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Replicative intermediates of human papillomavirus type 11 in laryngeal papillomas: Site of replication initiation and direction of replication

K. J. AUBORN*, R. D. LITTLE†, T. H. K. PLATT†‡, M. A. VACCARIELLO*, AND C. L. SCHILDKRAUT†

*Department of Otolaryngology, Long Island Jewish Medical Center, The Long Island Campus for the Albert Einstein College of Medicine, New Hyde Park, NY 11040; and †Department of Cell Biology, Albert Einstein College of Medicine, Bronx, NY 10461

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ABSTRACT **We have examined the structures of replication intermediates from the human papillomavirus type 11 genome in DNA extracted from papilloma lesions (laryngeal papillomas). The sites of replication initiation and termination utilized *in vivo* were mapped by using neutral/neutral and neutral/alkaline two-dimensional agarose gel electrophoresis methods. Initiation of replication was detected in or very close to the upstream regulatory region (URR; the noncoding, regulatory sequences upstream of the open reading frames in the papillomavirus genome). We also show that replication forks proceed bidirectionally from the origin and converge 180° opposite the URR. These results demonstrate the feasibility of analysis of replication of viral genomes directly from infected tissue.**

Human papillomaviruses (HPVs) are the etiological agents of papillomas (warts) and are associated with many cancers (see ref. 1 for review). HPV-11 is the most frequent cause of laryngeal papillomas and is commonly associated with condylomata (genital warts) (2). Two types of HPV infection have been documented: a maintenance state, where replication takes place in basal epithelial cells harboring <10 viral genomes per cell (3), and a much higher copy amplified replication, which occurs in the more differentiated layers of the epithelium. This high-copy replication precedes the production of virus particles (see ref. 4 for review) and is dependent on the differentiation of epithelial cells (5). The type of amplification observed in the outer layers of papillomas has been duplicated in organotypic cultures of a HPV-31-containing cell line (5, 6) and in explants of papillomas (7).

The specific differences between maintenance and amplified replication have not been defined. Both bidirectional (8–10) and rolling circle (11) modes of replication have been reported for bovine papillomavirus (BPV-1), which replicates in rodent fibroblasts, but the significance of this as it relates to the life cycle of the virus is not clear. In the Epstein–Barr virus, separate origins and different mechanisms of replication are used for latent (12) and lytic replication (13). Thus far, evidence for rolling circle replication has not been reported for HPV.

The site of replication initiation in papillomavirus genomes during the natural life cycle of the virus has not been determined. However, the upstream regulatory region (URR; ≈700 bp) of the papillomavirus genome contains enhancers and promoters and is strongly implicated as the origin of replication (see refs. 1, 4, and 14 for reviews). Physical mapping of the start site of replication has been performed for BPV-1, although some ambiguities exist. For example, electron microscopic analysis of replicating molecules mapped the site of initiation to the 5′ portion of the URR (15). In contrast, two-dimensional (2D) agarose gel electrophoresis was used to map the initiation site to the URR (8) and, more specifically, the middle portion of the URR (9). The possibility that many different origins may exist in BPV DNA was suggested after examination of DNA obtained from BPV virions by electron microscopy (16).

Several groups have subcloned portions of papillomavirus genomes and assayed for elements that permit autonomous replication of these plasmids in transfected cells (autonomous replicating sequences, ARS elements). Sequences conferring upon chimeric plasmids the ability to replicate autonomously have been identified in the 3′ portion of the URR of BPV-1. When sequences in this ARS are mutated in the otherwise intact BPV genome, replication was not detected after infection, suggesting that these elements are required for *in vivo* replication. Mutations in this ARS element render the complete BPV genome inactive for replication (17). ARS elements that contain the 3′ portion of the URR have also been described for some HPVs, including HPV-11 (18–20). The use of ARS assays for the identification of replication origins has some limitations in studies of chromosomal replication in eukaryotic cells. For example, only a subset of the ARS elements identified in the yeast *Saccharomyces cerevisiae* function as chromosomal origins of replication (21–23), although all functional chromosomal origins identified thus far are positive in ARS assays. The difficulty in obtaining comparable well-defined ARS sequences from mammalian chromosomes suggests that the genetic elements that specify the location of initiation sites may be much more complex than expected.

