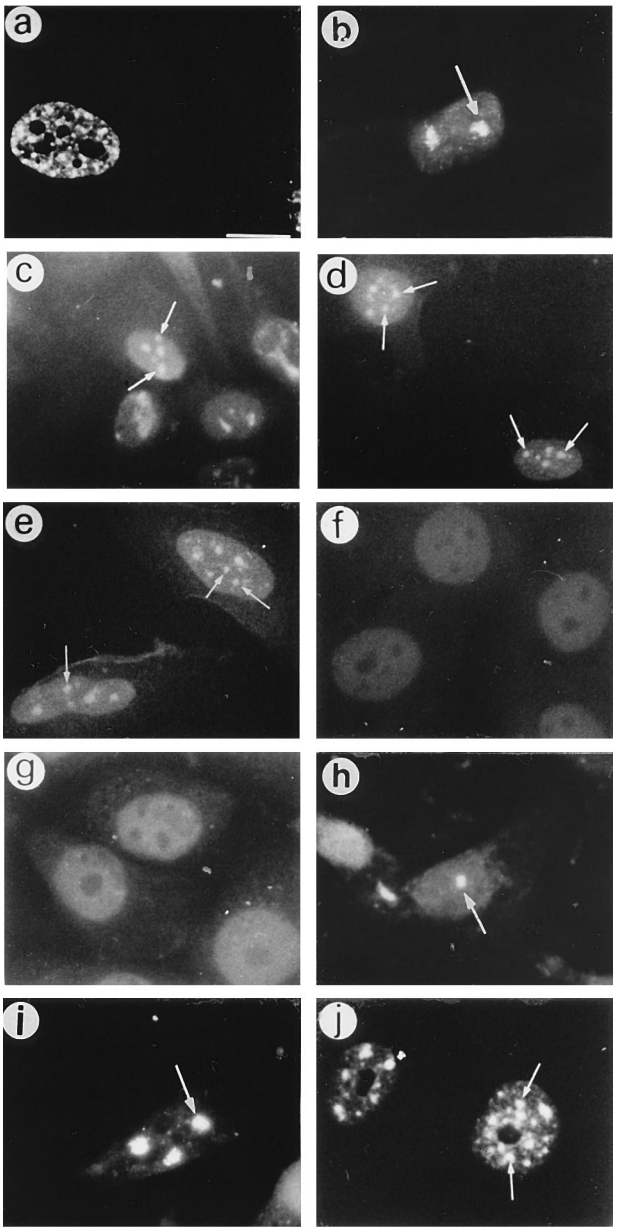

FIG. 1. Visualization of HSV-1 transcription sites. HeLa cells were infected with HSV-1 at a multiplicity of 10 PFU per cell for 0 (a), 2 (b), 6 (c), 8 (d), and 16 (e) h and incubated with Br-UTP for 15 min; incorporated label was detected with FITC-conjugated mouse anti-Br antibody. As a control, cells 16 h postinfection were labelled with Br-UTP in the presence of the transcription inhibitor α-amanitin (100 μg/ml) (f). HeLa cells infected with HSV-1 at multiplicities of 0.1 (g), 1 (h and i), and 100 (j) PFU per cell were labelled for sites of transcription at times between 0 and 16 h postinfection. At a multiplicity of 0.1 (g) or 1 (h), no or one focal site of transcription was observed at 4 h postinfection (arrow). The number of bright foci increased to a maximum of two or three by 8 h at a multiplicity of 1 (i). Infection at a multiplicity of 100 resulted in an upper limit of 18 transcription foci per nucleus at 16 h (j). Bar corresponds to 10 μm; arrows indicate focal sites of viral transcription.

or 10 focal sites (average, 7.7) were observed per nucleus (Fig. 1e). These data are represented in Fig. 4.

As a control, cells were treated with α-amanitin prior to and during the labelling procedure at a concentration sufficiently high to inhibit RNA polymerase II. Under these conditions, very little label was detected, confirming that the labelled foci represent sites of transcription (Fig. 1f). As an additional control, incorporation of UTP was not detected with a mouse anti-Br antibody, demonstrating antibody specificity (data not shown). Thus, these foci represent active sites of viral transcription which increase in number as incoming viral genomes are replicated, and cellular transcription is inhibited rapidly upon HSV-1 infection.

