The Periphery of Nuclear Domain 10 (ND10) as Site of DNA Virus Deposition

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Abstract. After DNA viruses enter the nucleus, they initiate a transcriptional cascade which is followed by replication. We investigated whether these processes take place at specific nuclear sites or, as suggested by the mode of entry, randomly throughout the nucleus. Three distinct nuclear domains, nuclear factor-1 sites, coiled bodies, and nuclear domain 10 (ND10), were used as markers to investigate the relative position of DNA virus replication sites. We found that all three nuclear domains had a very high spatial correlation with each other in uninfected cells. After adenoviral infection, nuclear factor 1 and coiled bodies were found associated with some viral replication domains. Simian virus 40 begins replication adjacent to ND10 but adenovirus 5 and herpes simplex type 1 modified ND10s before replication. Adenovirus E4orf 3 gene deletion mutants retain ND10 and begin replication at the peripheries of ND10. The same was found for the herpes simplex virus type 1 immediate early gene 1 mutants. That the deposition and replication of adenovirus 5 and herpesvirus type 1 at ND10 was not a mutant phenotype was confirmed by finding the input wild-type virus juxtaposed to ND10. The transport of viral genomes to ND10 does not require viral gene expression. Thus, the peripheries of ND10 represent preferred sites where early steps of transcription and replication of at least three DNA virus families take place, suggesting a new set of functional properties for this large nuclear domain.

Most DNA viruses must traverse the cytoplasm to enter the nucleus where they replicate and complete the encapsidation of progeny DNA. Viruses can enter the cell by membrane fusion (the enveloped herpes simplex virus type-1 [HSV-1]) (Batterson et al., 1983) and attach to and enter endosomes. The viruses are then either released by acidification of the unenveloped virus, adenovirus 5 (Ad5) (Chardonnet and Dales, 1970) or are enveloped by the cell membrane and enter the nucleus by a fusion and fission process with the nuclear envelope (SV-40) (Maul et al., 1978). The stepwise uncoating of adenovirus during entry into the cell results in a capsid that attaches to the nuclear pore complex (Dales and Chardonnet, 1973; Greber et al., 1993). HSV-1 also loses some of its structural proteins, including the tegument, and releases the viral transactivator Vp16 before attaching to the nuclear pore complex (Batterson et al., 1983). The mechanism of viral entry into the nucleus through the pore complex is poorly understood but is presumed to be active, selective, and energy dependent.

Entry of infectious viruses at any of the 2,000 nuclear pore complexes (Maul et al., 1972) should be reflected in randomly located replication sites. For HSV-1, however, a nonrandom distribution of replication sites has been reported (de Bruyn Kops and Knipe, 1994), and both HSV-1 and Ad5 use only a limited number of nuclear sites for replication (Quinlan et al., 1984; Voelkerding and Klessig, 1986; Jimenez-Garcia and Spector, 1993). These observations may suggest that preexisting nuclear domains could be used for viral replication.

The simplest self-regulating system that results in a nonrandom distribution of viral replication sites would be nuclear domains with high concentrations of insoluble nuclear matrix-bound proteins that are important for the viral replication cycle. Viruses arriving in these areas might have an increased chance of replicating. This possibility is testable for proteins known to participate in virus replication or to relocalize from distinct nuclear domains to viral replication domains. Nuclear factor 1 (NF-1, a CAAT transcription factor) appears to be distributed throughout the nucleus but is highly concentrated in a few domains. This protein, which is a cellular, single stranded

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DNA-binding protein (DBP) utilized by adenoviruses to facilitate the assembly of a nucleoprotein complex at the viral origin of DNA replication, becomes targeted to the Ad5 replication domains (Bosher et al., 1992). Therefore, Ad5 may be attracted to sites of high NF-1 concentration. Other domains called coiled bodies, which contain high concentrations of coilin (Chan et al., 1994), fibrillarin (Ochs et al., 1985), and certain splicing components (Huang and Spector, 1991; Carmo-Fonseca et al., 1992) are modified during Ad5 (Bridge et al., 1993) and HSV-1 (Phelan et al., 1993) infection, and proteins associated with them might be beneficial for Ad5 late splicing functions. Finally, there are nuclear domain 10 (ND10), nuclear domains with an average frequency of 10 per nucleus and a diameter of 0.3 μm to 0.5 μm in most cultured cells (Ascoli and Maul, 1991). The interchromatine matrix-bound nuclear bodies, which have been observed in various cell types since their first description (de Thé et al., 1960; Weber and Frommes, 1963), are part of ND10s (Koken et al., 1994; Maul et al., 1995). Presently, five different proteins have been identified to colocalize in ND10s. Sp100 is an autoantigen of primary biliary cirrhosis (Szostek et al., 1990). NDPS2 contains a LIM domain thought to be important for protein–protein interaction (Korioth et al., 1995). A third protein, PML (Dyk et al., 1994; Koken et al., 1994; Weiss et al., 1994), is a growth suppressor protein (Mu et al., 1994; Koken et al., 1995) that has been characterized as part of the fusion protein with the retinoic acid receptor α in acute promyelocytic leukemia due to the t(15;17) translocation (de Thé et al., 1991; Goddard et al., 1991; Kikizuka et al., 1991; Kastner et al., 1992). The PML-associated factor (PAF-1) has been identified as binding to PML (Chen, DJ., G.G. Maul, J.A. Dyck, and R.M. Evans, manuscript submitted for publication). NDPS5 (Ascoli and Maul, 1991) has not yet been molecularly characterized. All of them are upregulated by interferon (Guldner et al., 1992; Koken et al., 1994; Koriotth et al., 1995; Maul et al., 1995; Chen D.J., G.G. Maul, J.A. Dyck, and R.M. Evans, manuscript submitted for publication), suggesting that these proteins could be involved in antiviral defense mechanisms. Stress in the form of heat shock or CdSO4, distributes ND10-associated proteins throughout the nucleus, suggesting involvement of these proteins in ancient defense mechanisms (Maul et al., 1995). HSV-1 redistributes ND10-associated proteins (Maul et al., 1993), and genetic analysis revealed that the RING finger region of the immediate early protein ICP0 is sufficient to effect this modification (Maul and Everett, 1994; Everett and Maul, 1995). Thus, all three preexisting nuclear domains, the NF-1 accumulation, coiled bodies, and ND10, are in some way related to the viral replication cycle and can be used as position markers to study this process within the nucleus.

