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Human Papillomavirus DNA Replication Compartments in a Transient DNA Replication System

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Many DNA viruses replicate their genomes at nuclear foci in infected cells. Using indirect immunofluorescence in combination with fluorescence in situ hybridization, we colocalized the human papillomavirus (HPV) replicating proteins E1 and E2 and the replicating origin-containing plasmid to nuclear foci in transiently transfected cells. The host replication protein A (RP-A) was also colocalized to these foci. These nuclear structures were identified as active sites of viral DNA synthesis by bromodeoxyuridine (BrdU) pulse-labeling. Unexpectedly, the great majority of RP-A and BrdU incorporation was found in these HPV replication domains. Furthermore, E1, E2, and RP-A were also colocalized to nuclear foci in the absence of an origin-containing plasmid. These observations suggest a spatial reorganization of the host DNA replication machinery upon HPV DNA replication or E1 and E2 expression. Alternatively, viral DNA replication might be targeted to host nuclear domains that are active during the late S phase, when such domains are limited in number. In a fraction of cells expressing E1 and E2, the promyelocytic leukemia protein, a component of nuclear domain 10 (ND10), was either partially or completely colocalized with E1 and E2. Since ND10 structures were recently hypothesized to be sites of bovine papillomavirus virion assembly, our observation suggests that HPV DNA amplification might be partially coupled to virion assembly.

Papillomaviruses are small DNA viruses with circular, double-stranded genomes 7.9 kb long. They are tropic for mucosal and cutaneous squamous epithelia of their respective host species, where vegetative replication and the production of progeny virions are confined to the differentiated squamous cells in the upper strata (for a review, see reference 11). As a result, studies of human papillomavirus (HPV) or bovine papillomavirus (BPV) DNA replication have been limited largely to transient replication assays with transfected cells or to cell-free systems (for reviews, see references 10 and 63). These investigations have demonstrated that the E1 and E2 proteins are the only virus-encoded gene products that are necessary to initiate replication from the viral origin of replication (ori), which is comprised of several highly homologous E2 binding sites (E2BS) flanking a less conserved E1BS. The host cell provides all of the other necessary replication machinery and substrates. In vivo, host DNA replication is reactivated in the differentiated cells by the HPV *E*7 gene product, which binds to and inactivates the retinoblastoma susceptibility protein pRB and related proteins, thereby inducing the expression of host genes essential for viral DNA replication (6 and references therein).

The E1 protein binds weakly to the E1BS at the ori (33, 69, 74, 76). E1 is a DNA helicase that unwinds the ori (27, 57, 77). It interacts with the DNA polymerase/primase to initiate DNA replication (12, 39, 48). E1 is also required during elongation, suggesting that it also functions as a helicase during elongation (33). The E2 protein is a transcriptional regulatory protein (for reviews, see references 22 and 42) but also plays critical roles during the initiation of ori DNA replication. It prevents nucleosome formation around the ori (32). It associates with the E2BS with a high affinity and helps stabilize the association of E1 with the ori (20, 38, 43, 54–56, 76). E2 also facilitates the assembly of the preinitiation complexes with host proteins (33). For HPV, the E2 protein is the primary ori recognition protein, as the E2BS is essential but the E1BS is not (8, 29, 34, 35, 65). Furthermore, certain combinations of E1 and E2 proteins encoded by heterologous virus types support ori replication with a reduced efficiency or not at all (4, 9, 79). A direct interaction between pairs of E1 and E2 proteins of the same HPV types or BPV type 1 (BPV-1) has indeed been demonstrated (20, 24, 43, 44, 52). At least partly attributable to the relatively strong and nonspecific affinity of E1 for DNA, high concentrations of E1 support ori-independent replication in the absence of the E2 protein in cell-free assays. However, the specificity and efficiency of replication are significantly enhanced by collaboration with the E2 protein (29, 76). In contrast, in transfected cells, both E1 and E2 are required for ori replication, with few exceptions (4, 9, 54, 79). The reasons for the difference in the requirements for E2 between the two replication systems are not completely understood.

Cellular DNA replication of eukaryotic cells occurs within defined sites inside the nucleus that are identified in situ by colocalization of replication factors and nascent DNA, as revealed by bromodeoxyuridine (BrdU) labeling. These sites are distributed in a granular, punctate pattern throughout the nucleus (25, 45; reviewed in reference 14). The number of these sites has been estimated to be between 100 and 300, although the number and distribution change throughout the duration of the S phase, being more limited during the late S phase (46, 47). In situ detection of viral and host proteins has also defined

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intranuclear sites where the DNA replication of adenovirus (50, 72), herpesvirus (HSV) (51), and simian virus 40 (SV40) (23) occurs. Viral replication compartments are much fewer in number than those of cellular DNA synthesis and expand into larger, globular foci as replication proceeds.

The underlying structure responsible for the spatial organization of DNA inside the nucleus is believed to be the nuclear matrix (71). The nuclear matrix is implicated as the subnuclear structure on which most nuclear processes occur, including DNA replication, RNA transcription and processing, and ribosome biogenesis (for a review, see referene 70). Matrix attachment regions present on chromosomal DNA anchor chromatin to the nuclear matrix, creating loops of defined chromatin domains that confer a higher level of organization to chromosomal DNA beyond that which is provided by nucleosomes. The size of individual chromatin loops has been correlated to that of replication domains (for a review, see reference 14). Active cellular DNA replication complexes have been shown to fractionate with the nuclear matrix (60, 67). Similarly, the SV40 large-T antigen also fractionates with the SV40 DNA replication-competent nuclear matrix derived from SV40-infected cells (53, 62).

