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Evidence that Replication Initiates at Only Some of the Potential Origins in Each Oligomeric Form of Bovine Papillomavirus Type 1 DNA

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In a subclone of ID13 mouse fibroblasts latently infected with bovine papillomavirus type 1 (BPV-1) DNA, the viral genome occurred as a mixture of extrachromosomal circular monomers and oligomers. Multiple copies were also associated with the host cell genome, predominantly at a single site in a head-to-tail tandem array. We examined the replicative intermediates of extrachromosomal forms of BPV-1 DNA by using two-dimensional gel electrophoresis. The results obtained indicate that initiation of DNA replication occurred near the center of the *Eco*RI-*Bam*HI 5.6-kilobase fragment. In some molecules, however, this fragment was replicated from one end to the other by means of a single fork initiated elsewhere. Termination also occurred within this fragment. The *Eco*RI-*Bam*HI 2.3-kilobase fragment replicated as a DNA molecule containing a termination site for DNA replication and also by means of a single fork traversing the fragment from one end to the other. Thus, replication forks proceeded through these fragments in different manners, apparently depending on whether they were part of a monomer, a dimer, a trimer, or higher oligomers. These observations lead to the conclusion that initiation of DNA replication in BPV-1 DNA takes place at or close to plasmid maintenance sequence 1. From this point, replication proceeds bidirectionally and termination occurs approximately 180° opposite the origin. The results obtained are consistent with one or more replication origins being quiescent in BPV-1 DNA oligomers.

Viral genomes capable of being established as extrachromosomal elements are useful model systems to study the regulation of DNA replication in eucaryotic cells. Epstein-Barr virus (EBV) and bovine papillomavirus (BPV) are two such systems currently under investigation (for a review, see references 8, 19, and 26).

Papillomaviruses are widespread in nature. They are highly host specific and propagate primarily in the terminally differentiated epidermal cells of the warts they produce. A subset of the BPVs, among which the fully sequenced BVP-1 DNA is the most thoroughly studied (9), can readily transform established rodent fibroblast cell lines in culture (for a review, see reference 12). These transformed cell lines have been reported to carry multiple copies of circular extrachromosomal genomes of BPV-1; however, they produce no infectious virus (20, 21). This nonproductive, latent infection is characterized by the expression of viral genes encoded by early open reading frames but not those encoded by the late open reading frames. The late open reading frames are expressed during the lytic phase of BPV-1 infection (for a review, see references 19 and 26).

The ability to replicate autonomously as an extrachromosomal element is of particular interest for BPV-1 as a plasmid cloning vector (11, 31) and also as a model system to study the regulation of DNA replication in eucaryotic cells (8, 26). Examples of integrated viral DNA as well as the presence of extrachromosomal oligomeric copies have also been reported (2, 4, 15, 32, 39).

Genetic analysis led to the identification of two *cis*-acting sequences in the BPV-1 genome that, together with another *trans*-acting sequence, appear to allow the viral DNA to be maintained as an extrachromosomal element (23). These *cis*-acting sequences were designated plasmid maintenance sequences 1 and 2 (PMS-1 and PMS-2, respectively). PMS-1 was mapped to a 521-base-pair segment between positions 6945 and 7466 of the BPV-1 DNA genetic map (9). PMS-2 was mapped to a 140-base-pair segment between nucleotides 1514 and 1654 within the E1 open reading frame. The exact function of these sequences is still a matter of speculation. However, the location of PMS-1 is in good agreement with the site where DNA replication is initiated (position 6940 [±5%]), as determined by electron microscopy of replicative intermediates (37).

Recently, two different two-dimensional (2D) gel electrophoresis procedures have been developed, allowing the precise location of replication origins and termini as well as identification of the direction of fork progression along a DNA fragment (5, 18). These techniques have been successfully used to characterize the replication of the yeast 2μm plasmid (5, 18), to identify a replication fork barrier in the yeast chromosomal rDNA (6, 22), and to show that the EBV plasmid maintenance sequence *oriP* contains both the initiation and termination sites of DNA replication (14).

As a first step in an attempt to use BPV-1 as a model system to study the regulation of DNA replication in higher eucaryotes, we analyzed the replication of extrachromosomal forms of BPV-1 DNA in latently infected ID13 cells by means of 2D gel electrophoresis. Our main objectives in the studies reported here were as follows: (i) to determine whether the extrachromosomal circular forms of BPV-1 DNA occur as monomeric elements or as a series of oligomers, (ii) to identify the sites at which initiation and termination of DNA replication take place, (iii) to determine whether replication progresses in a uni- or bidirectional manner, and (iv) to locate potential replication fork barriers or pausing sites.

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MATERIALS AND METHODS

Cell culture and probes. Cell line ID13 (provided by P. Howley) derives from mouse C127 cells transformed by wild-type BPV (21). Cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin (GIBCO) per ml. Plasmid pML2-BPV1 (provided by P. Howley) contains the complete BPV-1 genome inserted into the *Bam*HI site of pML2, a derivative of pBR322 (for construction, see reference 30).

DNA isolation and restriction enzyme digestions. Extrachromosomal and genomic enriched DNAs were obtained from exponentially growing cells by the Hirt method (16). Cells were lysed in 0.01 M Tris (pH 9.0)–0.01 M EDTA–0.1% sodium dodecyl sulfate (SDS) at approximately 5.7×10^6 cells per ml at room temperature for 5 to 10 min, brought to 1 M with 5 M NaCl, and stored overnight on ice. Lysates were centrifuged at 12,000 rpm in an SW28 rotor at 4°C for 3 h. The pellet was dissolved in TE (0.01 M Tris [pH 9.0], 0.001 M EDTA), and both the Hirt pellet and supernatant fractions were separately incubated with 100 μg of proteinase K (Boehringer Mannheim Biochemicals) per ml at 37°C for 30 min. From this point on, the Hirt pellet and supernatant lysates were treated separately as follows. Lysates were extracted with an equal volume of NET (0.05 M Tris [pH 9.0], 0.15 M sodium chloride, 0.015 M EDTA)-saturated phenol-chloroform-isoamyl alcohol (PCI) (25:24:1), brought to 1 M with 5 M ammonium acetate, and precipitated with 2 volumes of 95% ethanol. Precipitates were centrifuged at 15,000 rpm in an SW28 rotor at 4°C for 1.5 h, lyophilized, and dissolved in 0.1× NET. These solutions were incubated with 100 μg of ribonuclease A per ml and 100 U of RNase T1 (Worthington Biochemical Corp.) per ml at 37°C for 30 min, extracted with PCI followed by chloroform-isoamyl alcohol (24:1), precipitated with 95% ethanol, centrifuged, and finally dissolved in 0.1× TE. Digestion with restriction enzymes (New England BioLabs, Inc.) was carried out as recommended by the manufacturer.