In this report, we documented the changes in specific nuclear domains during viral infections, location of viral replication sites, and the deposition of incoming virus relative to these domains using in situ hybridization, immunohistochemical techniques, and confocal laser microscopy. This morphological approach enabled us to define the preexisting nuclear structure at which the nucleic replication cycle of three very different DNA virus families begins and defined a new and unexpected property of virus–host interaction.

Materials and Methods

Antibodies and Cell Culture

ND10s were visualized using various antibodies. mAb 1150 recognizes Sp100 (Maul et al., 1995), while mAb 5E10 (Sturman et al., 1992) recognizes PML (Maul et al., 1995). Isotype-matched mAbs of unrelated specificity were used as controls. Polyclonal rabbit antiserum against PML (Dyk et al., 1994) was used. Rabbit antibodies against Sp100 were received from Dr. C. Szostek (Szostek et al., 1990). Anti-NDPS2, anti-NDPS5, and anti-PAF antibody were characterized in Korioth et al. (1995), Ascoli and Maul (1991), and Chen D.J., G.G. Maul, J.A. Dyck, and R.M. Evans, manuscript submitted for publication, respectively. Hybridomas producing HSV-1-specific antibodies against ICp8 and ICp4 to detect the single-stranded DBP and HSV-1-infected cells, respectively, were obtained from the American Type Culture Collection (Rockville, MD). mAb 11060 against ICp0 has been described (Everett et al., 1993). Anti-Ad5 DBP mAbs were obtained from Dr. A. Levine (Huang and Hearing, 1989), anticoilin and 72B9 antifibrillarin from R.L. Ochs (Reimer et al., 1987, Chan et al., 1994), and anti-NF-1 from Dr. K.T. Hay (Bosher et al., 1995). Anti-E1A and anti-large SV-40 T antibody PAB49 was purchased from Oncogene Science, Inc. (Manhasset, NY).

Hep-2 carcinoma cells were maintained in MEM supplemented with 10% FCS and antibiotics. All cells were grown at 37°C in a humidified atmosphere containing 5% CO2. Metabolic inhibitors were added at the time of infection at 100 μg/ml for cycloheximide and at 200 ng/ml for actinomycin D. Hep-2 cells were grown on round coverslips in 24-well plates (Corning Glass, Inc., Corning, NY) until approximately 80% confluency before infection.

Virus Infection and Mutants

At 2 d after plating, Hep-2 were infected with Ad5 or various mutants resulting in 10 to 95% infected cells (1–40 plaque-forming units) as determined by staining for E1A (or E4 orf's [open-reading frame 6] in the absence of E1A). Infected cells were fixed at different intervals after infection and assayed with the different antibodies or by in situ hybridization. Ad CB is a E1-deleted recombinant Ad5 expressing the lacZ gene (Berkner, 1992; Kozarsky et al., 1994). The E1B mutant H5 110 has only E1B 44 kD disrupted (Ibabiss and Ginsberg, 1984). The E2A mutant H5 802 contains a deletion in the 72-kD single-stranded DBP (Rice and Hearing, 1989). The E3 mutant H5 7001 is deleted in the sequences spanning 78.4 to 86 m.u. The E4 mutant H5 1004 has all E4 orf's deleted except orf1. E4 orf1–3 and E4 orf6 were deleted in H5 1006 and H5 1010 (Bridge and Ketner, 1989). In H5 351 and H5 352, the orf2 and E4 orf2 gene products were inactivated (Halbert et al., 1985). Mutant dl366 has E4 deleted and dl366 + 3 was constructed by inserting only E4 orf3 into a d1366 mutant virus (Huang and Hearing, 1989). All viruses with deletions were propagated on the Vero-derived complementing cell line W62 (Weinberg and Ketner, 1983). The wild-type Ad5, H5 7001, H5 1101, H5 338, and Ad CB were propagated in human 293 cells (Graham et al., 1977).

The E4 ORF3 expression vector was developed from a PCR-derived fragment by inserting it into a plasmid containing the cytomegalovirus promoter. Cells were transiently transfected by Ca-phosphate precipitation (Wigler et al., 1977) and assayed 16 h later using antibodies to various ND10 antigens. The same plasmid without insert was used as control.