It has been suggested that DNA viruses induce spatial reorganization of nuclear processes. For example, cellular DNA synthesis and a number of host nuclear proteins, including components of the cellular DNA replication apparatus, are localized exclusively within HSV and cytomegalovirus (CMV) replication compartments during the S phase (16, 19, 73). Another example is the disruption of nuclear domain 10 (ND10) by infection with adenovirus (17), HSV (18, 40, 41), or CMV (3, 28). ND10 structures, also known as POD (promyelocytic oncogenic domains), are intranuclear domains identified by the presence of one or more antigens, including the tumor suppressor protein promyelocytic leukemia protein (PML) (for a review, see reference 64). Components of ND10 are recruited into HSV replication compartments (36, 37). Moreover, adenovirus, HSV type 1, and SV40 DNAs are replicated at intranuclear sites that are located adjacent to ND10 (26).

In this study, the subnuclear topology of HPV-11 ori DNA replication was characterized in a transient replication system by indirect immunofluorescence methods. We identified intranuclear foci where E1, E2, the HPV origin containing plasmid (ori plasmid), and the host single-stranded DNA binding protein replication protein A (RP-A) were colocalized and showed that some of these sites were active for DNA synthesis by pulse-labeling with BrdU. We propose that these foci represent HPV replication compartments. Unexpectedly, in cells positive for E1 and E2, RP-A and BrdU were localized exclusively within E1 and E2 foci. The colocalization of E1 and E2 and of RP-A also occurred in the absence of the ori plasmid. Moreover, in a fraction of the transfected cells, PML was either partially or completely colocalized with the HPV replication compartments. The implications of these results are discussed.

MATERIALS AND METHODS

Cell line, plasmids, and DNA transfection. The HPV DNA-negative cervical carcinoma cell line C33A was used for all experiments. Cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% bovine calf serum. Plasmids pMTX-11EE-E1 (29) and pMT2-11E2 (9) were used for E1 and E2 expression, respectively. pMTX-11EE-E1 encodes the HPV-11 E1 protein tagged at its amino terminus with the glutamate-rich epitope (EE-E1) (21). pMT2-11E2 encodes the native HPV-11 E2 protein. Expression of both proteins was under the control of the adenovirus major late promoter. Plasmid p7730-99 contains a portion of the HPV-11 upstream regulatory region (URR) between nucleotides 7730 and 7933 and the contiguous nucleotides 1 to 99. This fragment contains the ori (8). The 7.9-kb-long plasmid pEGFP-11URR was constructed by inserting the HPV-11 *Bam*HI-*Pst*I DNA fragment (nucleotides 7072 to 7933 and 1 to 2350) into pEGFP-C1 (Clontech Laboratories, Inc., Palo Alto, Calif.) between the *Bgl*II and *Pst*I sites.

Plasmid DNA was transfected into C33A cells by electroporation (9) or by the calcium phosphate precipitation method as follows. C33A cells were seeded into four-well chamber slides (Nunc, Naperville, Ill.) at a density of 10^5 cells per well. Plasmids pMTX-11EE-E1 (125 ng), pMT2-11E2 (125 ng), and p7730-99 or pEGFP-11URR (12.5 ng) were mixed in a 25-μl total volume of BES-buffered saline–$CaCl_2$ solution (Stratagene, La Jolla, Calif.); sheared herring sperm DNA was used as carrier DNA when plasmids were omitted to maintain the total DNA concentration. DNA precipitates were allowed to form for 20 min at room temperature and then were added to the culture medium. Cells were incubated under standard growth conditions for 10 h, at which time the culture medium was replaced and incubation was continued for an additional 14 h. The cultures were then processed for indirect immunofluorescence as described below.

BrdU labeling of active replication sites. To identify active sites of DNA replication, transfected cells were metabolically pulse-labeled with 10 μM BrdU for 10 min immediately prior to harvest under standard growth conditions. Cells were processed for immunofluorescence as described below, except that slides were heated at 90°C for 30 min in 50 mM citrate (pH 5.2) to denature DNA and allow recognition by antibody BU-33.

Antibodies and indirect immunofluorescence. The dilutions for the various antibodies were as follows: 1:50 each for the mouse monoclonal antibody against the EE epitope tag of HPV-11 EE-E1, the rabbit polyclonal antiserum against the amino-terminal portion of HPV-11 E1 (9), the mouse monoclonal antibody BU-33 (Sigma Chemical Co., St. Louis, Mo.) for BrdU-labeled DNA, the mouse monoclonal antibody Ab-2 (Oncogene Research Products, Cambridge, Mass.) against the 34-kDa subunit of RP-A, and the mouse monoclonal antibody PG-M3 (Santa Cruz Biotechnology, Santa Cruz, Calif.) against PML; 1:200 for the rabbit polyclonal antibody against HPV-11 E2 (7); and 1:50 each for all secondary goat anti-rabbit or goat anti-mouse immunoglobulin G antibodies conjugated to either Texas red, fluorescein (Southern Biotechnology Associates, Birmingham, Ala.), or coumarin (Molecular Probes, Eugene, Oreg.).

Transfected cells in four-well chamber slides were washed twice with phosphate-buffered saline (PBS), prepermeabilized with 0.5% Triton X-100 in PBS at room temperature for 5 min, and fixed with 3% paraformaldehyde in PBS at room temperature for 15 min. Paraformaldehyde was removed by washing five times with PBS for a total of 5 min in a Coplin jar. After blocking was done with 50% normal goat serum (Life Technologies, Gaithersburg, Md.)–3% bovine serum albumin (BSA) in PBS at 37°C for 30 min, the cells were reacted with primary antibodies in antibody-binding solution (3% BSA, 0.5% Triton X-100, PBS) at 37°C for 30 min. Cells were then washed with PBS containing 0.5% Triton X-100 at room temperature for 1 h in a Coplin jar and reacted with secondary antibodies in binding solution containing 2 μM 4,6-diamidino-2-phenylindole (DAPI; Sigma) at 37°C for 30 min. Cells were washed as before and placed under coverslips with Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, Calif.). Cells were examined with an Olympus IX70 inverted microscope equipped with epifluorescence optics, Olympus U PlanApo ×40 and ×100 oil immersion lenses, a Photometrics cooled black-and-white video camera, a Ludl fluorescence filter wheel, and a *z*-axis step motor. Images were acquired and processed with IP LabSpectrum and converted to color images with Adobe Photoshop. Images were digitally deconvolved where indicated in the figure legends.

