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Origin of replication in episomal bovine papilloma virus type 1 DNA isolated from transformed cells

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The origin of replication of bovine papilloma virus type 1 (BPV-1) has been determined by isolating replicative intermediates (RI) of BPV-transformed hamster embryo fibroblasts (HEF-BPV). These RI were treated with single cut restriction enzymes to determine the start-position (origin) of the extending replication eyes using electron microscopic techniques. 'Cairns'-type RI molecules were shown to contain one replication eye in monomeric as well as in dimeric molecules. The position of this eye was localized at 6940 ± 5% bp in the physical map. In a second set of experiments BPV-1 DNA fragments cloned in pBR322 were tested for transient episomal replication. Transfected cells were harvested after increasing periods of time and screened for replication with isoschizomeric restriction enzymes to differentiate between input and replicated DNA. The part of the BPV genome harboring the replication origin spans the BPV *Cla*I-C restriction fragment corresponding to the non-coding region of the BPV genome and coincides with the DNase I-hypersensitive control region in the chromatin, isolated from transformed cells.

Key words: replicative intermediate/electron microscopy/episome/dominant selection marker/cloning vector

Introduction

In permissive systems, the viruses of the papova virus group replicate according to the 'Cairns' replication model as autonomous chromatin units until cell death and virus release. Several years ago papova viruses were detected which are able to replicate but do not continue replication after reaching a certain number of copies per cell, e.g., virus HD (Steffen *et al.*, 1980), and papilloma (Amtmann *et al.*, 1980). These viruses are present in the cells in an episomal state and usually do not integrate into the cellular genome (Steffen *et al.*, 1980; Amtmann *et al.*, 1980; Law *et al.*, 1981). A permissive system is not available. It is not yet clear which cellular or viral components in eukaryotes are responsible for maintaining a certain copy number.

Other members of the papova virus group such as SV40 and polyoma viruses start DNA replication at a well-characterized place in the genome (reviewed in DePamphilis and Wassarman, 1982). The DNA close to the origin of replication in the SV40 genome also has a function as a '*cis*-acting element' in integration (and excision) of viral molecules into the cellular genome (Conrad *et al.*, 1982). This region harbors particular DNA sequences which are able to form palindromic conformations and potential Z-DNA sequences (Nordheim and Rich, 1983). In the SV40 and polyoma genomes, these regions also have special structural properties which are responsible for a hypersensitivity to nucleases (Scott and Wigmore, 1978; Waldeck *et al.*, 1978; Varshavsky *et al.*, 1979) which seems to be a general feature of eukaryotic control regions (reviewed in Cartwright *et al.*, 1982).

In the case of papillomaviruses, the origin of DNA replication is not yet known. However, a DNase-hypersensitive region (DHSR) was detected in the bovine papilloma virus (BPV) genome (Rösl *et al.*, 1983). We tried to establish where the origin is located and whether there is a correlation with the DHSR or several control elements, such as palindromic sequences and AT-rich regions, in the non-coding part of the BPV genome (Chen *et al.*, 1982).

Two different approaches were used to determine the origin: (i) characterization by electron microscopy of replicative intermediates (RI) from BPV-transformed hamster cells cleaved with single cut restriction enzymes, and (ii) transfection of plasmids with inserted BPV DNA fragments followed by cleavage of the plasmid progeny with isoschizomeric restriction enzymes, recognizing methylated input DNA on the one hand and unmethylated replicated DNA on the other (DiMaio *et al.*, 1982).

Results

Purification of BPV RI and determination of the DNA size by comparison with standard DNA isolated from bovine warts

The BPV genome has been sequenced after molecular cloning and propagation in bacteria (Chen *et al.*, 1982). However, the determination of the origin of replication by electron microscopy requires information on the circumference of the unit length DNA molecules of the input virus used to transform the hamster cells. This allows a comparison with intracellular DNA forms including replicative intermediates. Virus particles were therefore purified from bovine warts, the virions were lysed, the virus DNA was extracted as described in Materials and methods and the BPV-1 DNA was characterized according to the restriction map (Chen *et al.*, 1982). The measurements of the circumference of the relaxed circular viral DNA (Figure 1) revealed a mean size of 2.25 μm ± 5%, as shown in the histogram in Figure 1.

