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Varying Efficiency of Long-term Replication of Papillomaviruses in *Saccharomyces cerevisiae*

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Abstract

Human papillomaviruses (HPVs) replicate in mitotically active basal keratinocytes. Two virally encoded proteins, E1, a helicase, and E2, a transcription factor, are important players in replication and maintenance of HPV episomes. We previously showed that HPV16 could replicate stably in *Saccharomyces cerevisiae* [Angeletti, P.C., Kim, K., Fernandes, F.J., and Lambert, P.F. (2002)] and we identified cis-elements that mediate replication and maintenance [J. Virol. 76(7), 3350-3358.; Kim, K., Angeletti, P.C., Hassebroek, E.C., and Lambert, P.F. (2005)]. Here, we demonstrate that although multiple HPV genomes replicate stably in yeast, they do so with differing long-term efficiency; HPV6-*Ura3* is replicated at the highest copy number, followed by HPV31-*Ura3* and HPV16-*Ura3* respectively, HPV11-*Ura3* and HPV18-*Ura3* were unable replicate without the presence of E2 expression and BPV-1-*Ura3* was unable to replicate, with or without the presence of E2. These studies suggest genotype-specific differences in HPV replication and maintenance.

Keywords

Extrachromosal DNA; Persistent infection; Human papillomavirus

Introduction

Human papillomaviruses (HPVs) are small, double-stranded, circular DNA viruses that infect squamous epithelial cells. During the course of epithelial cell differentiation, the virus shifts through three phases: establishment, maintenance; where the viral episome is maintained at low copy, and amplification; where the virus replicates at high-copy (Howley, 1996). The E1 protein functions as an ATP dependent helicase and recruits polymerase alpha and acts as an elongation factor (Fouts et al., 1999; Mohr et al., 1990; Patel and Picha, 2000). The viral proteins E1 and E2 function together in HPV genome amplification (DiMaio and Settleman, 1988; Ustav et al., 1991). In addition to transactivation and origin recognition, E2 is thought to serve as a maintenance factor by tethering newly synthesized HPV genomes to mitotic chromosomes or mitotic spindles, thereby allowing equal segregation to daughter cells (Bastien and McBride, 2000; Dao et al., 2006; Lehman and Botchan, 1998; Skiadopoulos and McBride, 1998). Our previous work indicated that HPVs could be stably maintained in the absence of E1 and E2, suggesting the existence of *cis*-acting maintenance elements (Angeletti et al., 2002; Kim et al., 2005; Zhao and Frazer, 2002b).

Having initially established that HPV genomes can replicate in *Saccharomyces cerevisiae*, the HPV/yeast system has proven easy to manipulate for study of many aspects of the HPV

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lifecycle, including transcription, replication and production of virus-like-particles (VLPs) (Angeletti, 2005). HPV6b, 11, 16, 18, and 31 have been reported to replicate in short-term assays when transformed into competent yeast (Angeletti et al., 2002; Kim et al., 2005; Zhao and Frazer, 2002a, 2002b). Furthermore, the Frazier lab has reported that BPV-1 replicates robustly in yeast, is transcriptionally and translationally active, and appears to create infectious virus particles (Zhao and Frazer, 2002a). Recently the Khan lab has reported that HPV1 could replicate in yeast, but requires a centromere to be maintained stably (Chattopadhyay et al., 2005). Kim et al. mapped both *ARS* and *CEN*-complementing functions in *S. cerevisiae* to the late region of HPV16 (Kim et al., 2005). Similar mapping studies done in Human cells identified the same regions of L1 and L2 for both replication and maintenance (Pittayakhajonwut and Angeletti, 2008). The great degree of conservation between the genomic replication and maintenance mechanisms of yeast and higher organisms creates the possibility that similar mechanisms could be involved in papillomavirus and maintenance in higher eukaryotes, especially during the maintenance phase when replication factor transcription is minimal.

