University of Nebraska - Lincoln
DigitalCommons@University of Nebraska - Lincoln

Virology Papers | Virology, Nebraska Center for

May 2005

Identification of *cis*-Acting Elements That Mediate the Replication and Maintenance of Human Papillomavirus Type 16 Genomes in *Saccharomyces cerevisiae*

Kitai Kim
University of Wisconsin Medical School, Madison, Wisconsin

Peter C. Angeletti
University of Nebraska - Lincoln, pangeletti2@unl.edu

Elizabeth C. Hassebroek
University of Wisconsin Medical School, Madison, Wisconsin

Paul F. Lambert
University of Wisconsin Medical School, Madison, Wisconsin

Follow this and additional works at: http://digitalcommons.unl.edu/virologypub

 Part of the Virology Commons

Kim, Kitai; Angeletti, Peter C.; Hassebroek, Elizabeth C.; and Lambert, Paul F., "Identification of *cis*-Acting Elements That Mediate the Replication and Maintenance of Human Papillomavirus Type 16 Genomes in *Saccharomyces cerevisiae*" (2005). *Virology Papers*. 32.
http://digitalcommons.unl.edu/virologypub/32

This Article is brought to you for free and open access by the Virology, Nebraska Center for at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Virology Papers by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.

JOURNAL OF VIROLOGY, May 2005, p. 5933–5942 Vol. 79, No. 10
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.10.5933–5942.2005
Copyright © 2005, American Society for Microbiology. All Rights Reserved.

Identification of *cis*-Acting Elements That Mediate the Replication and Maintenance of Human Papillomavirus Type 16 Genomes in *Saccharomyces cerevisiae*

Kitai Kim,†‡ Peter C. Angeletti,†§ Elizabeth C. Hassebroek, and Paul F. Lambert*

McArdle Laboratory for Cancer Research, University of Wisconsin Medical School, Madison, Wisconsin

Received 10 October 2004/Accepted 14 January 2005

Papillomaviruses contain small double-stranded DNA genomes that are maintained in persistently infected mammalian host epithelia as nuclear plasmids and rely upon the host replication machinery for replication. Papillomaviruses encode a DNA helicase, E1, which can specifically bind to the viral genome and support DNA synthesis. Under some conditions in mammalian cells, E1 is not required for viral DNA synthesis, leading to the hypothesis that papillomavirus DNA can be replicated solely by the host replication machinery. This machinery is highly conserved among eukaryotes. We and others found that papillomavirus DNA could replicate in a simple eukaryote, *Saccharomyces cerevisiae*. Specifically, papillomavirus DNA could substitute for the function of the autonomously replicating sequence (ARS) and centromere (CEN) elements that are normally both required for the stable replication of extrachromosomal DNAs in yeast. Furthermore, this form of replication in yeast was E1 independent. In this study, we map the elements in the human papillomavirus type 16 (HPV16) genome that can substitute for yeast ARS and CEN elements. A single element, termed *rep*, was identified that can substitute for ARS, and multiple elements, termed *mtc*, could substitute for CEN. The location of one of these *mtc* elements overlaps the location of *rep*, and this approximately 1,000-bp region of HPV16 was sufficient to support stable replication of a bacterial-yeast shuttle plasmid deleted of both ARS and CEN elements.

Papillomaviruses contain small double-stranded circular DNA genomes that are replicated in the natural mammalian host epithelial cells as nuclear plasmids. Two viral gene products, E1 and E2, contribute to the replication of papillomaviral genomes via their recognition and function at the E1-dependent origin of replication, a *cis*-element that maps to the 3′ end of the long control region (LCR) in the viral genome (30, 31). E1, a DNA helicase that assembles as a hexamer, recruits DNA polymerase α and unwinds DNA at the E1-dependent origin of replication (E1 ori) (7, 20, 26, 31, 34). E1 has functional and structural homology to cellular DNA helicases, including Werner's and Bloom's syndrome gene products, and the minichromosome maintenance family of helicases (23). E2, a transcriptional transactivator, also contributes to viral DNA replication by interacting with E1 and increasing the efficiency of E1 binding to the E1 ori (33), a function that can be overcome at high levels of E1 protein (8, 27). In addition, E2 can contribute to the stable inheritance of papillomavirus DNA to daughter cells during cell division (24), likely by tethering the viral genomes to host chromosomes during mitosis (3, 16, 29). The replication of the viral genome is tightly controlled during the viral life cycle, and this control is tied to the differentiation of the host epithelial tissue, and the regulation of expression and function of E1 and E2 likely contribute to the control of viral DNA replication during the viral life cycle (1, 22, 36).