Tyramide-enhanced FISH. Tyramide-enhanced fluorescence in situ hybridization (FISH) (61, 75) as modified by Philip Moen (42a) was conducted as follows. Transfected cells were processed for indirect immunofluorescence as described above, except that cells were not stained with DAPI and were refixed with 4% paraformaldehyde in PBS at room temperature for 15 min following immunostaining for antigens. (See Results and the legend to Fig. 1 for descriptions of the fluorochromes used to detect E1 and E2 proteins.) Cells were treated with RNase for 1 h at 37°C and then denatured for 2 min in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–50% formamide at 37°C. Enhanced green fluorescent protein (EGPF) fluorescence was quenched by denaturation.

The 1,700-bp *Age*I restriction fragment containing the HPV-11 URR and EGFP sequences was excised from pEGFP-11URR and labeled with biotin-N^6-dATP (NEN, DuPont, Boston, Mass.) by nick translation. After precipitation, the probe was dissolved in 2× SSC–50% formamide–10% dextran sulfate containing sheared salmon sperm DNA (60 μg/l μg of nick translation reaction mixture) and Cot-1 DNA (40 μg/l μg of nick translation reaction mixture). The probe was denatured and hybridized overnight at 37°C to slides. Slides were washed sequentially three times for 10 min each time in 2× SSC–50% formamide at 37°C, once for 30 min in 1× SSC at 37°C, once for 30 min in 1× SSC at room temperature, three times for 5 min each time in 4× SSC–0.5% Triton X-100, and once for 5 min in 4× SSC at room temperature. Slides were blocked with 4× SSC–1% BSA for 30 min at 37°C, incubated with streptavidin-conjugated horseradish peroxidase diluted 1:100 in 4× SSC–0.5% Triton X-100 for 30 min at 37°C, and washed three times for 15 min each time in 4× SSC–0.5% Triton X-100 at room temperature. Cyanine-3-tyramide diluted 1:100 in dilution buffer (Tyramide FISH kit; NEN) was allowed to deposit on the slides for 10 min. Slides were washed three times for 15 min each time in 4× SSC–0.5% Triton X-100 at room temperature and mounted with Antifade (Oncor, Gaithersburg, Md.). Images were acquired with a color CoolCam (SciMeasure Analytical Systems,

Inc., Decatur, Ga.) on a Nikon E-800 microscope and processed with Image-Pro Plus 3.0. Filters for analyses were either single pass or triple pass.

RESULTS

To identify sites of HPV DNA synthesis in situ, we used the previously described transient replication system and transfected human C33A cells (9, 68). Cotransfection of expression vectors encoding the HPV-11 E1 protein (pMTX-11EE-E1) and E2 protein (pMT2-11E2) supported the replication of either of the HPV-11 ori plasmids, p7730-99 (9) and pEGFP-11URR (this study), based on the generation of *Dpn*I-resistant plasmid DNA, as revealed by Southern blot hybridization (data not shown).

Colocalization of E1 and E2 proteins in intranuclear foci in the presence of an HPV ori plasmid. Indirect immunofluorescence with single-pass filters was used to characterize the subnuclear localizations of the E1 and E2 proteins. The E1 protein was detected by a fluorescein-conjugated secondary antibody, and the E2 protein was revealed by a Texas red-conjugated secondary antibody. Double immunostaining was also performed on mock-transfected cells, on cells transfected with either pMTX-11EE-E1 or pMT2-11E2 alone, and in the absence of either or both primary antibodies. These controls confirmed the specificity of the primary antibodies for the E1 and E2 proteins, as no cross-reactivity with each other or with host proteins was observed (data not shown). The specificity of the antibodies was further supported by the absence of staining in the majority of the cells that were not transfected.