These length measurements were the basis for the characterization of replicative intermediates used to determine the origin of replication by electron microscopy. For isolation of replicative intermediates, we purified the BPV episomes continuously present in transformed hamster cells with a copy number of ~200 copies/cell (Rösl *et al.*, 1983). One to five percent of these may be replicative intermediates in analogy to other papova viruses (Levine *et al.*, 1970). This results in an estimate of 2-10 RI molecules per cell. To minimize contamination with mitochondrial DNA during preparation of the RI, DNA was prepared from isolated nuclei of 10^9 cells by the NaCl-SDS isolation procedure (Hirt, 1967). The nuclear lysate was centrifuged to equilibrium in cesium chloride (CsCl) gradients without addition of

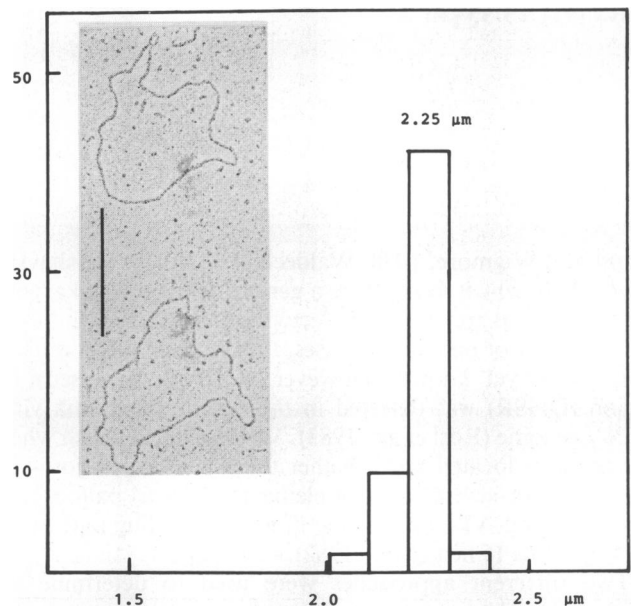

Fig. 1. Histogram of BPV-1 DNA lengths from isolated BPV-1 virions. The DNA was spread for electron microscopy as described (Waldeck *et al.*, 1983). Ordinate: number of molecules measured, abscissa: actual length of molecules. The insert shows an electron micrograph of characteristic BPV-1 virion DNA. The bar represents 0.5 µm.

ethidium bromide and the banded DNA was recentrifuged in a subsequent CsCl ethidium bromide gradient for separation of superhelical BPV-1 DNA. The BPV sequences contained in the gradient fractions were detected in both types of gradients by blot hybridization of aliquots analysed in agarose gels. The gradients were fractionated and the fractions containing the superhelical DNA were collected. Early replicative intermediates were assumed to be very close to the superhelical DNA, this enabled us to isolate preferentially 'young' replicative intermediates and avoided contamination with cellular DNA. Late replicative intermediates band close or together with relaxed or cellular DNA in the gradient, therefore late RI were rarely detectable. Early RI, however, are the best candidates for determination of the origin of replication.

Electron microscopic examination of the position of the replication eye

DNA obtained from the gradient fractions was examined by electron microscopy. Some examples of BPV replicating intermediates contained in the superhelical DNA pool are given in Figure 2. The length distribution of non-replicating DNA molecules and early replicating molecules contained in different preparations were in good agreement with the histogram shown for BPV-1 DNA initially isolated from virions derived from bovine warts (Figure 1). Examples for monomeric BPV DNA replicative intermediates with increasing sizes of the replicating eye (Figure 2a–f) and two molecules of nearly completely replicated monomeric BPV DNA (Figure 2g and h) are shown. Dimeric BPV DNA molecules are present in the transformed hamster cells at nearly the same frequency as monomeric DNA. Examples for replicative dimers are shown in Figure 3. Dimeric DNA molecules with two replication eyes could not be detected.

The replication eyes were localized by digestion of the replicative intermediates containing BPV DNA pool with the two single-cut restriction enzymes *Bam*HI and *Eco*RI, cleaving at map location 2113 (*Eco*RI) and 4450 (*Bam*HI) (Chen *et al.*, 1982). The resulting DNA fragments, when examined by electron microscopy, represented unit-length BPV DNA molecules contaminated with minor amounts of fragmented cellular DNA and mitochondrial DNA.