The purpose of this study was to investigate the replication of HPV *in vivo*. We used the approach of analyzing replicative intermediates directly from DNA isolated from bulky vocal cord papillomas. These lesions typically have low levels of capsid antigen (24–26), indicating that minimal amounts of mature virions are present in our samples. In these tissues there were at least 1000 copies of the viral genome per cell. Such high levels are characteristic of HPV infection in differentiated cells (27). Therefore, the mode of HPV-11 replication used in the tissues for this study should be primarily the amplified replication typical in differentiated cells and not the maintenance replication observed in basal cells. We used two complementary 2D agarose gel electrophoresis methods to examine populations of replicating viral molecules. Our results are consistent with initiation of replication in the URR of the viral genome. Replication forks

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Abbreviations HPV, human papillomavirus; BPV, bovine papillomavirus, URR, upstream regulatory region; ARS, autonomously replicating sequences; 2D, two dimensional; N/N, neutral/neutral; N/A, neutral/alkaline.

‡Present address: Department of Orthopedic Surgery, The Bronx Lebanon Hospital Campus of the Albert Einstein College of Medicine, Bronx, NY 10457.

proceed bidirectionally and termination usually occurs ≈180° from the origin. Thus, we show that the region containing the ARS element described for HPV-11 (19, 20) functions as the site of replication initiation *in vivo*. We demonstrate that this approach permits the direct analysis of viral DNA replication in the disease state. We also show that this analysis can be used to study viral DNA structure and organization directly from tissue samples.

MATERIALS AND METHODS

Tissues. Laryngeal papillomas were surgically removed (tissue discards) from two patients with recurrent juvenile-onset disease and frozen in liquid nitrogen. The tissues contained HPV-11, as determined by *Pst* I digestion and stringent hybridization with cloned HPV-11 DNA (28).

Plasmids. Cloned HPV-11 DNA (GenBank data base, accession no. M141119), a gift of H. zur Hausen (Heidelberg) was used to prepare hybridization probes.

Preparation of DNA from Tissue. Frozen tissue from HPV-11-induced laryngeal papillomas was pulverized while frozen and extracted (29) with phenol/chloroform followed by treatment with both RNase A and RNase T1.

Enrichment for Replication Intermediates. Restriction digests of DNA (≈100 μg) were enriched for replicative intermediates by benzoylated naphthoylated DEAE-cellulose chromatography (Serva) (30).

2D Gel Electrophoresis. The neutral/neutral (N/N) 2D agarose gel technique of Brewer and Fangman (31) was used to determine the molecular structures of replicating viral DNA. DNA fragments separated by this method give characteristic patterns, which identify fragments containing bubbles or single or double forks (31). Briefly, the first-dimension gels were 0.4% agarose (Seakem ME; FMC Bioproducts) in 1× TBE (89 mM Tris·HCl, pH 8/89 mM boric acid/2 mM EDTA) and were electrophoresed at 1 V/cm at room temperature for 17–19 hr depending on the size of the fragment being analyzed. After electrophoresis, gels were stained in 1× TBE containing 0.3 μg of ethidium bromide per ml for ≈15 min. The second-dimension gels (1% agarose in 1× TBE plus 0.3 μg of ethidium bromide per ml) were electrophoresed at 4°C in circulating 1× TBE containing 0.3 μg of ethidium bromide per ml at 4.8 V/cm for 4–7 hr. A second method, neutral/alkaline (N/A) electrophoresis, developed by Nawotka and Huberman (32), was used to determine the size of nascent strands and the direction of replication fork movement. The first-dimension conditions were identical to the N/N 2D gel method. The gels were treated as described above, except that 0.8–1.2% agarose in water was used for the second-dimension gel. The solidified gels were placed in an electrophoresis apparatus containing circulating alkaline electrophoresis buffer, 40 mM NaOH, 2 mM EDTA, and incubated at room temperature for 1 hr. Electrophoresis was performed at 0.57 V/cm for 24 hr. For both methods, HPV DNA segments of interest were detected by Southern transfer to GeneScreen*Plus* nylon membrane (New England Nuclear) and hybridization to specific ^{32}P-labeled fragments of cloned HPV-11.