One-dimensional gel electrophoresis. DNA samples were loaded into the wells of standard horizontal submerged 0.4% agarose gels in TAE (0.04 M Tris-acetate, 0.002 M EDTA) containing 0.1 μg of ethidium bromide per ml. Electrophoresis was carried out at 0.2 V/cm for 72 h at room temperature.

2D gel electrophoresis. The first dimension was run in a 0.4% agarose gel in TBE (0.089 M Tris-borate, 0.002 M EDTA) at 1 V/cm for 16 to 20 h at room temperature. Following electrophoresis, the gel was stained with 0.3 μg of ethidium bromide per ml, and only the lane containing the lambda DNA-*Hin*dIII marker sizes was exposed to UV light and photographed. The second dimension was in a 1% agarose gel in TBE containing 0.3 μg of ethidium bromide per ml, run at a 90° angle with respect to the first dimension. The dissolved agarose was poured around the excised lane from the first dimension, and electrophoresis was run at 5 V/cm in a 4°C cold room with circulating buffer.

Southern transfer and hybridization. Gels transferred to Zetaprobe membranes (Bio-Rad Laboratories) were washed twice for 7 min in 0.05 M HCl, soaked for 15 min in 0.4 M NaOH, and transferred to Zetaprobe membranes in 0.4 M NaOH for 18 to 24 h at room temperature. Gels transferred to Optibind-supported nitrocellulose membranes (Schleicher & Schuell, Inc.) were first washed twice for 15 min in 0.05 M HCl and then twice for 15 min in 0.5 M NaOH containing 1.0 M NaCl, followed by another 60-min wash in 1.0 M Tris [pH 8.0]–1.5 M NaCl. The DNA was transferred to Optibind-supported nitrocellulose membranes in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 18 to 24 h, and the membranes were baked in a vacuum oven at 80°C for 2 h. Zetaprobe as well as Optibind nitrocellulose membranes were prehybridized in 50% formamide–5× SSC–5× Denhardt solution (100× Denhardt contains 2% bovine serum albumin, 2% Ficoll [Pharmacia Fine Chemicals], 2% polyvinylpyrrolidone)–0.1% SDS–250 μg of sonicated salmon testes DNA per ml at 37°C for 4 to 18 h. Hybridization was carried out in 50% formamide–5× SSC–5× Denhardt solution–250 μg of sonicated salmon testes DNA per ml–10% dextran sulfate, with 2×10^7 cpm of [^{32}P]dCTP-probe labeled by nick translation, at 37°C for 24 to 48 h. After hybridization, the membranes were washed twice for 15 min in 2× SSC –0.1% SDS at room temperature and then two to four times for 15 to 20 min each time in 0.1× SSC–0.1% SDS at 55°C. Exposure of XAR-5 film (Eastman Kodak Co.) was carried out with 1 or 2 intensifying screens for 3 to 14 days at −70°C.

In situ hybridization. Logarithmically growing cells were incubated with 20 μg of 5-bromodeoxyuridine (BrdUrd) per ml for 7 h before harvesting. Chromosome spreads and in situ hybridization were carried out as described by Adolph et al. (1) with ^{3}H-labeled pML2-BPV1. Slides containing hybridized DNA were exposed for at least 2 weeks. Either before or after in situ hybridization, ID13 chromosomes in the slides were stained with 50 μg of acridine orange per ml for 5 min and photographs were taken. Then chromosomes were destained in methanol-acetic acid (3:1), restained with Giemsa, and rephotographed.

RESULTS

BPV-1 DNA sequences were present in both the Hirt pellet and supernatant fractions of ID13 cells. In order to determine the physical state of BPV-1 DNA in ID13 cells, extrachromosomal DNA as well as samples enriched with genomic DNA were obtained by the method of Hirt (16). DNA samples were digested with *Eco*RI and analyzed by electrophoresis in a 0.4% agarose gel run at 0.2 V/cm for 72 h at room temperature. Very large DNA molecules entered the gel and were readily separated according to size (13). The gel was photographed, transferred to nitrocellulose, and hybridized with labeled pML2-BPV1. The resulting autoradiogram is displayed in Fig. 1. A prominent signal of DNA molecules with an average size above 50 kilobases (kb) was detected in the undigested Hirt pellet DNA. This high-molecular-weight species was completely eliminated by digesting the Hirt pellet DNA with *Eco*RI (lanes 2 to 4). Two new signals appeared in these *Eco*RI-digested samples, a very prominent one migrating as a linear DNA molecule of approximately 8 kb (the size of a linear BPV-1 DNA molecule) and another weaker signal corresponding to a size of about 6.3 kb.

In contrast, the uncut Hirt supernatant DNA (Fig. 1, lane 5) revealed a series of discrete bands. These bands, detected in the uncut sample, disappeared after digestion with *Eco*RI (lanes 6 to 8), but only one new signal could be detected corresponding to a linear DNA molecule of about 8 kb.

The results of this experiment suggest that BPV-1 DNA existed in extrachromosomal and integrated forms in ID13 cells. The observation that undigested DNA in the Hirt pellet contained BPV-1 DNA that migrated predominantly in the high-molecular-weight region of the gel suggests that viral DNA copies might be integrated into the chromosomes of the host cell. The fact that *Eco*RI digestion converted this