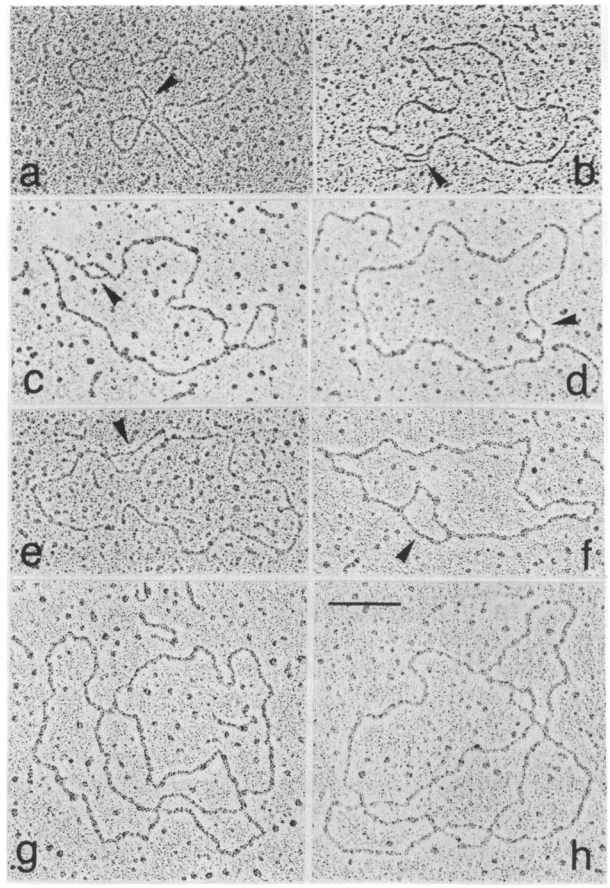

Fig. 2. Electron micrographs of BPV DNA replicative intermediates in spread preparations. Examples of different stages of replication. **(a–f)** show 'early' replicative intermediates with increasing size of the replication eye which are indicated by the arrows. **(g** and **h)** give examples for 'late' replicative intermediates. The bar represents 0.5 µm.

We were able to localize the position of the replicative eye in monomeric BPV-1 DNA relative to the restriction enzyme sites at the ends of the linearized molecules (Figure 4). The upper panel of Figure 4 shows electron micrographs of representative replicative intermediates (out of 50 molecules fitting the standard size of BPV DNA unit length) either cleaved with *Bam*HI (a) or *Eco*RI (b), respectively. The position of the replication eye within representative unit length molecules is shown schematically in Figure 4 (A: *Bam*HI; B: *Eco*RI). The distance from the opposite ends to the midpoint of the replication eye resulted in arm lengths of 33% and 67% using *Bam*HI and 40.5% and 59.5% in experiments using *Eco*RI, respectively. Summarizing the results, there is a coincidence of the position of the replication eye in a region near 6940 bp ± 5%, situated close to the single *Hind*III site (see Figure 5) which is localized at the 5′ end of the DHSR (Rösl *et al.*, 1983).

Further evidence for the presence of the replication origin in this region of the genome was received by cloning a DNA fragment including the *Hind*III site, the *Cla*I-C fragment (6834–7476 bp). The map positions of some characteristic