In this study, we investigate the long-term replicative and maintenance competence of five HPV types: 6b, 11, 16, 18, and 31 along with BPV-1. Replicons containing a *Ura3* nutritional marker were created for each papillomavirus, transformed into yeast, and analyzed by Southern blot to confirm that HPV6b, 16, and 31 genomes were replicating episomally while HPV11 and 18 were not. Notably, BPV-1, which had previously been reported to replicate in yeast (Zhao and Frazer, 2002b), failed to show significant long-term growth in selective media. The replicative ability of each HPV type was further analyzed by examining both growth rate in selective media as well as plasmid stability and loss rate.

RESULTS

Yeast transformed were with different HPV-*Ura3* constructs and grown on YNB minimal media omitting uracil (Ura) at 30 °C. yeast harboring HPV genomes demonstrated very different growth characteristics when plated on selective media (Figs. 1, 2). For example, pPA112 and pPA117 showed very little growth on the plates, with at best only one to two colonies growing. We had previously demonstrated HPV 16-Ura's long-term plasmid maintenance in yeast (Angeletti et al., 2002), and confirmed that result here. pPA116 and pPA106 transformed yeast seemed to grow as well, or better than pPA103 on selective media. Significantly, BPV-1-Ura (pPA118) showed no colony growth on selective media. Inclusion of a yeast centromere in an HPV1 plasmid construct had been previously been shown to allow for genomic maintenance (Chattopadhyay et al., 2005). Cloning of a *CEN* element into pPA118 resulted in small, slow growing yeast colonies containing the plasmid (Fig 2) which were very difficult to further propagate. Southern blotting confirmed that the HPVs which replicated successfully in yeast, HPVs 6, 16, and 31, were being maintained episomally at varying copy numbers (Fig 3).

We also found that E2 expression induced colony growth in yeast harboring extrachromosomal HPV6 (pPA116), HPV16 (pPA103), and HPV31 (pPA106), each containing the *Ura3* gene. However, HPV18 (pPA112), which had essentially no colony growth in the absence of E2, formed a small number of colonies when co-transformed with the E2 expression vector (Fig 4). Yet, BPV-1-Ura (pPA118) formed no colonies in the presence or absence of E2 (Fig 4). These data suggest that unlike HPV6b, 16, and 31 which can replicate autonomously yeast, HPV11 and 18, which form colonies only in the presence of E2 expression, do not contain sequences required for autonomous replication and maintenance. Similarly, BPV-1 does not replicate nor is it restored by E2.

Growth Rates and Plasmid Stability of HPV-Ura Constructs

Experiments with liquid media inoculations allowed for a closer examination of the growth characteristics of each HPV genome transformed yeast culture. By observing changes in OD_{600} over several hours, a doubling time for the mid-log phase of each yeast culture was obtained (Fig 3). HPV6-Ura (pPA116) had the longest doubling time, 7.5 h, while pPA103 had the shortest at 3.3 h. It is important to know that doubling times were calculated as an average of final and initial OD readings. The graphs of HPV6, 16 and 31 indicate a very similar growth rate over most of the time points. All three HPV transformed yeast strains grew more slowly than untransformed YPH500 in YPD complete media or pRS316 positive control transformed yeast.

Additionally, experiments were performed to compare the rate at which HPV episomes were lost from yeast in the absence of selection. All four plasmids which could replicate in yeast were grown first in selective media (−Ura) to mid-log phase and diluted to an OD_{600} of 0.15 into new cultures containing nonselective media (+Ura). The cultures were grown, and at fixed time points, an equivalent number of cells were plated from each culture onto both selective and nonselective media plates, allowing for determination of the percent loss rate per cell generation, All three HPV constructs replicated with a high degree of stability similar to that of the positive control, pRS316. The pPA116 (HPV6) constract had a loss rate of 2.63 per cell generation, and pPA106 (HPV31) had a loss rate of 2.40% per cell generation, and pPA103 (HPV16) had a loss rate of 0.64% per cell generation. The control ARS+ CEN+ plasmid, pRS316 was lost at a rate of 1.56% per cell generation. These data indicate that all of these HPV genomes have *cis*-acting maintenance function, which is likely to be independent of E2BSs.