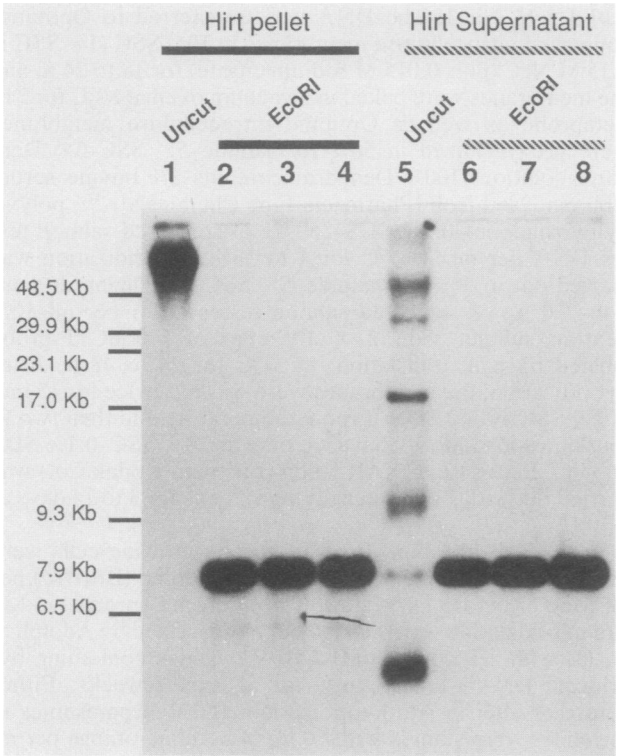

FIG. 1. Hirt pellet of ID13 cells was enriched in high-molecular-weight BPV-1 DNA which was probably integrated into the chromosomes of the host cell. The Hirt supernatant fraction contained extrachromosomal monomeric and oligomeric circular forms of BPV-1 DNA. Undigested (lanes 1 and 5), *Eco*RI fully digested (lanes 2 to 4 and 6 to 8), ID13 Hirt pellet (lanes 1 to 4), and supernatant (lanes 5 to 8) DNAs were analyzed by electrophoresis in a 0.4% agarose gel that was run at 0.2 V/cm at room temperature for approximately 72 h. After electrophoresis, the gel was transferred to nitrocellulose and hybridized with labeled pML2-BPV1. The sizes of the marker DNAs (a mixture of undigested, *Kpn*I-, and *Hin*dIII-digested lambda DNAs) are shown on the left side of the autoradiogram.

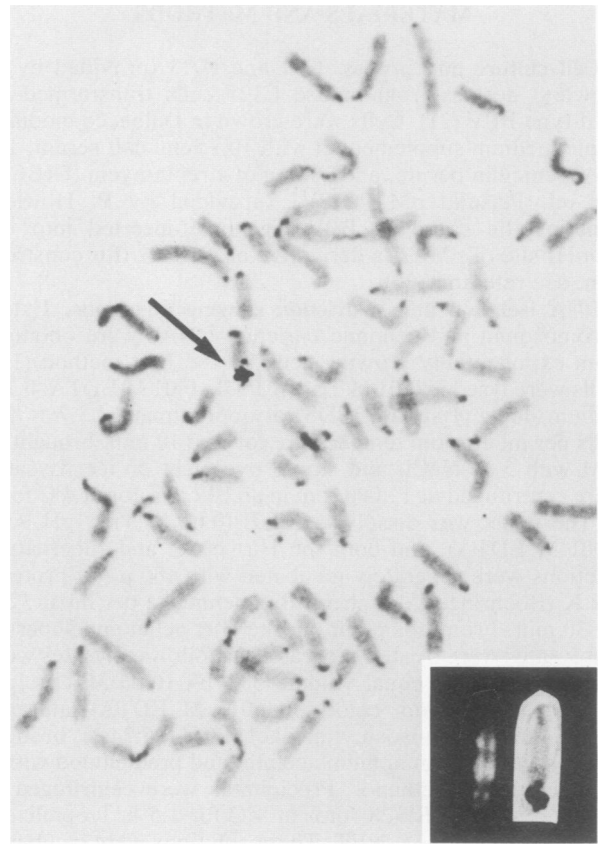

FIG. 2. BPV-1 DNA was associated primarily with a single chromosomal site of the host cell, probably as a result of integration. In situ hybridization with ^{3}H-labeled pML2-BPV1 of ID13 metaphase chromosomes stained with Giemsa after a 7-h treatment with BrdUrd before fixation. The arrow points to the single concentration of silver grains over the distal segment of chromosome 14. The insert shows chromosome 14 stained with acridine orange before (left) and after (right) autoradiography.

high-molecular-weight species into a very prominent linear DNA of about 8 kb suggests that the high-molecular-weight DNA observed in Fig. 1, lane 1, was probably formed by multiple copies of BPV-1 DNA organized in a head-to-tail tandem array. The observation that uncut Hirt supernatant was enriched in BPV-1 DNA migrating as a series of discrete-sized species and the fact that *Eco*RI digestion converted these species into a single band of about 8 kb strongly suggest that the Hirt supernatant of ID13 cells was enriched in extrachromosomal circular monomers and oligomeric forms of BPV-1 DNA in which the units were also organized in a head-to-tail configuration.

Multiple copies of BPV-1 DNA were associated with the chromosomes of this ID13 cell line, predominantly at a single site in a head-to-tail tandem array. Viral DNA can integrate into the chromosomes of the host cell in the following different forms: as interspersed monomers, as multiple copies integrated at a single site in a tandem array (which in turn can be organized in a head-to-tail configuration, a head-to-head configuration, or a combination of both), and also as multiple copies integrated at several different loci. In an attempt to determine whether or not multiple copies of BPV-1 DNA were associated with the chromosomes of ID13 cells, three different experiments were performed. In situ hybridization was used to determine whether BPV-1 DNA was present in a dispersed manner or was concentrated at a single or a few sites. Of the ID13 interphase nuclei that were scored, 80% showed a single very prominent concentration of silver grains (data not shown). When mitotic cells were studied, it was found that this concentration of silver grains specifically occurred over the distal segment of one chromosome 14 in the mouse complement. This was confirmed by BrdUrd banding and subsequent in situ hybridization (Fig. 2). This cytogenetic evidence indicates that multiple copies of BPV-1 DNA were associated with a single chromosomal site in ID13 cells, probably due to integration.

If multiple copies of BPV-1 DNA occurred at a single site in a head-to-tail tandem array, a partial digestion with a restriction enzyme that cuts the BPV-1 genome only once would generate a series of linear monomers, dimers, trimers, and higher oligomers. A series of *Bam*HI partial digests of ID13 Hirt pellet DNA was analyzed by electrophoresis in a 0.4% agarose gel. A ladder of BPV-1 linear DNA molecules of up to approximately 40 kb (equivalent to pentamers) could be clearly identified (Fig. 3A). This experiment showed that the Hirt pellet of ID13 cells was enriched in multiple copies of BPV-1 DNA organized in a head-to-tail tandem array. If these multiple copies were integrated at a unique site in the chromosomes of ID13 cells, complete digestions with different restriction enzymes that are known to cut BPV-1

