Identification of a 450-bp region of human papillomavirus type 1 that promotes episomal replication in *Saccharomyces cerevisiae*

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Abstract

Human papillomaviruses (HPVs) replicate as nuclear plasmids in infected cells. Since the DNA replication machinery is generally conserved between humans and *Saccharomyces cerevisiae*, we studied whether HPV-1 DNA can replicate in yeast. Plasmids containing a selectable marker (with or without a yeast centromere) and either the full-length HPV-1 genome or various regions of the viral long control region (LCR) and the 3′ end of the L1 gene were introduced into *S. cerevisiae* and their ability to replicate episomally was investigated. Our results show that HPV-1 sequences promote episomal replication of plasmids although the yeast centromere is required for plasmid retention. We have mapped the autonomously replicating sequence activity of HPV-1 DNA to a 450 base-pair sequence (HPV-1 nt 6783–7232) that includes 293 nucleotides from the 5′ region of the viral LCR and 157 nucleotides from the 3′ end of the L1 gene. The HPV-1 ARS does not include the binding sites for the viral E1 and E2 proteins, and these proteins are dispensable for replication in *S. cerevisiae*.
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Introduction

Human papillomaviruses (HPVs) are small (∼8 kb) double-stranded DNA viruses that cause mucosal as well as cutaneous lesions (zur Hausen and de Villiers, 1994). Cutaneous HPVs such as types 1, 2 and 3 cause plantar and palmar warts, whereas mucosal HPVs such as types 6, 11, 16 and 18 promote either benign or malignant growth of the infected tissue (zur Hausen and de Villiers, 1994). Replication of most HPVs in human cells requires the viral-encoded E1 helicase and the E2 protein (Chow and Broker, 1994; Lambert, 1991; Stenlund, 1996; Ustav and Stenlund, 1991; Wilson et al., 2002). Previous studies have shown that the E1 protein alone of the cutaneous HPV-1 can promote DNA replication in transfected human cell lines (Gopalak-rishnan and Khan, 1994). A major difficulty in the study of

HPVs is the lack of efficient in vitro systems for the vegetative replication of these viruses. Although raft culture systems allow limited replication and virus production for high-risk HPV types 16, 18 and 31 (Flores et al., 1999; Frattini et al., 1996; Meyers et al., 1992; Meyers et al., 1997), such systems have not been shown to promote vegetative replication of low-risk HPVs including types 1 and 2. Furthermore, while a few cell lines containing the DNA of high-risk HPVs such as types 16, 18 and 31 in an episomal state are available for biological studies, no cell lines containing benign, cutaneous HPVs such as types 1 or 2 have been described.

Recent studies have shown that bovine papillomavirus type 1 (BPV-1) and mucosal HPVs 6b, 11, 16, 18 and 31 DNA can replicate in *S. cerevisiae*, although the sequence requirements for this process are not known (Angeletti et al., 2002; Zhao and Frazer, 2002). It has also been shown that BPV-1 and several HPVs can also replicate in the absence of the E1 protein in mammalian cells (Kim and Lambert, 2002). This suggests an alternative mode of papillomavirus

replication in mammalian cells that does not require the viral helicase and is totally dependent on the host proteins. In an attempt to develop a system to study stable HPV-1 replication, we tested whether the HPV-1 genome can replicate in *S. cerevisiae*. Plasmids containing a yeast centromere (CEN) and a selectable marker along with either the full-length HPV-1 genome or sequences containing various portions of the long control region (LCR) and 3′ end of the L1 gene were introduced into *S. cerevisiae* and their ability for episomal replication and maintenance was tested. Our results demonstrate that HPV-1 contains sequences that promote its episomal replication in yeast. We also show that the autonomously replicating sequence (ARS) activity of the HPV-1 DNA in *S. cerevisiae* is contained within a 450-bp region (nt 6783–7232) containing the 5′ region of the LCR and 3′ end of the L1 gene, which do not include the E1 or E2 binding sites. The replication of HPV-1 plasmids was independent of the viral E1 and E2 proteins, and the
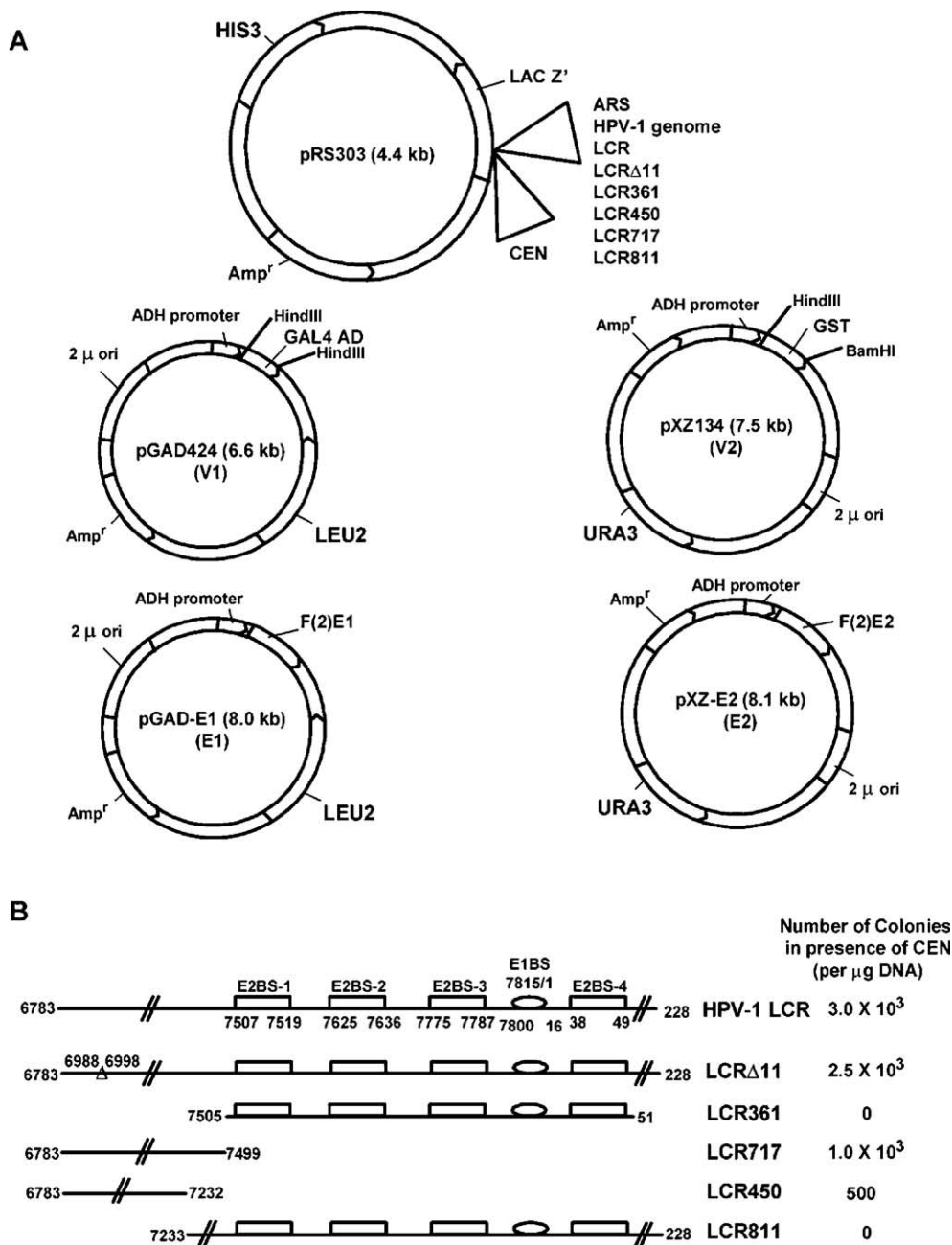

