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Sites of Herpes Simplex Virus Type-1

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The herpes simplex virus type 1 (HSV-1) nuclear replication cycle begins at localized sites, but it has remained unclear whether these sites are associated with any defined nuclear structure. We have previously shown that during infection, the HSV-1 immediate-early protein ICP0 dispersed proteins associated with ND10, nuclear sites that contain high concentrations of PML and other potentially regulatory proteins and correspond to the ultrastructurally defined nuclear bodies. Using *in situ* hybridization and immunohistochemical techniques, we found that ICP0 mutants of HSV-1 replicate in the close proximity with ND10, but increasing replication sites develop away from these nuclear structures. Input wild-type HSV-1 DNA was found preferentially adjacent to ND10 before ICP0 modified these nuclear structures and did not colocalize with ICP8 containing so-called prereplication sites. The sites where HSV-1 can begin replication then need to be redefined as preexisting potential replication sites. Viral RNA was also found associated with ND10 before early protein synthesis (ICP8), suggesting that input virus genomes are deposited at ND10 before they start replication. The deposition of input viral DNA at ND10 is virus gene expression independent, probably indicating cell regulation of this process. Taken together, these data demonstrate that some very early processes of the nuclear viral replication cycle happen in close proximity or at the periphery of ND10. The localization of input HSV-1 to ND10 represents a new host–virus interaction and provides an unexpected functional property for this large nuclear site.

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) infects the cell by fusion of its envelope with the cell membrane. Uncoating of the virus particle takes place in the cytoplasm, releasing the tegument-bound transcriptional activator VP16. The capsid is transported to the nuclear envelope and docks at fibers emanating from the nuclear pore complex (Morgan *et al.,* 1968). Since there are approximately 2000 nuclear pore complexes per cycling cell (Maul *et al.,* 1972), it is likely that viral DNA enters the nucleus at random sites. Little is known about the structural aspects of the steps that lead to the onset of the viral transcriptional cascade in the nucleus and where it begins. HSV-1 DNA replication begins at a limited number of sites which are located throughout the nucleus in an apparently nonrandom distribution (de Bruyn Kops and Knipe, 1994). These replication sites expand until they fuse and fill most of the nucleus. However, it is not known whether the initial sites of viral DNA replication associate with any preexisting nuclear structure. The fact that the replication sites appear to be nonrandom suggests that there are limited preexisting sites of virus–host interaction in the host nucleus. Prereplication sites were originally defined as numerous small sites of nuclear accumulation of the infected cell polypeptide 8 (ICP8) (Quinlan and Knipe 1984), the major viral DNA binding protein which associates with replicating viral DNA (Powell and Purifoy 1976; Knipe *et al.,* 1982). The term prereplication site presupposes that replication can begin there. This implies the presence of infecting HSV-1 DNA at all the ICP8-positive sites. This possibility is testable by combined immunohistochemical and *in situ* hybridization techniques. In this report, we demonstrate that viral DNA is not found at ICP8-defined prereplication sites but rather at preexisting nuclear domains, ND10.

ND10 (so named because of their average frequency in the nucleus) were first recognized as discrete matrix-bound nuclear sites with a diameter of 0.3–0.5 μm (Ascoli and Maul, 1991; Maul *et al.,* 1993) using autoantibodies from patients with primary biliary cirrhosis (Bernstein *et al.,* 1984); these autoimmune sera predominantly detect the ND10-associated protein Sp100 (Szostecki *et al.,* 1990). Another major component of ND10 is the protein PML, so named because the chromosomal translocation which results in a PML-RARα fusion protein causes the onset of promyelocytic leukemia (de Thé *et al.,* 1991; Fusconi *et al.,* 1991; Goddard *et al.,* 1991; Kakizuka *et al.,* 1991; Kastner *et al.,* 1992). This disease correlates with a dispersed distribution of the ND10 proteins (Dyck *et al.,* 1994; Koken *et al.,* 1994; Weis *et al.,* 1994). ND10

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correspond at least in part to "nuclear bodies" (Koken *et al.,* 1994; Maul *et al.,* 1995) which were first observed by electron microscopy (de Thé *et al.,* 1960; Hinglais-Guillaud *et al.,* 1961). As yet, no particular function has been assigned to ND10, although they increase in number after hormone treatment (reviewed in Brash and Ochs, 1992) and also in response to interferon (Guldner *et al.,* 1992; Koken *et al.,* 1994; Maul *et al.,* 1995). ND10-associated proteins are also dispersed during HSV-1 infection (Maul *et al.,* 1993), a process which requires functional immediate-early protein ICP0 (Maul and Everett, 1994; Everett and Maul, 1994). The finding that ND10 are retained during infection with ICP0-defective HSV-1 viruses made it possible to determine the relationship between ND10 and the sites of viral DNA replication. We found that HSV-1 ICP0 mutant DNA replication sites preferentially occur adjacent to ND10 and that input wild-type HSV-1 DNA is also preferentially deposited close to ND10 even in the absence of virus gene expression, suggesting that the nuclear replication cycle of HSV-1 begins at ND10.