RESULTS

HPV-11 DNA in Laryngeal Papillomas Is Episomal and Primarily Monomeric. Laryngeal papillomas from a single patient were pooled, frozen in liquid nitrogen, and pulverized, and DNA was isolated. Restriction enzyme analysis was used to demonstrate the episomal nature of the viral DNA (Fig. 1*A*). The electrophoretic migration of the viral DNA was unaffected by treatment with *Bcl* I, *Sac* I, or *Eco*RI, restriction enzymes that do not cleave HPV-11 DNA. The monomeric nature of the viral DNA was also established by N/N 2D gel analysis (Fig. 1*B*). The major portion of the viral DNA migrated as monomeric supercoiled or open-circle plasmids. All of the DNA samples used in this study were identical in this type of analysis.

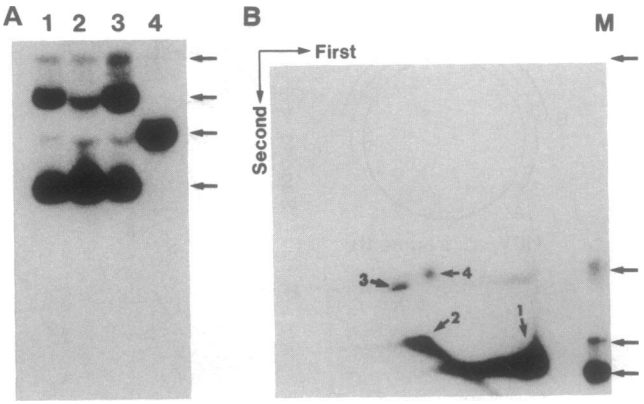

FIG. 1. HPV-11 DNA from papillomas is episomal. (*A*) Ten micrograms of DNA extracted from a laryngeal papilloma was digested with *Bcl* I, *Sac* I, and *Eco*RI, three restriction enzymes for which HPV-11 lacks sites, and *Bam*HI, which cleaves HPV-11 at one site, and electrophoresed (lanes 1–4, respectively). (*B*) Twenty micrograms of the undigested DNA was examined by 2D gel electrophoresis. Unlabeled arrows in *A* and *B* indicate positions from bottom to top of supercoiled, linear (migrating at 8 kb), and nicked circular HPV DNA and the wells. A control lane (M) with a sample of the same laryngeal papilloma DNA examined for replicative intermediates is electrophoresed in only the second dimension to indicate positions of supercoiled, linear, and nicked circular HPV DNA, respectively. In *B*, arrows 1 and 2 indicate positions at which the supercoiled monomeric and linear monomeric HPV-11 DNA migrate, respectively. Arrows 3 and 4 represent the positions at which nicked monomeric and supercoiled dimeric DNA migrate, respectively, based on the separation of oligomers of BPV from the ID13 murine cell line by Schvartzman *et al.* (8) and our own observations.

Replication of HPV-11 DNA Initiates at or Near the Upstream Regulatory Region of the Viral Genome. To determine the sites of replication initiation used by HPV-11 *in vivo*, we utilized the 2D gel method developed by Brewer and Fangman (31). Fig. 2 shows the analysis of two restriction endonuclease fragments containing the URR in a central location: a 3337-bp *Nci* I/*Xba* I segment and a 5135-bp *Xmn* I segment. Hybridizations were performed with a radiolabeled probe encompassing the URR (HPV-11, nt 7178–33). The 2D gel patterns observed from these segments demonstrated electrophoretic patterns typical of replication from an initiation site internal to the segment (bubble pattern). Molecules containing bubbles are best detected when the site of initiation is located in the central one-third of the fragment (33); thus, the initiation site is at or near the URR. In addition to the bubble pattern, a pattern characteristic of replicative intermediates containing a single replication fork (simple fork arc) was present in both fragments. A simple fork arc with no bubble pattern was detected in a *Sca* I fragment containing the URR (nt 5043–564) (data not shown). In this fragment, only the 5′ portion of the URR and not the 3′ region of the URR would be included in the central one-third of the fragment.