Fig. 1. (A) Schematic representation of the plasmids used in this study. Plasmid pRS303 was used as the backbone to clone the yeast CEN element, the yeast ARS, the full-length HPV-1 or LCR and its deletion derivatives. Plasmids pGAD424 (vector 1, V1) and pXZ134 (vector 2, V2) were used to clone the HPV-1 E1 and E2 genes with the FLAG epitope, respectively. (B) Schematic diagram of the HPV-1 LCR and its deletion mutants used in this study. The lines represent the regions of LCR cloned into the pRS303-CEN plasmid. The number of colonies obtained upon transforming yeast with these plasmids containing CEN is indicated on the right.

genetically-defined origin of replication of HPV-1 was dispensable for plasmid replication. The HPV-1 sequence did not encode an efficient segregation function since an *S. cerevisiae* CEN sequence was necessary for the generation of yeast transformants containing HPV-1 plasmids. The ability of HPV-1 DNA to replicate in *S. cerevisiae* may provide an important tool for the study of low-risk cutaneous HPVs, since no systems currently exist for long-term replication and amplification of the viral genome.

Results

The LCR of HPV-1 can promote retention of CEN plasmids in yeast

We investigated whether the genome of the cutaneous HPV-1 can replicate in *S. cerevisiae*, and if so, the sequence requirements for such replication. For this, we generated a series of plasmids containing the selectable histidine (HIS) marker and various HPV-1 sequences in the presence or absence of the yeast CEN element (Figs. 1A and B). The ability of these plasmids to transform the *S. cerevisiae* strain MSY182 was tested by plating the cells on minimal medium in the absence of histidine. As expected, the positive control plasmid pRS-ARS-CEN generated several thousand colonies, whereas the pRS-ARS plasmid which lacks the CEN element generated only a few colonies (Table 1). No colonies were obtained with the vector pRS303 and pRS-CEN plasmids (Table 1). Plasmids pRS-HPV-1 containing the full viral genome also failed to generate any colonies. The pRS-LCR plasmid (containing HPV-1 nt 6783–7815/1–228) which includes 157 nt from the 3′ end of the L1 gene, the full viral LCR and 125 nt from the 5′ end of the E6 gene also failed to form any colonies (Table 1). However, the pRS-HPV-1-CEN and pRS-LCR-CEN plasmids formed colonies at an efficiency that was only 1.33 to 2.66 fold lower than that observed with the pRS-ARS-CEN plasmid (Table 1). These results suggested that the HPV-1 genome may have a function similar to that provided by yeast ARSs, but it lacks the maintenance function provided by the CEN element. Furthermore, the ARS-like activity likely lies

within the region of HPV-1 (nt 6783–7815/1–228) included in the pRS-LCR plasmid.

In order to localize HPV-1 sequences that possess ARS-like activity, several sub-regions of the LCR (Fig. 1B) were cloned into the pRS303 plasmid either in the presence or absence of CEN. The full-length LCR is present within a 1261-bp *Sau*3A1 fragment of HPV-1 (nt 6783–7815/1–228) that also includes 157 nt from the 3′ end of the L1 gene and 125 nt from the 5′ end of the E6 gene. As expected, none of the LCR derivatives gave rise to colonies in the absence of a CEN sequence (data not shown). CEN-plasmids containing LCR717 (HPV-1 nt 6783–7499) and LCR450 (HPV-1 nt 6783–7232) which include sequences from the 5′ region of the LCR and 3′ region of the L1 gene were capable of forming colonies, although with a 3- to 6-fold lower efficiency than the complete LCR (Fig. 1B). Also, colonies containing the LCR717 and LCR450 plasmids grew more slowly than those containing the full LCR (5–6 days of incubation versus 3 days). On the other hand, a CEN plasmid containing the 3′ half of the LCR and the 5′ region of the E6 gene (LCR811, HPV-1 nt 7233–7815/1–228 that includes the functional HPV-1 origin in human cells and contains the E1 and E2 binding sites) failed to generate yeast transformants (Fig. 1B). Similarly, a plasmid containing the LCR361 region (HPV-1 nt 7505–7815/1–51) that also includes the E1 and E2 binding sites and the origin did not generate any colonies (Fig. 1B). These results suggested that a sequence within the 5′ region of HPV-1 LCR and 3′ end of the L1 gene, outside the previously defined HPV-1 origin of replication in human cells (Gopalakrishnan and Khan, 1994), is important for the retention of CEN plasmids in yeast. However, sequences in the 3′ half of the LCR may contribute to a better efficiency of replication. A search for the presence of a yeast ARS consensus sequence (ACS) in the above region of HPV-1 revealed a 100% match at position 6988–6998 (5′ TTTTATATTTT 3′) in an otherwise A/T-rich LCR. However, the LCRΔ11 plasmid lacking the above 11-bp sequence was similar to the LCR plasmid in its ability to form colonies in yeast (Fig. 1B). Taken together, these results indicate that sequences within the highly A/T-rich 5′ region of the HPV-1 LCR and at the 3′ end of L1 play an important role in the retention of CEN plasmids in yeast, and a sequence resembling the yeast ACS is not important for this activity.