HSV-1 17+ was used at 1–10 plaque-forming units, resulting in 40 to 90% infected cells at 5 h after infection as determined with anti-ICp4 antibodies. The ICp0 mutant FXE (Finger minus mutant) was used at pretitered infectious particle concentrations resulting in 50 to 90% infected cells at 8 h after infection as determined by immunofluorescence assay using anti-ICp4 antibodies. Wild-type SV-40 776 (obtained from Dr. J.C. Alwine, University of Pennsylvania, Philadelphia, PA) was used for SV-40 infections after dilution with medium without serum to 0.1 plaque forming unit/cell. Cells were infected for 1 h, and then washed and refed with complete growth medium.

Immunolocalization of Virus and Host Proteins

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 0.1% (vol/vol) Triton X-100 (Sigma Chemical Co., St. Louis, MO) in PBS. Antigen localization was determined after incubation of permeabilized cells with rabbit antiserum or with mAb diluted.
in PBS for 1 h at room temperature. Avidin-fluorescein or avidin-Texas red was complexed with primary antibodies through biotinylated secondary antibodies (Vector Laboratories, Inc., Burlingame, CA). Cells were double labeled with the respective secondary antibodies with either FITC or Texas red using biotin-avidin enhancement and with FITC for structures with the lowest staining intensity. Cells were then stained for DNA with 0.5 μg/ml of bis-benzimide (Hoechst 33258; Sigma Chemical Co.) in PBS and mounted with Fluoromount G (Fisher Scientific, Pittsburgh, PA). Cells were analyzed using a confocal scanning microscope (Leica, Inc., Deerfield, IL). The two channels were recorded simultaneously if no cross talk could be detected. In case of strong FITC labeling, sequential images were acquired with the more restrictive filters to prevent possible breakthrough of the FITC signal into the Red channel. Both acquisition modes gave the same results. The image enhancement software (Leica, Inc.) was used solely in balancing the signal strength. Because of the variability of the infectious cycle progression in any given culture, those most prevalent were photographed and presented as single nuclear images at high magnification. The number of cells studied in each sample exceeded 500 and the experiments were repeated at least three times.

**In Situ Nuclear Run-on Experiment**

The nuclear run-on assay was conducted as described (Wansink et al., 1993) using Ad5-infected HEp-2 cells at 12 h after infection. Briefly, cells were washed in glycerol buffer and permeabilized in glycerol buffer containing 0.05% Triton X-100 for 3 min at room temperature. After the removal of the detergent-containing buffer, transcription was initiated by adding transcription buffer (100 mM KC1, 50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 0.5 mM EGTA, 25% glycerol, 25 mM S-adenosyl-L-methionine [Boehringer Mannheim Corp., Indianapolis, IN], 5 U/ml RNase inhibitor from human placenta, 1 mM PMSF, 0.5 mM ATP, CTP, GTP, and 0.2 mM BrUTP). All washes and incubations with antibodies including anti-BrdU antibody (Boehringer Mannheim Corp.) solutions contained RNase inhibitors. Maximal incorporation occurred after 10 min. Control experiments included 200 ng/ml of actinomycin D.

**In Situ Hybridization of Viral DNA**

For the localization of virus DNA, fluorescent in situ hybridization techniques were adapted from those described by Lawrence et al. (1989) and Huang and Spector (1991). For Ad5, the hybridization probe was prepared using total Ad5 DNA. For HSV-1, DNA of cosm id 56 (Cunningham and Davison, 1993) was used, which includes the region between 79442 and 115152 bp of the HSV-1 genome. The probe for SV-40 was a plasmid (p776) containing the entire SV-40 genome (courtesy of Dr. J.C. Alwine). DNA was nick translated in the presence of biotinylated-dl dUTP (Sigma Chemical Co.). 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 Ad5, HSV-1, SV-40, or their respective mutants were fixed with freshly prepared 1% paraformaldehyde and permeabilized with 0.2% Triton X-100. Cellular and probe DNA were denatured simultaneously for 3 min at 90°C on a heat block and hybridized for 2 h at 37°C in a hybridization mixture containing 50% deionized formamide, 10% dextran sulfate, 1 × SSC, 10 ng of biotinylated viral DNA, 1 μg of salmon sperm DNA, and 1 μg of human DNA per μl. After hybridization, cells were washed three times in 50% formamide/2 × SSC prewarmed to 42°C followed by three washes with 5 μl each in 0.1 × SSC prewarmed to 60°C. Washed cells were then stained with fluoresceinated avidin (Vector Laboratories, Inc.) in 3% BSA/2 × SSC. The signal was enhanced with an additional round of biotinylated anti-avidin antibodies followed by fluoresceinconjugated 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 mM Mg²⁺ and RNase inhibitor (1 mg/ml RNasin; Promega Corp., Madison, WI) for 2 h at room temperature. 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 Corp.) for 2 h at room temperature.

**Electronmicroscopy and Immunohistochemistry**

Infected and uninfected HEp-2 cells were fixed in situ and embedded either according to standard procedures (Maul, 1971) or in 1% paraformaldehyde and 0.1% glutaraldehyde after trypaninization and pelleting and embedded in LR White Resin (EM Sciences, Ft. Washington, PA). After sectioning and incubation with primary antibody, the reaction was visualized with 15-nm gold-labeled antitissue antibodies. (Auroprobe; American Life Sciences, Chicago, IL)