Infections were performed at multiplicities of 0.1, 1, 10, 50, and 100 PFU per cell, and the number of transcription foci per nucleus was determined. Cells infected at multiplicities of 0.1 and 1 PFU demonstrated either no or one focal site of viral transcription at 4 h (Fig. 1g and h), increasing to a maximum of two to three foci by 8 h (Fig. 1i) and 16 h (not shown). Cells infected at multiplicities of 50 and 100 demonstrated an upper limit of 18 transcription foci per nucleus even at 8 h (not shown) and 16 h (Fig. 1j), compared to 7.7 foci at a multiplicity of 10 (Fig. 1e). Thus, the number of transcription foci is limited to fewer than 20 per nucleus regardless of the multiplicity of infection; furthermore, the increasing number of transcription foci with increased multiplicity of infection indicates that the foci are of viral origin.

Visualization of viral DNA replication foci. HSV-1-infected HeLa cells were pulse-labelled with biotin-11-dUTP to label synthetic sites of DNA replication (Fig. 2a to e). The labelled foci were fluorescently detected with avidin-conjugated FITC or rhodamine as appropriate and examined by fluorescence microscopy. Cells were labelled at 0, 4, 6, 8, and 16 h postinfection. Uninfected cells demonstrated a general diffuse staining pattern comprised of hundreds of small cellular replication foci (18) (Fig. 2a). At 4 h, three to four additional spots of intensely labelled DNA were observed (Fig. 2b), consistent with the number of transcription foci at this time point. By 6 h postinfection, the number of replication foci had increased to six to seven (Fig. 2c). At 8 h, this number approached 10 to 12 (Fig. 2d), and by 16 h postinfection, more than 20 sites of DNA replication were observed (Fig. 2e). These data are represented in Fig. 4. The disparity between the number of transcription foci and the number of replication foci at later times suggests that there are viral genomes which are committed to DNA replication, which are either poorly transcribed or not transcribed.

As controls, replication labelling was performed in the presence of aphidicolin (an inhibitor of DNA polymerase I) at 1 μg/ml (17). With wild-type infections, only faint background label was obtained and no viral replication foci were seen (data not shown). As a further control, and to determine that these foci were of viral and not cellular origin resulting from infection, infections were performed with the aphidicolin-resistant HSV-1 mutant aph^R. This approach enabled us to perform replication label experiments in the presence of an aphidicolin concentration high enough to inhibit cellular DNA replication while allowing viral replication to proceed. Cells infected for 7 h prior to labelling were pretreated with 1 μg of aphidicolin per ml for 5 h prior to and during the labelling procedure. In both the presence (Fig. 2f) and absence (not shown) of aphidicolin, six to eight replication foci were observed with HSV-1 aph^R. Only when the aphidicolin concentration was increased to 10 to 100 μg/ml were there a total inhibition of replication label and no focal sites (Fig. 2g). Thus, the brightly staining foci were indeed sites of viral DNA replication. Transcription label with HSV-1 aph^R in the presence and absence of aphidicolin (1 μg/ml) demonstrated wild-type patterns (not shown).

Infections were performed at multiplicities of 50 and 100 PFU per cell, and the number of replication foci per nucleus was determined. By 6 to 8 h postinfection, the number of foci