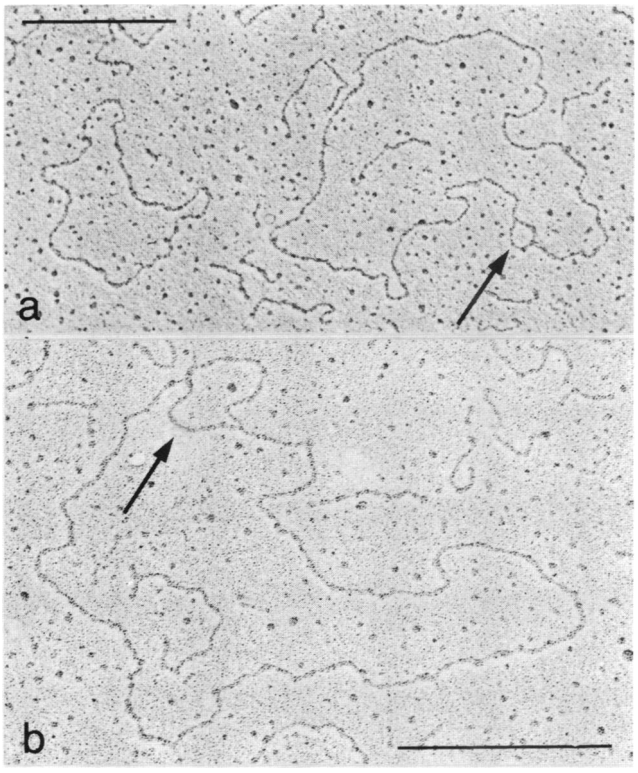

Fig. 3. Electron microscopy of spread preparations of replicating dimeric concatemers of BPV-1 DNA. In (**a**) an example of a monomeric, non-replicating and a dimeric, replicating DNA molecule is shown. Another example for a replicating dimeric molecule is given in (**b**). The arrows indicate the replication eyes. The bars represent 0.5 μm.

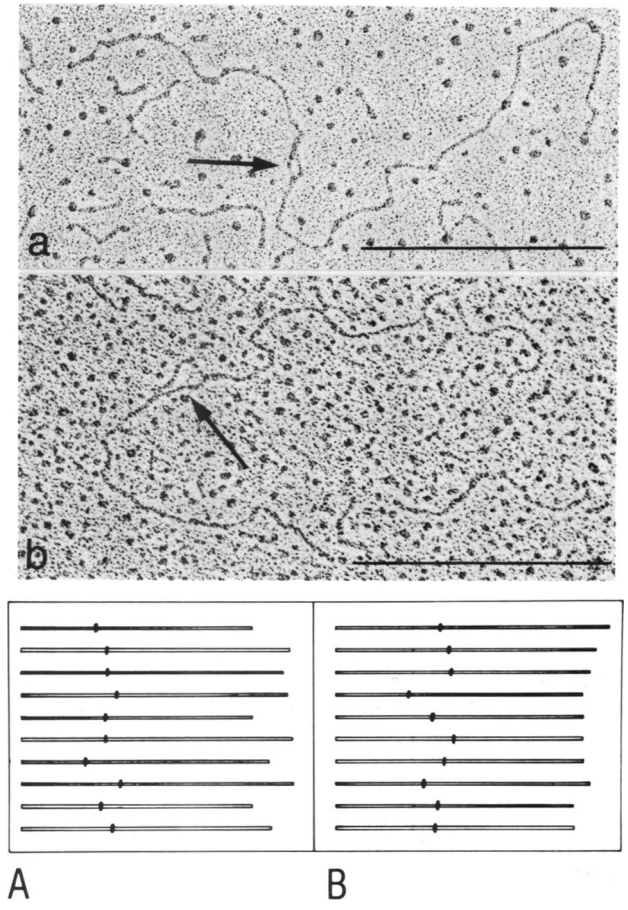

Fig. 4. Mapping of the replication start region in BPV-1 DNA by electron microscopy. After cleavage with the single-cut restriction enzymes *Bam*HI and *Eco*RI, the DNA was spread and the position of the eye structure was determined by length measurements. In the upper panel, micrographs of cleaved RI molecules are given (*Bam*HI-digested, **a**; *Eco*RI-digested, **b**). The lower panels show a series of DNA molecules in schematic presentation. **A** = *Bam*HI-cleaved; **B** = *Eco*RI-cleaved. The arrows in **a** and **b** indicate the replication eyes. The bars represent 0.5 μm.

enzyme cleavage sites and the position of the replication origin are indicated in Figure 5.

Molecular cloning of the 'ori' fragment and propagation in prokaryotes and eukaryotes

The fragment containing the origin of DNA replication, as determined by electron microscopy, was inserted into pBR322 plasmids and was propagated and characterized in bacteria (Rösl *et al.*, 1983). It was assayed for replication properties by transfection onto BPV-transformed hamster embryo fibroblasts (HEF-BPV) (Wigler *et al.*, 1978). In these cells the plasmid could be retained in an episomal state, whereas in other cell lines tested the plasmid became integrated (e.g., C127; unpublished data), see also (Lusky and Botchan, 1984).