Prior studies of bovine papillomavirus type 1 first established the role of E1 in viral DNA replication. Specifically, E1 was found to be essential for the establishment of the viral genome as a nuclear plasmid in C127 cells (9, 25), a mouse cell line that is transformed by bovine papillomavirus type 1 (6) and supports the long-term maintenance of the bovine papillomavirus type 1 genome as a nuclear plasmid (15). Using a series of temperature-sensitive mutants of bovine papillomavirus type 1 E1 that had been generated previously (18), we confirmed the requirement for E1 in the establishment of the viral genome as a nuclear plasmid but made the surprising discovery that once the viral genome was established as a nuclear plasmid and had transformed C127 cells, E1 was no longer required (12). We also found that human papillomavirus (HPV) DNA genomes, while dependent upon E1 for their establishment as nuclear plasmids in their natural host cells (human keratinocytes), are able to replicate independently of E1 in certain highly transformed human epithelial cell lines, including a cervical cancer cell line (K.Kim and P. F. Lambert, unpublished observations). Thus, under certain conditions, the cellular replication machinery can substitute for the requirement for E1.

Because the eukaryotic cellular DNA replication machinery is highly conserved, we tested whether papillomaviral genomes could replicate in a simple eukaryote, *Saccharomyces cerevisiae*. We and others found that the genomes of multiple HPV genotypes (2) as well as bovine papillomavirus type 1 (37) can stably replicate as nuclear plasmids in yeast. This HPV plasmid

* Corresponding author. Mailing address: McArdle Laboratory for Cancer Research, University of Wisconsin-Madison, 1400 University Ave., Madison, WI 53706. Phone: 608-262-8533. Fax:. 608-262-2824. E-mail: Lambert@oncology.wisc.edu.

† Kitai Kim and Peter C. Angeletti contributed equally to this paper.

‡ Present address: Childrens Hospital, Harvard Medical School, New Research Building, 300 Longwood Ave., 7th floor, Boston, MA 02115.

§ Present address: Nebraska Center for Virology, E130 Beadle Center, University of Nebraska-Lincoln, 1901 Vine Street, Lincoln, NE 68588-0666.

DNA replication in yeast was not dependent upon any viral gene, including E1 and E2 (2). In those studies, papillomaviral sequences functionally substituted for yeast ARS and CEN elements. We therefore concluded that *cis*-acting elements present in the papillomaviral genomes are recognized by cellular factors that in turn support both the replication and maintenance of the viral genome as an extrachromosomal element in yeast.

In the present study, we map the *cis*-acting elements in the HPV16 genome that substitute for ARS and CEN in yeast. We define these elements as the replication (*rep*) and plasmid maintenance (*mtc*) elements. For these studies, we generated plasmid libraries containing segments of the HPV16 genome inserted into bacterial/yeast shuttle vectors in which the yeast ARS and/or CEN elements were deleted. Segments of the viral genome that could substitute for these yeast *cis*-acting elements were selected by virtue of their ability to confer stable plasmid replication in yeast. Recombinant ARS^-/CEN^+, ARS^-/CEN^+ and ARS^-/CEN^- plasmids containing segments of HPV16 that could replicate stably in yeast were recovered by transformation back into bacteria and sequenced to identify the HPV sequences contained within them. Using this assay, we mapped a single *rep* element to an approximately 1,000-bp region overlapping the 3′ end of the L1 translational open reading frame and the 5′ end of the long control region (LCR). Using an independent assay for identifying plasmid maintenance elements, we identified two distinct regions of the viral genome to efficiently confer *mtc* function. One of these *mtc* elements overlaps the *rep* element described above; this region of HPV16 substituted for both ARS and CEN elements in yeast (i.e., conferred stable plasmid replication on an ARS^-/CEN^- plasmid vector). The *rep* and *mtc* elements functioned in the absence of E1 or E2 protein, confirming that an E1- and E2-independent mode of plasmid replication and maintenance is functional in yeast. The biological significance of these studies with regard to the papillomavirus life cycle is discussed.