Replication of HPV-11 DNA Is Bidirectional. To determine whether replication proceeded bidirectionally from the site of replication initiation, we examined segments whose midpoint is ≈180° from the URR (Fig. 3). If bidirectional replication occurred, these segments would be expected to give rise to replicative intermediates containing two converging replication forks (double Y pattern), each entering the segment from opposite directions and meeting to form a replication termination region. Two different segments, a 4594-bp *Nci* I/*Xba* I segment and a 2323-bp *Xmn* I segment, were examined.

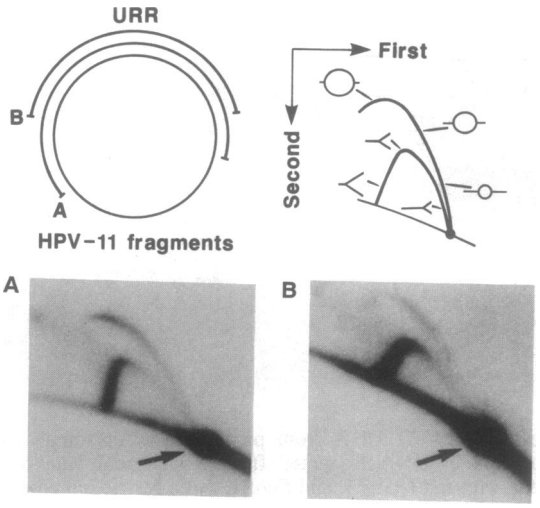

FIG. 2. Initiation of replication occurs in segments containing the URR. An aliquot of the DNA sample analyzed in Fig. 1 was cleaved with *Xmn* I (segment A) or *Nci* I and *Xba* I (segment B) and examined by N/N 2D gel electrophoresis. (*Upper left*) Position of the 5135-bp *Xmn* I segment (A, nt 4988–2191 in HPV-11) and the 3337-bp *Nci* I/*Xba* I segment (B, nt 5885–1291) are indicated. Both segments contain the URR (nt 7273–33). (*Upper right*) Schematic indicating migration of molecules containing internal bubbles indicative of a site of initiation of DNA replication (bubble pattern). In addition, the migration of molecules containing a single replication fork is shown (simple Y pattern). (*Lower*) 2D agarose gel analysis of segments A and B. Arrows indicate positions of nonreplicating linear segments. Hybridizations were performed with a probe (HPV-11, nt 7178–33) containing the URR.

Replicative intermediates from both segments contained two opposing forks, indicating bidirectional replication. These DNA segments also exhibited patterns characteristic of a single replication fork traversing the fragment.

Replication Initiates in Downstream Sequences of the URR. To provide more information about the site of replication initiation and the direction of fork movement, we used the N/A 2D gel method developed by Nawotka and Huberman (32). A schematic diagram of the electrophoretic patterns observed for a fragment with an internal initiation site using several probes is shown (Fig. 4). A DNA preparation distinct from the one used in Figs. 1–3 was first analyzed by N/N 2D gel electrophoresis to confirm the integrity of replication intermediates. The N/N 2D gel pattern obtained from a 5135-bp *Xmn* I restriction fragment containing the URR displayed a bubble arc indicating initiation internal to the segment (Fig. 5*A Upper left*). Aliquots of this same DNA preparation, also cleaved with *Xmn* I, were examined on N/A 2D gels. Probes located along the length of the *Nci* I/*Xba* I segment were used to determine the sizes of nascent strands at various positions along the length of the fragment. The same Southern transfer was hybridized to the following probes sequentially: an 897-bp probe at the 5′ end of the fragment (nt 4988–5885; Fig. 5*A1*), a 135-bp sequence encompassing the ARS element (nt 7833–33, Fig. 5*A2*), a 1616-bp sequence at the 3′ end of the fragment (nt 576–2192; Fig. 5*A3*), and an 804-bp sequence at the 3′ end of the fragment (nt 1388–2192; Fig. 5*A4*). As shown in Fig. 5*A* (*Lower*), probes located near either end of the fragment detect large nascent strands. In contrast, the probe located near the central portion of the fragment detects both small and large nascent strands, indicating that the probe is very close to the initiation site.