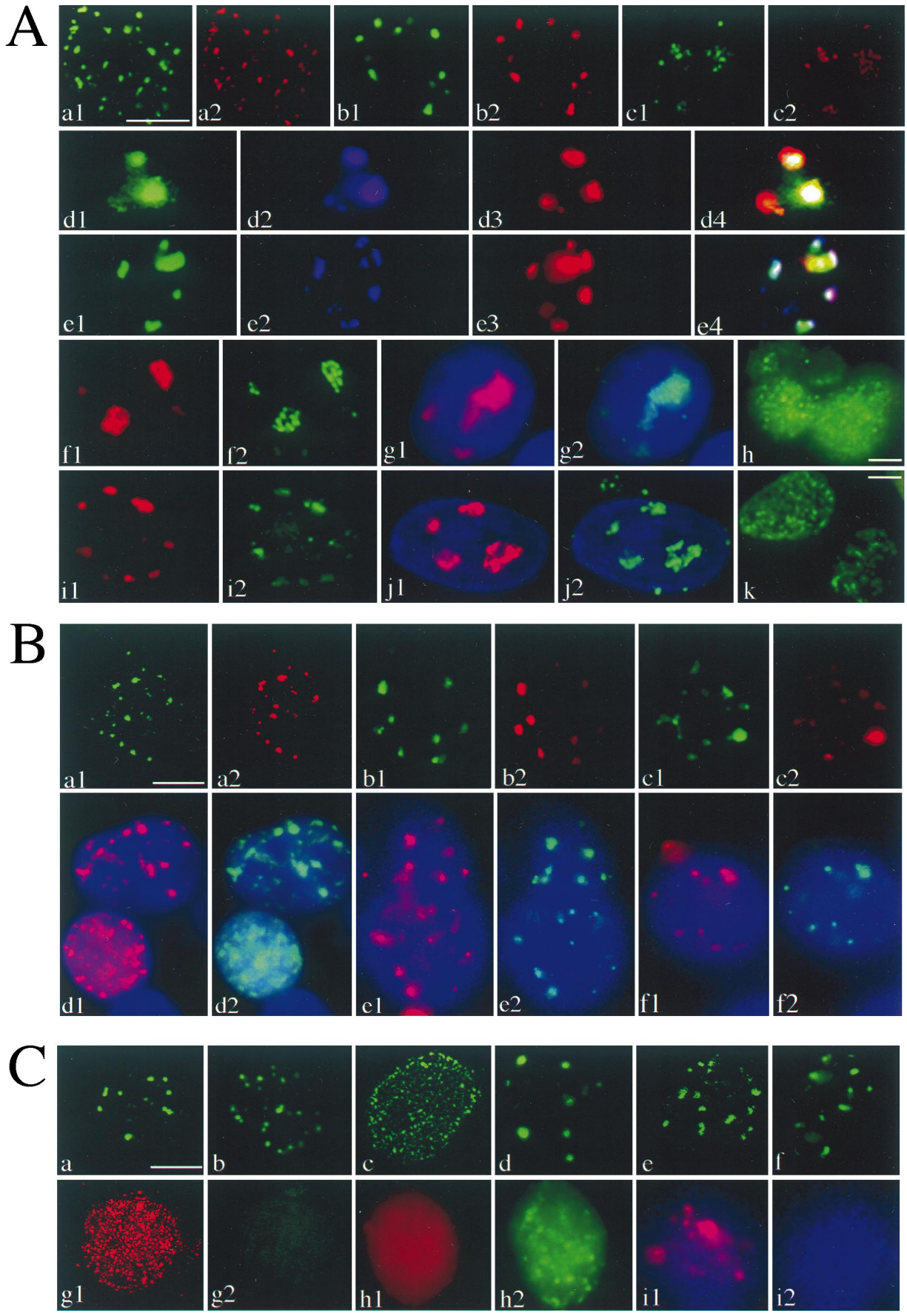
Plasmid pEGFP-11URR was used as the HPV ori plasmid in these experiments, since it contained a convenient restriction fragment that was of sufficient length for use as a probe for FISH. EGFP also provided a means for the initial assessment of successful transfection into C33A cells. To allow the use of fluorescein reagents for the detection of viral proteins, EGFP was removed by prepermeabilization with Triton X-100 prior to fixation. E2 was detected in all cells in which E1 was observed, while E1 was detected in 83% of cells in which E2 was observed. In all cells in which the coexpression of E1 and E2 was observed, the two proteins invariably were colocalized to focal structures within the nucleus (Fig. 1A, panels a, b, and c). The number of these focal structures per nucleus varied from cell to cell. Of cells in which E1 expression and E2 expression were both observed, 51% contained fewer than 5 foci, 25% contained between 5 and 20 foci, and 24% contained more than 20 foci. The sizes of the E1 and E2 foci also varied and were largely inversely proportional to the number of foci in the nucleus. Large E1 and E2 foci often resembled clusters of smaller substructures, as evident in Fig. 1A, panels c, f, g, and j. The E1 and E2 foci were often distributed toward the periphery of the nucleus (Fig. 1A, panels b and i). Similar results were obtained with cotransfection of the ori plasmid p7730-99 (data not shown).

On the basis of these and additional observations to be described, we suggest that, whenever E1 is observed in focal structures, E2 is also present in these structures when the colocalization of a third antigen with E1 is assessed. This assumption is necessary, since our reagents limit us to detecting two antigens at a time. For example, when RP-A or BrdU was colocalized with E1 in focal structures in cells cotransfected with both pMTX-11EE-E1 and pMT2-11E2 as described below, the presence of E2 in those structures was assumed. This assumption was corroborated by parallel experiments that showed the colocalization of E1 and E2, of E2 and RP-A, or of E2 and BrdU.

Colocalization of the HPV ori plasmid with E1 and E2 foci. To determine if the E1 and E2 foci indeed contained the HPV ori plasmid, we assessed the localization of the HPV ori plasmid in relation to the viral proteins by using a tyramide-enhanced FISH method as described in Materials and Methods. The probe was derived from the contiguous URR and EGFP coding sequences in pEGFP-11URR and did not cross-react with either pMTX-11EE-E1 or pMT2-11E2. RNase treatment of the fixed cells precluded hybridization of the probe to EGFP mRNA. The fluorescence of EGFP was abolished by the hybridization procedure and, consequently, did not interfere with the immunostaining with fluorescein reagents. A fluorescein- or coumarin-conjugated secondary antibody was used for the detection of E1 or E2, respectively. Cyanine 3-tyamide was deposited for the detection of the ori plasmid (Fig. 1A, panels d and e). The images in panels d4 and e4 were obtained through a triple-pass filter for fluorescein, coumarin, and rhodamine (which allows cyanine-3 to bleed through). The individual colors in Fig. 1A, panels d4 and e4, were enhanced to reveal white color when all three signals were colocalized, yellow when red and green were colocalized, or red-purple when red and blue were colocalized. The results demonstrated that most, if not all, of the ori plasmid was colocalized with the E1 and E2 foci. This colocalization was specific in two aspects: ori signals were not observed outside the E1 and E2 foci in the positive nuclei, nor were they detected in the nuclei of any cells in which E1 and E2 expression was not observed. Signals were occasionally seen on the glass not associated with cells or at the periphery of the cells (data not shown) and may have represented DNA that was not taken up by the cells or transported into the nucleus. We concluded that the ori-containing foci represent replicated ori DNA and that most of the unreplicated, intranuclear ori DNA was quickly degraded to below the level of detection.