MATERIALS AND METHODS

Yeast strains, yeast plasmid isolation, and transformation methods. The haploid yeast strain YPH500 (*MAT ura3-52 lys2-801 ade2-101 trp1-63 his3-200 leu2-1*) was a gift from Paul Ahlquist (University of Wisconsin–Madison) (28) and was used in the experiments. Yeasts were grown on minimal medium omitting tryptophan (tryptophan) or uracil (uracil), as needed, to select for plasmids. The EZ yeast transformation kit (Zymo Research, Orange, Calif.) was used for transformation of yeast, and the Zymoprep kit (Zymo Research, Orange, Calif.) was used for yeast plasmid minipreps according to company specifications.

Plasmids and library constructions. To generate a plasmid library with which to screen for *rep* elements in HPV16, we first created a bacterial/yeast shuttle vector that was deleted specifically for the yeast ARS element. pΔYac (14), which contains the yeast *TRP1* gene, an ARS, and a CEN element, was digested with StuI and BsaBI to cut out the DNA segment containing the ARS element, and the remaining plasmid DNA fragment was religated. The resulting ARS^-/CEN^+ plasmid was named pKT330. To generate a library of pKT330 derivatives containing subgenomic fragments of HPV16, 20 μg pEF399 (a derivative of pUC19 containing the entire HPV16 genome cloned into the unique BamHI site) was diluted into a total volume of 270 μl, containing 5 units of NlaIII and 1X NlaIII restriction enzyme buffer, and incubated at 37°C for 0, 1, 2, 4, 8, 16, 32, 60, and 120 min to partially digest the DNA. Restriction enzyme digestion was stopped by adding EDTA to a final concentration of 50 mM and heating the samples to 65°C for 15 min. The NlaIII partially digested DNA was size selected for fragments between 0.5 kb and 4 kb on a 1% low-melting agarose gel. The eluted DNA fragments were cloned into pKT330 that was linearized at the unique SphI site to generate ends compatible to NlaIII sites. The ligated DNAs were transformed into *Escherichia coli.* The size of the resulting plasmid library was estimated as 1×10^4 by subtraction of the estimated number of transformants that contained self-ligated plasmids (assessed by miniprep analysis). Sequence analysis of multiple independent clones verified that the vast majority of plasmids in this library had no more than one insert, as evidenced by the paucity of clones with two noncontiguous HPV16 sequences present (data not shown).

To generate a plasmid library with which to screen for *mtc* elements, Sau3AI fragments of pEF399 were cloned into BglII-cleaved, phosphatase-treated pPA94 (2), a bacterial/yeast shuttle vector that contains the yeast *TRP1* gene and a yeast ARS element but no CEN element. The ligated DNAs were transformed into *E. coli* to construct the library. The size of the library was estimated at 1.5×10^3 and was found to contain primarily plasmids with no more than one Sau3AI-generated fragment of HPV16 inserted into it. The library also contained the original plasmid pPA94 vector without any insert (data not shown; presumably arising due to incomplete phosphatase treatment); this fortuitously provided an internal negative control for assessing the robustness of our *mtc* selection scheme (see Results).

To generate a plasmid library with which to screen simultaneously for both *rep* and *mtc* elements, pKT275 (2), which contains the yeast *URA* gene, an HPV16 LCR, and a yeast CEN element, was digested with XmaI to cut out the HPV16 LCR and the yeast CEN element, and the remaining plasmid DNA fragment was religated to create the bacterial/yeast shuttle vector pBH104, which does not contain either an ARS or CEN element. The size selected, NlaIII partially digested, pEF399 DNA that was described above was then ligated into SphI-linearized pBH104 to generate the plasmid library. This plasmid library, in addition to containing clones with one insert, also contained many clones with more than one fragment inserted into it, as judged by the presence of noncontiguous HPV16 sequences within individual clones (see Results).