**Results**

Ad5 replication domains appear as round or goblet-shaped structures in infected nuclei (Pombo et al., 1994). The number of replication domains can be determined by counting the single-stranded DBP-positive sites (Brough et al., 1992). When approximately 50% of cells are infected with Ad5, two to six viral replication sites are present at 12 h after infection. However, when the infectious virus input is increased more than 10-fold, the average number of replication sites remains below 10, suggesting that the number of viral replication sites is restricted. During S-phase, 150-300 cellular DNA replication sites exist (Nakayasu and Berezney, 1989), making it unlikely that these sites restrict the number of viral replication sites. Domains with a high concentration of the splicing factor SC35 have a frequency of 30-50 (Fu and Maniatis, 1990), which is much higher than the number of viral replication sites. Thus, we compared the position of viral replication sites to nuclear sites which have a low frequency. Nuclei of HEp-2 cells contain about one to six sites of high NF-1 concentrations, although there is faint NF-1 staining throughout the nucleus as shown in Figs. 1, A and B. Coiled bodies (labeled with anticoilin antibodies) are also present with a frequency of one to six (Figs. 1, A and C). Both the NF-1 sites and coiled bodies are mostly found adjacent to each other or partially overlapping (Fig. 1, A, yellow). ND10, as indicated by labeling with α-PML antibodies, are present with an average frequency of 10 (range 5-20) (Figs. 1, B and C). Often large NF-1 sites were found juxtaposed to ND10s (Fig. 1 B; arrows). Coiled bodies are also often attached to ND10s (Fig. 1 C). The total volume of ND10 per nucleus is <0.05% if the diameter of 0.4 μm is used to calculate the average volume of ND10s. The obvious side by side location of a large number of coiled bodies and NF-1 sites as well as both domains with ND10 is therefore not coincidental. These three sites provide precisely circumscribed markers throughout the nucleus that can be used to correlate the position of viral replication sites.

**Ad5 Modifies the Preexisting Nuclear Domains**

To determine where Ad5 replication domains are located relative to these three nuclear domains, we infected HEp-2 cells with Ad5. 16 h after infection, cells were stained for the nuclear domains and the single-stranded DBP. DBP take part in viral DNA replication and early and late gene expression (Horowitz, 1990; Williams and Chase, 1990) and can be used as a marker for viral replication domains. Early viral replication sites as determined by small DBP accumulation were found in association with some sites of high NF-1 concentration (Fig. 1 D, left nucleus, arrows), but the number of replication sites was higher than the number of NF-1 sites. These findings show that wild-type Ad5 can start replication at NF-1 sites but that they are not dominant sites. In cells where replication has progressed further and where the viral replication sites expanded, NF-1 accumulated in the replication domains (Fig. 1 D; right nucleus).
Ad5 replication domains, as determined by DBP localization, were often found juxtaposed to coiled bodies, as shown by the double labeling with coilin (Fig. 1 E; left nucleus, arrow). Not all coiled bodies had a replication site attached, and the number of replication sites was higher than coiled bodies. Thus, coiled bodies as well as NF-1 sites are not dominant sites where wild-type Ad5 replication domains are located. These domains are modified at later stages of replication, and coilin was recruited into distinct sites within the replication domains (Fig. 1 E; right nucleus). The apparent amount of coilin in the infected cell seems to be greatly increased over the uninfected cell, possibly due to the redistribution of this protein from high concentrations in coiled bodies. Fibrillarin is also present in the coiled bodies and in the nucleolus, where it is thought to be involved in the posttranscriptional assembly of preribosomes (Tollervey et al., 1993). Although coilin redistributes into Ad5 replication domains, fibrillarin does not (Fig. 1 F; arrow shows coiled body).

A surprising redistribution of ND10-associated proteins occurred after Ad5 infection. At 8 h after infection, all ND10-associated proteins had distributed to track-like structures that had no obvious positional correlation with DBP-positive sites (shown for PML; Fig. 1 G). The tracks were present in larger numbers than the average of ND10s. Analysis of other ND10-associated proteins such as Sp100, NDP52, NDP55, and PAF-1, normally colocalized in ND10s (shown for Sp100 and PML; Fig. 1 H, left nucleus), revealed that at early stages of infection, all proteins redistribute to tracks before viral DNA replication began. After replication progressed, segregation from tracks to replication domains (determined by DBP staining) was seen for most ND10-associated proteins (shown for Sp100; Fig. 2). Only PML was not present in the viral replication domains throughout the replication cycle, and it remained in the tracks (Fig. 1 H, right nucleus; arrows indicate Sp100 in characteristically shaped viral replication domains - compare with Fig. 2). Thus, it was not possible to directly determine the spatial relationship of ND10 and the viral replication domains due to redistribution of ND10-associated proteins.

To correlate the punctate distribution of ND10-associated proteins within viral replication domains with viral transcription, we used an in situ nuclear run-on procedure incorporating BrUTP and compared the location of Sp100 (or other ND10-associated proteins) and transcripts. Surprisingly, nearly all transcripts localized to the viral domains (marked by Sp100) but mostly did not colocalize with ND10-associated proteins inside these domains (Fig. 1 F; right nucleus). The uninfected nucleus (Fig. 1 F, left) shows the normal distribution of Sp100 in ND10 and transcripts throughout the nucleus except the nucleoli, phenomena already observed by others (Wansink et al., 1993). Thus, Sp100 and other ND10-associated proteins do not appear to be directly associated with transcripts although all except PML are segregated in viral replication domains. Comparison of viral single-stranded DNA (visualized by DBP) and Sp100 indicated localization in the same domain, although DBP was more diffusely distributed than Sp100 (Fig. 2, A and B). Thus, within the resolution limits of confocal microscopy, neither nascent viral transcripts nor single-stranded DNA can be assumed to colocalize with the nuclear matrix-associated protein Sp100 or the other ND10 proteins, although they were recruited into the viral replication domains.