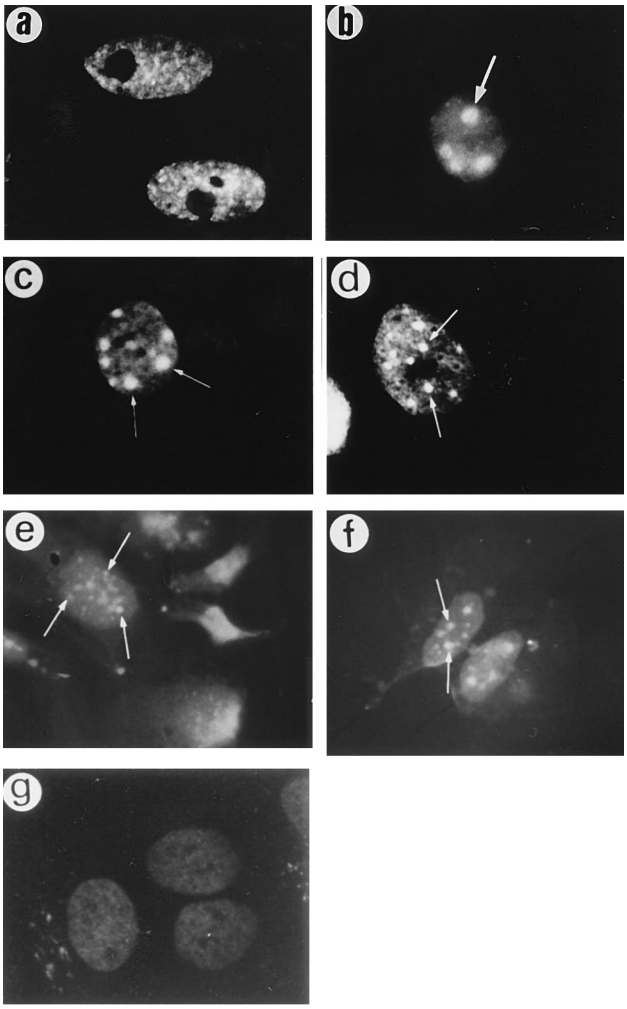

FIG. 2. Visualization of HSV-1 DNA replication foci. HeLa cells infected with HSV-1 at a multiplicity of 10 PFU per cell for 0 to 16 h were incubated with biotin-11-dUTP for 15 min at 33°C to label sites of DNA replication; the incorporated label was detected with avidin conjugated to FITC. Cells were labelled at 0 (a), 4 (b), 6 (c), 8 (d), and 16 (e) h postinfection. Note that the number of viral DNA replication foci increases in a near-exponential manner with time postinfection. HeLa cells infected with the HSV-1 aphidicolin-resistant mutant aph^R for 7 h were treated for 5 h prior to and during the labelling procedure with aphidicolin at either 1 (f) or 100 (g) μg/ml. Bar corresponds to 10 μm; arrows indicate focal sites of viral DNA replication.

increased to more than 25 at both multiplicities, becoming indistinguishable as distinct foci (data not shown).

Transcription-replication double label of HSV-1-infected cells. Infected and mock-infected HeLa cells were simultaneously labelled with Br-UTP and biotin-11-dUTP to label sites of transcription and DNA replication in the same nucleus. The two labels were detected with FITC and rhodamine, respectively, which allowed labelled sites to be spatially oriented relative to each other. At early times, intensely labelled sites of transcription and replication almost perfectly colocalized (Fig. 3a and b). However, at 16 h, while all of the transcription foci (Fig. 3c) colocalized with sites of replication (Fig. 3d), there were additional replication foci which showed no detectable transcription (Fig. 3c and d). To ensure that this result was not due to differences in sensitivity of the two labelling procedures, the relative concentration of Br-UTP was increased fivefold to enhance the detection of transcription foci; however, no additional foci were detected (data not shown). Thus, at later times postinfection, sites of viral DNA synthesis at which active transcription cannot be detected are present.

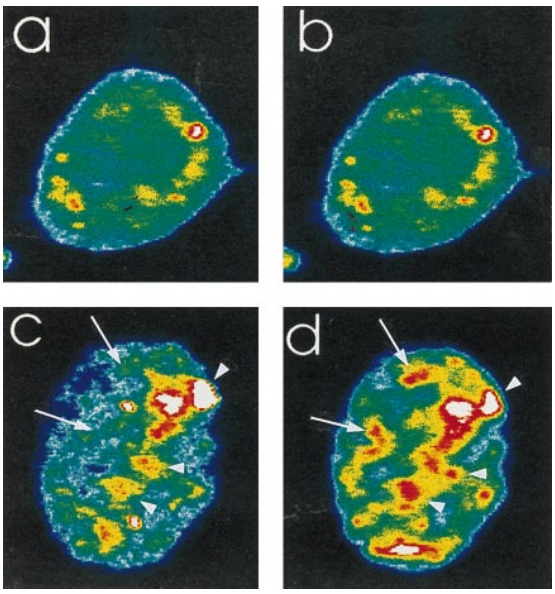

FIG. 3. Simultaneous visualization of viral replication and transcription foci. HeLa cells infected with HSV-1 at a multiplicity of 10 PFU per cell at times up to 16 h were labelled simultaneously for sites of transcription (Br-UTP) and DNA replication (biotin-11-dUTP) and detected with FITC- and rhodamine-conjugated antibodies, respectively. At 4 h postinfection, sites of transcription (a) and DNA replication (b) perfectly colocalize. By 16 h postinfection, sites of transcription (c) colocalize with replication foci (d) (arrowheads), but there are many additional sites of DNA replication at which no transcription was detected (arrows). Bar corresponds to 10 μm. These cells are presented in both red/green and false color. False color enhances the relative contrast of the fluorescent label. Red and white represent regions of most intense label; blue and black represent regions of least intense label.