DISCUSSION

Our results indicate that certain HPVs (6, 16 and 31) replicate stably in yeast, while some require expression of E2 (HPV18 and 11). The definition of stable replication is based primarily on the ability to form colonies on selective media, which can be successfully restreaked and subcultured onto a new solid media plate as well as liquid media (equating to at least 50 cell generations). Previous studies performed by Angeletti et al. as and Frazer et al. have established that HPV11, 18, and BPV-1 are capable of replicating successfully in yeast during short term assays utilizing complete, non-selective liquid media (Angeletti et al., 2002; Zhao and Frazer, 2002a, 2002b). Growth on solid, selective media characteristically includes a 1–3 day growth period wherein yeast colonies are either not visible or very small, resembling petite mutants. Consequently, replicons with a defect in replication or maintenance will not develop to the point of visible detection as colonies and, thus, would be scored as not replicating stably. This differs from transient experiments performed previously, wherein transformed yeast were grown in non-selective liquid media for 20 cell doublings or more (Angeletti et al., 2002; Zhao and Frazer, 2002a, 2002b).

Notably, there seemed to be some degree of difference when comparing the Southern blot versus the liquid culture growth curves. Specifically HPV16 appeared to have a reduced copy number compared to the other HPV replicons but demonstrated a similar growth rate in transformed yeast. Presumably an increased copy number would lead to greater accumulation the *Ura3* nutritional marker and, thus, more growth. The reason for this discrepancy is not currently apparent, but one potential explanation could be an underestimation of HPV16's copy number by the assay (Angeletti et al., 2002). In any case, differences between the growth rates of the HPV replicons as compared to controls were not appreciably different.

The great degree of conservation between the DNA replication systems of yeast and higher eukaryotes suggests the possibility that the same factors may be involved in papillomavirus

genome replication and maintenance for both systems. As stated previously, papillomaviruses spend the majority of their life cycle in a maintenance phase, replicating episomally at low copy number, with only minimal expression of the virus's replication factors E1 and E2. We have previously demonstrated that HPV16 is capable of replicating with *cis*-acting factors alone in the absence of E1 and E2 (Angeletti et al., 2002). Also, Hoffman et al. have previously demonstrated that HPV16 replicates in a once-per-S-phase manner in certain cell lines, depending on presence or absence of viral replication factors, while HPV31 was shown to only replicate randomly under the study conditions (Hoffmann et al., 2006).

All of these results point to a large degree of reliance upon host factors for regulation of viral genome replication and maintenance than previously expected. The differences in replication and/or maintenance success of different HPV types likely represents genotype-dependent differences in *cis*-acting elements in the late region of the genomes as compared to that of HPV16, which was recently shown to provide both functions by Kim et al. (Kim et al., 2005; Pittayakhajonwut and Angeletti, 2008). Completing similar mapping experiments of other HPV types should help identify which *cis*-acting factors are playing a role in yeast replicative success. In addition, the experiments described by Hoffman et al., if performed in yeast, should shed light on the mode of replication being utilized. Furthermore, while all the HPVs in this study replicate successfully in host cells, the difference in replicative success shown in this study may imply a greater or lesser degree of utilization of host replication factors between varying HPV types and differing dependence on E1 and/or E2 (Fig. 4).

We found that BPV-Ura was incapable of long-term replication in yeast under selective conditions, yet in short-term experiments, we previously showed that BPV-1 could generate Dpn I resistant DNA products, indicating that replication is initiated but that DNAs are most likely not stably maintained (Angeletti et al., 2002). In agreement with this possibility, the addition of a centromere region (pPA119 plasmid), resulted in partial complementation of maintenance function. Reports by the Frazer lab that BPV-1 DNA transformation, or native virion infection of yeast protoplasts could result in replication (Zhao and Frazer, 2002a, 2002b), may be explained by differing approaches to the experiments. For, example BPV-1 virions may have intrinsic properties that facilitate establishment of stable replication. Also, our experiments were done with strict nutritional selection, which might exclude observation of replicons that require long establishment times under non-selective conditions.