LCR sequences promote episomal replication of CEN plasmids in yeast

The results of the above experiments showed that HPV-1 sequences promote retention of CEN plasmids in *S. cerevisiae*. We wished to determine if these sequences promote episomal replication of the above plasmids. For this, the physical state of the CEN plasmids containing various HPV-1 sequences was investigated by Southern blot analysis of DNA isolated from two independent yeast

Table 1
Transformation efficiency of *S. cerevisiae* with various plasmids

Plasmids	Plasmid features	Number of colonies per μg DNA in *S. cerevisiae* MSY182
pRS303	ARS⁻ CEN⁻	0
pRS-CEN	ARS⁻ CEN⁺	0
pRS-ARS	ARS⁺ CEN⁻	50
pRS-ARS-CEN	ARS⁺ CEN⁺	4.0×10^3
pRS-HPV-1	ARS⁻ HPV-1⁺ CEN⁻	0
pRS-HPV-1-CEN	ARS⁻ HPV-1⁺ CEN⁺	1.5×10^3
pRS-LCR	ARS⁻ LCR⁺ CEN⁻	0
pRS-LCR-CEN	ARS⁻ LCR⁺ CEN⁺	3.0×10^3

colonies. The control plasmid, pRS-ARS-CEN, as well as all the other CEN plasmids that formed colonies showed the presence of both supercoiled (SC) and open circular (OC) forms of the DNA (Fig. 2A). Furthermore, treatment of the DNAs with *Eco*RV or *Xho*I which cleave the various plasmids only once generated a single band, confirming that the HPV-1 sequences were present in an episomal form as observed with the ARS-CEN plasmid (Fig. 2B). Based on the intensity of the bands obtained with plasmids containing

various HPV-1 sequences, the copy number of these plasmids appeared to be approximately 2-fold lower than that of the ARS-CEN plasmid (Fig. 2B). This analysis also showed that none of the plasmids were integrated into the yeast genome. Note that treatment of the pRS-HPV-1-CEN plasmid with *Eco*RV is expected to generate two bands of 12.2 kb and 1 kb, the smaller band having migrated out of the gel. Experiments using DNA from several additional, independent colonies gave similar results (not shown),

Fig. 2. Episomal replication of HPV plasmids in yeast. DNA was isolated from yeast colonies and 25 μg of each sample was subjected to Southern blot hybridization. (A) Southern blot analysis of undigested DNA from two independent yeast colonies containing various plasmids. C represents 100 pg of control plasmid DNAs. SC, super-coiled plasmid DNA; OC, open-circular DNA. (B) Southern blot analysis of yeast DNA samples digested with *Eco*RV (Ec) and *Xho*I (Xh) that linearize the various plasmids (*Eco*RV digestion of HPV-1 plasmid releases a 1-kb fragment which is not shown). The first two lanes of each set contain 100 pg of control plasmid DNAs. (C) The HPV-1 plasmids undergo replication in yeast as demonstrated by *Dpn*I resistance of the isolated DNA. Various DNA samples isolated from yeast colonies were linearized with *Xho*I and one half of each sample was also treated with *Dpn*I. Control DNA corresponds to 1 μg of input plasmid DNA isolated from *E. coli*; this lane is underexposed.

confirming the episomal nature of the HPV-1 plasmids in *S. cerevisiae*. We also introduced the above CEN plasmids containing various HPV-1 sequences isolated from yeast into *E. coli* with selection for the ampicillin marker. DNA isolated from such *E. coli* transformants contained plasmids of the appropriate size (data not shown).

In order to ascertain that the HPV-1 plasmids underwent rounds of replication in yeast, plasmid DNA was treated with *Dpn*I which is expected to cleave only the methylated input DNA from *E. coli*. Plasmid DNA obtained from yeast colonies containing various plasmids was linearized with *Xho*I, one half of each sample was also digested with *Dpn*I and Southern blot analysis was carried out (Fig. 2C). The resistance of various DNAs to digestion with *Dpn*I showed that the plasmids containing the HPV-1 sequences replicated in *S. cerevisiae*. The control input plasmid DNA isolated from *E. coli* was digested by *Dpn*I (Fig. 2C). The above results showed that a 450-bp HPV-1 sequence (nt 6783–7232) containing the 5′ region of the LCR (nt 6940–7232) and 3′ end of the L1 gene (nt 6783–6939) can support episomal replication in yeast in the absence of the viral E1 and E2 proteins.