In situ hybridization to detect sites of viral DNA. To directly label the incoming viral genomes in cells infected at a multiplicity of 10, in situ hybridization was performed with HSV-1 genomic DNA as a probe. Two to three spots were detected at 1 and 2 h postinfection (Fig. 5b); at 8 h, six to eight spots per nucleus were seen (Fig. 5c); by 16 h postinfection, the intense labelling was distributed throughout the nucleus and distinct

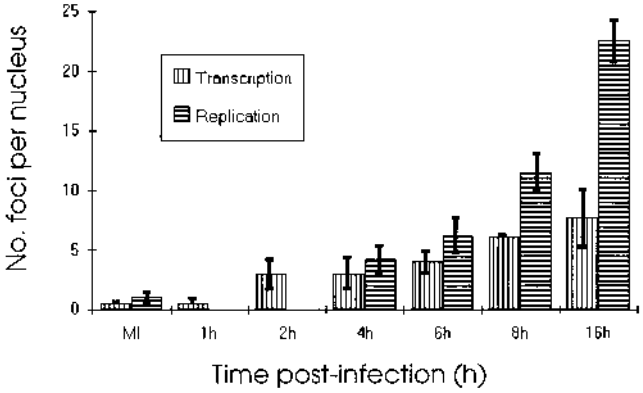

FIG. 4. Number of transcription foci (as illustrated in Fig. 1) and number of replication foci (as illustrated in Fig. 2) per nucleus with time after HSV-1 infection, at a multiplicity of 10 PFU per cell. Error bars represent maximum variation in the number of foci observed at the different time points. Each average value was determined from more than 2,500 cells.

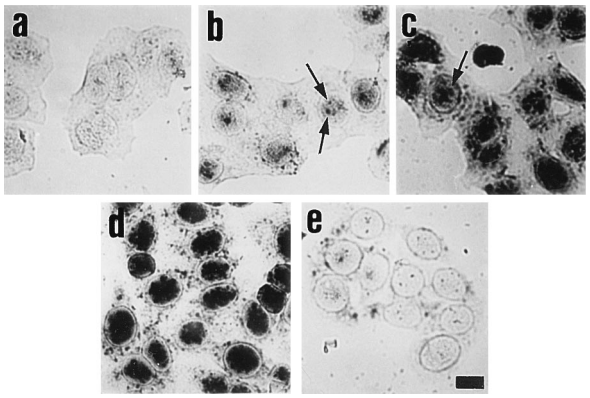

FIG. 5. Visualization of viral DNA. HeLa cells were infected with HSV-1 at a multiplicity of 10 PFU per cell and labelled for sites of viral DNA, using a hydrolyzed fluorescein-labelled HSV genomic DNA probe, which was then detected by alkaline phosphatase color development. Cells were fixed at 0 (a), 2 (b), 8 (c), and 16 (d) h postinfection. As a control, cells at 16 h postinfection were treated with DNase prior to labelling (e).

spots were difficult to distinguish (Fig. 5d). Uninfected HeLa cells did not label with this probe (Fig. 5a), and although a limited amount of virus DNA replication could have occurred by 2 h postinfection, the sites of DNA at 1 and 2 h would predominantly represent input parental virus DNA.

Replication label and immunodetection of snRNP and DNA replication proteins. We performed double immunolabelling in which infected cells labelled for sites of viral DNA replication were also labelled by using a monoclonal antibody directed against either the B″ component of the U2 snRNP or HSV-1 DNA polymerase (UL30) or the UL19 virus protein required for DNA packaging. Experiments to label both viral DNA replication sites (Fig. 6c and e) and the HSV-1 DNA polymerase (Fig. 6d) or the packaging protein (Fig. 6f) demonstrated that the three colocalized, indicating that sites of DNA replication and packaging of newly synthesized genomes are coincident in the nucleus. Replication label combined with B″ snRNP detection demonstrated that the majority (70 to 80%) of viral DNA replication sites were distinct from the redistributed snRNPs at later times; however, 20 to 30% of replication foci were coincident with high snRNP concentrations (compare Fig. 6a and b). It is possible that the regions of replication label and snRNP colocalization represent foci which are undergoing both transcription and DNA replication to which the snRNPs have been recruited. Thus, a proportion of snRNPs, although redistributed, may perform cotranscriptional splicing. Transcription label and monoclonal antibody detection of virus cell proteins cannot be performed together, as both rely on a mouse monoclonal detection step.