MATERIAL AND METHODS

Antibodies and cell culture

ND10 were visualized using various antibodies that detect ND10-associated proteins. Monoclonal antibody (MAb) 1150 recognizes Sp100 (Maul *et al.,* 1995), while MAb 5E10, which was generated against a nuclear matrix protein (Stuurman *et al.,* 1992), recognizes PML (Koken *et al.,* 1994; Maul *et al.,* 1995). Isotype-matched MAbs of unrelated specificity were used as controls. Polyclonal rabbit antisera against PML (Dyck *et al.,* 1994) were used. Rabbit antibodies against Sp100 were provided by Dr. J. Frey. Hybridomas producing HSV-1-specific antibodies to detect ICP8 and ICP4 (indicative of single-stranded DNA binding protein and HSV-1-infected cells, respectively) were obtained from the American Tissue Culture Collection (ATCC, Rockville, MD). MAb 11060 against ICP0 has been described (Everett *et al.,* 1993). HEp-2 carcinoma cells and MRC5 diploid human fibroblasts were maintained in MEM supplemented with 10% FCS and antibiotics. All cells were grown at 37° in a humidified atmosphere containing 5% CO_2. For immunofluorescence, cells were grown on round coverslips in 24-well plates (Corning Glass, Inc., Corning, NY).

Herpes virus infection

HEp-2 and MRC5 cells were infected 2 days after plating when they were approximately 80% confluent. HSV-1 17^+ was added to the medium at 1–10 PFU, resulting in 40–90% infected cells as determined with anti-ICP4 antibodies. The ICP0 mutants D22, FXE, D8, and the ICP0 deletion mutant dl1403 were used (Everret, 1989) at pretitered infectious particle concentrations resulting in 50–90% infected cells as determined by immunofluorescence assay using anti-ICP4 antibodies. Metabolic inhibitors were added at the time of infection at 100 μg/ml for cycloheximide and 200 ng/ml for actinomycin D (Act D).

Immunolocalization of virus and host proteins

HSV-1 and mutant-infected HEp-2 cells were fixed at room temperature for 15 min with freshly prepared 1% paraformaldehyde in PBS, washed with PBS, and permeabilized for 20 min on ice with 0.2% (v/v) Triton X-100 (Sigma Chemical Co., St. Louis, MO) in PBS. Antigen localization was determined after incubation of permeabilized cells with rabbit antiserum or MAb diluted in PBS for 1 hr at room temperature. Avidin–fluorescein was complexed with primary antibodies through biotinylated secondary antibodies (Vector Labs Inc., Burlingame, CA). Cells were double-labeled with the respective second antibodies labeled either with FITC or Texas Red using the biotin–avidin enhancement and FITC for structures with the lowest staining intensity. Cells were then stained for DNA with 0.5 mg/ml of bis-benzimide (Hoechst 33258; Sigma, St. Louis, AL) in PBS and mounted with Fluoromount G (Fisher Scientific, Pittsburgh, PA). Cells were analyzed using a Leica confocal scanning microscope, and single optical sections were photographed with a Focus Graphics Inc. device and printed with a Kodak Color Ease printer.

In situ hybridization of HSV-1 DNA

For the localization of HSV-1 DNA or RNA, fluorescent *in situ* hybridization techniques were adapted from those described by Lawrence *et al.* (1989) and Huang and Spector (1991). The hybridization probe was prepared using DNA of cosmid 56 (Cunningham and Davison, 1993), which contains the region between 79,442 and 115,152 bp of the HSV-1 genome. As a control, DNA of adenovirus type 5 (Ad5) was nick-translated in the same way as HSV-1 DNA in the presence of biotinylated-11-dUTP (Sigma, St. Louis, AL). The DNase concentration and the duration of nick-translation was adjusted to obtain a probe fragment 200–500 bp in size. Cells infected for various time periods with HSV-1 or its mutants were fixed with freshly prepared 1% paraformaldehyde and permeabilized with 0.2% Triton X-100. Cellular and probe DNA (or only the DNA probe for viral RNA detection) were denatured simultaneously for 3 min at 90° on a heat block and hybridized for 2 hr at 37° in a hybridization mixture containing 50% deionized formamide, 10% of dextran sulfate, 1× SSC, and 20 ng of biotinylated probe, 1 μg of salmon sperm DNA, and 0.5 μg of human DNA per milliliter. After hybridization, cells were washed three times in 50% formamide–2× SSC prewarmed to 42°, followed by three washes for 5 min each in 0.1× SSC pre-