Stability of the LCR-CEN plasmids in S. cerevisiae

We assessed the mitotic stability of the pRS-LCR-CEN and pRS-LCRΔ11-CEN plasmids in yeast in the absence of nutritional selection. Log phase cultures of yeast growing in selective medium (without His) were diluted, and grown further for 10 cell generations (approximately 24 h) in the absence of selection. Due to its slow growth, the strain containing the ARS plasmid grew for only 5 generations during the same duration. The growth of spots at 0 or 10 cell

generations (5 cell generations in the case of the ARS plasmid) on selective versus nonselective medium were generally comparable (Fig. 3). Appropriately diluted cultures (from G_0, G_5 or G_{10}) were also plated on nonselective and selective solid media for quantification. Plasmid stability was calculated as described in materials and methods. As expected, the ARS^+CEN^+ plasmid was found to be stably maintained with a percent plasmid loss of 1.07 per cell generation, while the ARS^+CEN^- plasmid was very unstable and lost at a much higher rate (Fig. 3). The LCR-CEN and LCRΔ11-CEN plasmids were less stable than the ARS^+CEN^+ plasmid, having a plasmid loss of approximately 2.2% per cell generation (Fig. 3). Since all the above plasmids share the CEN sequence, the stability differences presumably arise from less efficient replication from the HPV-1 LCR as compared to the yeast ARS. Southern blot analysis performed on DNAs isolated from multiple colonies retained on the selective medium (without His) again revealed the presence of episomal plasmids indicating that the LCR-CEN plasmids were maintained extrachromosomally (data not shown). Plasmid retention assays could not be performed with the LCR717-CEN or LCR450-CEN plasmids since in both the cases we obtained small colonies which were very slow growing and did not form sufficient colonies at the highest dilution.

E1 and E2 proteins do not affect the replication or physical state of LCR-CEN plasmid in yeast

Since replication of the HPV-1 plasmids in *S. cerevisiae* did not require the origin of replication that is functional in human cells (Gopalakrishnan and Khan, 1994), we tested whether the E1 and/or E2 proteins of HPV-1 affected the
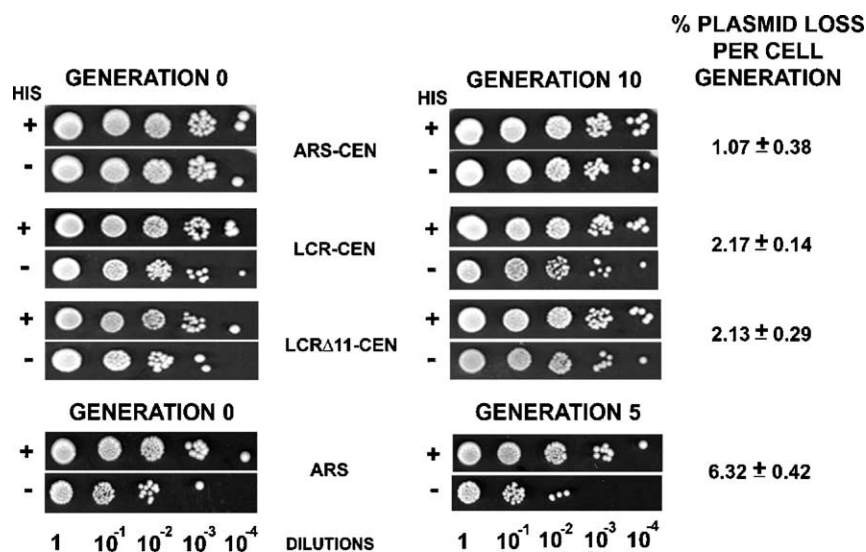

Fig. 3. Plasmid retention assay in wild-type yeast. Yeast cells harboring ARS-CEN, LCR-CEN and LCRΔ11-CEN plasmids were grown in the absence of growth selection (+His) for approximately 10 cell generations. The ARS plasmid was grown for 5 cell generations. Five microliters of serially diluted samples (for each set) from generation 0 and 10 (or 5) were spotted onto selection (−His) and non-selection (+His) plates to assess the growth characteristics of cells containing the His plasmids. Plasmid loss was determined by plating equal amounts of appropriately diluted cultures on selective and nonselective plates and the colonies were counted after 3 to 4 days. The values indicated on the right are average of three independent experiments.
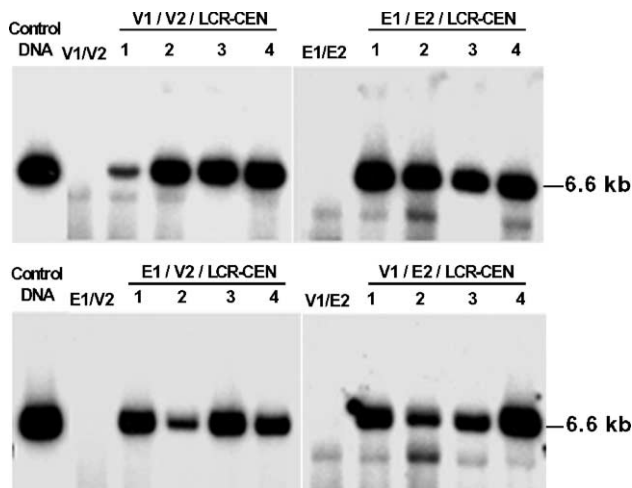
Fig. 4. HPV-1 E1–E2 expression does not alter the episomal state of viral DNA in yeast. Yeast cells expressing HPV-1 E1 and/or E2 genes were transformed with the LCR-CEN plasmid and total yeast DNA was isolated from colonies grown on minimal medium plates lacking leucine, uracil and histidine. DNA was digested with *Xmn*I which linearizes the LCR-CEN plasmid while digesting the vector plasmids into multiple smaller fragments that either migrate ahead of the LCR-CEN band or run out of the gels. Yeast cells containing the two empty vectors pGAD424 (V1) and pXZ134 (V2) were used as controls. The size of the linear LCR-CEN plasmid is indicated. Control DNA corresponds to 500 pg of the control LCR-CEN plasmid DNA digested with *Xmn*I.

replication of the pRS-LCR-CEN plasmid in yeast. First, plasmids pGAD-E1 and pXZ-E2 expressing the E1 and E2 proteins fused to the FLAG epitope were introduced into yeast and expression of the viral proteins confirmed by Western blot analysis using anti-FLAG monoclonal antibodies (data not shown). Four independent transformants containing the pRS-LCR-CEN plasmid along with various combinations of empty vector plasmids (V1 and V2), E1 or E2 expressing plasmids or both (Fig. 1A) were analyzed for the presence of plasmid DNA after linearization of the pRS-LCR-CEN plasmid with *Xmn*I. A single band of approximately similar intensity for the LCR-CEN plasmid was observed in all cases, indicating that the presence of the E1 and/or E2 proteins did not affect the replication or the episomal state of this plasmid in yeast (Fig. 4).