In a second set of experiments, several papillomas from a second patient were pooled; DNA was isolated and digested with *Xmn* I. After N/A 2D gel electrophoresis, we again used the 135-bp URR probe and two additional probes flanking the URR. This experiment shows that the 135-bp URR probe detects nascent strands as small as 300 nt, but probes flanking the URR detect increasingly longer nascent strands. Taken

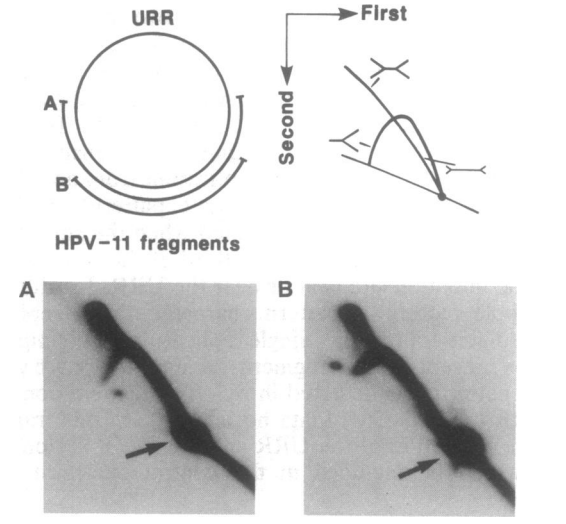

FIG. 3. Termination of DNA replication in HPV-11 occurs ≈180° from the URR. (*Upper left*) Positions of the 4594-bp *Nci* I/*Xba* I segment (A, nt 1291–5885), and the 2323-bp *Xmn* I segment (B, nt 2665–4988). (*Upper right*) Schematic indicating migration of molecules containing two opposing replication forks that converge in the center of the fragment (double Y pattern). (*Lower*) 2D agarose gel analysis of segments A and B. Arrows indicate positions of nonreplicating linear segments. Hybridizations were performed with a probe (HPV-11, nt 2665–4988) containing sequences located 180° from the URR.

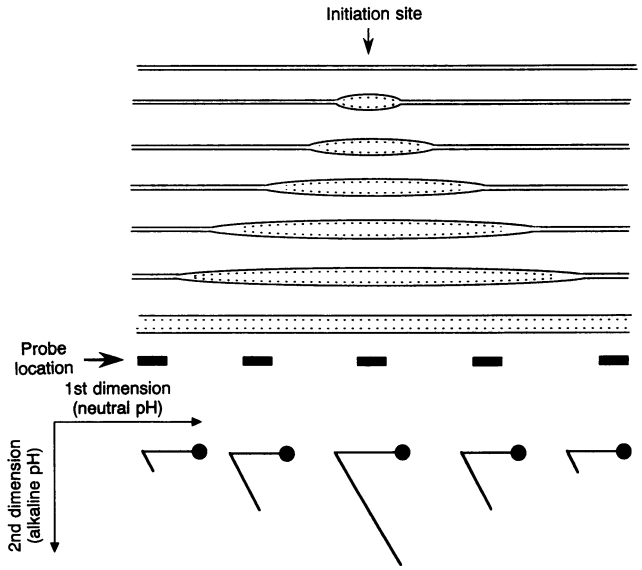

FIG. 4. Schematic illustrating N/A 2D gel analysis using probes at multiple locations within a fragment. (*Upper*) Replication intermediates resulting from site-specific replication initiation and bidirectional fork movement. Nascent strands are shown as dotted lines, parental strands are shown as solid lines. Location of five probes distributed across a particular fragment is shown below the replicative intermediates. (*Lower*) Five schematic diagrams of a N/A gel hybridized with each of the five probes sequentially. In each diagram, the round spot represents nonreplicating linear segments, horizontal line represents parental strands (which are all of the same mass), and diagonal line represents nascent strands (which vary in size depending on the location of the probe with respect to the initiation site). The closer a probe is to the origin, the smaller the nascent strands that will be detected.