Our experiments demonstrate that plasmids containing the *Cla*I-C origin region are able to replicate in HEF-BPV cells in contrast to plasmids that do not contain this ori region (Figure 6), or plasmids containing the *Cla*I-B fragment (unpublished data). This transient replication assay is based on the fact that methylated plasmids derived from dam$^+$ bacteria become resistant to the restriction enzyme *Dpn*I, if they replicate in eukaryotic cells (DiMaio *et al.*, 1982). In a control experiment, pBR322 DNA was completely cleaved by *Dpn*I, whereas SV40 DNA was rendered completely resistant by incubation of DNA with an excess of enzyme. *Dpn*I was used, however, in combination with its isoschizomer *Sau*3A which cuts both methylated and unmethylated DNA. Therefore we could exclude the possibility that the DNA is resistant *per se* by screening the DNA after various periods of time with these two enzymes. The DNA was extracted from the eukaryotic hamster cell line at different time points after

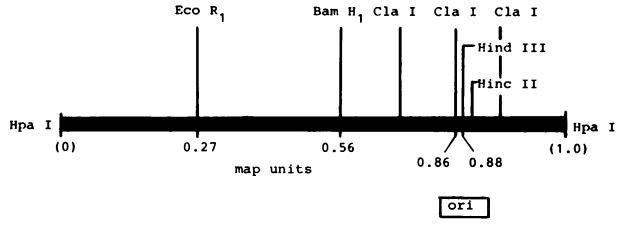

Fig. 5. Physical map of the localization of the BPV-1 replication origin. The linear map represents the BPV-1 genome opened at the *Hpa*I site giving the map position 0 and 1.0. All restriction enzyme cleavage sites are in accordance with Chen *et al.* (1982). The box 'ori' marks the origin of DNA replication determined by electron microscopy.

transfection and analysed by agarose gel electrophoreses (Figure 6). Panel c and f show the DNA transfected, with (c) and without (f) the insertion of the *Cla*I-C fragment. An at least 5-fold increase of DNA copies could be observed from 40 h after transfection where form II DNA is detectable (Figure 6a), up to 120 h after transfection where form II and III can be seen (Figure 6b) using pBR322 with the inserted *Cla*I-C fragment. pBR322 itself, however, does not replicate. The DNA isolated 40 h after transfection is shown in Figure 6d and 120 h post-transfection is shown in Figure 6e. To en-

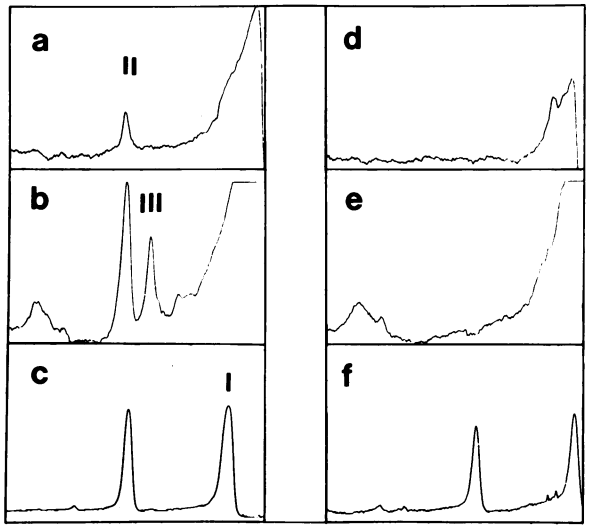

Fig. 6. Replication of plasmids with and without an inserted BPV *Cla*I-C fragment. pBR322 with and without inserted BPV *Cla*I-C fragment was transfected onto HEF-BPV cells. Cells were harvested (**a** and **d**) 40 h after transfection and (**b** and **e**) 120 h after transfection. The DNA was digested with *Dpn*I and run into a 1% agarose gel. (**c** and **f**) show the marker positions of the plasmids with and without insert, respectively. The DNA from the gel was transferred to nitrocellulose filters and hybridized with nick-translated plasmid DNA as described in Materials and Methods. After exposure of the gel to X-ray film, the densitometer tracings were performed using a Joyce Loebl Chromoscan III densitometer. The positions of form I, II and III are indicated.

sure that the DNA in the densitometer tracings (a) and (b) is replicated DNA, we digested the samples with *Dpn*I in order to remove the methylated, bacteria-derived input plasmid DNA. In a parallel assay, the DNA was completely digested with the isoschizomer *Sau*3A to prove the availability of the restriction sites. In a separate set of experiments, a comparable sensitivity to the isoschizomeric restriction enzymes was observed after passaging of the cells (unpublished data).