Selection of the library in yeast. Two hundred ng of the bacterially amplified HPV16 plasmid libraries generated using the pKK330, pPA94, or pBH104 vectors were transformed into the yeast strain YPH500, and growth of transformed cells was selected for on plates minus tryptophan or uracil. As a control, the plasmid library vector without insert (negative control) and pΔYAC (positive control, ARS^+/CEN^+) were transformed independently. For the pKK330 and pBH104 libraries, yeast colonies that grew on the selection medium were purified by streak culture on the solid agar plate. Low-molecular-weight DNAs were isolated from the individual yeast transformants and transformed into *E. coli.* Plasmid DNAs were isolated from *E. coli*, and sequenced to learn the region of HPV16 in the insert.

The size of plasmid DNAs were also determined by electrophoresis on an agarose gel. For the pPA94-based library, the colonies of transformed yeast that grew on tryptophan-minus plates were pooled and cultured en masse in the absence of selection (in defined medium including tryptophan) for 30 cell generations. Low-molecular-weight DNAs was isolated from this yeast culture and plasmids were rescued back into *E. coli* by transformation and selection on ampicillin. Plasmid DNAs were isolated from *E. coli*, confirming the plasmid replication competence of the individual clones in yeast, and sequenced to learn the region of HPV16 in the insert. The sizes of plasmid DNAs were also determined by agarose gel electrophoresis.

DNA replication assays in yeast. The pKT330:rep plasmid containing the predicted minimal NlaIII fragment that can substitute for the yeast ARS was transformed back into yeast strain YPH500. Transformed cells were grown in continuous culture on solid medium for more than 3 weeks prior to analysis. DNA was isolated from liquid cultures as described above. The DNA samples were digested with DpnI for 24 h and loaded onto a 0.8% agarose gel. The DNA was electrophoresed, transferred to nitrocellulose, and probed with ^{32}P-radiolabeled pKT330. Radiolabeling was achieved by use of the Rediprime kit (Amersham, Piscataway, N.J.) in accordance with the manufacturer's instructions. The replicated DNAs were visualized and quantified by use of a PhosphorImager (Molecular Dynamics, San Jose, Calif.).

Plasmid stability assay. The plasmid stability experiment was performed as described by Angeletti et al. (2). Yeast colonies harboring episomal copies of pΔYac (ARS^+ CEN^+), pPA94 (ARS^+ CEN^-), pPA95 (ARS^+ CEN^-: HPV16), pPA94:mtc1, pPA94:mtc2, or pPA94:mtc3 were analyzed for plasmid stability in the absence of nutritional selection. First cultures were grown in selective medium to mid-log phase and diluted to an optical density at 600 nm of 0.1 into nonselective medium (with tryptophan). Cultures were grown from 0 to 10 cell generations. Cultures at either 0 or 10 generations were diluted to an optical density at 600 nm of 0.1 and further serially diluted by 10-fold dilutions. Five-μl aliquots of each dilution were spotted to selective (no tryptophan) and nonselective (with tryptophan) medium.

In order to determine the percentage of cells retaining plasmid, equal amounts of an appropriate dilution of the cultures were plated on selective and nonselective medium and counted after 3 days of growth. The percent of cells retaining

plasmid was given by the ratio of the number of colonies present on selective versus nonselective medium. The rate of plasmid loss was calculated, as described by Kramer et al. (13), by use of the following formula: $X = [1 - (F/I)^{1/N}][100]$, where X is the percent plasmid loss per cell generation. I is the initial percent plasmid containing cells under selective growth. F is the percent plasmid containing cells after growth in nonselective medium. N is the number of cell generations in the absence of selection. The rates of loss per cell generation were calculated from triplicate data sets.