Transcription and replication label in cells infected with HSV-1 mutants. Incorporation of Br-UTP into nascent RNA transcripts in cells infected with the IE63 mutant virus 27-lacZ (Fig. 7A) demonstrated an initial increase in the number of transcription foci from one to two at 2 h postinfection (not shown) to four to five at 4 h postinfection (Fig. 7A, panel b). This result was similar to that for wild-type infection (compare Fig. 7A, panel b, with Fig. 1c); however, with 27-lacZ, no further increase in the number of foci occurred at later times (Fig. 7A, panels c and d). Infection of the IE63-complementing 2-2 cell line with 27-lacZ resulted in wild-type labelling patterns (data not shown).

Sites of viral transcription were labelled in HeLa cells infected with HSV-1 DNA packaging (UL6:lacZ-UL6-1) and DNA replication (UL8:ambUL8 and UL9:7-2) mutants; the results are shown in Table 1. The UL6 packaging mutant, which shows wild-type DNA replication levels, demonstrated a wild-type increase in the number of transcription foci, whereas nuclei infected with the UL8 and UL9 replication mutants exhibited only limited numbers of transcription foci, namely, three to four at 2 h and not more than four to five even at 16 h. This lack of viral DNA replication in cells infected with the UL8 and UL9 mutants is reflected in a reduced number of transcription foci.

The HSV-1 mutants in genes required for viral DNA replication and packaging (UL6) were used to infect both HeLa cells and, as a control, the complementing A26 cell line, which expresses all three deficient virus functions. Replication label in HeLa cells or the A26 parental Vero cells infected with the replication mutants produced only two to three bright foci per nucleus throughout the course of infection (Fig. 7B, panels b and c); uninfected cells demonstrated the normal diffuse replication label pattern (Fig. 7B, panel a). Infection of the complementing A26 cell line with these replication mutants demonstrated wild-type labelling patterns (Fig. 7B, panel d). Absence of ongoing viral DNA replication with these mutants is reflected in the lack of intensely labelled sites, again verifying that the foci observed with wild-type virus represent sites of viral DNA replication. Infections with the UL6 packaging mu-

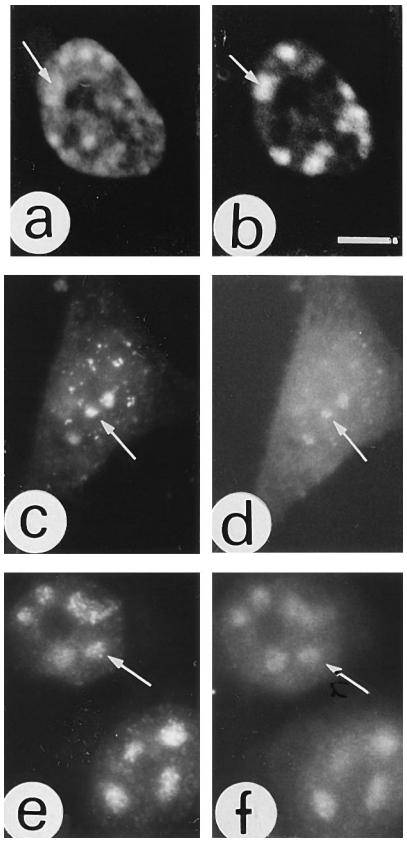

FIG. 6. Localization of DNA replication foci in relation to replication and processing proteins. HeLa cells infected with HSV-1 at a multiplicity of 10 PFU per cell for 8 h (e and f) or 16 h (a to d) were double labelled for sites of viral DNA replication (a, c, and e) and with antibodies against the U2 snRNP (b), UL30 DNA polymerase (d), and packaging protein UL19 (f). The viral replication and packaging proteins colocalize with the sites of replication; the snRNP was largely distinct from replication complexes. Bar corresponds to 10 μm; arrows indicate regions of colocalization.