In a further approach, we ligated the 'origin fragment' into the single *Cla*I site of a dominant selection marker containing plasmid (pAG60) which allows cells to grow in the presence of geneticin (G418) (Colbère-Garapin *et al.*, 1981). These plasmids with and without the BPV-1 *Cla*I-C insertion were transfected onto HEF-BPV cells as described and the cells were challenged for growth in G418 after replating. G418-resistant colonies were trypsinized, expanded to cultures and the DNA was extracted. Even after five passages, replication of the plasmids containing the *Cla*I-C fragments could be shown by density labeling with BrdUrd and analysis in CsCl gradients (Figure 7).

Figure 7A shows the distribution of pAG60 plasmid DNA containing the *Cla*I-C fragment after centrifugation in a density gradient. Aliquots of every second fraction were analyzed in an agarose gel. As can be seen, the plasmid is able to replicate and the DNA distribution results in heavy-heavy (HH; both strands replicated), heavy-light (HL; one strand replicated) bands after a 20 h BrdUrd incorportion. The light-light (LL) DNA represents unreplicated material. These data are characteristic of a semi-conservative mode of DNA replication. In addition, dimeric and linear (FoIII) DNA is present which is characteristic for molecules involved in replication.

In Figure 7B the DNA distribution of the same plasmid without the inserted origin fragment is analyzed. A statistical

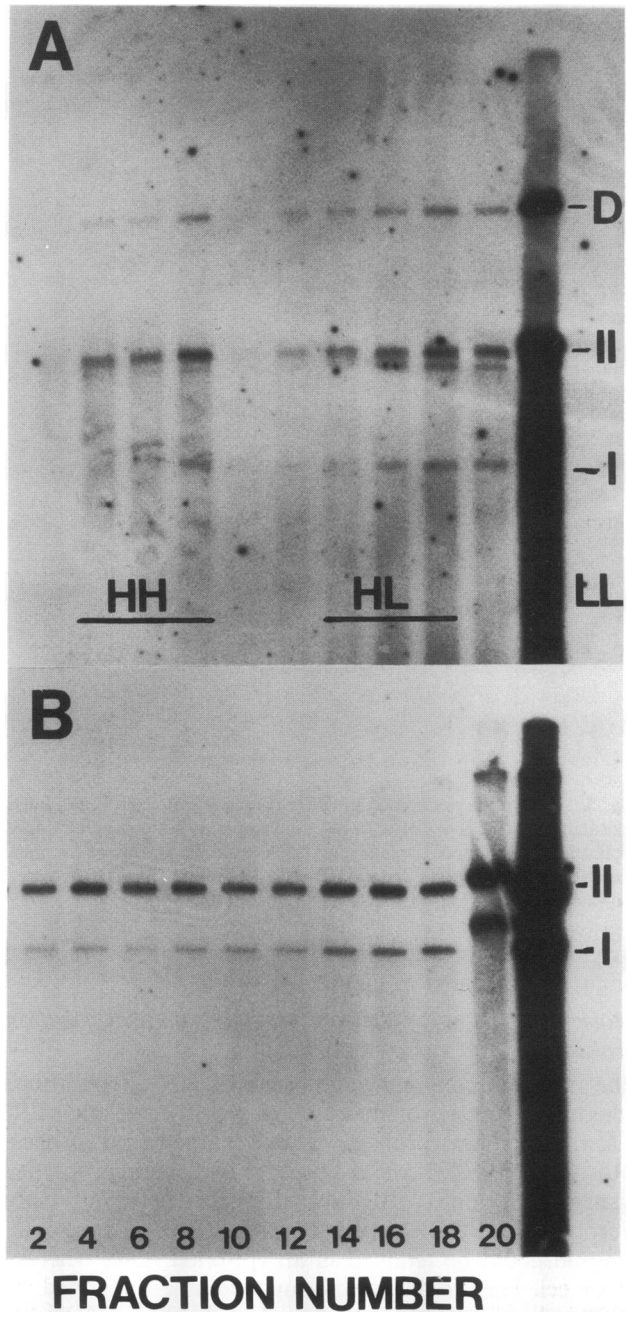

Fig. 7. Replication analysis by density labeling with bromodeoxyuridine. Plasmids with and without the origin fragment, both including the neomycin resistance gene (pAG60), were transfected to HEF-BPV cells. After five passages in the presence of 500 μg/ml G418, the cells were diluted 1:10 and exposed to BrdUrd 12.5 μg/ml for 20 h. After isolation, the DNA was centrifuged in an appropriate CsCl-gradient (initial refractive index 1.4150) in a Beckman VTI 65 vertical rotor for 15 h at 35 000 r.p.m. at 30°C. Fractions were collected, diluted 1:1 with H_2O and aliquots analyzed in 1% agarose gels. The DNA from the gels was blotted and hybridized with the nick-translated pAG60 plasmid. The marker positions of supercoiled (I), relaxed (II), linear (III) and dimeric (D) DNA forms are indicated. HH, HL and LL represent the expected density regions by BrdUrd incorporation.