Sequence Analysis. The HPV16 genome was analyzed for the presence of ARS (5′-TTTAT[A/G]TTT[A/T]-3′) and CEN sequences, CDE I (5′-[G/A]CAC[G/A]TG-3′) and CDE III (5′-[T/A]TG[T/A]TTTCC-3′). Sequences which deviated from the consensus by at most one nucleotide were determined using the Fuzznuc program (http://bioweb.pasteur.fr/seqanal/interfaces/fuzznuc.html). Further analysis for potential ARS-likes sequences was performed using a scanning algorithm (Oriscan), which compares the query sequences with ARS elements of known function and ranking (4).

RESULTS

Mapping of *rep*. Our prior studies had indicated that the HPV16 genome contains one or more *cis*-acting *rep* elements that can substitute for a yeast ARS element, permitting the replication of the viral genome in *S. cerevisiae* and in the absence of E1 protein (2). To map the *rep* element(s) in the HPV16 genome, we generated a plasmid library containing subgenomic fragments of the HPV16 genome cloned into a bacterial/yeast shuttle vector, pKT330, containing a yeast CEN element but lacking a yeast ARS element. pKT330 is a derivative of pΔYAC and contains the *TRP1* biosynthetic gene that confers growth on a tryptophan-auxotrophic strains of yeast cultured in tryptophan-minus medium. The subgenomic fragments of HPV16 DNA used in making this plasmid library were generated by partial digestion with restriction enzyme NlaIII, for which there are 28 sites distributed across the viral genome (Fig. 1A).

The partially digested HPV16 DNA was size selected for fragments between 0.5 kb and 4 kb, and these fragments were cloned into pKT330 linearized with SphI to generate compatible ends. As such, all portions of the viral genome are represented by overlapping fragments, with the exception of the region spanning the unique BamHI restriction enzyme site in HPV16, which was used originally to clone it into the bacterial vector. The ligated DNAs were transformed into *E. coli* and amplified. The amplified plasmid library DNA was then transformed into the yeast strain YPH500, and stable transformants were selected for growth on tryptophan-minus medium. As controls, pKT330 without an insert (negative control: ARS^-/CEN^+) and pΔYAC (positive control: ARS^+/CEN^+) were transformed into yeast in parallel.

As expected, the negative control plasmid failed to give rise to transformants, while the positive control allowed efficient colony formation. The yeast cells transformed with the library DNA formed colonies efficiently (more than 200 transformants per agar plate). Individual yeast colonies from the transformants were purified by streaking cultures on selective agar medium. Low-molecular-weight DNAs were isolated from the individual yeast transformants and transformed back into *E. coli*, which was then selected on ampicillin to identify colonies harboring the bacterial shuttle vector. By this means, only those plasmid DNAs replicating extrachromosomally in the yeast transformants would be rescued back into bacteria.

The rescued plasmid DNAs were isolated from *E. coli*, the competence of the individual clones to replicate as plasmids in yeast was retested, and the positive clones were sequenced to identify the region of HPV16 present. Figure 1B summarizes the data obtained from this analysis. The original vector-minus insert, pKK330, which was represented in the library owing to self-ligation (data not shown), was not recovered in bacteria, consistent with its inability to confer growth on YPH500 in the absence of tryptophan and verifying that we selected against plasmids that did not contain a *rep* function.

All plasmid clones that could replicate in yeast and were rescued back in *E. coli* contained NlaIII restriction enzyme fragments of HPV16 that were overlapping. The region common to all replication-competent plasmids clones, and therefore containing the *rep* element, was from HPV16 nucleotides 6732 to 7793. This region includes the 3′ portion of the L1 open reading frame (ORF) that encodes the major capsid protein and the 5′ half of the LCR but does not contain *E1 ori*, which is located at the 3′ end of the LCR. No other region of HPV16 was identified to contain a *rep* element based on this assay. The library was generated by partial digestion with NlaIII in order to eliminate the possibility that a *rep* element might be disrupted by cleavage at any particular NlaIII site. Given the evidence that most clones did contain partially digested HPV16 DNA (Fig. 1B), we conclude that no other *rep* element exists within this viral genome.