The above experiments show that wild-type Ad5 replication sites are situated close to coiled bodies and to sites of NF-1 concentration, but the number of these sites is less than the number of viral replication domains. However, ND10 are apparently eliminated and all presently known ND10-associated proteins redistribute into many tracks before replication begins. Thus, the initial site of wild-type viral replication cannot be correlated with the original ND10. We therefore used two approaches to determine the spatial relationship of ND10 and Ad5 DNA: a) identification of mutants that replicate but do not modify ND10; and b) determination of input wild-type viral DNA localization before ND10 modification.

The E4 orf3 Gene Product Redistributes ND10 Proteins into Tracks

To determine the position of Ad5 replication domains relative to ND10, we searched for a viral gene that induces the modification of ND10 in order to use a deletion mutant of this gene to retain ND10 during replication. The rich genetic background information and availability of deletion mutants allowed us to eliminate most early proteins as modifying ND10. Cells infected with virus mutants deleted in the E1A, E1B, E2, and E3 had a phenotype ex-

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Figure 1. Immunohistochemical definition of various nuclear domains before and during Ad5 infection. HEp-2 cells were double labeled with antibodies against different nuclear domains to demonstrate their relative position. (A) High concentrations of NF-1 (α-NF-1 antibodies; green) are present at coiled bodies (red) resulting in a yellow signal. (B) High NF-1 concentrations (green) lay adjacent to ND10s (α-PML antibodies, red, arrows). (C) Some coiled bodies (α-coilin antibodies; green) are found attached to ND10 (α-PML antibodies; red). (D) HEp-2 cells infected with Ad5 wild type showing an early stage of replication (left nucleus; arrows indicate large NF-1 sites; green) and an advanced replicative state (right nucleus) based on the distribution of the Ad5 single-stranded DBP (red) and NF-1 (green); yellow indicates overlap of these two proteins. (E) Same as (D), but showing association of a coiled body with replication domain (left nucleus) and loss of coiled bodies and recruitment of coilin (green) into viral replication domains (DBP, red, right nucleus). (F) Same as (D), only coilin (green) localizes to the viral replication domain whereas fibrillarin (red) remains in the nucleolus and a single remaining coiled body (arrow). (G) Same as (D), but comparing PML (green) and DBP (red) showing loss of ND10 and segregation of PML into tracks away from the replication domain. (H) ND10-infected HEp-2 cell at an early stage of viral replication showing PML (red) and Sp100 restricted to tracks, and Sp100 (green, arrows) segregating to the round viral replication domains (right nucleus; compare with Fig. 2). The uninfected cell (left nucleus) shows colocalization of the two proteins in ND10. (I) A nuclear run-on experiment with the newly transcribed RNA (red) and Sp100 (green) indicating their presence but not colocalization in the same viral replication domain (right nucleus). In the left uninfected nucleus, transcripts are distributed throughout the nucleus except the nucleolus. Bar, 10 μm.
cept PML to accumulate in the viral replication domains. These data correlated with those recently presented by Carvalho et al. (1995) and Doucas et al. (1996). Therefore, we determined at higher resolution whether any other structures are interposed between nuclear bodies and the replication domains of Ad5. By standard electron microscopy, coiled bodies are identified by their characteristic coiled structure (Brasch and Ochs, 1992), simple nuclear bodies are identified by their homogeneous round shape, which has previously been shown to contain PML and Sp100 (Koken et al., 1994; Maul et al., 1995; Puvion-Dutilleul et al., 1995), and Ad5 viral replication domains are identified by fine fibrillar substructure not present in uninfected cells (Puvion-Dutilleul et al., 1992). Simple nuclear bodies and viral replication domains were found frequently to be closely asso-

Ad5 Replication Domains Are Located Adjacent to ND10s

To determine the position of Ad5 DNA replication domains, we double labeled H5 1004-infected HEP-2 cells (12 h after infection) for DBP and PML (Fig. 3 B). Both signals were found to be juxtaposed to each other. The same results were obtained using in situ hybridization under denaturation conditions for labeling of viral DNA combined with immunohistochemical localization of PML (Fig. 3 C). ND10 and viral DNA (and possibly viral transcripts as the section has not been RNase treated) are located near each other but do not colocalize. Many cells had more ND10 than replication domains. From the images obtained, we conclude that Ad5 replication domains are not randomly distributed, as they are located close to or at the periphery of ND10 expanding away from these nuclear domains.

Due to the resolution limits of confocal microscopy, we cannot decide whether ND10 and replication domains are directly apposed or whether other structures interpose. Ultrastructurally, some ND10 correspond to nuclear bodies (Maul et al., 1995). Therefore, we determined at higher resolution whether any other structures are interposed between nuclear bodies and the replication domains of Ad5. By standard electron microscopy, coiled bodies are identified by their characteristic coiled structure (Brasch and Ochs, 1992), simple nuclear bodies are identified by their homogeneous round shape, which has previously been shown to contain PML and Sp100 (Koken et al., 1994; Maul et al., 1995; Puvion-Dutilleul et al., 1995), and Ad5 viral replication domains are identified by fine fibrillar substructure not present in uninfected cells (Puvion-Dutilleul et al., 1992). Simple nuclear bodies and viral replication domains were found frequently to be closely asso-

Figure 2. Redistribution of ND10-associated proteins into viral replication domains. HEP-2 cells (infected with Ad5 wild type and fixed 12 h after infection) were double labeled with Sp100 (A) and DBP (B). The superimposed image (C) shows localization of Sp100 in viral replication domains, but some Sp100 still remain in tracks. Bar, 10 μm.