incorporation of BrdUrd by nick-repair can be deduced because, instead of characteristic peaks, a broad DNA distribution from HH to LL appears. Furthermore neither dimers nor form III DNA are propagated, solely input form II and I were modified by repair synthesis.

Discussion

Our aim was to map the position of the replication origin of extrachromosomal BPV DNA. These episomes are continuously present in ~200 copies/nucleus (Rösl *et al.*, 1983). As a rough estimation, we expected to find ~1–10 replicative intermediates per cell, using the replication cycle of papova viruses as a basis for calculation (Levine *et al.*, 1970). Viral DNA replication can be studied using different approaches. However, recently it has been shown that in transfection experiments of cloned viral restriction fragments to study DNA replication, the transfected DNA tends in some cases to recombine (Pomerantz *et al.*, 1983). Furthermore, in replication studies of SV40 and polyoma DNA using the *Xenopus* egg system it became clear that there is still viral replication after deletion of the origin sequences (Harland and Laskey, 1980).

We used a different approach to study DNA replication. Earlier data obtained from our laboratory reveal that the BPV-1 genome has a DHSR (Rösl *et al.*, 1983). In analogy to SV40 and polyoma, where the origin of replication coincides with the DHSR, we surmised that the situation in the BPV genome might be similar.

As a basis for the determination of the replication origin, we determined the size of virion-derived BPV-1 DNA, compared it with episomal monomeric DNA from transformed hamster cells and used it as a standard for characterization of unit length replicative intermediates. The DNA pool containing the replicative intermediates was examined for all DNA structures known to be involved in replication. We were unable to detect any 'rolling circle'-like molecules, but we found 'Cairns'-type molecules which have also been demonstrated for the other papova viruses. In these replicative intermediates replication starts at one single point namely the 'origin'. In case of BPV-RI we observed either monomeric or dimeric molecules but they all had only one replication eye. The position of the eye in the physical map of the BPV genome was localized by measuring the length of the arms in unit length molecules and determined to be near map position 6940 ± 5%. From these data it was not clear whether the replication follows a uni- or bidirectional mode.

To use the origin region in episomal vectors, we cloned a DNA fragment as already described (Rösl *et al.*, 1983) in pBR322 and demonstrated by digestion with an isoschizomeric set of restriction enzymes that the molecules detected in a transient replication assay are really replicated. After transfection of the pBR *Cla*I-C plasmid, the amount of DNA in the recipient cells increased at least 5-fold from 40 to 120 h and was *Dpn*I resistant, whereas the control plasmid without the BPV *Cla*I-C fragment was cleaved with *Dpn*I. In the meantime Lusky and Botchan (1984) reported two 'plasmid-maintaining sequences' ('PMS') in the BPV-genome. We therefore reexamined two separately purified preparations of BPV RI DNA molecules for coincidence of the origin region with the PMS-2 region near the *Bgl*II site (1515) (Chen *et al.*, 1982). The PMS-2 region has an unknown function and is situated in the hypothetical reading frame of protein E_1. We did not find any molecules with a replication eye in this region. The PMS-I region, however, is in good agreement with the position of the origin of replication in BPV DNA described here.

Further experiments enabled us to assign the origin of replication of BPV to the *Cla*I-C fragment by connecting it to a dominant selection marker (pAG60), which allows cells containing these plasmids to grow in the presence of G418 antibiotic. Exposure of G418-resistant cells to BrdUrd for 20 h, and subsequent analysis of the DNA in CsCl gradients showed that only plasmids containing the BPV-1 insertion accumulate in the characteristic HH and HL positions. Plasmids without insertion smear through the gradient indicating a statistical repair synthesis. Additionally, the presence of dimeric molecules confirms ongoing replication.