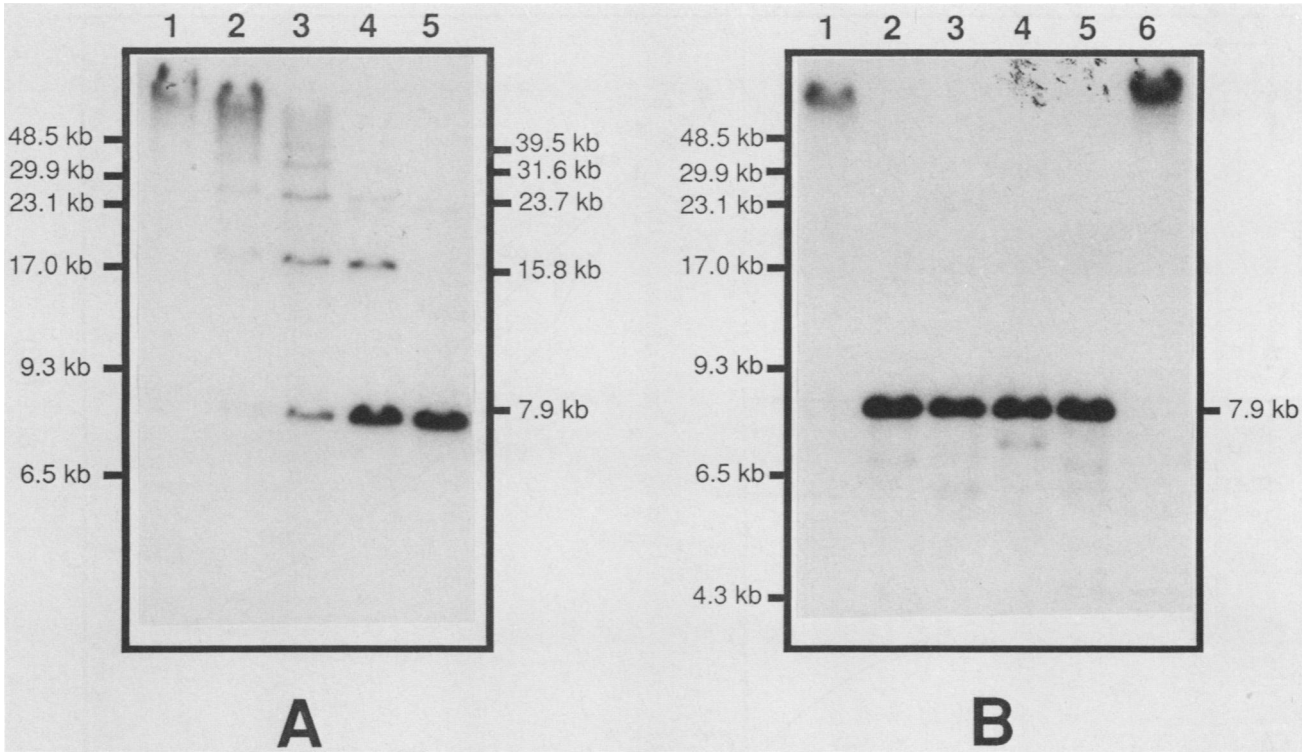

FIG. 3. Multiple copies of BPV-1 DNA were likely to be integrated into the chromosomes of ID13 cells, predominantly in a head-to-tail tandem array. (A) Partial digestion of ID13 Hirt pellet DNA with *Bam*HI revealed a ladder of bands (lane 3), the size of which corresponds to BPV-1 DNA multimers. ID13 Hirt pellet DNA was analyzed by electrophoresis in a 0.4% agarose gel undigested (lane 1) or after a 1-h digestion with 0.06 (lane 2), 0.12 (lane 3), 0.25 (lane 4), and 0.5 (lane 5) U of *Bam*HI per μg at 37°C. The sizes of the marker DNAs (a mixture of undigested, *Kpn*I-, and *Hin*dIII-digested lambda DNAs) are shown on the left side of the autoradiogram, while the position expected for linear BPV-1 DNA multimers is indicated at the right side. (B) Complete digestion of ID13 Hirt pellet DNA with different restriction enzymes that cut BPV-1 DNA only once revealed the appearance of one prominent band of about 8 kb, the size of a linear BPV-1 DNA unit, and other weaker bands of variable sizes. ID13 Hirt pellet DNA was analyzed by electrophoresis in a 0.4% agarose gel undigested (lanes 1 and 6) or after an overnight digestion with *Eco*RI (lane 2), *Bam*HI (lane 3), *Hin*dIII (lane 4), or *Kpn*I (lane 5) at 37°C. The sizes of the marker DNAs (a mixture of undigested, *Kpn*I-, and *Hin*dIII-digested lambda DNAs) are shown on the left side of the autoradiogram. In both cases (A and B), electrophoresis was run at 0.2 V/cm at room temperature for approximately 72 h. After electrophoresis, the gels were transferred to nitrocellulose and hybridized with labeled pML2-BPV1.

DNA only once would produce single prominent bands of about 8 kb, the size of a complete BPV-1 unit and two weaker signals of variable size corresponding to the flanking segments. ID13 Hirt pellet DNA, fully digested with *Eco*RI, *Bam*HI, *Hin*dIII, and *Kpn*I, generated one prominent band corresponding to a linear DNA molecule of about 8 kb and other weaker bands (Fig. 3B). Longer exposures of this film revealed several less prominent bands of different intensities that varied in size, depending on the restriction enzyme used (data not shown). These weaker signals, detected only in longer exposures, could be due to integration at additional sites in the same cells, to subpopulations of cells in which integration occurred at sites different from that identified in Fig. 2, to occasional deletions and/or rearrangements within the BPV-1 DNA repeat, or to infrequent head-to-tail configurations. These experiments suggest that most of the BPV-1 genomes are integrated into the chromosomal DNA of ID13 cells at a single site in a head-to-tail tandem array. They also suggest that a smaller number of additional copies might be integrated at different sites.

Hirt supernatant DNA of ID13 cells was enriched in extrachromosomal circular monomers and oligomeric forms of BPV-1 DNA. In order to better characterize the nature of the series of BPV-1 discrete-sized DNA species detected in the undigested ID13 Hirt supernatant (Fig. 1, lane 5), a portion of this undigested DNA was analyzed by electrophoresis in a 2D agarose gel system (5, 7). Briefly, the first dimension of this 2D gel system was run under conditions that separated molecules predominantly according to their mass, minimizing the effect of their shape. The second dimension maximized the effect of retardation by molecular shape. This method allows identification of linear DNA molecules, covalently closed circles, open circles, and different forms of catenanes (5, 7). The autoradiogram of this experiment is shown in Fig. 4A. Comparison of the pattern obtained in this film with those obtained with the yeast 2μm plasmid (5, 7), led us to conclude that the species detected above the arc of linear molecules in Fig. 4A were covalently closed and open circle forms of different sizes.