warmed to 60°. Washed cells were then stained with fluorescinated avidin (Vector Lab, Inc., Burlingame, CA) in 3% BSA–2× SSC. The signal was enhanced with an additional round of biotinylated anti-avidin antibodies followed by fluorescinated avidin treatment. All preparations were stained with Hoechst 33258. For DNase treatment, coverslips were incubated in RNase-free DNase I (10 μg/ml) in the presence of 5 m*M* Mg^{2+} and RNase inhibitor (1 unit/ml RNasin; Promega, Madison, WI). The effectiveness of DNase treatment was judged based on residual Hoechst 33258 staining. RNase digestion was carried out using 20 μg/ml RNase (Boehringer, Mannheim, Germany) for 2 hr at room temperature. Immunohistochemical analysis and *in situ* hybridization were combined using FITC to label the weaker *in situ* hybridization signal and Texas Red to label the proteins.

RESULTS

Parental HSV-1 DNA preferentially localizes adjacent to ND10

ND10 were visualized in uninfected HEp-2 cells by immunofluorescence using antibodies against the autoantigen Sp100 and by antibodies that recognize PML. The signals from these two proteins completely colocalize as shown in a pair of daughter cells with similar ND10 distribution (Fig. 1A). Staining of HSV-1-infected cells with an antibody that detects the major viral single-stranded DNA binding protein ICP8 at 7 hr p.i. revealed a similar arrangement of viral replication sites in another pair of daughter cells (Fig. 1B), consistent with previous observations in binucleated cells (de Bruyn Kops and Knipe, 1994), suggesting a nonrandom distribution of these viral sites. An average of 7 viral replication sites were found early during viral DNA replication (5 hr p.i.) in cells infected with 40 PFU of virus/cell. To determine whether the number and apparent nonrandom distribution of ND10 induced the nonrandom distribution of HSV-1 DNA replication sites, we examined these two sites using double-labeling techniques. However, ND10 are not recognizable in the infected cells (ICP8 positive; Fig. 1C; right) as they are in the apparently uninfected cell (Fig. 1C; top left). The two cells identified by the phase contrast image of their nuclei were neither labeled with ICP8 nor PML. This indicates that ND10 become unrecognizable before early viral protein synthesis (ICP8) consistent with our previous work (Maul *et al.,* 1993; Maul and Everett 1994; Everett and Maul 1994). In ICP8-positive nuclei one often recognizes two distinct distributions, larger aggregates (arrows) considered to be replication sites and unit sized dots previously named prereplication sites (Quinlan and Knipe, 1984).

The apparent absence of ND10 shortly after infection made it impossible to determine directly whether HSV-1 begins its replication at ND10 or at the ICP8-defined prereplication sites. To circumvent this problem, we determined: (i) the location of wild-type HSV-1 DNA before ND10-associated protein dispersal, and (ii) the replication sites of the ICP0 mutant virus which does not modify ND10 before replication begins. We optimized the *in situ* hybridization technique for HSV-1 DNA detection and combined it with immunohistochemistry for simultaneous demonstration of input viral DNA and ND10 in the same cell, since only this single cell assay in conjunction with the resolving power of confocal microscopy can address the relationship of the two signals. As shown in Fig. 1D, the location of viral DNA and ICP8 in the nucleus overlap, verifying that the *in situ* hybridization procedure recognizes the site of replicated viral DNA. The left pointing arrow in Fig. 1D indicates point-size signals representing input viral DNA. The right pointing arrow indicates so-called prereplication sites none of which presents a viral DNA signal. This is interpreted to mean that the so-called prereplication sites do not contain HSV-1 DNA.

At any given time after infection individual cells are at somewhat different stages of the viral replication cycle for several reasons: virus stocks have a high particle-to-PFU ratio; infection is not precisely synchronous; a proportion of the viral genomes remains uncoated or releases viral DNA very slowly. Therefore, only images that represent the predominant finding are shown. However, at least 500 cells were evaluated for each experiment which was repeated at least three times. High magnification was used to definitively identify the point-size DNA signals of input virus in the cell. At very early times of wild-type virus infection (1.5 hr), the bulk of the input viral DNA was found either in the cytoplasm or at the periphery of the nucleus (Fig. 2A). However, even at that time, some small discrete signals from viral DNA could be detected in the nucleus, and some of these were very closely associated with ND10 (arrow). At 3 hr after infection, up to 50% of HSV-1 DNA could be detected in the nucleus in the immediate vicinity of ND10 (Fig. 2B). This represents a strong correlation because all ND10 in a nucleus represent $<0.05\%$ of the total nuclear volume. The images reveal that the viral DNA does not colocalize with ND10 but is located directly adjacent to these structures probably binding to its periphery. Viral entry into the nucleus at random sites, and the subsequent association of the input viral DNA with the periphery of ND10, implies movement of the viral DNA to ND10 rather than movement of ND10 to the input virus. ND10 represent nuclear bodies at the ultrastructural level, large nuclear matrix-bound structures (0.3–0.5 μm) (Maul *et al.,* 1995), which are unlikely to move as such. Furthermore, the number of ND10 does not change for either low or very high particle infections relative to uninfected cells. A change would be expected if ND10 proteins moved to the sites of input virus attachment at the nuclear matrix before replication begins. At later times, dis-