HPV16 nucleotides 6732 to 7793 contains a functional rep element that efficiently substitutes for a yeast ARS element. The derivative of the ARS^-/CEN^+ pKT330 plasmid containing the minimal overlapping region of HPV16 (nucleotides 6732 to 7793) identified in Fig. 1B, named pKT330:rep, was transformed back into yeast strain YPH500 to confirm that this minimal region confers plasmid replication in yeast and to assess its efficiency of function. The transformation efficiency of pKT330:rep (more than 200 transformants per plate) was similar to that observed with pΔYAC, the parent to pKT330, which contains a functional yeast ARS element. In contrast, pKT330, lacking both the yeast ARS and HPV16 sequences, failed to transform yeast.

To verify that pKT330:rep can support long-term extrachromosomal DNA replication, transformed cells were grown in continuous culture for more than approximately 230 cell generations prior to further analysis, allowing extensive dilution of any input bacterially synthesized DNA. Low-molecular-weight DNAs were isolated from these cultured yeast transformants, digested with DpnI to cleave selectively any remaining input bacterially synthesized plasmid, and subjected to Southern analysis using pKT330 as the probe (Fig. 2). Ten independent transformants were analyzed; all contained extrachromosomal forms of pKT330:rep. This experiment confirmed that a *rep* element lies within HPV16 (nucleotides 6732 to 7793) and that this element confers plasmid replication in yeast with efficiency similar to that seen with a yeast ARS.

Mapping *mtc* elements in HPV16. In our prior studies, we discovered that the HPV16 genome contains one or more *cis*-acting elements (defined here as maintenance or *mtc* elements) that could functionally substitute for a yeast CEN element and that the *mtc* element(s) function in the absence of any viral *trans*-acting factor (2). To identify the *mtc* elements in HPV16, we generated a library of plasmids in which subgenomic fragments of the HPV16 genome, generated by digestion with Sau3AI, were introduced into pPA94, a bacterial/