Our experiments have revealed intrinsic differences among HPVs and BPV-1 in their ability to replicate stably in yeast. These differences may be related to the number of E2 binding sites in PV genomes (17 for BPV-1, as compared to 4 E2BS for most α-HPVs) (Li et al., 1989), implying varying reliance of PVs on E2. However, despite positive effects on HPV colony formation, expression of E2 in the yeast cells did not restore replication of BPV-1. After careful analysis, We conclude that, under selective conditions, BPV-1 does not appear to be stably maintained in yeast to the extent of other PVs investigated in this study. It is still possible that this is the result of differences between the mammalian Brd4 protein and its yeast homologue, Bdf1, which lacks the C-terminal domain that has been shown to interact with E2 and facilitate tethering of the BPV genome to host chromosomes (Brannon et al., 2005). Fusion proteins of Bdf1 and the C-terminus of Brd4 have been shown to restore E2 maintenance function (Brannon et al., 2005), and it is possible that such a fusion would restore BPV-Ura replication.

We have concluded that HPVs are utilizing cellular factors for replication and maintenance in yeast. In a recent study we determined that several Human cellular factors had predicted binding sites in HPV16; Centromere-binding protein B (CENP-B), topoisomerase II (Topo II), matrix attachment regions (MARs), telomere-repeat binding factors (TRF), and histone-related factors (HMG and H1) (Pittayakhajonwut and Angeletti, 2008). Many of these factors are well

conserved from yeast to Humans, suggesting that they may be good candidates as maintenance and/or replication factors.

Materials and Methods

Yeast strains, plasmid isolation and transformation methods

The haploid yeast strain YPH500 (*MAT ura3-53 lys2-801 ade2-101 trp1-63 his3-200 leu2-1*) was used for all transformation and Southern experiments, while YPD complete media was utilized additionally to examine plasmid stability and loss rate. The EZ Yeast Transformation kit (Zymo Research, Orange, Calif.) was used for transformation with the experimental plasmids according to the kit protocol and the Zymoprep kit (Zymo Research, Orange, Calif.) was used for yeast plasmid minipreps according to company specifications.

Plasmid constructions

Numerous constructs were created using similar methods. The *Ura3* gene was ligated into unique restriction sites in either the papillomavirus genome or vector sequence, selected in *E. coli* grown on Luria Broth media containing Ampicillin, and the DNAs were isolated using the Qiaprep Spin Miniprep Kit (Qiagen Sciences, Maryland 20874) in accordance with the manufacturer's instructions. The *Ura3* markers were cloned into the various constructs as follows: pPA102 (pGEMT, Age I), pPA103 (HPV16, Xho I), pPA104 (Puc18, Sal I), pPA106 (HPV31, Spe I), pPA112 (PPR 322 with HPV18 ligated into Nco I site, Avr II), pPA116 (HPV6, Age I), pPA117 (HPV11, Age I), and pPA118 (BPV-1, Mlu I). Construct PA119 was created by digesting pΔYac with AvrII, releasing the CEN element, and then ligating this fragment into a unique AvrII site in pPA118. pRS 316, an ARS+ CEN+ *Ura3*+ yeast replicon, was included as a positive control.

DNA replication in yeast

Two-hundred ng of each plasmid were transformed into YPH500 yeast, plated on Ura-selective agar, and incubated for 3 days at 30 °C. Plates were then scored for number of colonies formed and restreaked on selective solid agar, grown for an additional 3 days, and inoculated into 5 ml of Ura-liquid media and allowed to grow for approximately 40–50 cell generations. OD_{600} was recorded for each sample to obtain approximate number of cell equivalents per milliliter, and samples were diluted in order to equalize this number, allowing for a determination of copy number by Southern blot. Low molecular weight DNA was isolated from liquid culture as described above. The DNA was then loaded onto a 1% agarose gel, electrophoresed, transferred to nitrocellulose, and probed with ^{32}P-radiolabeled pPA104. Radiolabeling was performed using the Amersham Rediprime II kit (GE Healthcare UK, Buckinghamshire) in accordance with the manufacturer's instructions.

Growth curves

2 ml of liquid Ura- media were inoculated to an OD_{600} of 0.15 with each of yeast harboring the papillomavirus genomes or a control (pRS316). In addition, 2 ml of YPD media were inoculated to an equivalent OD_{600} with untransformed YPH500 yeast to establish a standard doubling time. OD_{600} for each sample was recorded at time points 0, 3, 6, 8, 24, and 30 h after inoculation. Growth rate was then determined by calculating doubling time during the mid log growth phase using the formula $T_d=(t_f-t_i)*[\log(2) / \log(q_f/q_i)]$ where T_d is doubling time, t_f and t_i are time final and initial, and q_f and q_i are the OD_{600} values at the t_f and t_i.