Figure 3. Localization of Ad5, HSV-1, and SV-40 replication sites relative to preexisting nuclear domains. (A) HEP-2 cell infected with H5 1004 18 h after infection double labeled with PML (red) and Sp100 (green) showing circumscribed ND10 (yellow) and viral replication domains outlined by Sp100 (compare with Fig. 2). (B) Same as A, but fixed 12 h after infection and labeled to show ND10 (PML; green) juxtaposed to DBP (red). (C) HEP-2 cell infected with H5 1004 the E4-1 mutant of Ad5 and labeled at 12 h after infection for PML (red) and hybridized in situ with Ad5 DNA (green) showing the DNA signal in close contact with ND10. (D) HSV-1-infected HEP-2 cell labeled with antibodies against the single-stranded DBP ICP8 and in situ hybridized with Ad5 DNA probe 7 h after infection. No DNA signal is present. (E) Ad5-infected HEP-2 cell labeled with α-DBP antibodies and in situ hybridized with HSV-1 DNA.
probe 12 h after infection. No DNA signal is present. (F) Shows Ad5 infected cell in situ hybridized with Ad5 DNA probe (green) colocalizing perfectly with α-DBP antibodies (red), resulting in yellow staining. (G) HEp-2 cell infected with Ad5 wild type (1.5 h after infection) labeled with α-PML antibodies (red) and in situ hybridized with Ad5 DNA probe (green). Many point-sized DNA signals are found, of which only an occasional one is localized adjacent to an ND10. (H) HEp-2 cell nucleus 4 h after infection (Ad5 wild type) showing most DNA signals juxtaposed to PML-positive structures (red). (I) Two HEp-2 cells infected with the HSV-1 ICP0 mutant FXE 8 h after infection. In situ hybridized HSV-1 DNA probe (green) localizes adjacent to ND10 (PML; red). (J) HEp-2 cells infected with wild-type HSV-1 2 h after infection in situ hybridized with HSV-1 DNA probe (green). Most of the signal is found adjacent to or overlapping with ND10 (PML, red). (K) SV-40 infected HEp-2 cells labeled 20 h after infection for large T-antigen (red) and PML (green). ND10 are retained and partially juxtaposed to high concentrations of large T-antigen. (L) SV-40-infected large HEp-2 cell nucleus 20 h after infection hybridized in situ with SV-40 DNA probe (green) and labeled with α-PML antibodies (red) showing the DNA/RNA signal juxtaposed to ND10s. Bar, 10 μm.
associated in H5 1004 infected cells (Fig. 4 A). In addition, a few coiled bodies were found adjacent to but not directly associated with the viral replication domains and nuclear bodies. This finding is in agreement with results obtained from immunofluorescence microscopy and suggests that viral replication domains are in direct contact with nuclear bodies in the mutant virus infected cells. Viral replication domains are only recognizable at an advanced stage by direct electron microscopy. The earliest stages, however, should be recognizable by the presence of DBP identified by immunohistochemical techniques. We used the postembedding method and located the mAb against DBP with gold-labeled second antibodies. At approximately the same stages as shown in Fig. 4 A, large areas are labeled by DBP antibodies (Fig. 4 B). Nuclear bodies are attached to these sites, although nuclear bodies without DBP-containing domains are present, which is in agreement with immunofluorescent data. As shown in Fig. 4, C and D, small, gold-labeled areas are also found in direct juxtaposition with nuclear bodies. Immunohistochemical localization at the ultrastructural level then places even the smallest DBP accumulation directly adjacent to simple nuclear bodies.

Input Ad5 Is Preferentially Located at ND10

It may be argued that the localization of early Ad5 replication domains juxtaposed to ND10 is a mutant-specific function. Therefore, we determined the location of input wild-type viral DNA before ND10-associated proteins redistributed into tracks due to E4 ORF3 expression, assuming that transcription takes place from viral genomes bound to the nuclear matrix (Sarnow et al., 1982; Schaack et al., 1990) and that the virus may not be repositioned for the beginning of replication. To assess the localization of input Ad5 DNA relative to ND10, we adapted an in situ hybridization technique that detects input viral DNA in the nucleus and retains the proteinaceous structure comprising the matrix and ND10. Negative (Figs. 3, D and E) and positive (Fig. 3 F) control experiments indicated the specificity of the DNA probe. In cells fixed early after Ad5 infection (1.5 h after infection), most viral DNA was recognized as small, distinct signals that were distributed throughout the nucleus and that had no recognizable correlation with ND10s (Fig. 3 G). Controls include in situ hybridization in nondenaturing conditions and DNase treatment. At later times (4 h after infection), approximately 50% of the input viral DNA was found close to ND10 (Fig. 2 H). Considering the volume occupied by ND10 relative to that of the nucleus (<0.05%), this represents a preferential association of the input virus or their transcripts with ND10. Still later (8 h after infection), this association was not convincingly demonstrable because ND10 proteins were redistributed into many tracks. The same experiment was repeated in the presence of cycloheximide and actinomycin D from the time of infection to inhibit protein and RNA synthesis, respectively; input viral DNA arrived at ND10 in the presence of the inhibitors as in the uninhibited cells. Control experiments included RNase and DNase treatment before hybridization. Our findings demonstrate that input wild-type viral DNA is preferentially located at ND10s and that transcription and protein synthesis are not necessary for the positioning of input Ad5 DNA to their preferential attachment sites, ND10.