E1 and E2 foci as active HPV DNA replication compartments. To identify active sites of DNA synthesis in the transfected cells, we assessed the intranuclear localization of RP-A, the eukaryotic single-stranded DNA binding protein required for replication initiation and elongation for both cellular and papillomavirus DNAs (29, 76, and references therein). Cells were cotransfected with the E1 and E2 expression vectors and the ori plasmid p7730-99. The E1 or E2 protein was detected by a Texas red-conjugated secondary antibody, while RP-A was revealed by a fluorescein-conjugated secondary antibody. In cells negative for the E1 or E2 protein, RP-A staining was either undetected, diffuse, punctate, or granular (Fig. 1A, panel h, and data not shown), similar to the RP-A staining observed for mock-transfected cells (data not shown). These different patterns may represent cells in different phases of the cell cycle. In contrast, RP-A was colocalized with E1 foci in all E1-positive cells (Fig. 1A, panel f1 and f2) and with E2 foci in 87% of E2-positive cells (panels g1 and g2). As discussed above, the E1 foci always also contained E2. The degree of colocalization between RP-A and E1 or E2 varied and was completely superimposable in 84% of the cells.

As a more definitive indicator for sites of DNA synthesis, we performed coimmunostaining for BrdU and E1 or E2. BrdU was added to the transfected cells for 10 min immediately prior to harvesting to label nascent DNA in active centers of DNA synthesis. In this experiment, the E1 or E2 protein was detected by a Texas red-conjugated secondary antibody, while BrdU was revealed by a fluorescein-conjugated secondary antibody. BrdU incorporation was observed in 23% of E1-positive cells and in 18% of E2-positive cells. In cells negative for the E1 or E2 protein, BrdU was distributed in a granular or punctate manner throughout the nucleus (Fig. 1A, panel k),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similar to the BrdU pattern observed for untransfected cells (data not shown). However, in cells positive for E1 or E2, the BrdU foci were largely, if not entirely, colocalized within the E1 and E2 structures (Fig. 1A, panels i and j). These foci were relatively large and limited in number in comparison to the E1 or E2 foci in cells that were negative for BrdU incorporation. We infer that the E1 and E2 foci in BrdU-positive cells represent HPV ori replication compartments and that those in BrdU-negative cells may represent pre- or postreplication foci.

Colocalization of E1 and E2 proteins in nuclear foci in the absence of an HPV ori plasmid. It has been suggested that host or viral DNA contains matrix attachment sequences (2, 66). To determine whether the ori plasmid or its active replication is the driving force for the complete colocalization of E1, E2, and RP-A, we determined the nuclear distribution of these three proteins in cells cotransfected with pMTX-11EE-E1 and pMT2-11E2 in the absence of the ori plasmid. The E1 protein was detected by a fluorescein-conjugated secondary antibody, while the E2 was protein revealed by a Texas red-conjugated secondary antibody. E2 was detected in all E1-positive cells in which the two proteins were colocalized in structures similar to those observed in the presence of the ori plasmi (Fig. 1B, panels a, b, and c). E1 was detected in 53% of E2-positive cells in similar structures. Of cells in which E1 expression and E2 expression were both observed, 7% contained fewer than 5 foci, 32% contained between 5 and 20 foci, and 61% contained more than 20 foci. The foci were irregular in size and shape and were rarely as large as the replication factories shown in Fig. 1A, panels d, e, f, g, and j. These data suggest that the ori plasmid is not essential for the colocalization of E1 and E2. Rather, the E1 and E2 proteins interact in vivo and are cotargeted to certain nuclear domains.

We then performed coimmunostaining for RP-A and E1 or E2. The E1 or E2 protein was detected by a Texas red-conjugated secondary antibody, while RP-A was revealed by a fluorescein-conjugated secondary antibody. Interestingly, in the absence of the ori plasmid, RP-A was colocalized with E1 in all E1-positive cells (Fig. 1B, panels d1 and d2) and with E2 in 46% of E2-positive cells (panels e and f). The degree of colocalization between RP-A and E1 or E2 varied and was completely superimposable in 74% of the cells. These data suggest that the colocalization of RP-A with E1 and E2 foci is not dependent on the presence of the ori plasmid or active HPV DNA synthesis.

Independent localizations of E1 and E2. Since E1 was completely colocalized with E2 in E1 foci, whereas only a fraction of focal E2 contained E1, we were curious to learn whether the E1-E2 colocalization was directed by E2. Furthermore, we also wondered how RP-A was recruited into the E1 and E2 foci. Therefore, we assessed the localizations of the E1, E2, and RP-A proteins in cells separately transfected with the E1 or E2 expression vector alone. To determine the percentages of cells in which E1 or E2 might display diffuse nuclear staining, prepermeabilization with Triton X-100 prior to fixation was omitted in these experiments.

The E1 protein exhibited a focal localization pattern in only 13% of positive cells (Fig. 1C, panels a and b). In the remaining cells, a variety of E1 staining patterns were observed. In some cells, E1 was distributed throughout the nucleus in a diffuse manner (Fig. 1C, panel h1). In other cells, there was a punctate pattern of numerous small foci (Fig. 1C, panels c and gl). In yet others, mixed patterns were observed. The reason for these varied patterns is not known. In 66% of E2-positive cells, E2 exhibited a diffuse, nuclear distribution (data not shown). However, in the remaining E2-positive cells, E2 was localized either in foci that were similar in size and distribution to the compartments formed by E1 and E2 together (Fig. 1C, panels d, e, and f) or in foci that were superimposed on diffuse nuclear staining (data not shown). These data demonstrated that only a fraction of either the E1 or the E2 protein exhibited a focal, intranuclear localization pattern similar to that observed when both proteins were present.