Plasmid loss rate assay

The plasmid loss rate per cell generation was calculated utilizing a method similar to that described by Marahrens and Stillman (1992) Briefly, approximately 100–200 cells were plated

onto YPD and YNB-Ura plates after being removed from selection at time points of 0, 4, and 8 h post inoculation. All sets of plates were then incubated for 3 days and scored for colony growth. Plasmid loss rate was then calculated using the formula $L=P_f-P_i/T$ where L is the percent loss rate per cell generation, P_f is the percentage of growing yeast which contained the plasmid at the final time point, P_i is the percentage of growing yeast with the plasmid at the initial time point, and T is the number of cell generations.

HPV colony formation assay in E2-expressing yeast

Yeast containing either an empty vector (pPD2) or an E2-expressing construct (pPD-16E2) were cultured under –Leu selection in liquid media overnight. Two-hundred ng of each plasmid construct (pPA116 (HPV6-Ura), pPA117 (HPV11-Ura), pPA103 (HPV16-Ura), pPA112 (HPV18-Ura), pPA106 (HPV31-Ura), pPA118 (BPV-1-Ura) were transformed into yeast either expressing E2 or not. Positive control plasmid, pRS316, or negative control plasmid, pPA104 (puc18-Ura), were transformed into the same strains. Yeast were then plated onto selective media (YNB-Leu, –Ura) and allowed to grow for 4 days. Plates were then scanned and scored for growth.

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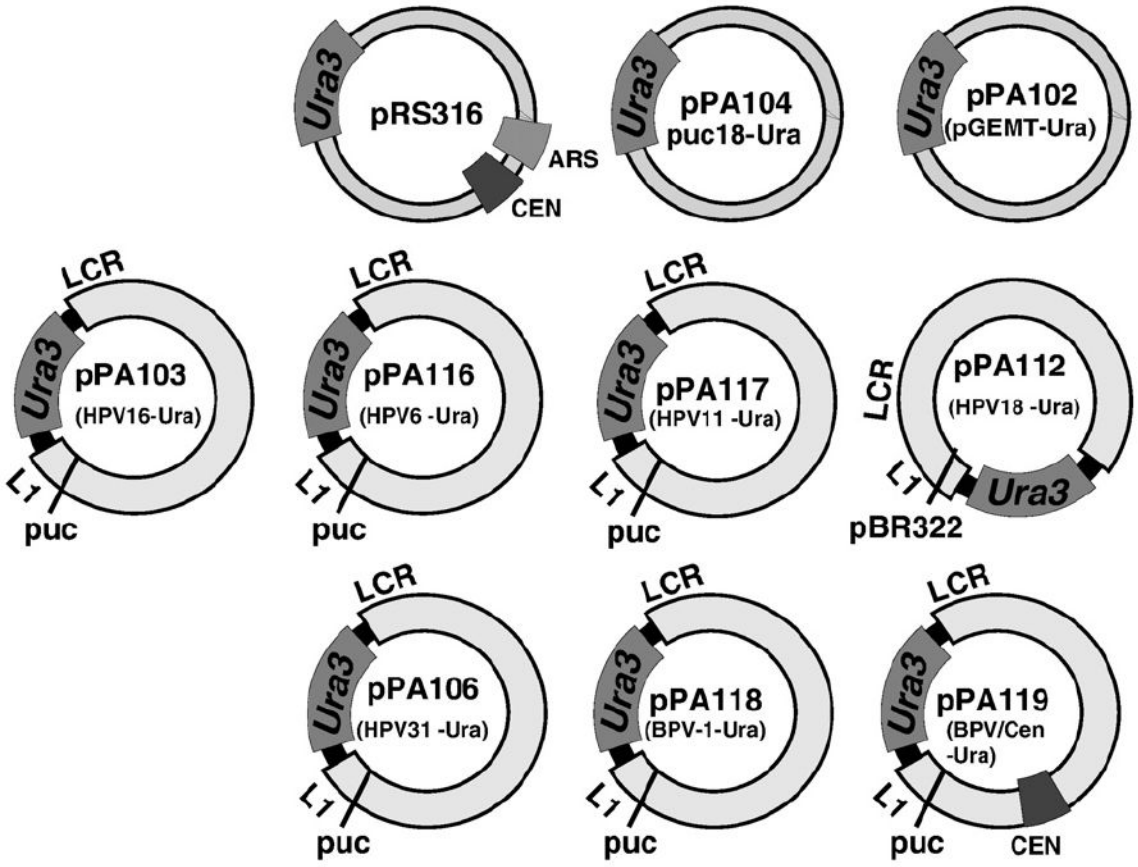