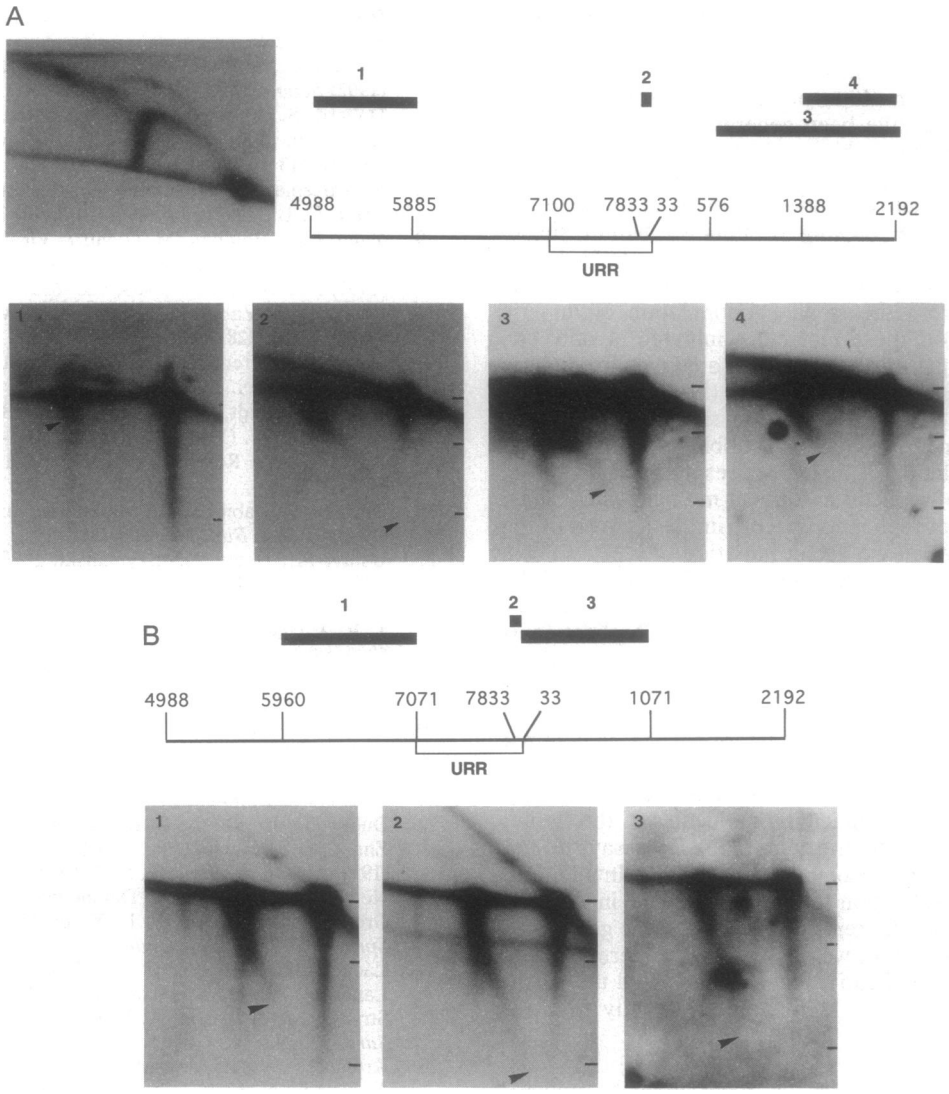

FIG. 5. Replication initiates in or very close to the URR and proceeds bidirectionally. (*A*) (*Upper right*) Map illustrating the *Xmn* I fragment analyzed (thin solid line) with relevant nucleotide markers. Open box below map shows location of the URR. Location of the four probes used in N/A 2D gel analyses is shown above the map (thick solid lines: probe 1, nt 4988–5885; probe 2, nt 7833–33; probe 3, nt 576–2192; probe 4, nt 1388–2192). (*Upper left*) N/N 2D gel shows evidence for initiation within the central one-third of this fragment. (*Lower*) Four separate hybridizations of the same Southern transfer of a N/A 2D gel using four probes at various locations within the fragment. (*B*) (*Upper*) Map identical to that shown in *A* except for the probes and their corresponding nucleotide numbers. (*Lower*) Results of three separate hybridizations of the same Southern transfer of a N/A 2D gel with the three probes noted above. Arrowheads in *A* and *B* indicate positions of the smallest nascent strands detected with each probe. Vertical spike below the spot of unreplicated segments results from nicks in the DNA prior to the second (alkaline) dimension. To the right of each autoradiograph are horizontal lines representing size markers of 0.3, 1.3, and 5 kb from bottom to top.

together, these results suggest that initiation occurs at or near the 3′ portion of the URR and that replication forks proceed bidirectionally.