The distance migrated by a series of supercoiled oligomers in a standard agarose gel is inversely related to the logarithm of the molecular weight of each of the members of the series (24). Figure 4C shows a comparison of the electrophoretic mobility in the first dimension of the 2D gel system shown in Fig. 4A and the sizes of monomeric and oligomeric forms of BPV-1 DNA. This graph was made by assuming that the fastest-migrating species corresponded to monomeric, covalently closed circles of BPV-1 DNA. These observations, together with the results shown in Fig. 1, strongly suggest that in ID13 cells, extrachromosomal BPV-1 DNA occurred

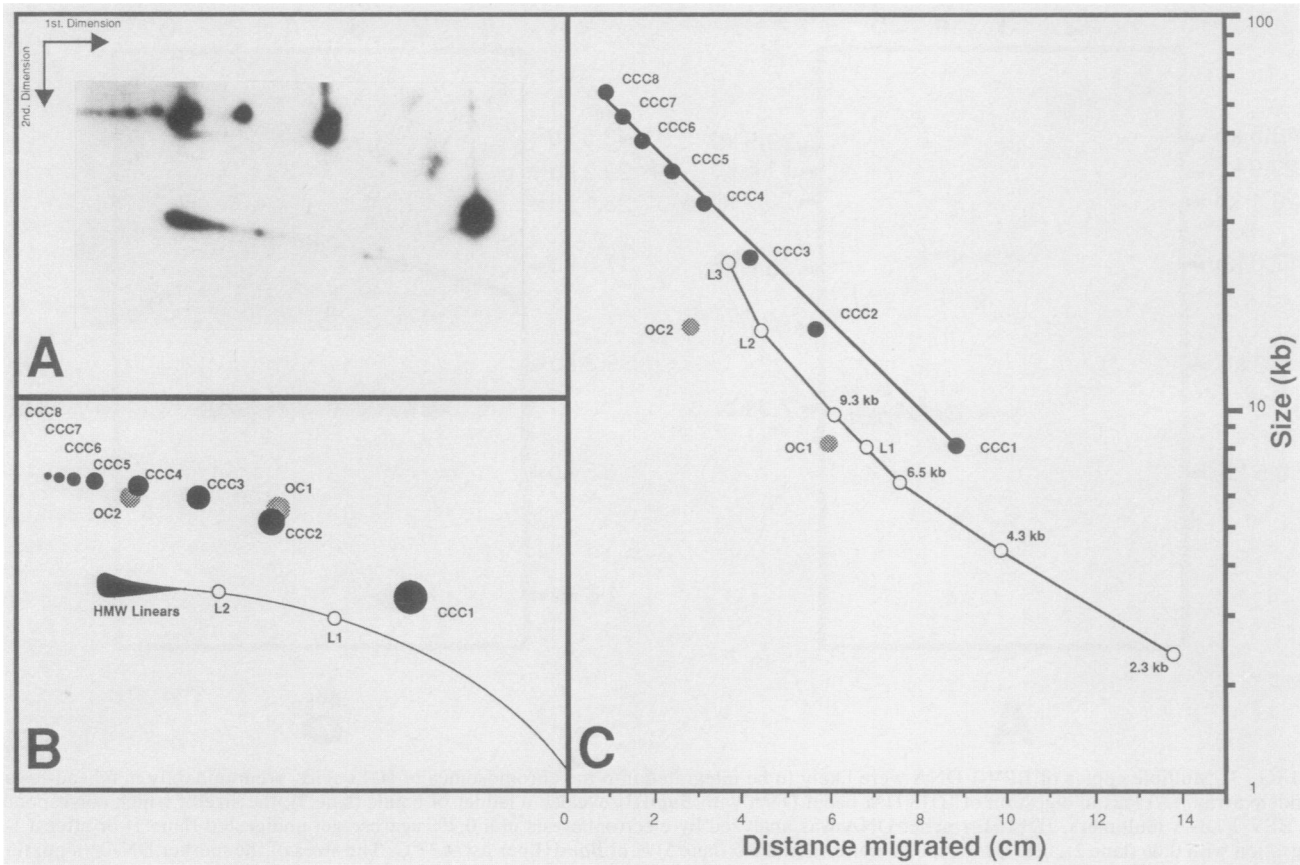

FIG. 4. Hirt supernatant DNA of ID13 cells was enriched in extrachromosomal circular monomers and oligomers of BPV-1 DNA. Undigested ID13 Hirt supernatant DNA was analyzed by 2D gel electrophoresis. After electrophoresis, the gel was transferred to a Zetaprobe membrane and hybridized with labeled pML2-BPV1 DNA. (A) Autoradiogram of the filter; (B) interpretation of the autoradiogram; (C) comparison of the mobility in the first dimension of the 2D gel shown in panel A of the different species observed, with the sizes of the BPV-1 DNA monomer and oligomers. Abbreviations: CCC, covalently closed circles; OC, open circles; L, linear forms. 1, 2, 3, etc. refer to monomers, dimers, trimers, and higher oligomers, respectively.

as a mixture of monomers and a series of oligomers in which the units were organized in a head-to-tail configuration.

The *Eco*RI-*Bam*HI 5.6-kb fragment of BPV-1 DNA replicated as a molecule containing an internal origin, by means of a single fork traversing the fragment, and also as a molecule containing a termination site for DNA replication. In order to determine the mode of replication of extrachromosomal forms of BPV-1 DNA in ID13 cells, a portion of the Hirt supernatant was double digested with *Eco*RI and *Bam*HI and analyzed by 2D gel electrophoresis. This double digestion generated two linear fragments of 5.6 and 2.3 kb. Despite the presence of some partially digested molecules, replicative intermediates of the 5.6-kb fragment were readily identified (Fig. 5). The maximally retarded arc arising from the 5.6-kb spot on the curve of linear molecules corresponds to the pattern expected for replicative intermediates whose migration in the second dimension is increasingly retarded as they increase in size. This arc did not return to the curve of linear molecules, and the signal ended beyond the site to which linear molecules of 11.2 kb migrated in the first dimension. This pattern is consistent with that expected for DNA molecules where initiation of DNA replication occurs at a site near the middle of the fragment (5).

The other arc arising from the 5.6-kb linear spot returned to the curve of linear molecules at 11.2 kb, the position at which 5.6-kb molecules that are almost completely replicated would be expected to migrate (Fig. 5). This pattern is consistent with that expected for linear DNA molecules in which a single replication fork progresses from one end to the other, generating a series of Y-shaped replicative intermediates.