Fig. 1.

Maps of HPV/yeast replicons. Into each HPV genome a *Ura3* gene cassette was introduced as shown in each of the plasmid maps. The pPA103 vector contains the HPV16 genome with the Ura gene inserted at nt 7309 of the genome, as indicated. PPA106 contains the HPV31 genome, pPA112 contains the HPV18 genome, pPA116 contains the HPV6 genome, pPA117 contains the HPV11 genome, pPA118 contains the BPV genome, and pPA119 contains the BPV-1 genome with a yeast centromere inserted. Control plasmids included pPA104 (puc-*Ura3*), PgemT-*Ura3* as negative controls and pRS316, as a positive control.

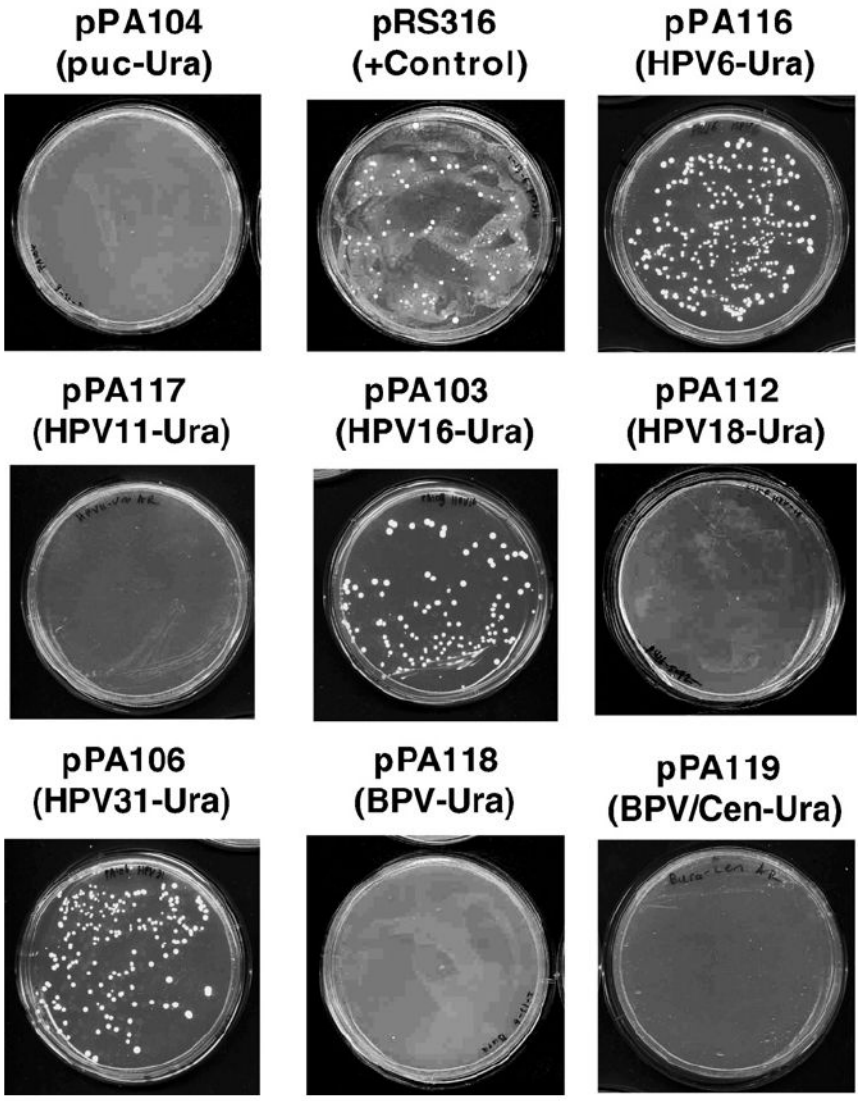

Fig. 2.