In agreement with Lusky and Botchan (1984), we observed that plasmids containing certain BPV sequences connected to a dominant selection marker usually remain episomal after transfection onto BPV-1-transformed cells, but integrate into the cellular genome in the absence of BPV genomes (unpublished data). This suggests that protein(s), (presumably E_1) transcribed from the BPV genomes present in the transformed cells is able to function in *trans* and retain transfected plasmids carrying certain BPV sequences (ori) in an episomal state. When plasmids contain this *cis*-essential BPV element (ori), the BPV protein can recognize it and the plasmid, even with other inserted genes such as the neomycin-resistance gene coding for the resistance to G418, is able to replicate together with the wild-type BPV DNA. The BPV *Cla*I-C fragment may be a good candidate for the construction of an origin-shuttle vector. We are currently investigating the properties of such constructs in this 'cos-like' (Gluzman, 1981) BPV system by testing the maintenance of several genes as stable episomes co-existing with wild-type BPV genomes. Furthermore, *Bal*31 deletion mutants have been prepared to destroy the transforming potential of the endogenous wild-type BPV genomes and therefore allow reselection for transformation by introducing into the cells BPV ori vectors containing cellular or viral oncogenes.

Materials and methods

Virus purification and extraction of virion DNA

BPV-1 virions were prepared as described (Koch *et al.*, 1967) after separating the cell debris of the wart tissue. The virus containing supernatant was concentrated by centrifugation at 40 000 r.p.m. for 2 h at 4°C in a Beckman 60 Ti rotor. The pellet was resuspended in phosphate-buffered saline and adjusted to a density of 1.34 g/cm^3 with CsCl. The banded material was recentrifuged at 40 000 r.p.m. for 40 h at 15°C in a Ti 65 rotor. The BPV-1 virions were collected and dialyzed against 20 mM Tris-HCl pH 7.9 at 4°C overnight.

The viral DNA was isolated after treatment of the virions with SDS (0.5%) and proteinase K (100 μg/ml) for 2 h, extracted with phenol/chloroform, precipitated with ethanol and resuspended in 20 mM Tris-HCl pH 7.3, 1 mM EDTA.

Cells

BPV-1 transformed HEF cells (Amtmann and Sauer, 1982) were grown in Eagle's basal medium (BME) containing 10% fetal calf serum (FCS), 1% penicillin, streptomycin and amphotericin, respectively. The antibiotic geneticin (G418) was purchased from Gibco and stored in stock solutions (20 mg/ml in 0.1 M Hepes pH 7.5) at −20°C.

Transfection of DNA into eukaryotic cells

DNA was introduced into HEF-BPV cells by the calcium-phosphate coprecipitation technique as described (Wigler *et al.*, 1978).

Plasmid DNA (1 μg) and salmon sperm carrier DNA (10 μg) was applied to 10 cm plates containing 5 x 10^5 cells in BME. Cells were incubated overnight, washed with Hank's balanced salt solution and incubation was continued with 20 ml BME containing 10% FCS, 1% penicillin, streptomycin and amphotericin, respectively.

In case of G418 selection, the cells were replated 48 h after transfection with a density of 1 x 10^6 cells/plate. 24 h later the cells were challenged for growth in medium containing 250–750 μg/ml G418.

DNA manipulation

The commercially available restriction enzymes *Eco*RI, *Bam*HI, *Dpn*I, *Sau*3A were used as specified by the suppliers (Boehringer, Mannheim; BRL,

Bethesda).

The BPV *ClaI* fragments were subcloned in the *ClaI* site of pAG60 according to standard procedures (Maniatis *et al.*, 1982). DNA transformation of $CaCl_2$-treated *Escherichia coli* strain HB101 and plasmid extractions were performed as described (Dagert and Ehrlich, 1979; Birnboim and Doly, 1979).

DNA extractions from cells were carried out by the NaCl-SDS procedure (Hirt, 1967).

Gel electrophoresis, blot analysis and hybridization

Electrophoresis was carried out in 1% horizontal agarose gels as described (Tegtmeyer and Macasaet, 1972). The DNA was transferred to nitrocellulose filters (Schleicher and Schüll) according to the method of Southern (1975). After pre-incubation in Denhardt's solution (Denhardt, 1966), the filters were hybridized with ^{32}P-labeled nick-translated pBR322 or cloned BPV-1 DNA (Rigby *et al.*, 1977). The washing conditions were described recently (Rösl *et al.*, 1983). The filters were exposed with Kodak XAR-5 X-ray films using intensifying screens.

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