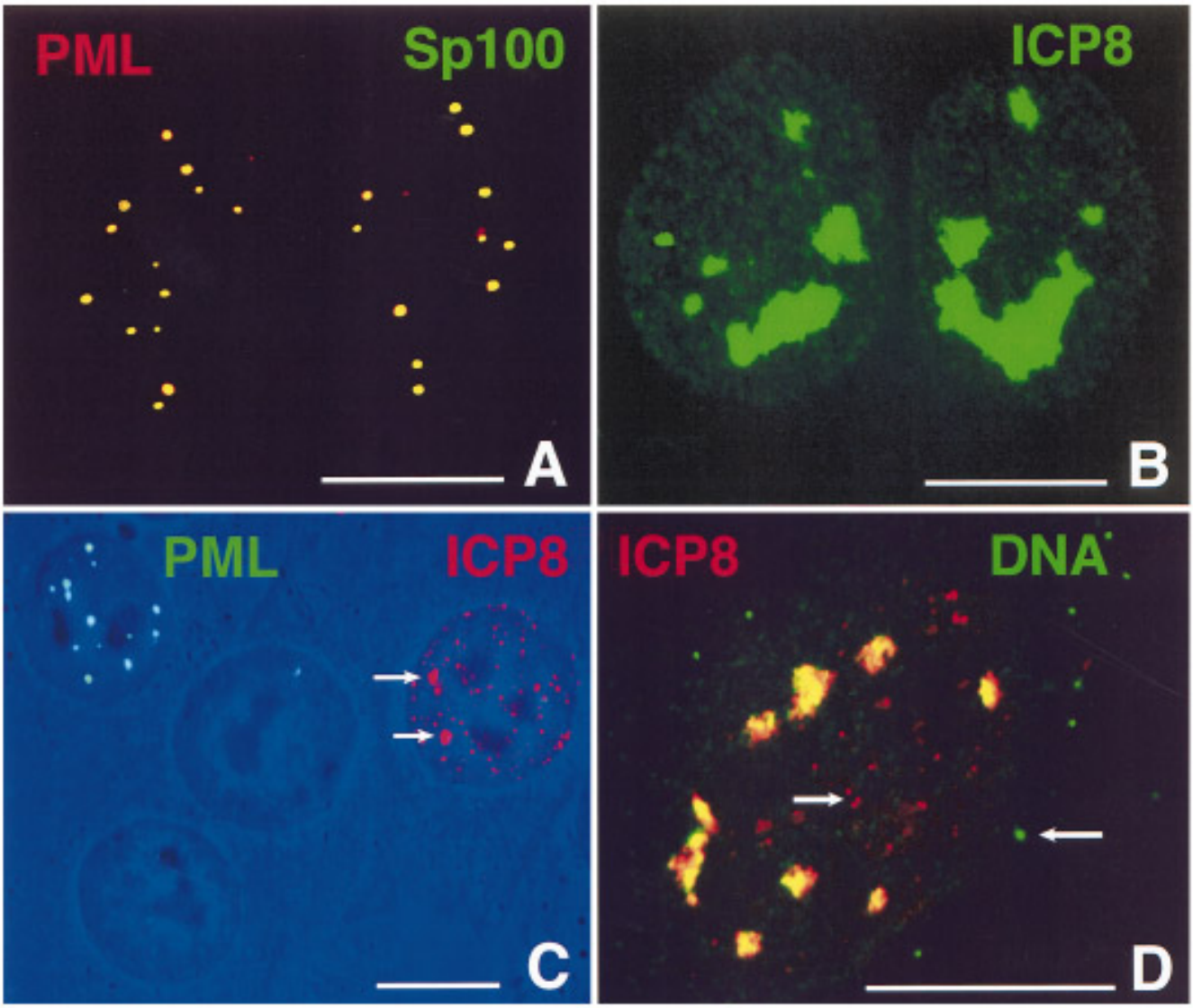

FIG. 1. Distribution of ND10 and HSV-1 DNA replication sites. (A) Uninfected HEp-2 daughter cells probed with the rabbit antibody against Sp100 (green) and anti-PML MAb 5E10 (red) recognizing ND10. (B) Two HEp-2 daughter cells infected with HSV-1 at 7 hr p.i. and stained with anti-ICP8; both cells show a similar arrangement of replication sites. (C) HEp-2 cells infected with HSV-1 4 hr p.i., double-labeled with PML (green) and ICP8 (red). The larger ICP8 accumulations represent replication sites (arrows). (D) HEp-2 cell infected with HSV-1 4 hr p.i., double-labeled by *in situ* hybridization with biotinylated HSV-1 DNA (green) and ICP8 (red). The yellow color denotes colocalization within the single optical section. Arrows pointing left indicate DNA signals in the cytoplasm that are presumably derived from an input viral genome. Arrow pointing right indicates unit size dots. Bar marker represents 10 μm.

persion of ND10-associated proteins by ICP0 made it impossible to determine the relative position of the viral DNA. The controls for probe specificity included mock- and Ad5-infected cells hybridized with HSV-1 DNA probe and HSV-1-infected cells hybridized with adenovirus DNA probe (Fig. 2C). No DNA signal was obtained, indicating probe specificity.

To allow more time for the release of input viral DNA into the nucleus before ICP0 expression disperses ND10 in wild-type infections, the experiment was repeated in the presence of either cycloheximide (Fig. 2D) or actinomycin D (Fig. 2E). In both cases, there was a strong correlation between the location of the virus-derived signal and ND10. The enlarged hybridization signal in the cycloheximide-treated cell (Fig. 2D) relative to the small signal in the Act D-treated sample (Fig. 2E) may reflect the accumulation of immediate early transcripts under these conditions, i.e., represent the combined RNA and DNA signal. These results strongly suggest that ND10 are the preferred sites of localization of input HSV-1 DNA and that viral gene expression is not necessary for the deposition of the viral genome to ND10.