Discussion

In the present study, we have shown that HPV-1 sequences promote episomal replication of plasmids containing the CEN element in *S. cerevisiae* and the ARS activity of the HPV-1 genome is contained within a 450-bp segment that includes the 5′ region of the LCR (nt 6940–7232) and the 3′ end of the L1 gene (nt 6783–6939). However, HPV-1 sequences do not encode a CEN-like function in yeast.

It is well known that stable replication of extrachromosomal DNA in *S. cerevisiae* requires the ARS and CEN elements (Fangman et al., 1983; Fitzgerald-Hayes et al.,

1982; Marahrens and Stillman, 1992; Stinchcomb et al., 1979). A plasmid containing the HIS marker and the CEN element cannot be established in yeast. Attachment of the HPV-1 genome to such a plasmid (Fig. 1A) resulted in colony formation and episomal maintenance of the plasmid (Figs. 1B, 2A and B). Since no colonies were observed with the pRS-HPV-1 plasmid lacking the CEN element (Table 1), our results demonstrate that the HPV-1 genome contains ARS activity in yeast although it lacks the CEN function. Since the origin of replication of HPV-1 as demonstrated by transient replication analysis in human cells is located within its LCR (Gopalakrishnan and Khan, 1994), we investigated whether the ARS-like activity of the HPV-1 genome is also contained within the LCR. A plasmid containing a 1,261-bp fragment of HPV-1 (nt 6783–7815/ 1–228) promoted efficient episomal replication and retention of a CEN plasmid (Figs. 1 and 2), demonstrating that the ARS activity of HPV-1 is present within a region that contains the full LCR and the 3′ end of L1 and 5′ end of E6 genes. Deletion analysis showed that plasmids containing the LCR717 and LCR450 regions which contain the 157-bp from the 3′ end of L1 and 560 or 293 bp from the 5′ end of the LCR (Fig. 1B) also replicated episomally in yeast (Figs. 2A and B). However, they formed very slow-growing colonies, suggesting that their ARS activity was reduced. *Dpn*I resistance of plasmid DNA isolated from all the above strains (Fig. 2C) demonstrated that these plasmids underwent rounds of replication in yeast. CEN plasmids containing the 3′ half of LCR (LCR361 and LCR811) that includes the origin of replication functional in human cell lines did not form any yeast colonies (Fig. 1B). Taken together, our results suggest that the ARS-like activity of HPV-1 in yeast is contained within a 450-bp sequence in the 5′ region of its LCR and 3′ end of the L1 gene, and is independent of sequences that function as the origin of replication in human cells. However, the 3′ half of LCR may be important for the efficiency of replication as revealed by the very slow colony growth phenotype of cells with LCR717 and LCR450 plasmids that lack this region. It is possible that the 3′ region of LCR contains DNA unwinding elements and/or auxiliary sequences such as the binding sites for transcription factors that are known to contribute to efficient replication from yeast origins of replication (Coverley and Laskey, 1994). In *S. cerevisiae*, the sequence 5′ (A/T)TTTAT(A/G)TTT(A/T) 3′ corresponds to the ARS consensus sequence, ACS (Fangman et al., 1983; Marahrens and Stillman, 1992; Stinchcomb et al., 1979). Since the sequence, 5′ TTTTA-TATTTT 3′ located within the 5′ region of LCR (HPV-1 nt 6988–6998) has a perfect match with the ACS, we tested if this sequence may provide the ARS function in *S. cerevisiae*. This 11-bp region was deleted to generate plasmid pRS-LCRΔ11-CEN. This plasmid replicated in yeast with efficiency similar to that of the pRS-LCR-CEN plasmid (Figs. 1B and 2B). Two additional ARS-like sites (nt 6980–6990 and 6996–7006) that differ from the ACS by one nucleotide partially overlap the above perfect ACS.

When the 11-bp perfect ACS was deleted, it also changed the two ARS-like regions such that they now differed from the ACS at two positions making it unlikely that these would promote replication of the pRS-LCRΔ11-CEN plasmid. Taken together, our results suggest that the ARS activity of LCR is not due to the presence of an *S. cerevisiae* ARS-like element and future studies should identify sequences that are critical for HPV-1 replication in yeast.

Recent studies suggest that replication of BPV-1 and some mucosal HPV types can occur independently of the viral E1 proteins in both *S. cerevisiae* and mammalian cells (Kim and Lambert, 2002; Angeletti et al., 2002). While this paper was under review, a paper was published demonstrating that a 1-kb region of HPV-16 can provide both replication and maintenance functions in yeast (Kim et al., 2005). Two additional maintenance regions in the HPV-16 genome were also identified (Kim et al., 2005). Results presented here demonstrate that HPV-1 sequences located in the 3′ region of the L1 gene and the 5′ region of the LCR can also promote plasmid replication in *S. cerevisiae*. However, we did not identify any maintenance sequences in the HPV-1 genome since a yeast CEN sequence was required for plasmid maintenance. The sequences in the HPV-1 and HPV-16 genomes that provide the replication function in *S. cerevisiae* are located in similar regions of the viral genome. Future studies should reveal whether there are common elements in HPVs that support plasmid replication in yeast. The papillomavirus E1 and E2 proteins bind to the viral origin of replication and E1 acts as a helicase that unwinds the origin and promotes initiation of replication (Chow and Broker, 1994; Ustav and Stenlund, 1991; Wilson et al., 2002). In the absence of E1, it is likely that the replication of HPV-1 plasmids in yeast involves conserved factors such as the origin recognition complexes (ORCs) that bind to the ARSs, and the MCM proteins that act as a helicase to unwind the DNA during the initiation of replication (Bell and Dutta, 2002; Coverley and Laskey, 1994). Future studies should reveal whether the 450-bp region of HPV-1 (nt 6783–7232) that promotes episomal replication in yeast contains sequences that are recognized by ORCs. Angeletti et al. (2002) reported that E2 expression in *S. cerevisiae* leads to an increase in the copy number of HPV-16 plasmids. However, we did not observe any significant differences in the copy number of HPV-1 plasmids in the presence of E2 and/or E1 (Fig. 4). This may be due to differences in the expression levels of E2 or in the activities of different E2 proteins. It is also possible that the presence of a yeast CEN sequence may interfere with the induction of replication by the E2 protein. Future studies are expected to provide more information on this issue.