To assess the localization of RP-A in relation to the E1 or E2 protein in C33A cells, the E1 (Fig. 1C, panels g1 and h1) or E2 (panel i1) protein was detected by a Texas red-conjugated secondary antibody, while RP-A was revealed by a fluorescein-conjugated secondary antibody (panels g2, h2, and i2). The distribution of RP-A was apparently unaltered by the expression of either E1 or E2 alone, exhibiting a diffuse, punctate, or homogeneous pattern, or occasionally was below detectable levels, analogous to those observed in untransfected cells (Fig. 1C, panels g2, h2, and i2). Interestingly, in cells in which E2 (Texas red) was localized in focal structures, RP-A staining was either below the threshold of detection or distributed homogeneously throughout the nucleus (Fig. 1C, compare panel i1 to panel i2). Although the pattern of distribution of E1 in many of the cells (Fig. 1C, panels c and g1) apparently mimicked that of cellular replication sites, as inferred from the patterns of BrdU labeling and RP-A antibody reactivity in untransfected cells (Fig. 1A, panels h and k), we were unable to demonstrate definitive colocalization of E1 and RP-A. Indeed, a homogeneous nuclear distribution was often observed for E1 in cells in which RP-A was localized in punctate structures (Fig. 1C, compare panel h1 to panel h2) and vice versa (compare panels g1 to panel g2). These observations suggest that, if either E1 or E2 alone is indeed targeted to cellular replication sites or to a subset thereof, its association with these sites is not mediated by RP-A. Thus, we conclude that the colocalization of RP-A with E1 and E2 foci occurs only when the E1 and E2 proteins are expressed together.

Localization of PML relative to HPV DNA replication compartments. ND10 was recently proposed as the site of BPV-1 virion assembly (15). From the perspective of the virus, colocalization of at least some of the sites at which vegetative viral

FIG. 1. Localization of HPV-11 E1, E2, ori, BrdU, and RP-A. Individual cells viewed with different filters are denoted by the same lowercase letters and different numbers. (A) Cells were cotransfected with pMTX-11EE-E1, pMT2-11E2, and pEGFP-11URR and pulse-labeled with BrdU. With the exception of d4 and e4, all images were recorded with a single-pass filter. Panels were as follows: a, b, and c, coimmunostaining for E1 (1, fluorescein) and E2 (2, Texas red), d and e, coimmunostaining for E1 (1, fluorescein) and E2 (2, coumarin [blue]) and FISH with the ori plasmid-specific probe (3, cyanine-3 [red]); d4 and e4, images obtained through a triple-pass filter (red, green, and blue) and enhanced to reveal the colocalization of the three colors, which then appeared as white; f and g, coimmunostaining for RP-A (2, fluorescein) and E1 (f1, Texas red) or E2 (g1, Texas red); h, untransfected cell stained for RP-A (fluorescein); i and j, coimmunostaining or BrdU (2, fluorescein) and E1 (i1, Texas red) or E2 (j1, Texas red); and k, BrdU staining of untransfected cells (fluorescein). (B) Cells were cotransfected with pMTX-11EE-E1 and pMT2-11E2. Panels were as follows: a, b, and c, coimmunostaining for E1 (1, fluorescein) and E2 (2, Texas red); d, e, and f, coimmunostaining for RP-A (2, fluorescein) and E1 (d1, Texas red) or E2 (e1 and f1, Texas red). In e1 and f1, the E2 antibody reactivity at the edge of the nuclei appears to be the centrosome to which rabbit polyclonal antibodies often react (13). (C) Cells were separately transfected with pMTX-11EE-E1 (a, b, c, g, and h) or pMT2-11E2 (d, e, f, and i). Panels were as follows: a to f, immunostaining for E1 (a, b, and c) or E2 (d, e, and f) (fluorescein); g to i, coimmunostanining for RP-A (2, fluorescein) and E1 (g1 and h1, Texas red) or E2 (i1, Texas red). Images in panels a, b, c, f, g, i and j of A and panels d, e, and f of B were deconvolved. All cells were costained with DAPI (blue), but the DAPI fluorescence is not included in every panel. Bars 10 μm. The bar in panel a1 of A applies to all panels of A except for panels h and k, which are of a lower magnification and have a different bar scale.