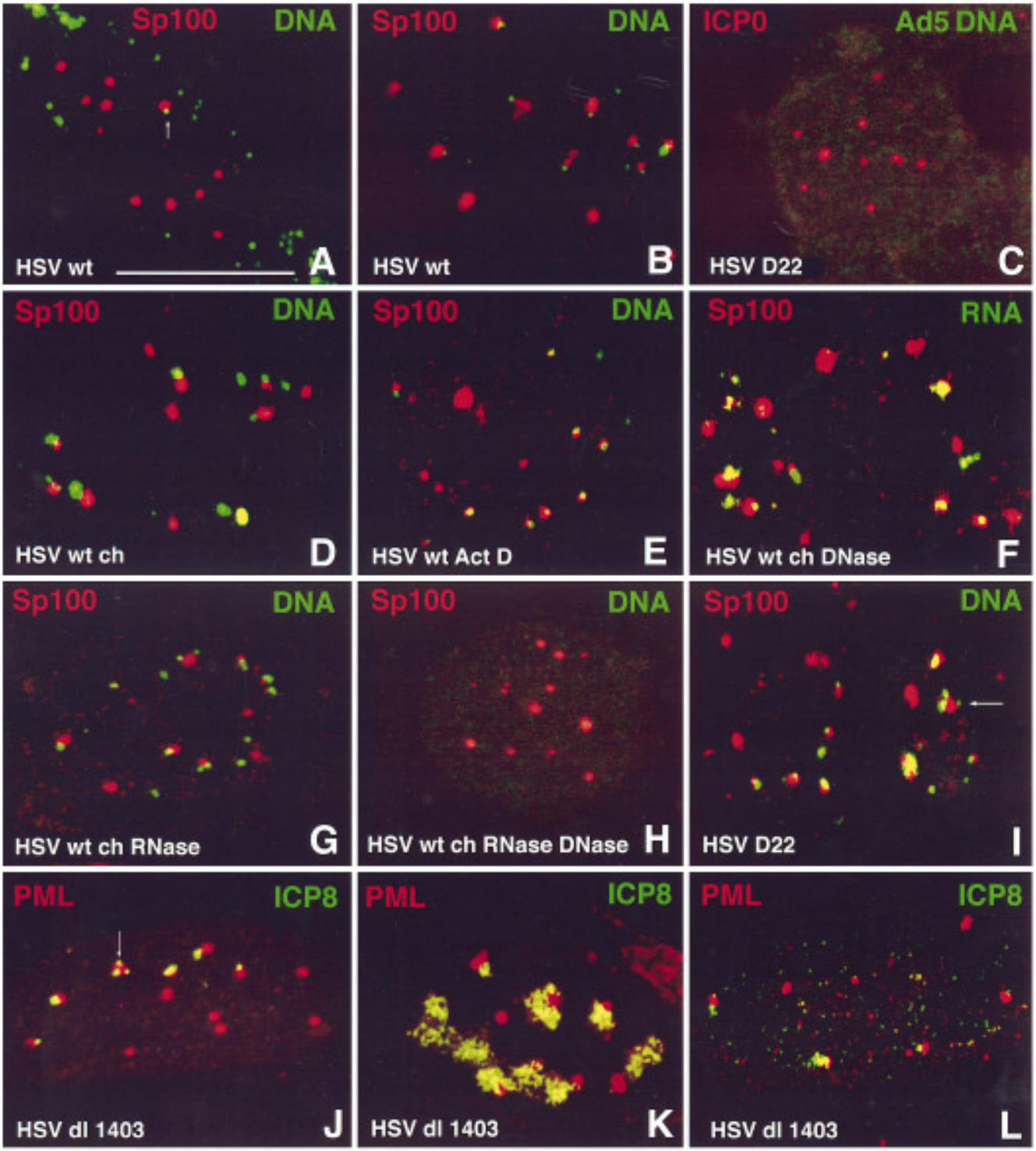

FIG. 2. Localization of HSV-1 DNA relative to ND10. HEp-2 cells were infected with HSV-1 and at various times postinfection (p.i.) were probed for ND10 using either anti-PML or anti-Sp100 antibodies (red) followed by *in situ* hybridization with biotinylated HSV-1 DNA probe labeled with FITC–avidin (green). All images are single optical sections selected to show representative position of virus DNA and ND10. High magnification was chosen to reveal the signals of input viral DNA. (A) HEp-2 cell 1.5 hr p.i. shows viral DNA (green) in the cytoplasm and one precise signal at ND10 (red; Sp100) (arrow). (B) Same as A but 3 hr p.i. showing most input viral DNA (green) juxtaposed to ND10 (red; Sp100). (C) *In situ* hybridization control showing HSV-1 mutant D22-infected HEp-2 cell 3 hr p.i., using biotinylated Ad5 DNA for *in situ* hybridization and MAb 10060 to label ICP0 (located in case of this mutant at ND10; Maul and Everett, 1994), demonstrating that this cell is infected; no DNA signal was visible. (D) HEp-2 cell infected with HSV-1 17^+ 5 hr p.i. and cycloheximide-treated (ch) at the beginning of infection; most of the hybridization signals (green) are juxtaposed to ND10 (red). The larger size of the viral hybridization signal (compare with B) is probably due to the cycloheximide block. (E) Same as D but

To determine whether the *in situ* hybridization signals are derived from viral DNA or RNA transcripts or both, we treated cells infected in the presence of cycloheximide with either DNase or RNase prior to hybridization. Treatment with DNase did not remove the hybridization signal and a significant proportion of the RNA-derived signal was again closely adjacent to ND10 (Fig. 2F). Cellular DNA was completely digested as judged from the