HSV-1 Replication Compartments Are Located at the Periphery of ND10

If the positioning of Ad5 to ND10 is a cell-induced phenomenon, i.e., independent of viral gene expression, another virus presumably entering the nucleus in the same manner as Ad5 would also be expected to be localized to ND10. Our previous studies showed that HSV-1 modifies ND10 by distributing all known ND10-associated proteins throughout the nucleus and that ICP0, an immediate early gene product, is responsible for this modification (Maul et al., 1993; Maul and Everett, 1994). HSV-1 ICP0 mutants defective in the ability to modify ND10 can therefore be tested for the location of viral DNA replication compartments relative to ND10. HEp-2 cells infected with the ICP0 RING finger deletion mutant FXE (8 h after infection) showed PML and the replication compartments adjacent to each other (Fig. 3 J). As for Ad5, HSV-1 replication domains developed away from ND10. Both the wild-type and mutant HSV-1 had a similar number of replication compartments (two to eight) at 50% productively infected cells. When the viral input was increased 10 times, an average of approximately seven viral domains was found for both. Also, wild-type HSV-1-infected cells (3 h after infection) showed preferential localization of input HSV-1 DNA and their transcripts in close proximity to ND10s (Fig. 3 J). Preventing protein and RNA synthesis at the time of infection did not inhibit the positioning of the input viral DNA to ND10. These observations parallel those for Ad5, i.e., both viruses locate to the periphery of ND10.

SV-40 Begins Replication Adjacent to ND10

If Ad5 and HSV-1 deposition is a common feature important for the DNA virus replication cycle, it should also be the case for small DNA viruses such as SV-40. We found that this virus does not modify ND10 noticeably at the beginning of replication and that some higher concentrations of the large T-antigen are found juxtaposed to ND10 after viral infection (Fig. 3 K) as well as after transfection by large T-antigen expression vectors (Carvalho et al., 1995, and our observation). Thus, we tested whether SV-40 begins replication at ND10 or close to the nuclear envelope through which it enters. In SV-40-infected HEp-2 cells, where approximately 10% of cells became positive for large T-antigen (20 h after infection), the average number of replication sites per nucleus, as determined by in situ hybridization, was approximately seven. The high number

Figure 4. Electron micrograph of HEp-2 cells infected with ad5 E4-mutant 1004 10 h after infection. A shows the intimate association of nuclear bodies (NB) with a viral replication domain (VD). Also included is a coiled body (CB) close to but not directly associated with the viral replication domain. B shows an area similar to A where the gold labeled DBP indicates the viral replication domain (NO, nucleolus). C and D show small areas of gold-labeled DBP in direct association with nuclear bodies. Bar, 0.5 µm.
of replication sites and low number of productively infected cells suggest that substantially more than one infectious particle must penetrate the nucleus to start the replicative process. Surprisingly, ND10 were not modified over the time period investigated (48 h) and none of the ND10-associated proteins or collin were recruited into the viral replication domains during this period. Double labeling of viral replication domains by in situ hybridization and immunolocalization of ND10 by anti-PML antibodies showed that most SV-40 replication domains localized adjacent to ND10 (Fig. 3 L). Thus, SV-40 replication sites are located at the periphery of the same preexisting nuclear domain as those of Ad5 and HSV-1.

**Discussion**

The possibility that Ad5 and HSV-1 start replication and transcription at specific and preexisting nuclear sites was suggested by the observation that an increasing input of infectious virus did not substantially increase the number of replication sites, which was on average below 10. We initially argued that the number of such preexisting nuclear sites should be similar to the average number of viral replication sites and that sites with high concentrations of nuclear matrix-bound proteins useful in viral replication/transcription may provide a simple selection mechanism for successful replication of those viral genomes that reach these sites. We identified three nuclear domains that fit this description. NF-1 is essential for the Ad5 viral replication and enters the viral replication domains (Bosher et al., 1992). Coiled bodies are present in the nucleus at a frequency of one to six and contain two known proteins, collin and fibrillarin (Chan et al., 1994; Ochs et al., 1985) as well as splicing components (Carmo-Fonseca et al., 1992; Chan et al., 1994). Coiled bodies disappear after HSV-1 and Ad5 infection (Bridge et al., 1993; Phelan et al., 1993), but for reasons unknown, only collin appears in the Ad5 replication domains. The third nuclear domain with a low frequency is ND10, which is eliminated early after HSV-1 infection, i.e., before replication begins (Maul et al., 1993). These three domains had a high spatial correlation with each other. This correlation of nuclear domains was surprising and may be the basis for the variety of nuclear bodies observed by electron microscopy (see Brasch and Ochs, 1992 for review). All three nuclear domains served as structural markers with which to correlate viral replication domains.