A straight signal that started near the inflection point of the arc of Y-shaped replicative intermediates and extended diagonally upwards was observed (Fig. 5). This straight signal is consistent with the pattern expected for replicative intermediates generated by two converging replication forks approaching a termination site located within the fragment. Another straight signal with a size of 11.2 kb started at the arc of linear molecules and extended almost to the point where maximally retarded molecules containing a termination site migrated. This second pattern is consistent with that expected for a series of nonreplicative recombinants of 11.2 kb (3, 6). The vertical straight signal arising from the arc of linear molecules at 15.8 kb is likely to be due to replicative intermediates derived from partially digested BPV-1 DNA molecules. Resolution was significantly reduced in this high-molecular-weight portion of the gel.

The presence of these patterns demonstrated that some of the *Eco*RI-*Bam*HI 5.6-kb fragments of BPV-1 DNA were replicated from an origin located at or near the middle of the fragment. Other 5.6-kb fragments were replicated by a single fork that progressed from one end of the fragment to the

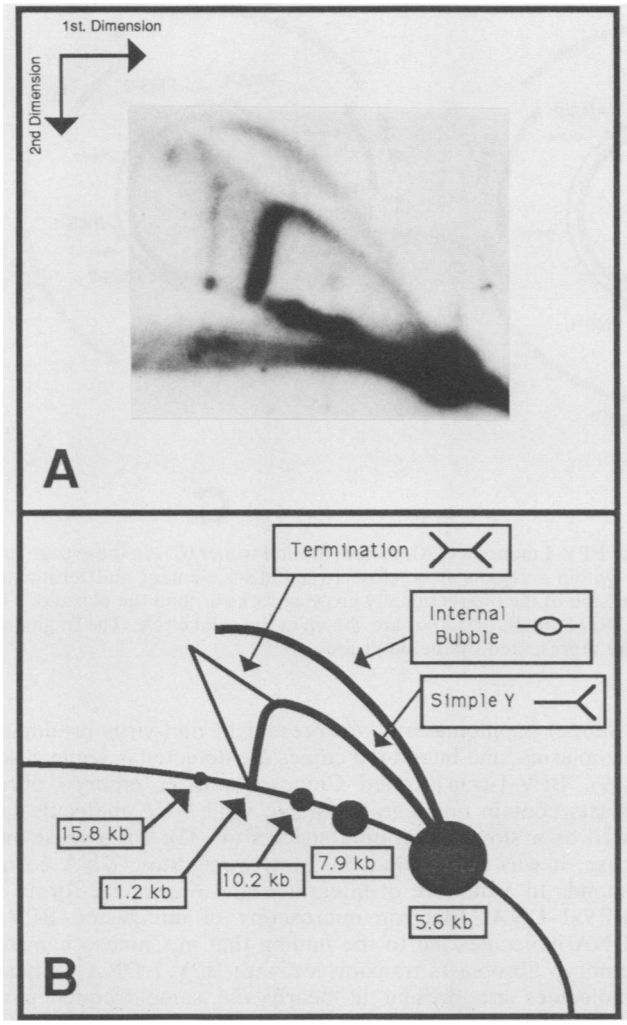

FIG. 5. Replicative intermediates of the BPV-1 DNA *Eco*RI-*Bam*HI 5.6-kb fragment as visualized after 2D gel electrophoresis. ID13 Hirt supernatant DNA was digested with a mixture of *Eco*RI and *Bam*HI and analyzed by 2D gel electrophoresis. After electrophoresis, the gel was transferred to a Zetaprobe membrane and hybridized with labeled pML2-BPV1 DNA. (A) Autoradiogram of the filter; (B) interpretation of the autoradiogram. Examples of the molecules that produce each pattern are shown in boxes.

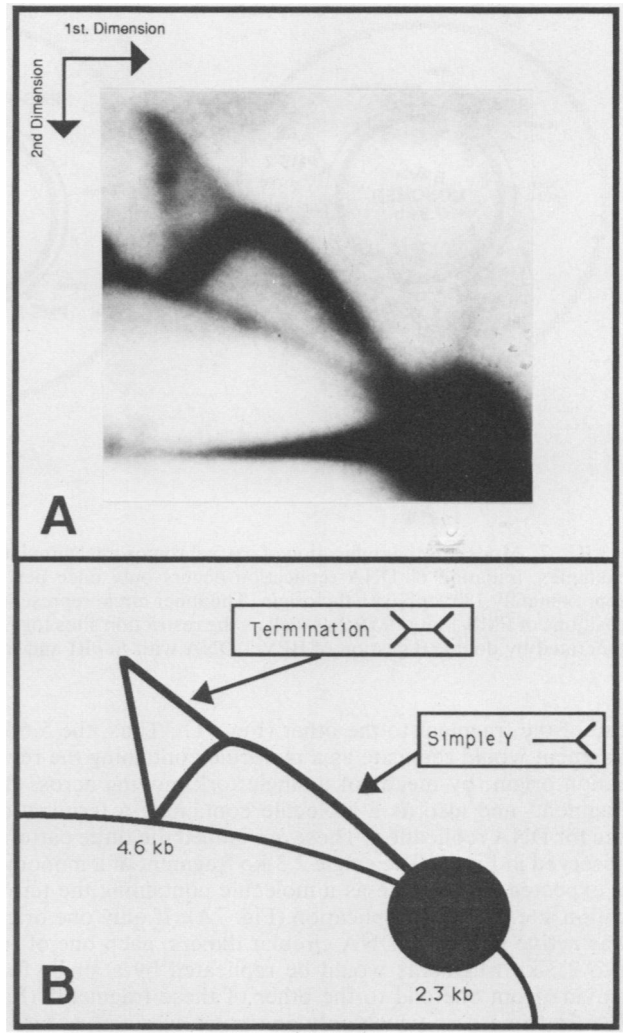

FIG. 6. Replicative intermediates of the BPV-1 DNA *Eco*RI-*Bam*HI 2.3-kb fragment as visualized after 2D gel electrophoresis. ID13 Hirt supernatant DNA was digested with a mixture of *Eco*RI and *Bam*HI and analyzed by 2D gel electrophoresis. After electrophoresis, the gel was transferred to a Zetaprobe membrane and hybridized with labeled pML2-BPV1 DNA. (A) Autoradiogram of the filter; (B) interpretation of the autoradiogram. Examples of the molecules that produce each pattern are shown in boxes.

other. Finally, some 5.6-kb fragments were replicated by two converging forks that met within this fragment.