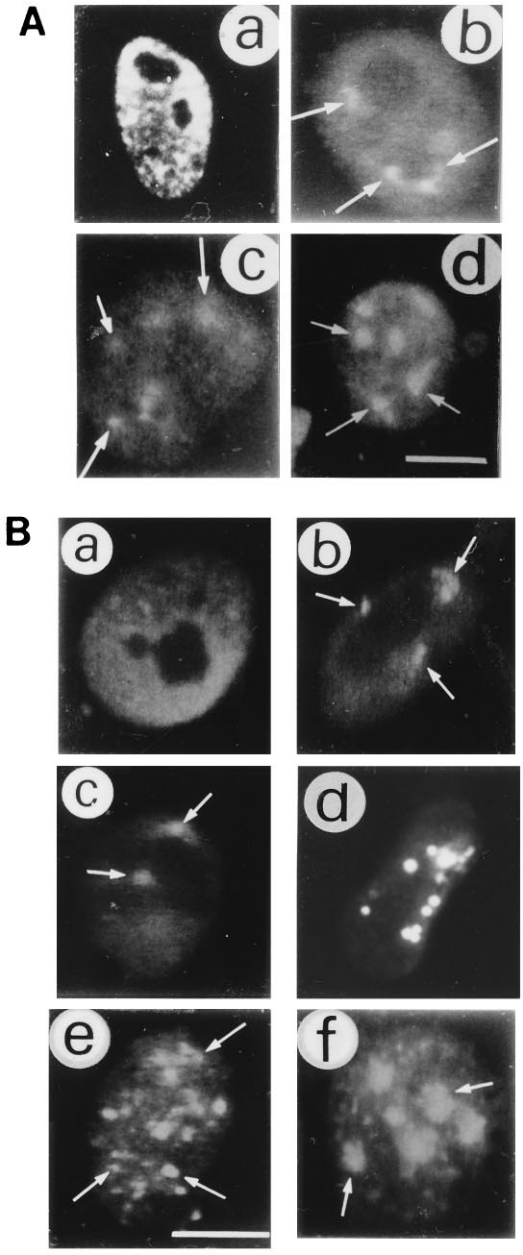

FIG. 7. Localization of transcription and DNA replication foci in HSV mutant-infected cells. (A) Transcription foci in cells infected with IE63 mutant virus 27-lacZ. HeLa cells infected with 27-lacZ at a multiplicity of 10 PFU per cell were labelled for sites of transcription by the incorporation of Br-UTP, with fluorescence immunodetection at 0 (a), 4 (b), 8 (c), and 16 (d) h postinfection. Bar corresponds to 10 mm; arrows indicate sites of transcription label. (B) Visualization of DNA replication foci in mutant HSV-1-infected cells. HeLa cells infected with HSV DNA replication and packaging mutants at a multiplicity of 10 PFU per cell from 0 to 16 h were labelled for sites of DNA replication by the incorporation of biotin-11-dUTP. Mock-infected cells demonstrated general nuclear label (a). Infections with UL9 (origin binding protein) (b) and UL8 (helicase-primase) (c) virus mutants demonstrated only one to two replication foci at 16 h postinfection. Infection of the complementing A26 cell line with the UL8 mutant demonstrated wild-type labelling patterns at 16 h postinfection (d). Infection with the UL6 packaging mutant (e) demonstrated more than 20 replication foci at 16 h postinfection. Infections with the IE63 virus 27-lacZ resulted in only three to four replication foci even at 16 h postinfection (f). Bar corresponds to 10 μm; arrows indicate sites of viral DNA replication.

TABLE 1. Number of viral transcription foci in HeLa cells infected with HSV-1 DNA packaging (UL6) and DNA replication (UL8 and UL9) mutants over a time course of infection

Time (h) postinfection	No. of transcription foci in cells infected with:		
	UL6 mutant	UL8 mutant	UL9 mutant
0 (mock infection)	1–2	1–2	1–2
2	4–6	3–4	3–4
4	4–6	3–4	3–4
8	6–7	4–5	4–5
16	9–10	3–4	4–5

tant demonstrated replication foci similar to those for the wild type, with 5 to 6 foci at 8 h and 20 to 25 foci at 16 h (Fig. 7B, panel e). Thus, viral DNA packaging is not a prerequisite for continued viral DNA synthesis.

Viral DNA replication sites in cells infected with the IE63 mutant virus 27-lacZ revealed a limited number of replication foci throughout infection; even by 16 h, only five to six foci were seen in most cells (Fig. 7B, panel f); a number of smaller foci were present, but these may represent abortive replication sites. Southern blot analysis demonstrated an initial increase in the amount of 27-lacZ DNA to around 4 h postinfection; levels then remained constant, in contrast to wild-type infection, in which a continued increase in DNA levels was observed (data not shown).

DISCUSSION

We have shown that sites of viral transcription and replication can be identified as discrete domains in the nuclei of HSV-1-infected HeLa cells by direct pulse-labelling. The numerical data (unless otherwise stated) were determined by infection of HeLa cells at a multiplicity of infection of 10 PFU per cell. The number of viral transcription sites, initially three to four per nucleus, increases from 4 h postinfection, rising to a final number of seven to eight sites by 16 h postinfection. In contrast, the number of DNA replication sites increases throughout the course of infection from 3 to more than 15 in a near-exponential manner. The increase in transcription foci observed at early times would be consistent with an early amplification of input virus DNA which then undergoes transcription. Interestingly, at early times, transcription can be detected at all replication sites, whereas at later times, additional foci which appear solely committed to virus DNA replication are present.