Very little information is currently available on how DNA replication is regulated during different stages of the papillomavirus life cycle. What might be the biological significance of E1- and viral origin-independent replication of HPV-1 DNA in *S. cerevisiae*? Studies with BPV-1 and several HPVs have suggested an alternative, E1-independent mode of viral replication in mammalian cells (Kim and Lambert, 2002). It is possible that low-level, maintenance stage replication of HPV-1 DNA in the basal cells of the epithelium may be independent of E1 and the viral origin of replication containing the E1 and E2 binding sites. On the other hand, productive, high level replication of HPV-1 DNA during terminal differentiation may involve a more efficient E1-dependent mode in which the 3′ region of the viral LCR that contains the E1 and E2 binding sites functions as the origin as observed in transiently transfected human cell lines (Gopalakrishnan and Khan, 1994). Other viruses, such as the Epstein–Barr virus are known to replicate from alternative origins of replication requiring different initiator proteins during the stable and productive modes of their replication (Tsurumi et al., 2005; Yates, 1996). Future studies are expected to clarify the sequence requirements and modes of replication of HPV-1 during its stable and productive replication in infected cells.

Materials and methods

Yeast strains, media and transformation methods

The *S. cerevisiae* strain MSY182 (*MAT*a *ura3-52 leu2Δ1 trp1Δ63 his3Δ200*) was used in this study (Ganster et al., 1998). Yeast was grown at 30 °C (Rose et al., 1990) in either standard YEPD medium or minimal medium lacking either leucine (Leu), uracil (Ura) or histidine (His) as needed to select for the appropriate plasmids. Plasmid DNAs were introduced into yeast using the standard lithium acetate method (Gietz et al., 1995).

Plasmid constructs

The yeast integrative plasmid pRS303 (4.4 kb, ARS⁻CEN⁻) containing the *HIS3* gene was used as the backbone to clone either the *S. cerevisiae* ARS sequence (from pRS313) or the CEN element (from pYAC4), or both to generate ARS⁺CEN⁻, ARS⁻CEN⁺ and ARS⁺CEN⁺ plasmids, respectively, to be used as controls in this study. The full-length HPV-1 genome and the viral LCR or its deletion derivatives were cloned into the pRS303 plasmid either containing or lacking the CEN sequence. The CEN4 region of pYAC4 (Burke et al., 1987) was PCR amplified with primers containing either *Bam*HI or *Not*I ends and cloned into *Bam*HI- or *Eag*I-digested pRS303, respectively. The appropriate pRS-CEN plasmids (5.4 kb) were subsequently used to clone different viral sequences or the *S. cerevisiae* ARS. The full-length HPV-1 genome was released with *Bam*HI from a pBR-HPV-1 plasmid and cloned into the *Bam*HI site of pRS303 or pRS-CEN to generate pRS-HPV-1 (12.2 kb) and pRS-HPV-1-CEN (13.2 kb), respectively. Since the *Bam*HI site is internal to the E2 gene of HPV-1, the above cloning resulted in the inter-

ruption of the E2 gene in the plasmid. An 1261-bp *Sau*3AI fragment containing the LCR of HPV-1 (nt 6783–7815/1–228, including the full LCR, 157-bp from the 3′ end of L1 and 125-bp from the 5′ end of E6 gene) was isolated from pBR-HPV-1 and initially cloned into *Bgl*II-digested yeast integrative plasmid pJJ246 containing the tryptophan gene (Jones and Prakash, 1990). The PCR amplified CEN4 sequence was then cloned into the pJJ246/LCR plasmid at the *Bam*HI site. Subsequently, the LCR-CEN cassette was isolated from this plasmid by *Sac*I–*Bgl*II digestion and ligated into *Sac*I–*Bam*HI digested pRS303 to generate the pRS-LCR-CEN plasmid (6.6 kb). We also generated two plasmids containing either 717 (HPV-1 positions 6783–7499) or 450 nt (positions 6783–7232) from the 5′ region of the LCR and 3′ region of L1. This was done by PCR amplification of the appropriate regions using primers that contained *Eco*RI ends, and ligation of the DNA fragments into *Eco*RI-digested pRS-CEN or pRS303 plasmids. This generated plasmids pRS-LCR717-CEN (6.1 kb) and pRS-LCR450-CEN (5.8 kb) containing the CEN, and plasmids pRS-LCR717 (5.1 kb) and pRS-LCR450 (4.9 kb) lacking the CEN. We also generated plasmids that lack the 3′ end of L1 and 5′ region of the LCR. A 361-bp region of HPV-1 LCR (nt-7505–7815/1–51) containing its optimal origin of replication that includes one E1 and four E2 binding sites (Gopalakrishnan and Khan, 1994) was PCR amplified with primers having *Sal*I ends and cloned into *Sal*I-digested CEN⁻ or CEN⁺ vectors to generate pRS-LCR361 (4.8 kb) and pRS-LCR361-CEN (5.8 kb) plasmids, respectively. An 811-bp region from the 3′ end of the LCR and 5′ end of E6 (nt 7233–7815/1–228) was amplified with primers containing *Bam*HI ends and cloned into the *Bam*HI site of RS303 or pRS-CEN plasmids to generate pRS-LCR811 (5.3 kb) and pRS-LCR811-CEN (6.3 kb) plasmids, respectively. The pRS-LCRΔ11-CEN plasmid contains a deletion of 11 bp (nt 6988–6998) in the LCR which was done by using the Excite PCR based Site-directed Mutagenesis kit (Stratagene). Appropriate deletion mutants were identified and their sequence confirmed by automated DNA sequencing.