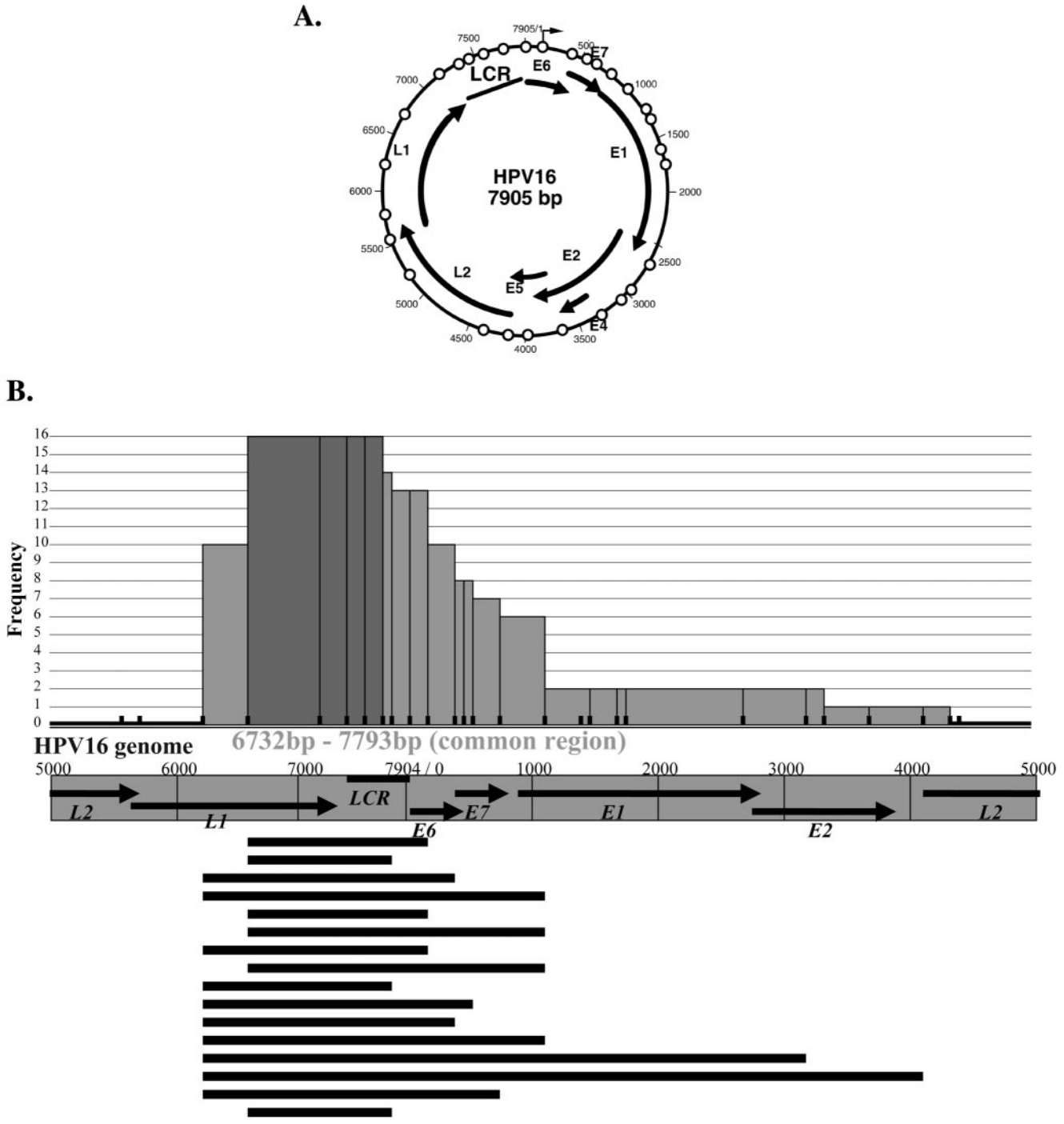

FIG. 1. Mapping of *rep* elements in HPV16 genome. (A) Shown are the locations of restriction enzyme sites for NlaIII (small circles) in the HPV16 genome. (B) Shown is the HPV16 genome linearized at nucleotide 5000. Arrows indicate the positions of the translational open reading frames, and the bar indicates the location of the LCR. Shown below are the subgenomic HPV16 fragments, identified from individual clones. The histogram above shows the frequency of recovery of each of the subgenomic HPV16 fragments which contain *rep* function. The darker area indicates the minimal overlapping region. Hatchmarks and vertical lines indicate the positions of NlaIII sites.

yeast shuttle vector containing a yeast ARS element and the *TRP1* gene but lacking a yeast CEN element.

Sau3AI cleaves the HPV16 genome at 11 sites (Fig. 3A). The complexity of the library is evident based upon the ethidium bromide staining pattern of the plasmid DNAs isolated from pools of *E. coli* that had been transformed with the plasmid library (Fig. 3B). Also highly represented in the plasmid library was the parental pPA94 vector that had self-ligated onto itself without an insert. The amplified plasmid library was transformed into the yeast strain YPH500 and grown under

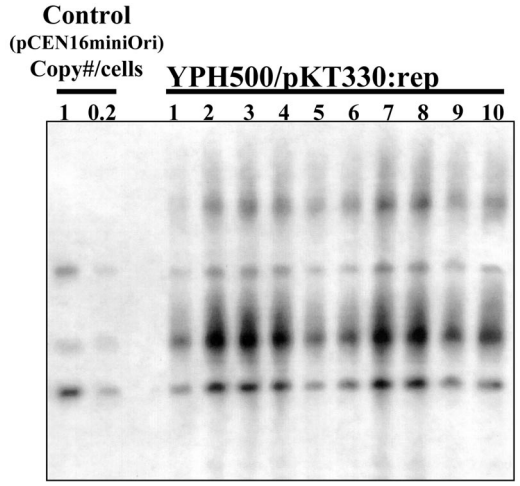

FIG. 2. Plasmid replication of the ARS$^-$/CEN$^+$ plasmid containing the minimal overlapping fragment of HPV16 harboring *rep* function. Shown is the Southern analysis of low-molecular-weight DNAs from ten independent colonies of yeast transformed with pKT330:rep. Low-molecular-weight DNAs were isolated and digested with DpnI for 24 h. Southern analysis was performed using pKT330 as a probe.

selection on tryptophan-minus plates. In order to select plasmids that have *mtc* elements and therefore are stably inherited during cell division, these transformed yeast cells that grew on tryptophan-minus plates were pooled and grown in liquid culture in the absence of nutritional selection for 30 cell generations. Low-molecular-weight DNA was isolated from the resulting yeast population and transformed into *E. coli*, and the plasmid DNAs present in individual, ampicillin-resistant bacterial colonies were characterized.