Shortly after HSV-1 infection, the genome circularizes and DNA replication is believed to occur via a rolling-circle mechanism (7, 40, 43). However, rolling-circle replication from input genomes alone is regarded as insufficient to account for the amount of viral DNA synthesis that takes place, and Zhang et al. (53), on the basis of field inversion electrophoretic studies, and Skalliter and Lehman (43) have suggested that a plasmid-like amplification of circular templates precedes initiation of rolling-circle replication.

Rixon et al. (39) detected virus DNA replication initially restricted to a few well-defined sites at the nuclear membrane which increased in size to involve the entire nucleus. More recently, de Bruyn Kops and Knipe (9) detected replication foci deeply buried within the nucleus, and this finding is confirmed by our analysis. Using in situ hybridization, we have visualized viral DNA as an initial one to three sites which presumably represent incoming viral genomes. While we should bear in mind that in general only a proportion of input

genomes will be replicated or expressed, this number corresponds well to the initial number of transcription and replication sites under our infection conditions. The amount of detectable viral DNA increases until individual spots cannot be differentiated; note that double-label experiments where sites of HSV DNA and sites of viral replication are visualized simultaneously have not been performed, as the alkaline phosphatase color development method used for the detection of HSV genomic DNA cannot be combined with fluorescence techniques.

At early times, the numbers of transcription and replication foci per nucleus are comparable and must represent transcription and replication of the incoming genomes. Double labelling of DNA replication and transcription sites in the same nucleus at early times has demonstrated that the two processes almost perfectly colocalize, which indicates that input genomes are transcribed and replicated simultaneously. This is consistent with the finding of Rice et al. (37) that cellular RNA polymerase II localizes to HSV replication compartments. The proposal that transcription and replication can occur simultaneously on cellular DNA is a contentious issue. Hassan and Cook (15) found that replication initiates only at transcription sites and that the two activities colocalize, whereas others (50) have illustrated that transcription and replication domains do not colocalize and believe that DNA replicated late in the cell cycle is not generally transcribed.

At later times, there are more viral replication sites than transcription foci, and double-label experiments demonstrate that the transcribing sites are undergoing replication, while other foci are undergoing replication in the absence of transcription even in circumstances where we have imbalanced the procedure to favor transcription. We do not rule out the possibility that low levels of transcription occur at exclusive replication sites. However, these domains are committed predominantly to DNA replication; additionally, an individual site may not be committed exclusively to transcription or replication throughout its lifetime. The labelling of discrete DNA replication sites at later times may represent sites of replication located within the mass of replicated DNA which has expanded throughout the nucleus. We speculate that at later times when the virus is committed to replication and packaging of its genome, these nontranscribing sites undergo rolling-circle DNA replication. Indeed, Rice et al. (37) observed regions of the nucleus which labelled with the major DNA binding protein but did not colocalize with RNA polymerase II.

Replication label pulse-chase experiments (data not shown) have demonstrated that newly replicated viral DNA does not move far from its synthetic site, even during a 2-h chase period. We have shown that a DNA packaging protein colocalizes with the replication sites and virus DNA polymerase; thus, these sites presumably represent sites of viral DNA replication and of DNA packaging. Ward et al. (51) describe an HSV gene product, UL43.5, which associates with viral capsid and scaffold proteins; they state that these proteins are distinct from sites of DNA synthesis.

The number of viral transcription foci is limited throughout infection, initially doubling between 2 and 8 h as a result of replication of parental genomes but then increasing only slightly between 8 and 16 h postinfection (Fig. 4). We envisage that the increase in transcription site number as infection proceeds could be the result of (i) input genomes becoming transcriptionally active as infection proceeds, (ii) single input viral genomes registering as more than one transcriptionally active focus, or (iii) input viral genomes which are replicated during the first few rounds being subsequently transcribed (our preferred option).