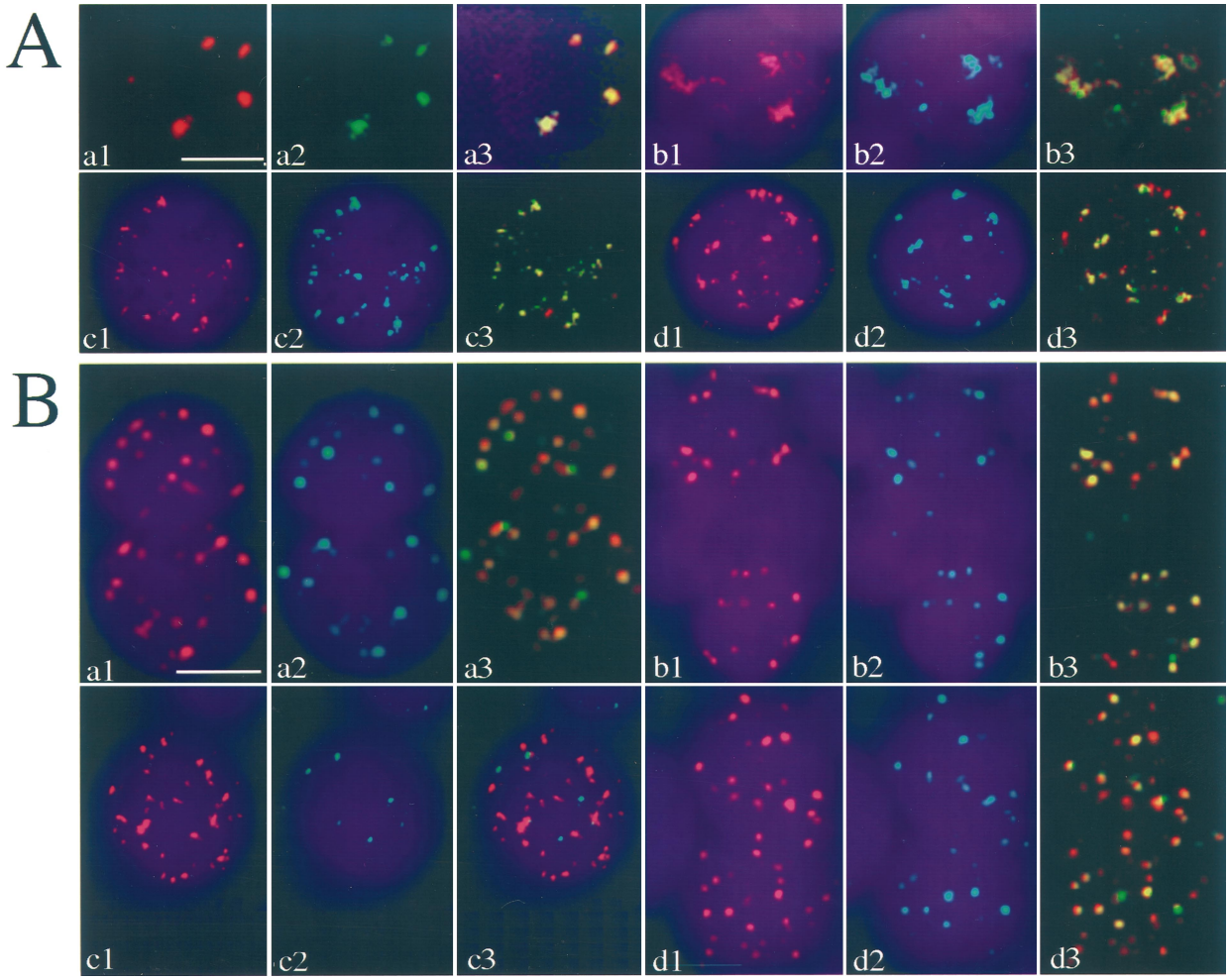

FIG. 2. Localization of E1 and E2 compartments in relation to ND10. Individual cells viewed with different filters are denoted by the same lowercase letters and different numbers. (A) Cells were cotransfected with pMTX-11EE-E1, pMT2-11E2, and p7730-99 and coimmunostained for PML (2, fluorescein) and E1 (a1 and b1, Texas red) or E2 (c1 and d1, Texas red); 3, red and green merged images. (B) Cells were cotransfected with pMTX-11EE-E1 and pMT2-11E2 and coimmunostained for PML (2, fluorescein) and E1 (a1 and b1, Texas red) or E2 (c1 and d1, Texas red); 3, red and green merged images. All images were deconvolved. All cells were costained with DAPI (blue), but the DAPI fluorescence is not included in every panel. Bars, 10 μm.

DNA replication and progeny virion assembly take place is an attractive hypothesis. To test this possibility, we assessed the localization of E1 and E2 compartments in relation to PML, a component of ND10. The E1 or E2 protein was detected by a Texas red-conjugated secondary antibody, while PML was revealed by a fluorescein-conjugated secondary antibody. Expression of either the E1 or the E2 protein alone had no apparent effect on the ND10 structures (data not shown). However, when E1 and E2 expression vectors were cotransfected with the HPV ori plasmid, PML was either partially or completely colocalized with E1 or E2 in 42% of E1-positive cells and in 37% of E2-positive cells (Fig. 2A). When the ori plasmid was omitted from the transfection, PML was still colocalized with E1 or E2 (Fig. 2B), but at a much reduced frequency, suggesting that the colocalization was influenced by HPV DNA synthesis. The degree of colocalization of PML and E1 or E2 in individual foci varied.

The green and red signals did not always overlap perfectly, suggesting that PML and E1 or E2 are localized to juxtaposed or partially overlapping nuclear domains. In cells negative for E1 or E2, ND10 structures were small, round, and distinctive. However, in some of the cells in which PML was colocalized with E1 or E2 in the presence or in the absence of the ori plasmid, the structures resembled the E1 and E2 compartments described above, in that their numbers commonly exceeded 20 per nucleus and that they were larger, globular structures or irregular in shape. In other E1- or E2-positive cells, only a fraction (Fig. 2B, panels a and d) or none (panels c) of the PML structures were colocalized with the viral proteins, and the structures appeared unaltered in morphology. Thus, additional factors besides the expression of the viral proteins may be important for colocalization with ND10.

DISCUSSION

Using immunofluorescence, we characterized the subnuclear topology of HPV-11 DNA replication by transient cotransfection of an ori plasmid and expression plasmids for the E1 and E2 proteins. The HPV-11 ori DNA replication compartments were identified on the basis of the colocalization of the E1 and E2 proteins and the HPV ori plasmid and the pairwise colocalization of viral proteins and the host RP-A protein and BrdU incorporation. Although 100% of the E1 and E2 foci were positive for RP-A, only 18% were positive for

BrdU. This difference could have been a matter of the relative sensitivities between assays or the short duration of the BrdU incorporation. However, that HPV replication is restricted to a period of time relative to the S phase is also possible. The E1-, E2-, and RP-A-positive and BrdU-negative foci could then represent preinitiation or postreplication centers.

Intriguing observations were made after only one or two of the three plasmids were transfected. The colocalization of E1, E2, and RP-A foci in the absence of an ori plasmid suggests that E1 and E2, but not ori DNA, determine the intranuclear locations where HPV ori replication occurs. E2 has been reported to associate with RP-A (31). However, the vast majority of RP-A did not appear to be colocalized with either E1 or E2 when each viral protein was individually expressed (compare Fig. 1B and C). These observations indicate that E1 and E2 jointly recruit RP-A or are jointly targeted to a limited number of domains where RP-A is normally found.