E1 and E2 expression plasmids were generated using the 2 micron based yeast shuttle plasmids pGAD424 and pXZ134. HPV-1 E1 and E2 genes, each containing two tandem copies of the FLAG epitope at their 5′ ends, were PCR amplified using pSGMF2E1-1 and pSGMF2E2-1 template DNAs (Van Horn et al., 2001) with a pair of primers having 50-nt sequence overlap near the termini of *Hin*dIII digested pGAD424 and *Hin*dIII–*Bam*HI digested pXZ134 plasmids, respectively. Treatments of pGAD424 with *Hin*dIII and pXZ134 with *Hin*dIII and *Bam*HI released DNA fragments encoding the Gal4 activation domain and GST, respectively. The PCR amplified E1 gene was co-transformed with *Hin*dIII-digested pGAD424 (5.9 kb) while the E2 fragment was co-transformed with *Hin*dIII–*Bam*HI digested pXZ134 (6.8 kb) into yeast cells. This resulted in the cloning of the FLAG-tagged viral E1 and E2 open reading frames downstream of the yeast *ADH1* promoter in

the above vectors as a result of yeast gap repair mechanism in vivo (Orr-Weaver et al., 1981, 1983). The above strategy resulted in the generation of the pGAD-E1 (8 kb) and pXZ-E2 (8.1 kb) plasmids that are expected to express FLAG-E1 and FLAG-E2, proteins, respectively. A schematic representation of all the plasmids used in this study is shown in Fig. 1A.

DNA isolation from yeast

Yeast cells harboring plasmids with different HPV-1 sequences were grown in 10 ml of the appropriate selective medium overnight to obtain saturated cultures having an OD_{600} of greater than 1.0. The cells were harvested and re-suspended in 800 μl of lysis buffer [10 mM Tris–HCl (pH 8.0), 100 mM NaCl, I mM EDTA, 2% Triton-X-100 (v/v), 1% sodium dodecyl sulfate (w/v)]. Cells were lysed by vortexing in the presence of glass beads. The DNA was then recovered by phenol/chloroform/isoamyl alcohol extraction and alcohol-precipitation (Sambrook et al., 1989). Contaminating RNA was removed by treatment of the samples with RNaseA (Sigma) at 37 °C for 30 min and the DNA recovered by alcohol-precipitation.

Southern blot hybridization

To determine the physical state of the HPV-1 plasmids, total DNA from various yeast colonies was isolated and subjected to Southern blot analysis either with or without treatment with various restriction enzymes. Twenty-five micrograms of total DNA from various yeast colonies were digested with either *Eco*RV, *Xho*I or *Xmn*I which cut the HPV-1 plasmids only once (except for the plasmid containing the full HPV-1 genome which generates two fragments upon treatment with *Eco*RV). When the yeast strains also contained plasmids expressing the E1 and/or E2 proteins, the DNA was digested with *Xmn*I which linearizes the pRS303 derivatives containing various HPV sequences while digesting the high copy number 2 micron plasmid or its derivatives containing the HPV-1 E1 or E2 gene into multiple smaller fragments. Uncut or restriction enzyme digested samples from various yeast strains were run on 0.7% agarose gels using 1X TBE buffer, and following electrophoresis, the DNA was transferred nylon membranes (Sambrook et al., 1989). The Southern blots were probed with ^{32}P-labeled pUC19 probe generated by the random primer labeling method (Sambrook et al., 1989).

DNA replication assays in yeast

Yeast cells harboring plasmids with different HPV-1 sequences were maintained on solid medium for 3–4 weeks and then DNA was extracted from liquid cultures of the colonies as described above. To confirm that the HPV-1 plasmids underwent rounds of replication in *S. cerevisiae*, *Dpn*I resistance assay was performed (Peden et al., 1980).

Twenty-five micrograms of each DNA preparation were digested with *Xho*I which is expected to linearize the plasmids. One half of the digested samples were also treated with *Dpn*I to digest the input methylated plasmid DNA isolated from bacterial cells. The DNA samples were subjected to agarose gel electrophoresis and Southern blot analysis as described above using the pUC19 DNA as the probe.

Plasmid retention assays

The stability of the HPV-1 plasmids in *S. cerevisiae* was analyzed by the method of Kapoor et al. (2001). Yeast colonies harboring plasmids containing regions of the viral LCR were analyzed for plasmid loss in the absence of nutritional selection. The ARS^+CEN^+ plasmid was used as the positive control in this assay. Cultures were grown in selective medium (without His) until the mid-log phase, diluted to an OD_{600} of 0.1 in non-selective medium (with His) and grown for 10 or 5 generations as indicated in Fig. 3. The starting point of growth in nonselective medium was taken as generation zero (G_0). Cultures from both G_0 and the end of 10 or 5 generations (G_{10} or G_5) were diluted to an OD_{600} of 0.1 and each sample was further diluted by 10-fold serial dilutions. Five microliters of each dilution samples were then spotted on either nonselective (with His) or selective (without His) agar medium for a qualitative analysis of the retention of the His plasmids. To quantify the percentage of cells retaining the HPV-1 plasmid, equal amounts of appropriately diluted cultures were also plated on nonselective and selective plates at both G_0 and G_{10} (or G_5) and the colonies were counted. The percentage plasmid loss per cell generation was measured as $[(R_i - R_f)/n]$, where R_i is the percent of cells retaining the plasmid at G_0, R_f is percent of cells retaining the plasmid at G_{10} or G_5, and n is the number of generations of yeast growth in the absence of nutritional selection.

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References

Angeletti, P.C., Kim, K., Fernandes, F.J., Lambert, P.F., 2002. Stable replication of papillomavirus genomes in *Saccharomyces cerevisiae*. J. Virol. 76, 3350–3358.