DISCUSSION

In this study, we have analyzed replicating HPV-11 molecules in DNA isolated from laryngeal papillomas. We demonstrate that replication bubbles originate from the URR and that forks proceed bidirectionally from this initiation site and converge in a region 180° from the URR. In this analysis, the probe that best defines the initiation site of HPV-11 DNA replication spans nt 7833–33. The ARS assays (19, 20) have thus proven to be a good indicator for the location of the replication initiation site of HPV-11 DNA in papillomas. This report examines viral replicative intermediates isolated directly from tissue by using 2D gels. Although BPV replicative intermediates have been studied in cultured cells, this appears to represent latent replication, whose mechanism may differ from BPV replication that occurs in warts. It is not known whether the replication of the BPV genome in papillomas proceeds by the same mechanism observed *in vitro* (10) or in ARS assays (17, 18). However, this study indicates that the site of replication initiation for HPV-11 in lesions is similar to the replication origin detected in ARS assays.

Although a majority of HPV genomes appear to replicate *in vivo* by the mechanism described above, our analysis indicates that alternative modes of replication may exist. First, we detected patterns for molecules containing a single fork initiated from an external origin superimposed on the replication initiation and termination patterns (Figs. 2, 3, and 5). The replicative intermediates containing single forks with a mass greater than half replicated molecules probably originate because initiation is not perfectly symmetrical within the fragment (i.e., one fork from a bubble-containing molecule migrates off one end of the segment before the other fork). Some of these molecules may result from forks proceeding bidirectionally at different rates from symmetrically

placed origins. In addition to the relatively strong signal from molecules containing a single fork of large mass, it is clear that some replicative intermediates span the entire spectrum of a simple Y pattern. It is possible that some of these intermediates could have been generated by low levels of rolling circle replication. Alternatively, some of the molecules that contain a single fork may have initiated at other origins of bidirectional replication, perhaps at multiple origins. In addition, the nicking of bubbles within single-stranded regions of replication forks can generate Y-shaped molecules. Finally, intermediates containing single forks may have originated from the small subpopulation of dimeric molecules (Fig. 1) if these molecules initiated at only one copy of the origin per molecule, as was observed for dimeric and higher oligomeric forms of BPV-1 (8). Second, a triangular pattern that emanates from the simple fork arc was observed for segments containing bubble patterns (Figs. 2 and 5). This population of molecules results primarily from the convergence of replication forks at multiple sites. Thus, some termination of replication occurs in the segment of the HPV genome in which we detect initiation.

In the present study, we demonstrate by N/N and N/A 2D gel electrophoresis that replication initiates within or very close to the URR of the HPV-11 genome. Replication is bidirectional and usually terminates 180° opposite the origin. The 2D gel approach has several advantages over other methods. For example, this method examines steady state levels of the sum of all replicative intermediates, in contrast to techniques using electron microscopy, where conclusions about the mechanism of replication are based on a relatively small percentage of the replicating molecules in the tissue. Also, as pointed out in the Introduction, ARS assays do not necessarily reflect chromosomal origin activity. In addition, it is possible that replication of the viral genome in cultured cells is different from the replication of the same genome in tissues. The approach that we have described permits characterization of viral replicative intermediates and the mechanism of replication of the HPV genome directly from infected tissue.