The *Eco*RI-*Bam*HI 2.3-kb fragment of BPV-1 DNA was replicated by means of a single fork traversing the fragment and also as a molecule containing a termination site for DNA replication. Only two patterns of replicative intermediates derived from the 2.3-kb fragment (Fig. 6). An arc of simple Y-shaped forms started at the 2.3-kb spot on the curve of linear molecules and returned to this curve at 4.6 kb, the position at which 2.3-kb molecules that are almost completely replicated would be expected to migrate. A straight signal consistent with the pattern expected for replicative intermediates containing a termination site was also evident, together with another straight signal corresponding to nonreplicative recombinants. However, no signal indicating the presence of molecules containing a replication origin was detected. This experiment demonstrated that some of the 2.3-kb fragments generated by double digestion of BPV-1 DNA with *Eco*RI and *Bam*HI were replicated by a single fork traversing the fragment. Other fragments were replicated by two converging forks, indicating that termination of DNA replication also occurred within this fragment.

There is evidence suggesting that some potential replication origins are not functional in tandemly repeated DNA sequences (see Discussion). If only one origin was active in BPV-1 DNA circular dimers, termination would occur 180° opposite the functional origin, near the site where the nonfunctional origin is located (Fig. 7B). One of the patterns of replicative intermediates of the 5.6-kb fragment observed in Fig. 5 is consistent with molecules replicated by two converging forks initiated elsewhere. This observation indicates that in some BPV-1 DNA plasmids, termination occurred within this fragment. In trimeric forms, in which only one origin was functional, one of the three 5.6-kb fragments would replicate from an internal origin. Each of the other two would be replicated by a single fork moving from one

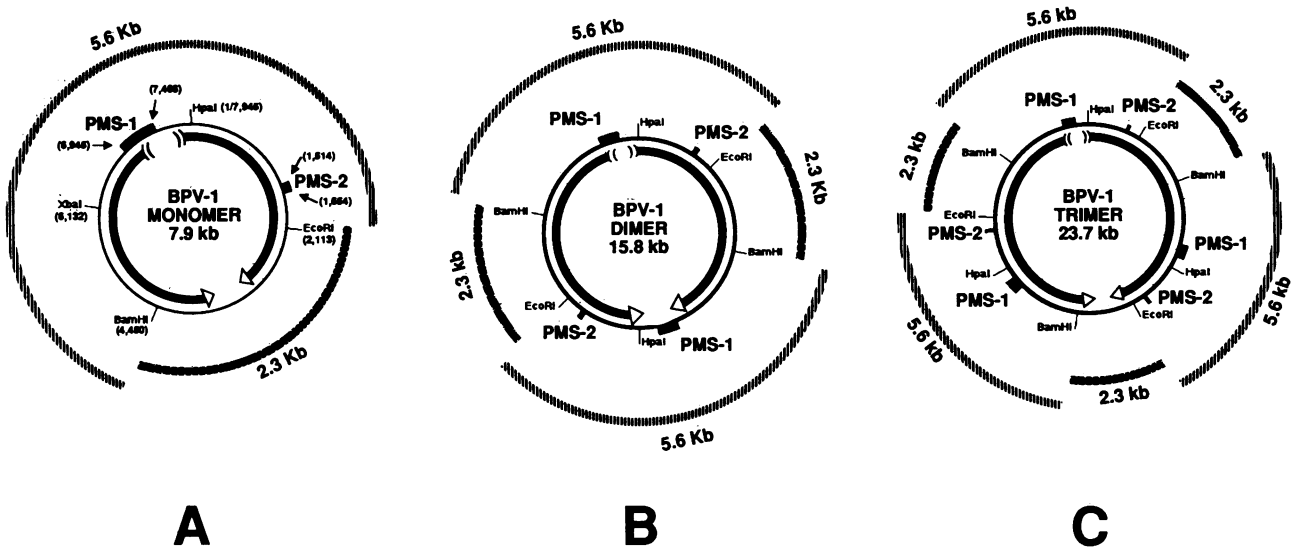

FIG. 7. Model for the replication of extrachromosomal circular forms of BPV-1 monomer (A), dimer (B), and trimer (C). In these particular examples, initiation of DNA replication occurs only once per plasmid within a region at or close to a PMS-1 element and terminates approximately 180° opposite the origin. The inner circle represents progression of the bidirectionally growing forks around the plasmid. The positions of PMS-1 and PMS-2 as well as the restriction sites for *Hpa*I, *Eco*RI, *Bam*HI, and *Xba*I are shown in the solid circle. The fragments generated by double digestion of BPV-1 DNA with *Eco*RI and *Bam*HI are represented by the outer arcs.

end of the fragment to the other (Fig. 7C). Thus, the 5.6-kb fragment would replicate as a molecule containing the replication origin, by means of a single fork moving across the fragment, and also as a molecule containing a termination site for DNA replication. These are indeed the three patterns observed in Fig. 5. The single 2.3-kb fragment of a monomer is expected to replicate as a molecule containing the termination site for DNA replication (Fig. 7A). If only one origin was active in BPV-1 DNA circular dimers, each one of the two 2.3-kb fragments would be replicated by a single fork moving from one end to the other of these fragments (Fig. 7B). In trimers, in which only one origin was active, one of the 2.3-kb fragments would replicate as a molecule containing the termination site for DNA replication while the other two would be replicated by a single fork moving from one end to the other (Fig. 7C). Thus, the 2.3-kb fragment would be replicated by a single fork traversing the fragment and also by two converging forks that meet at the termination site for DNA replication. Figure 6 shows that the *Eco*RI-*Bam*HI 2.3-kb fragment indeed replicated as a series of simple Y-shaped forms and also as a molecule containing the termination site for DNA replication. Thus, one or more replication origins remain quiescent in BPV-1 DNA oligomers.

DISCUSSION

Integrated copies and extrachromosomal circular oligomeric forms of BPV-1 DNA in ID13 cells. Transformation of mammalian cells by simian virus 40 (SV40), polyomavirus, adenoviruses, or retroviruses usually involves integration of viral DNA into the chromosomes of the host cell (for a review, see reference 17). Two reported exceptions are EBV and BPV-1, which are capable of being maintained as stable extrachromosomal elements in the nuclei of infected cells (for a review, see references 8, 19, and 26). In addition to extrachromosomal monomers, integrated genomes of some papillomaviruses and extrachromosomal oligomers have been reported. Variable-sized free episomes of the rabbit (Shope) papillomavirus are present in non-virus-producing neoplasms, and integrated copies are detected in some cases (39). BPV-1-transformed Chinese hamster embryo fibroblasts contain unintegrated single viral DNA molecules as well as a slowly migrating species (4, 32). In at least one case, it was shown that this slowly migrating DNA corresponds to a mixture of integrated and oligomeric forms of BPV-1 DNA. Electron microscopy of undigested BPV-1 DNA molecules led to the finding that in Chinese hamster embryo fibroblasts transformed with BPV-1 DNA, dimeric molecules are present at nearly the same frequency as monomeric forms (37). In several C127 mouse cell lines latently infected with BPV-1 DNA, a mixture of high-molecular-weight forms occurs (2). One component of this mixture consists of discrete-sized oligomeric extrachromosomal circular forms, while another component appears to be viral genomes integrated into the chromosomes of the host cell. We have found that ID13 cells latently infected with BPV-1 DNA contain extrachromosomal and likely integrated copies of the virus as well. Multiple copies of BPV-1 DNA appeared to be integrated in a head-to-tail tandem array on one chromosome 14 of the mouse complement (Fig. 2). The extrachromosomal component consisted of monomers and a series of oligomeric forms in which the units were also organized in a head-to-tail configuration. All of these observations strongly suggest that genomes of papillomaviruses tend to form oligomers as well as integrate into the chromosomes of the host cell.