absence of residual Hoechst 33258 staining. This result was confirmed by hybridization under undenaturing conditions, indicating that the hybridization signal at Fig. 2D is in part derived from viral transcripts. Hybridization after RNase treatment gave results similar to the previous experiments using Act D (Fig. 2E), i.e., the viral DNA signal was very close to ND10. The images presented also show that not all viral DNA signals were recognizably close to ND10, which is in part due to the recording of single optical sections leaving some ND10 out of the optical plane. Combined DNase and RNase digestion reduced the hybridization signal to undetectable levels (Fig. 2H).

HSV-1 DNA replicates in close association with ND10

The above experiments illustrate that the input viral genomes are deposited very close to ND10. However, the use of wild-type virus made it difficult to determine whether the viral replication sites are also associated with ND10, because ICP0 expression prior to DNA replication leads to dispersion of ND10-associated proteins. Accordingly, we used ICP0-deficient virus mutants which replicate effectively after high multiplicity infection (Stow and Stow, 1986) and which fail to modify ND10 before viral replication starts. ICP0 mutant D22 has a deletion close to the RING finger motif which prevents the mutant virus from dispersing ND10-associated proteins (Everett, 1988; Maul and Everett, 1994). Cells were infected with HSV-1 D22 and *in situ* hybridization was combined with staining for ND10. At 7 hr after infection, there was a striking association of the viral replication sites with ND10 (Fig. 2I). Other ICP0 mutant viruses (FXE, D8) with phenotypes similar to that of D22 or the ICP0 deletion mutant dl1403 gave equivalent results in this experiment (data not shown).