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1. Pfister, H., ed. (1990) *Papillomaviruses and Human Cancer* (CRC, Boca Raton, FL).
2. Gissmann, L., Wolnik, L., Ikenberg, H., Koldovsky, U., Schnurch, H. G. & zur Hausen, H. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 560–563.
3. Mungal, S., Steinberg, B. M. & Taichman, L. B. (1992) *J. Virol.* **66**, 3220–3224.
4. McCance, D. J. (1986) *Biochim. Biophys. Acta* **823**, 195–205.
5. Bedell, M. A., Hudson, J. B., Golub, T. R., Turyk, M. E., Hosken, M., Wilbanks, G. D. & Laimins, L. A. (1991) *J. Virol.* **65**, 2254–2260.
6. Meyers, C., Frattini, M. G., Hudson, J. B. & Laimins, L. A. (1992) *Science* **257**, 971–973.
7. Dollard, S. C., Wilson, J. L., Demeter, L. M., Bonnez, W., Reichman, R. C., Broker, T. R. & Chow, L. T. (1992) *Genes Dev.* **6**, 1131–1142.
8. Schvartzman, J. B., Adolph, S., Martin-Parras, L. & Schildkraut, C. L. (1990) *Mol. Cell. Biol.* **10**, 3078–3086.
9. Yang, L. & Botchan, M. (1990) *J. Virol.* **64**, 5903–5911.
10. Yang, L., Li, R., Mohr, I. J., Clark, R. & Botchan, M. R. (1991) *Nature (London)* **353**, 628–632.
11. Dasgupta, S., Zabielski, J., Simonsson, M. & Burnett, S. (1992) *J. Mol. Biol.* **228**, 1–6.
12. Yates, J., Warren, N., Reisman, D. & Sugden, B. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 3806–3810.
13. Hammerschmidt, W. & Sugden, B. (1988) *Cell* **55**, 427–433.
14. Lambert, P. F. (1991) *J. Virol.* **65**, 3417–3420.
15. Waldeck, W., Rosl, F. & Zentgraf, H. (1984) *EMBO J.* **3**, 2173–2178.
16. Burnett, S., Zabielski, J., Moreno-Lopez, J. & Pettersson, U. (1989) *J. Mol. Biol.* **206**, 239–244.
17. Ustav, M., Ustav, E., Szymanski, P. & Stenlund, A. (1991) *EMBO J.* **10**, 4321–4329.
18. Chiang, C. M., Ustav, M., Stenlund, A., Ho, T. F., Broker, T. R. & Chow, L. T. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 5799–5803.
19. Chiang, C. M., Dong, G., Broker, T. R. & Chow, L. T. (1992) *J. Virol.* **66**, 5224–5231.
20. Lu, J. Z.-J., Sun, Y.-N., Rose, R. C., Bonnez, W. & McCance, D. J. (1993) *J. Virol.* **67**, 7131–7139.
21. Umek, R. M., Linskens, M. H., Kowalski, D. & Huberman, J. A. (1989) *Biochim. Biophys. Acta* **1007**, 1–14.
22. Dubey, D. D., Davis, L. R., Greenfeder, S. A., Ong, L. Y., Zhu, J. G., Broach, J. R., Newlon, C. S. & Huberman, J. A. (1991) *Mol. Cell. Biol.* **11**, 5346–5355.
23. Newlon, C. S., Collins, I., Dershowitz, A., Deshpande, A. M., Greenfeder, S. A., Ong, L. Y. & Theis, J. F. (1993) *Cold Spring Harbor Symp. Quant. Biol.* **58**, 415–423.
24. Lack, E. E., Vawter, G. F., Smith, H. G., Healy, G. B., Lancaster, W. D. & Jenson, A. B. (1980) *Lancet* **ii**, 592.
25. Strauss, M. & Jenson, A. B. (1985) *Otolaryngol. Head Neck Surg.* **93**, 342–346.
26. Kreider, J. W., Howett, M. K., Stoler, M. H., Leure-Dupree, A. E., Lill, N. L. & Zaino, R. J. (1987) in *Cancer Cells 5*, eds. Steinberg, B. M., Brandsma, J. L. & Taichman, L. B. (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 215–221.
27. Stoler, M. H. & Broker, T. R. (1986) *Hum. Pathol.* **17**, 1250–1258.
28. Dartmann, K., Schwarz, E., Gissmann, L. & zur Hausen, H. (1986) *Virology* **151**, 124–130.
29. Krieg, P., Amtmann, E. & Sauer, G. (1983) *Anal. Biochem.* **134**, 288–294.
30. Gamper, H., Lehman, N., Piette, J. & Hearst, J. E. (1985) *DNA* **4**, 157–164.
31. Brewer, B. J. & Fangman, W. L. (1987) *Cell* **51**, 463–471.
32. Nawotka, K. A. & Huberman, J. A. (1988) *Mol. Cell. Biol.* **8**, 1408–1413.
33. Linskens, M. H. & Huberman, J. A. (1990) *Nucleic Acids Res.* **18**, 647–652.