The number of transcription foci did not increase proportionately with increasing multiplicity of infection. When cells were infected at very high multiplicities, a limited number of transcription foci was observed, with an upper limit of 18 sites per nucleus. Similarly, the number of replication foci was limited at high multiplicities of infection, with a maximum of 30 to 40 sites (data not shown). This restriction on viral transcription sites likely reflects a limitation of cellular transcription factors or perhaps space constraints within the nucleus. Maul et al. (24) have proposed that HSV DNA replication foci localize to predefined nuclear sites, correlating to ND10 sites which are occupied by the regulatory cellular proteins PML, Sp-100, and NDP55. HSV RNA also was found associated with ND10, suggesting that viral transcription takes place at these sites. During HSV infection, the IE protein ICP0 interacts with and disperses ND10 domains, and it is possible that preexisting ND10 domains determine the number of HSV transcription and DNA replication foci which can form. By contrast, adenovirus infection results in a reorganization of ND10 domains as their cellular factors are relocated into viral replication centers (10). Adenovirus replication factories do not form at ND10 sites, and consistent with this finding, there does not appear to be an upper limit on the number of transcription foci that an adenovirus infected cell can support.

Our use of virus mutants has allowed verification that focal sites of label incorporation are of viral origin and further has shown that the essential DNA replication functions of the origin binding protein and the helicase-primase activities are required for replication focus formation. In cells in which packaging of virus DNA was inhibited, normal replication foci were formed; hence, genomic packaging is not a prerequisite for continued DNA synthesis, which is consistent with evidence that a significant proportion of replicated viral DNA is never packaged (1). Transcription label of cells infected with viral DNA replication mutants demonstrated a reduced number of foci throughout infection, representing transcription from input virus DNA. The small increase in the number of transcription foci observed with virus replication mutants (Table 1) could represent either extremely limited DNA replication occurring in the absence of a full complement of viral replication proteins or delayed transcription from incoming parental genomes. The DNA packaging mutant showed a wild-type increase in the number of transcription foci, thus demonstrating that a failure to package DNA does not affect transcription site formation.

IE63 protein is required for efficient HSV DNA replication; the effect is cell cycle dependent, and it has been suggested that IE63 promotes formation of the DNA replication complex (8) and regulates expression of viral replication proteins (26, 48). Using our labelling methods, we found that in cells infected with the IE63 mutant virus 27-lacZ, there was a small initial increase in replication site number (not quite a doubling) but no subsequent increase from 9 to 16 h. We found that the number of transcription sites in cells infected with the IE63 mutant increased in line with wild-type infections.

Studies by us and others (23, 42) have demonstrated that HSV infection causes a clumping of the splicing snRNPs and splicing auxiliary factors, and we have shown that IE63 is both necessary and sufficient to cause this effect (31). IE63 could, by redistributing the snRNPs to the nuclear periphery, facilitate the formation and spatial arrangement of viral transcription and replication complexes. As some five HSV transcripts require to be spliced, this finding raises the question of where HSV splicing takes place. Analyses of HSV-infected cells double labelled for sites of DNA replication (therefore indirectly for transcription at early times), and with antibodies against

snRNP proteins, show that the redistributed snRNPs predominantly do not colocalize with sites of viral transcription and replication. However, 20 to 30% of replication sites colocalize with the snRNP clumps, and it is possible that the 20 to 30% of DNA replication foci at late times which also undergo transcription colocalize with, or are adjacent to, the redistributed snRNPs. This would suggest that cotranscriptional splicing of intron-containing viral transcripts may occur at these sites; however, only 30% of the total redistributed snRNP colocalizes to these replication foci. Evidence for splicing occurring at late times is provided by the detection of UL15 transcripts in the cytoplasm (32); however, 30% of the cytoplasmic UL15 was found to be unspliced (14). HSV infection causes a slight but noticeable clumping of polyadenylation factors as they become concentrated presumably toward active sites of viral transcription and 3′ processing (26).

In comparing the organization of nuclei infected with HSV with that of nuclei infected with adenovirus, distinct differences are apparent. With HSV infection, viral DNA replication and transcription sites are coincident at early times, and the splicing machinery is largely removed from these sites. In contrast, Pombo et al. (33) have shown that sites of adenovirus transcription and replication, while adjacent, do not colocalize, as transcription foci form on those newly replicated DNA viral templates which have moved from their synthetic sites. With adenovirus-infected cells, as with uninfected cells (52), snRNPs localize to sites of transcription, suggesting that cotranscriptional splicing occurs and that snRNPs associate with nascent viral transcripts (33, 34). Furthermore, the adenovirus replication cycle requires a major postreplicative transcriptional burst to produce the late virus mRNAs; therefore, snRNPs in adenovirus-infected cells also associate with newly replicated virus DNA. Thus, HSV and adenovirus infections both cause reorganization of the host cell nucleus but in ways which reflect their different requirements for virus replication.

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