If we accept that the demonstration of single-stranded DBAs or viral DNA accumulation in the nucleus is indicative for the place of viral replication domains, then small signals of either should indicate the nuclear position where these domains start developing. Wild-type Ad5 then began replicating in association with NF-1 sites and some coiled bodies, but the number of viral replication sites was substantially higher than the number of nuclear domains in each optical section. Although the association of ND10 with coiled bodies and NF-1 sites suggested ND10 as likely sites of Ad5 initial transcription/replication, this possibility could not be directly documented because Ad5, like HSV-1, modified ND10 before replication began. Thus, we determined the mutants of Ad5 and HSV-1 that replicate but do not modify ND10. For HSV-1, we found that ICP0 mutants with a deletion in the RING finger region were unable to remove the proteins from ND10 (Maul and Everett, 1994; Everett and Maul, 1994). For Ad5, the deletion of E4 orf3 leaves ND10 intact (Carvalho et al., 1995, Ducaas et al., 1996). Both viral mutants begin transcription and replication adjacent to ND10 because single-stranded DBP as well as viral DNA were found at ND10s. Additionally, suggestive evidence at the ultrastructural level is the close association of the Ad5 replication domains with nuclear bodies showing no other structural elements interposed. The most direct evidence that DNA viruses begin their replication cycle adjacent to ND10 is the finding of SV-40 replication domains juxtaposed to these nuclear structures. Thus, the periphery of this preexisting nuclear structure is apparently the preferred start site of nuclear replication cycles for viruses of three quite different families. The juxtaposition of Ad5 wild-type virus replication domains with NF-1 sites and coiled bodies, on the other hand, is apparently a consequence of the association of these two nuclear domains with ND10.

Wild-type Ad5 and HSV-1 DNA replication could not be documented at ND10s because all known ND10-associated proteins were redistributed early after infection. Assuming that the nuclear matrix attachment site of the viral genome for transcription does not differ from the one for replication, the replication start site should be reached by the virus before modification of ND10. Early after infection, incoming DNA of both viruses is preferentially accumulated at these sites. The peripheries of ND10 are therefore preferred attachment sites for incoming Ad5 and HSV-1 DNA, which should result in replication at these positions even after ND10 modification. This contention is supported by the finding that the mutant replication domains of Ad5 and HSV-1, as well as wild-type SV-40, expand from ND10.

Our findings raise the question of how this host–virus interaction is initiated. HSV-1 and Ad5 enter the nucleus through the pore complex (Dales and Chardonnet, 1973; Batterson et al., 1983) or, in the case of SV-40, through the nuclear membranes (Maul et al., 1978) and after binding to the periphery of ND10, begin the nuclear replication cycle. This implies movement of the virus to ND10 from a specific entry site rather than movement of ND10 to the input virus. In the simplest case, diffusion would drive the viral genome to ND10 and a nondiffusing factor not accessible elsewhere would provide the virus that arrives at ND10s with a selective replicative advantage. Neither SV-40 nor HSV-1 recruit any of the ND10 proteins into the replication domains, as has been shown for other cellular proteins (Wilcock and Lane, 1991; Bosher et al., 1992). Only Ad5 replication domains recruit ND10 proteins, excluding PML, which is considered to be a growth suppressor (Mu et al., 1994). This reduces the likelihood of a general involvement of ND10 proteins in the transcription and replication cycle that takes place in the viral domains. This is supported by the expansion of the viral replication domain of all three viruses away from the ND10. Accumulation of input DNA adjacent to ND10s might be due to diffusion and selective binding, although targeted transport of these large genomes cannot be excluded. The question of whether the viral genome binds directly at the periphery of ND10 or if there is another structure inter-
vening depends on the definition of the borders of ND10s and what surrounds ND10s. Electron micrographs of Ad5 E4orf3 mutants show an intimate association of simple nuclear bodies with the replication domains partially surrounding them, a structural precondition for formation of the reported goblet-shaped replication domains in wild-type Ad5 virus (Pombo et al., 1994). The ultrastructural data then suggest that replication sites form directly adjacent to simple nuclear bodies.

Any potential advantage derived from the usage of ND10-associated proteins by the three viruses is likely to differ because HSV-1 apparently removes ND10-associated proteins completely from this structure before replication begins and Ad5 modifies ND10 by depositing all ND10 proteins into tracks, but SV-40 induces no detectable change in ND10, suggesting that modification of these structures is not a prerequisite for viral replication. Thus, the only similarity among the three viruses is their localization and binding at specific nuclear sites, ND10. One explanation for our observation is that a common, very early function of these disparate viral families must take place at ND10 and is enhanced by the release of ND10 proteins.

Several observations about ND10-associated proteins and their response to environmental changes suggest an alternative explanation. The movement to and retention of viral DNA at ND10 may not be for the benefit of the virus and is a more complex and potentially host-regulated process. ND10-associated proteins are interferon-upregulated (Guldner et al., 1992; Koken et al., 1994; Koriath et al., 1995; Maul et al., 1995), which may suggest that the proteins and the structure in which they aggregate are part of an inducible intracellular defense mechanism. At present, only PML of all the known ND10-associated proteins has been studied functionally and is considered to have growth suppressor activity (Mu et al., 1994; Koken et al., 1995). PML is also the only known ND10-associated protein not recruited into the Ad5 viral replication domain, and preliminary observations in our laboratory suggest that PML suppresses expression of cotransfected proteins. These observations are consistent with the possibility that ND10 are part of a nuclear defense mechanism. We postulate that as part of this defense mechanism, viral DNA is attracted to these sites where some early and as yet unknown functions common to at least three viral families are suppressed. The finding that two virus families each have a gene that modifies ND10 can be interpreted as a mechanism to circumvent this cellular defense. With this premise, targeted transport of the infecting virus genome from the pore complex to ND10 might be contemplated.

The remarkable deposition of SV-40, Ad5, and HSV-1 at ND10 and the beginning of replication/transcription at the periphery of these sites represent a new and general interaction of DNA viruses and their respective hosts during the early stages of the viral nuclear replication cycle and provide a function as potential viral transcription/repllication start sites for this large nuclear domain.

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