Growth of HPV replicons. Two-hundred ng of each plasmid construct were transformed into YPH500 yeast, plated onto YNB-Ura media, and allowed to grow for 72 h. Plates were then scanned and scored for growth.

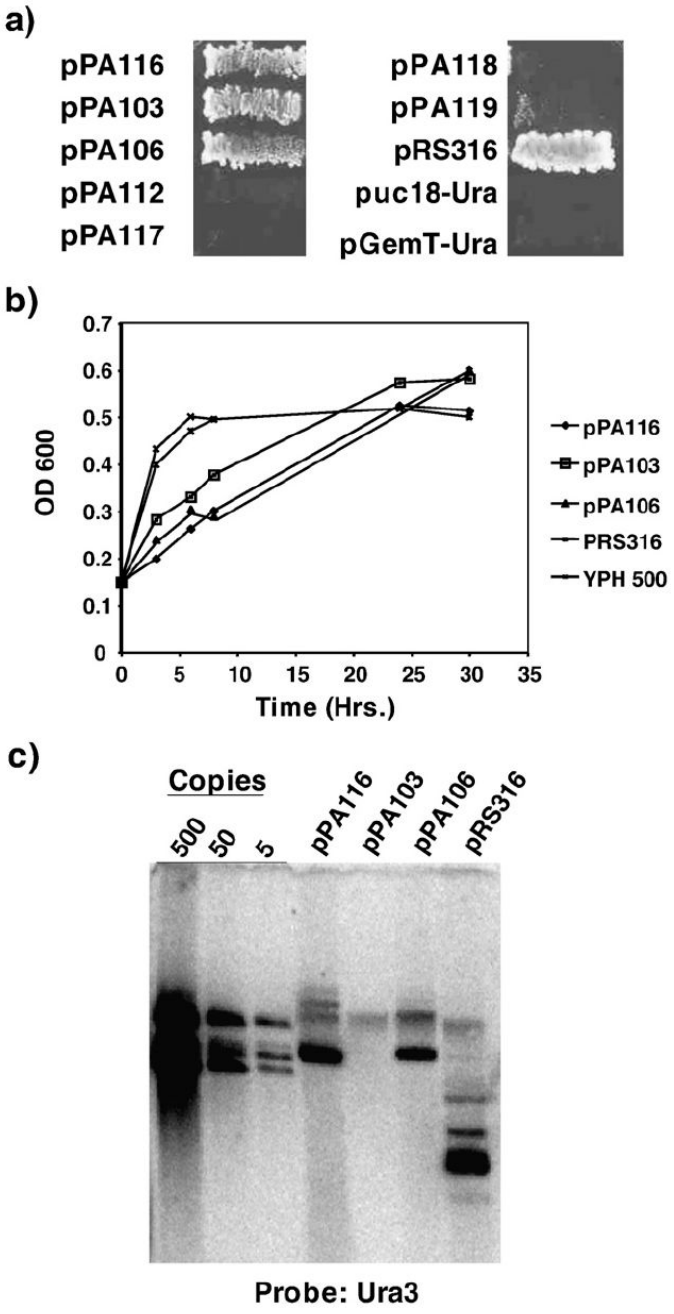

Fig. 3.