The major DNA binding protein of HSV-1, ICP8, colocalized with replicated viral DNA (Fig. 1D); therefore we used ICP8 as a marker for the viral replication sites. MRC5 cells were infected with ICP0 deletion mutant dl1403 and probed 5 hr later with antibodies against ICP8 and PML (Fig. 2J). Again, a striking juxtaposition of ICP8 with ND10 was observed, and, as after *in situ* hybridization with viral DNA, multiple HSV–DNA replication sites were found associated with ND10 (Figs. 2I and 2J; arrow). As infection progressed, the viral DNA replication sites became larger and they appear to develop away from ND10, but retained their association with ND10 (Fig. 2K).

In some HSV-1-infected cells, numerous small ICP8-positive sites were observed, so-called "prereplication sites," and it has been suggested that these are precursors of the viral DNA replication sites (Quinlan *et al.*, 1984). These prereplication sites were not found in association with ND10, and in cells containing both these and the larger replication sites, only the latter were adjacent to ND10 (Fig. 2L). One interpretation of this observation is that, unlike the ICP8-positive sites in close proximity to ND10, the small ICP8 prereplication sites do not generally develop into replication sites.

If HSV-1 DNA replication begins at the periphery of ND10, most replicating viral DNA should be found near these sites. A semiquantitative evaluation of 100 HSV-1 D22-infected cells with more than one replication site revealed that 81% of the *in situ* hybridization signals were associated with ND10. This finding suggests that either ND10 are not the exclusive but rather the preferred sites of viral DNA replication, or that some ND10 are too small or faint to be detected after *in situ* hybridization. Consistent with the latter possibility is the finding that ND10-derived signals are generally smaller after the heat treatment necessary for *in situ* hybridization than after immunohistochemical visualization alone. When the number of viral DNA replication sites was determined using the ICP0 mutant D22 at early times after infection, i.e., before the replication sites had fused (4–6 hr p.i.), an average of 7 was found using 40 PFU/cell for infection. This number corresponds to that for wild-type virus confirming the similarity between the mutant and wild-type virus replication site formation.

DISCUSSION

The experiments reported here demonstrate that the earliest events in the HSV-1 nuclear replication cycle start in close proximity or at the periphery of ND10. We have shown that a large proportion of the input viral DNA

treated with Act D at the start of infection. Most of the viral DNA signals are located at ND10. (F) Same as D but DNase-treated before *in situ* hybridization showing RNA-derived signal (green) at ND10. (G) HEp-2 cell as in D but RNase-digested before *in situ* hybridization, resulting in very precise DNA-derived signals (green), preferentially located at ND10. (H) Same as in D but double-digested with DNase and RNase, a treatment that removed all of the hybridization signal. (I) Two HEp-2 cells infected with the D22 mutant at 7 hr p.i. showing that the *in situ* hybridization signals (green) of this virus mutant are located adjacent to ND10. Arrow indicates multiple replication sites at single ND10. (J) HSV-1 dl1403-infected MRC5 cells at 5 hr p.i. labeled for ICP8 (green) and PML (red), showing all viral ICP8-positive sites at ND10 (multiple sites, arrow). (K) Same as in J at 9 hr p.i. showing larger replication sites located near ND10. (L) Same as in J but with larger numbers of very small ICP8-positive sites throughout the nucleus and a few early replication sites at ND10. Bar marker represents 10 μm.

is deposited close to ND10 prior to viral gene expression, and that the viral DNA replication starts adjacent to ND10. Previous work suggested that HSV-1 DNA replication sites are distributed nonrandomly; inhibition of cytokinesis by cytochalasin B showed that their distribution in binucleated cells is partially symmetrical (de Bruyn Kops and Knipe, 1994). This nonrandom distribution can now be attributed to the location of the preexisting ND10 which also show a partially similar distribution in some daughter cells. The absence of precise synchrony of virus infection, and the fact that virus stocks have a high particle-to-PFU ratio, make it impossible to determine whether all infectious viral genomes locate to ND10, but the results clearly implicate ND10 as a preferred site of input virus localization. Since both the input and replicated viral genomes are juxtaposed to ND10, it is likely that viral transcription also occurs at these locations, a contention supported by the finding that after DNA digestion as well as under nondenaturing conditions, viral RNA was located adjacent to ND10. We did not, however, observe the track-like signals reported for one of the EBV transcripts (Lawrence *et al.,* 1989).