The mechanism by which single circular elements can lead to oligomers and multiple copies integrated into the chromosomes of the host cell in a head-to-tail tandem array remains to be determined. One possibility is that generation of oligomeric forms is due to recombination. Another possibility is that oligomers arise during DNA replication by sister chromatid exchange (25). After integration of an oligomer, unequal crossing-over can lead to multiple copies organized in a head-to-tail tandem array (34).

Initiation and termination of DNA replication. The BPV-1 genome lacks a long dyad symmetry element that is found at

or near the replication origin in other viral genomes such as EBV or SV40 (10, 26). We have found, however, that the replicative forms derived from the 5.6-kb fragments generated by double digestion of BPV-1 DNA with *Eco*RI and *Bam*HI produce a pattern that corresponds to molecules containing a replication origin (Fig. 5). This is consistent with initiation of DNA replication taking place near PMS-1 at position 6940 (±5%), as determined by electron microscopy of replicative intermediates (37). It was not possible to determine in Fig. 5 whether some 5.6-kb fragments initiated DNA replication at a site near the PMS-2 element. To test this possibility, we used 2D gel electrophoresis to examine the replicative forms derived from another fragment generated by double digestion of BPV-1 DNA with *Hpa*I and *Xba*I (data not shown). As in the case of the *Eco*RI-*Bam*HI 2.3-kb fragment, only two kinds of replicative intermediates derived from this 6.1-kb fragment. Some molecules replicated as a family of simple Y-shaped forms. Other molecules showed the pattern expected for molecules in which termination occurs within the fragment. This observation confirmed previous findings indicating that no initiation of DNA replication seems to take place near the PMS-2 element in BPV-1 DNA (37).

The site at which termination of DNA replication occurs in circular plasmids is indicative of whether replication proceeds in a unidirectional or bidirectional manner. In the SV40 genome, termination occurs within a region located approximately 180° opposite the initiation site of DNA replication (35). A different situation is found in plasmid p174 that contains the EBV DNA replication origin. Here the family of direct repeats, which is part of *oriP*, acts as a replication fork barrier. Thus, in this instance, replication proceeds from a site at or near the dyad symmetry element in a predominantly unidirectional manner (14). We have found termination patterns in both the 5.6- and the 2.3-kb fragments generated by double digestion of BPV-1 DNA with *Eco*RI and *Bam*HI. These observations indicate that termination of DNA replication occurred at a site located approximately 180° opposite the origin, leading to the conclusion that replication of monomeric and oligomeric forms of BPV-1 DNA is bidirectional. The faint shadow that was sometimes observed within the triangle delimited by the high-molecular-weight portion of the family of Y-shaped forms, the straight signal of terminating replicative intermediates, and the straight signal corresponding to nonreplicative recombinants could be due to molecules terminating DNA replication at different but closely spaced sites. Subtle variations in the rate of fork progression are likely to produce such an event.

The DNA analyzed by 2D gel electrophoresis in this study was prepared from the Hirt supernatant fraction of ID13 cells. This fraction is enriched in low-molecular-weight DNA molecules (16). It is conceivable that the relative number of extrachromosomal oligomeric forms of BPV-1 DNA in ID13 cells was higher than that observed in Fig. 1 and 4. Thus, the relative proportion of the different forms and replicative patterns observed in Fig. 1, 4, 5, and 6 do not necessarily reflect the proportion of these forms in ID13 cells. It is also possible that some of the multimeric forms of BPV-1 DNA that are present in ID13 cells do not replicate or replicate less efficiently than the monomers. This is not the case, since it has been shown that monomeric and oligomeric forms of BPV-1 DNA replicate throughout the S phase in these cells (15). Gilbert and Cohen (15) treated ID13 cells with BrdUrd for either 1.5 or 14 h and isolated the unlabeled DNA (light/light) and the DNA labeled with BrdUrd in either one (heavy/light) or both strands (heavy/heavy). They found that the relative proportion of BPV-1 DNA present as monomers and oligomers was similar in these light/light, heavy/light, and heavy/heavy fractions. This constancy of the monomer/multimer ratio clearly indicates that all forms replicate in ID13 cells.

Replication origin inactivation and its consequences in extrachromosomal circular oligomers of BPV-1 DNA. Some multimeric forms contain several copies of the sequence that may serve as a replication origin. However, initiation of DNA replication takes place in only some of these sequences. There is evidence suggesting that one or more of these potential origins remain quiescent. The rDNA of *Saccharomyces cerevisiae* consists of approximately 120 identical repeat units arranged in a head-to-tail configuration on chromosome XII (28, 29). Each one of these units contains a potential origin located in the nontranscribed spacer-2 (33). Only one of 3 to 10 origins, however, is actually used in each round of DNA replication (6, 22, 38). For chromosomal DNA, this provides an example of many identical origins very close to each other in which only some are actually used. SV40 DNA linearized at a site near the replication origin with *Bgl*I was analyzed by using neutral-alkaline 2D gel electrophoresis (27). Results showed that the maximum length of the nascent strands derived from dimeric forms is equivalent to the size of a complete monomer. This is consistent with the notion that SV40 circular dimers replicate bidirectionally from one of the two origins. Electron microscopy of undigested BPV-1 DNA led to the finding that all the dimeric forms also have only one replication bubble (37). Thus, it is likely that one or more origins of replication are not functional in extrachromosomal circular multimeric forms.

The mechanism responsible for the quiescence of some replication origins present in circular oligomeric forms remains to be determined. It has been suggested that the frequency of origin usage is stochastically controlled by the concentration of a variety of *trans*-acting factors in eucaryotic cells (36). In addition, once an origin fires, the positive supercoiling generated ahead of the bidirectionally growing forks could make it more difficult for other neighboring origins to open.

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