Bell, S.P., Dutta, A., 2002. DNA replication in eukaryotic cells. Annu. Rev. Biochem. 71, 333–374.

Burke, D.T., Carle, G.F., Olson, M.V., 1987. Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors. Science 236, 806–812.

Chow, L.T., Broker, T.R., 1994. Papillomavirus DNA replication. Intervirology 37, 150–158.

Coverley, D., Laskey, R.A., 1994. Regulation of eukaryotic DNA replication. Annu. Rev. Biochem. 63, 745–776.

Fangman, W.L., Hice, R.H., Chlebowicz-Sledziewska, E., 1983. ARS replication during yeast S phase. Cell 32, 831–838.

Fitzgerald-Hayes, M., Buhler, J.M., Cooper, T.G., Carbon, J., 1982. Isolation and subcloning analysis of functional centromere (*CEN11*) from *Saccharomyces cerevisiae* chromosome XI. Mol. Cell. Biol. 2, 82–87.

Flores, E.R., Allen-Hoffmann, B.L., Lee, D., Sattler, C.A., Lambert, P.F., 1999. Establishment of the human papillomavirus type 16 (HPV-16) life-cycle in an immortalized human foreskin keratinocyte cell line. Virology 262, 344–354.

Frattini, M.G., Lim, H.B., Laimins, L.A., 1996. In vitro synthesis of oncogenic human papillomaviruses requires episomal genomes for differentiation-dependent late expression. Proc. Natl. Acad. Sci. U.S.A. 93, 3062–3067.

Ganster, R.W., McCartney, R.R., Schmidt, M.C., 1998. Identification of a calcineurin-independent pathway required for sodium ion stress response in *Saccharomyces cerevisiae*. Genetics 150, 31–42.

Gietz, R.D., Schiestl, R.H., Willems, A.R., Woods, R.A., 1995. Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast 11, 355–360.

Gopalakrishnan, V., Khan, S.A., 1994. E1 protein of human papillomavirus type 1a is sufficient for initiation of viral DNA replication. Proc. Natl. Acad. Sci. U.S.A. 91, 9597–9601.

Jones, J.S., Prakash, L., 1990. Yeast *Saccharomyces cerevisiae* selectable markers in pUC18 polylinkers. Yeast 6, 363–366.

Kapoor, P., Shire, K., Frappier, L., 2001. Reconstitution of Epstein–Barr virus-based plasmid partitioning in budding yeast. EMBO J. 20, 222–230.

Kim, K., Lambert, P.F., 2002. E1 protein of bovine papillomavirus 1 is not required for the maintenance of viral plasmid DNA replication. Virology 293, 10–14.

Kim, K., Angeletti, P.C., Hassebroek, E.C., Lambert, P.F., 2005. Identification of cis-acting elements that mediate the replication and maintenance of human papillomavirus type 16 genomes in *Saccharomyces cerevisiae*. J. Virol. 79, 5933–5942.

Lambert, P.F., 1991. Papillomavirus DNA replication. J. Virol. 65, 3417–3420.

Marahrens, Y., Stillman, B., 1992. A yeast chromosomal origin of DNA replication defined by multiple functional elements. Science 255, 817–823.

Meyers, C., Frattini, M.G., Hudson, J.B., Laimins, L.A., 1992. Biosynthesis of human papillomavirus from a continuous cell line upon epithelial differentiation. Science 257, 971–973.

Meyers, C., Mayer, T.J., Ozbun, M.A., 1997. Synthesis of infectious human papillomavirus type 18 in differentiating epithelium transfected with viral DNA. J. Virol. 71, 7381–7386.

Orr-Weaver, T., Szostak, J., Rothstein, R., 1981. Yeast transformation: a model system for the study of recombination. Proc. Natl. Acad. Sci. U.S.A. 78, 6354–6358.

Orr-Weaver, T., Szostak, J., Rothstein, R., 1983. Genetic applications of yeast transformation with linear and gapped plasmids. Methods Enzymol. 101, 228–245.

Peden, K.W.C., Pipas, J.M., Pearson-White, S., Nathans, D., 1980. Isolation of mutants of an animal virus in bacteria. Science 209, 1392–1396.

Rose, M.D., Winston, F., Hieter, P. (Eds.), 1990. Methods in Yeast Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 177–186.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, New York.

Stenlund, A., 1996. Papillomavirus DNA replication. In: DePamphilis, M.L. (Ed.), DNA Replication in Eukaryotic Cells. Cold Spring Harbor Laboratory Press, New York, pp. 679–697.

Stinchcomb, D.T., Struhl, K., Davis, R.W., 1979. Isolation and characterization of a yeast chromosomal replicator. Nature 282, 39–43.

Tsurumi, T., Fujita, M., Kudoh, A., 2005. Latent and lytic Epstein–Barr virus replication strategies. Rev. Med. Virol. 15, 3–15.

Ustav, M., Stenlund, A., 1991. Transient replication of BPV-1 requires two viral polypeptides encoded by the E1 and E2 open reading frames. EMBO J. 10, 449–457.

Van Horn, G., Sheikh, S., Khan, S.A., 2001. Regulation of human papillomavirus type 1 replication by the viral E2 protein. Virology 287, 214–224.

Wilson, V.G., West, M., Woytek, K., Rangasamy, D., 2002. Papillomavirus E1 proteins: form, functions and features. Virus Genes 24, 275–290.

Yates, J.L., 1996. Epstein–Barr virus DNA replication. In: DePamphilis, M.L. (Ed.), DNA Replication in Eukaryotic Cells. Cold Spring Harbor Laboratory Press, New York, pp. 751–773.

Zhao, K.N., Frazer, I.H., 2002. Replication of bovine papillomavirus type 1 (BPV-1) DNA in *Saccharomyces cerevisiae* following infection with BPV-1 virions. J. Virol. 76, 3359–3364.

zur Hausen, H., de Villiers, E.M., 1994. Human papillomavirus. Annu. Rev. Microbiol. 48, 427–447.