(a) Patch plate analysis of growth of initial isolates. After initial growth upon being transformed into yeast, colonies were restreaked onto an additional –Ura plate both to provide for ease of manipulation and also to examine long-term maintenance. All plasmids which grew initially continued to grow stably on the master plate. Additionally, BPV-1/CEN-Ura showed signs of limited growth, evident as small colonies. (3b) Growth curve analysis of HPV replicons. 5 ml of liquid media were inoculated to an OD600 of .15 with each of the successfully replicating plasmid constructs (HPV6, 16, and 31-Ura as well as pRS316.) Additionally, a similar OD600 was generated with YPH500 alone grown in YPD complete media. OD600 was recorded at time points 0, 3, 6, 8, 24, and 30 h post inoculation. Growth rate was then determined by

calculating doubling time during the mid log growth phase. (3c) Extrachromosmal replication of HPVs in yeast. All successfully replicating constructs (HPV6-Ura, HPV16-Ura, HPV31-Ura, and PRS316) were inoculated into 5 ml of YNB-Ura media and incubated for 2 days. Small molecular weight DNA was then harvested via yeast miniprep, loaded into 1% agarose gel, and electrophoresed. DNA was then transferred to a nitrocellulose membrane and analyzed via Southern blot pPA116 (HPV6-Ura), pPA103 (HPV16-Ura), pPA106 (HPV31-Ura), and pRS316 were probed utilizing ^{32}P radiolabelled Ura3. Control volumes of HPV6-Ura were utilized in the first 3 lanes to provide an idea of relative copy number.

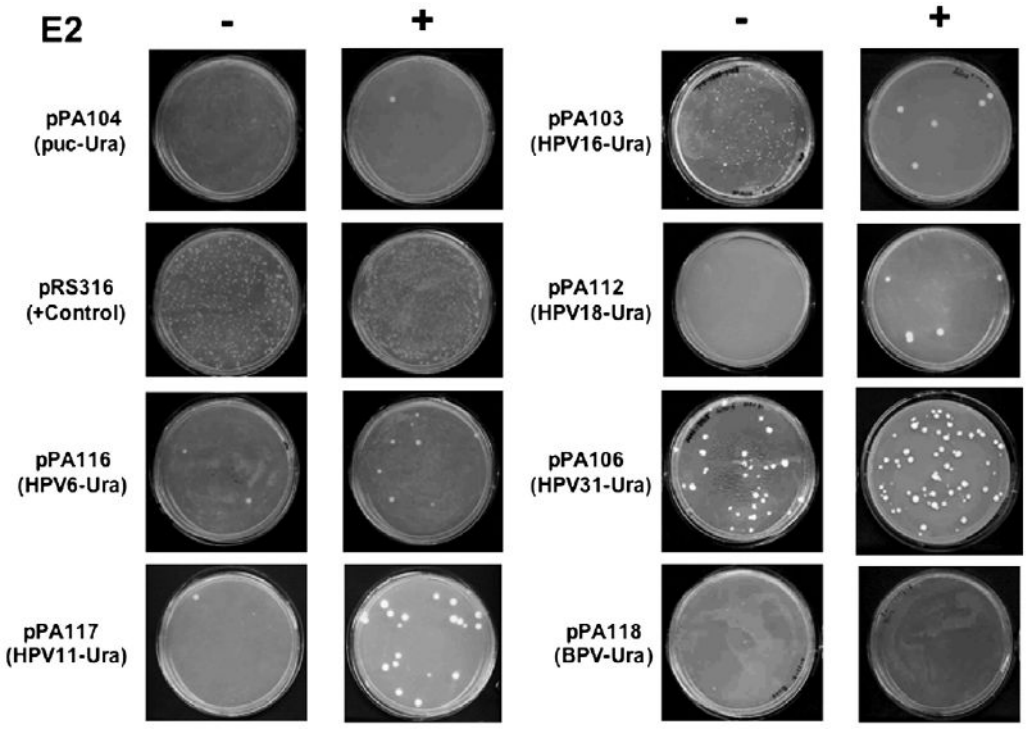

Fig. 4.

The effect of E2 on HPV colony formation in yeast. Two-hundred ng of each plasmid construct (pPA116 (HPV6 Ura), pPA117 (HPV11 Ura), pPA103 (HPV16 Ura), pPA112 (HPV18 Ura), pPA106 (HPV31 Ura), pPA118 (BPV-1-Ura) were transformed into yeast containing either pPD2 (−) or pPD2-16E2 (+). As a negative control, pPA104 (puc18-Ura), and a positive control, pRS316, were transformed into the same strains. Yeast were then plated onto YNB-Leu, –Ura media and allowed to grow for 4 days. Plates were then scanned and scored for growth.