A couple of observations suggest that the E2 protein plays a major role in recruiting the E1 protein to replication sites. (i) In a substantial fraction of the cells, E2 was localized independently of E1 or of the ori plasmid in nuclear structures that were characteristic of E1 and E2 foci. In contrast, in the absence of E2, E1 was usually found dispersed throughout the nuclei rather than within larger foci. (ii) E1 invariably was colocalized with E2 foci, whereas some E2 foci were negative for E1, consistent with the interpretation that E1 is stabilized by its recruitment into E2 foci. Thus, the ability to target E1 to nuclear foci could be a new role for the E2 protein beyond its previously demonstrated functions in HPV ori replication. Together with the alleviation of nucleosomal repression of HPV DNA replication by E2 (32), this putative new function for E2 may account for the difference in the requirement for the E2 protein between cell-free and transient transfection replication systems. We also note that a lower percentage of E2-positive cells also contained E1 when the ori plasmid was not cotransfected (53% versus 83%). This difference may signify a contribution of the ori plasmid to E1 and E2 colocalization, but the trivial explanation of differential cotransfection efficiencies between these two experiments cannot be ruled out. Based on the data presented, we conclude that the formation of replication compartments requires the cooperative assembly of E1 and E2 and that the presence of an ori plasmid is not necessary but may increase the efficiency of colocalization.

Day et al. (15) recently reported a diffuse nuclear localization pattern for the BPV-1 E2 protein and a requirement of L2 for the localization of E2 in foci. Perhaps the discrepancy between that study and ours is due to differences in the two papillomavirus systems or in the levels of protein expressed from the two systems. Day et al. expressed the BPV-1 E2 protein from a recombinant Semliki Forest virus in cells which harbor endogenous BVP-1 DNA, whereas we expressed the HPV-11 E2 protein from plasmids transfected into naive cells.

Two additional observations were unexpected. (i) The localization of RP-A exclusively in E1 and E2 foci, even in the absence of an ori plasmid, and (ii) the incorporation of BrdU exclusively in E1, E2, and RP-A foci in the presence of an ori plasmid. There are at least two alternative explanations for these observations. First, the cellular DNA replication machinery is grossly reorganized by E1 and E2 and is recruited into HPV DNA prereplication compartments, as has been proposed for herpesviruses (19, 73). However, we doubt that the amounts of the E1 and E2 proteins in transfected cells are sufficient to cause a quanitative redistribution of the entire intranuclear RP-A reservoir. Nevertheless, we cannot rule out the possibility that less abundant host proteins, such as cyclin E and the cyclin-dependent kinase Ckd2, which are essential for S-phase entry and efficient HPV replication (38a), are recruited to the foci, creating a condition in which only the E1 and E2 foci are capable of replicating viral or host DNA. We suggest that the transient replication system is akin to vegetative replication in differentiated squamous karatinocytes in benign lesions. In these cells, viral DNA is present at a high copy number per cell, presumably on the order of hundreds up to one or several thousands. Since differentiated karatinocytes soon undergo programmed cell death, such a gross redistribution of the replication machinery could be tolerated. However, extensive host DNA replication does occur in these differentiated cells (6), and polyploidy has been observed in benign papillomas and condylomata (58). Furthermore, such a hypothetical dramatic alteration in the host DNA replication machinery is not compatible with the viability of proliferating cells.

We propose another scenario to explain the exclusive localization of the host replication machinery in the viral replication centers. We propose that the E1 and E2 proteins are targeted to restricted host replication sites at a certain stage(s) of the cell cycle. We favor the late S phase as a likely time for the occurrence of this event, when host DNA replication is largely completed and the number of active cellular replication sites is restricted. In *Xenopus* sperm nuclei, RP-A has been shown to be localized in prereplication centers prior to the S phase and the onset of DNA synthesis (1). Following replication at a particular site, RP-A dissociates from that site. Thus, late in the S phase, after chromosomal DNA replication is complete, RP-A exhibits a diffuse or undetectable staining pattern that persists through mitosis. We suggest that the replication machinery, including RP-A, that dissociates from host replication sites upon the completion of cellular DNA synthesis is retargeted to HPV replication compartments, accounting for the very bright signals in these foci.

Attractive attributes of this latter hypothesis are a reduced opportunity for newly replicated viral DNA to dissociate from the host chromosome scaffolding and an increased probability of proper segregation of viral genomes with host chromosomes during the mitosis that soon follows. Clearly, proper segregation of viral DNA is essential for long-term maintenance of the viral genome in the dividing cells of a papilloma. Interestingly, recent reports suggest that the maintenance of BPV genomes in cycling cells is accomplished by their association with host chromosomes during mitosis mediated by the E2 protein and E2BS in the URR (30, 49, 59). A similar observation has been made for the HPV-11 E2 protein (78).

Our data also demonstrate that, in a fraction of the cells, the PML antigen was partially or completely colocalized with E1 and E2 (Fig. 2). PML and NDP55, both components of ND10, have been shown to be colocalized with HSV type 1 replication compartments (36, 37). Thus, papillomaviruses and herpesviruses may each interact with a common ND10-associated activity. The HSV ICPO, adenovirus E4 ORF3, and CMV IE1 proteins have been shown to induce the disruption of ND10 structures (3, 5, 40). Without a third antigen as a reference point, we do not know whether a similar disruption of ND10 structures is induced by the expression of the E1 and E2 proteins. However, the PML, E1, and E2 foci often no longer resembled ND10 (Fig. 2). The BPV-1 minor capsid protein L2 was recently reported to localize to ND10, where it recruited E2 and the major capsid protein L1 (15). Taken together, these data strongly suggest that papillomaviral DNA amplification and progeny virion assembly may be partially coupled and occur at common sites.

In summary, we have conducted an extensive investigation of HPV DNA replication complexes in transfected cells in rela-

tion to the host replication protein RP-A, BrdU incorporation, and ND10 distribution. Our results suggest that E2 plays a major role in ori replication and, together with E1, is targeted to prereplication foci, where replication subsequently occurs. The apparently exclusive association of the host replication machinery with HPV replication centers is provocative and invites further investigation.

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