The association of replication sites with ND10 cannot be observed with wild-type virus since the expression of functional ICP0 leads to the rapid dispersal of the ND10-associated antigens. However, the use of ICP0-deficient viruses made it possible to observe this association during mutant virus infection, which clearly indicated that these mutants started replication mostly juxtaposed to ND10. Since input wild-type genomes localize predominantly adjacent to ND10, it is highly likely that the wild-type virus starts its DNA replication at these positions, i.e., adjacent to those previously occupied by ND10-associated proteins. The possibility of residual structural elements of ND10 remains to be investigated but the results clearly indicate that the structural integrity is not essential for wild-type HSV-1 DNA replication. The localization of the mutant virus DNA at ND10 and the equal number of wild-type and mutant replication sites per cell is consistent with the idea that both viruses use the same sites for replication. These results also explain the existence of a limited number of replication sites.

The concept of prereplication sites (Quinlan *et al.,* 1984) implies that such sites later develop into replication sites. Prereplication sites had been defined as ICP8-positive sites, are often present substantially more numerously than early replication sites, and as shown, do not contain viral DNA. As an early protein, ICP8 can only be visualized after expression of immediate early proteins, the transcription of which takes place at the nuclear matrix. Under the assumption that the viral DNA is not transported from its site of transcription to a new site for replication, the attachment of the viral DNA to the nuclear matrix is establishing potential replication sites and no new sites of potential relication are established with the expression of ICP8 ND10, the 0.3- to 0.5-μm aggregates of proteins (Maul *et al.,* 1995), to the periphery of which viral input DNA can be deposited, may therefore be redefined as preexisting potential replication sites. Those ND10 to which HSV-1 DNA becomes attached during immediate early protein phase of infection are potential replication sites, and they do not depend on the presence of ICP8 for their definition. In this sense most of the small ICP8 positive sites which can be detected at early times of infection are not potential replication sites since they are not associated with viral DNA.

The demonstration that HSV-1 DNA transcribes and begins replication in association with ND10 raises the question of whether these sites have particular advantages for the virus or whether the cell directs the input genomes to these locations. Finding a similar deposition of Ad5 and SV40 to ND10 (Ishov and Maul, submitted) suggests that the localization of the input viral genomes is a cell-directed rather than a herpes virus-specific function. The localization of input viral genomes to ND10 in the absence of viral gene expression may be a function of the cell or of some component of the infecting virus particle. Several observations on ND10-associated proteins and their reaction to environmental stimuli suggest that the movement to and retention of the viral genomes at ND10 is a complex process. ND10-associated proteins are upregulated by interferons (Guldner *et al.,* 1992; Koken *et al.,* 1994; Korioth *et al.,* 1995; Maul *et al.,* 1995), and their distribution is radically altered by stress, such as heat shock or $CdSO_4$ treatment (Maul *et al.,* 1995). These observations are consistent with the idea that ND10 are involved in an intranuclear defense mechanism. In this regard, it has been reported that overexpression of PML, one of the ND10-associated proteins, leads to suppression of cell growth (Mu *et al.,* 1994). Consistent with this possibility is the expression of a single protein by both HSV-1 (Everett and Maul, 1994; Maul and Everett, 1994) and Ad5 (Doucas *et al.,* 1996) that redistributes ND10-associated proteins. In the case of HSV-1, failure to express functional ICP0 leads to retention of ND10 and inefficient onset of viral growth during the more physiological low-multiplicity infections (Stow and Stow *et al.,* 1986; Everett, 1989; Cai and Schaffer, 1991; Cai *et al.,* 1993). That this defect can be overcome by high multiplicities of input virus in cultured cells suggests that any hypothetical defense mechanism associated with ND10 is effective only in low viral multiplicity infections typical in the organism.

HSV-1 has the intriguing and important ability to establish a latent state in sensory neurons during which the viral genome is sequestered as an episome in the nucleus and expresses only a limited part of its genome (latency-associated transcripts), the functions of which are unknown (reviewed in Fraser *et al.,* 1992). Reactivation occurs as a result of various stimuli, including stress

in the form of heat shock (Sawtell and Thompson, 1992; Stow and Stow, 1986) and Cd^{2+} exposure (Fawl and Roizman, 1993). Interestingly both stress factors disperse ND10-associated proteins, an effect also induced by ICP0 during normal infection. It is possible that input genomes in these latency systems migrate to ND10 in the same way as demonstrated by the present experiments which suggests the possibility that ND10 comprise sites where the latent genomes reside in neurons. If they do, disruption of ND10 by environmental stimuli, as shown for heat shock and $CdSO_4$, or the provision of exogenous ICP0 by superinfecting virus could result in reactivation of viral replication.

The localization of input HSV-1 DNA close to ND10 is independent of viral gene expression and replication at these sites and represents a new host–virus interaction. It also provides unexpected functional properties for a large nuclear site that has resisted elucidation since its discovery (de Thé *et al.,* 1960). These properties might be investigated in the context of an intranuclear defense mechanism that involves the segregation of infecting virus genomes to ND10 where some as yet unknown very early viral function may be inhibited.

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