Using this approach, only extrachromosomally replicating plasmid DNAs that were efficiently retained in yeast in the absence of selection for at least thirty cell generations could be recovered in *E. coli*. To assess whether this selection scheme was sufficiently robust to select for plasmids that harbor *mtc* elements, we first monitored the frequency of recovery of the ARS$^+$/CEN$^-$ parental vector pPA94 from the plasmid library, which was highly represented in the library due to self-ligation (see above). Of the plasmids rescued in bacteria from the yeast that had been outgrown in the absence of selection for 30 cell generations, none (0 out of 13) of the characterized clones were pPA94 itself (i.e., all 13 clones contained HPV16 sequences). In contrast, pPA94 was readily rescued from the pooled yeast transformants that had been outgrown in the absence of selection for only 10 cell generations at a frequency of one out of three clones. This result proved to us that the selection process that included outgrowth for at least 30 cell generations was robust and is consistent with other reports monitoring the rate of loss of ARS$^+$/CEN$^-$ plasmids in *S. cerevisiae* (11, 17, 35).

To identify the inserts present in the plasmids rescued from yeast following outgrowth for 30 cell generations in the absence of selection, we sequenced the HPV16 inserts. Among the 13 clones sequenced, three of the eleven Sau3AI restriction fragments of HPV16 (nucleotides 871 to 3480, nucleotides 4538 to 5072, and nucleotides 6151 to 6951) were identified to support long-term maintenance of the ARS$^+$/CEN$^-$ plasmid vector pPA94. Therefore, we conclude that three noncontiguous regions of the HPV16 genome contain *mtc* elements. We named these elements *mtc1*, *mtc2*, and *mtc3* (see Fig. 3B). Two of the Sau3AI restriction fragments (nucleotides 4538 to 5072 overlapping the L2 ORF, and nucleotides 6151 to 6951 overlapping the L1 ORF) were recovered in multiple ($n = 7$ and 5, respectively) independent clones, whereas one (nucleotides 871 to 3480, overlapping the E1 ORF) was recovered in only one clone (Fig. 3B). We interpret these findings to indicate that the HPV16 genome contains at least two efficient *mtc* elements (*mtc2* and *mtc3*), which are located within the late region of the viral genome, with possibly a third, less efficient *mtc* element (*mtc1*) located within the early region. Interestingly, the *mtc3* element maps to a region that partially overlaps the region of HPV16 to which we mapped the *rep* element in the pKT330 library (Fig. 1B).

Three HPV16 *mtc* elements are distinguished by their effect on plasmid copy number. A hallmark of yeast CEN elements is their effect on plasmid copy number. Plasmids containing CEN elements are maintained at levels of one copy per cell (5, 10). In contrast, removal of CEN elements, while causing decreased plasmid stability in the absence of selection, leads to increased plasmid copy number as long as selection for retention of the plasmid is maintained (21). This phenomenon was recapitulated in our hands (Fig. 4, lanes labeled pΔYac versus pPA94). In our prior studies, we noted that the HPV16 genome was maintained as a plasmid in yeast at a low copy number, similar to that seen with yeast vectors containing CEN elements (2). This is likewise seen in the present study (Fig. 4, lane pPA95).

Interestingly, the capacity of the HPV16 *mtc* elements identified in the above selection scheme to affect plasmid copy number varied. Whereas *mtc3* and to a lesser degree *mtc2* elements could lead to low plasmid copy number, *mtc1* showed little capacity to suppress copy number, having copy numbers similar to that seen with the ARS$^+$/CEN$^-$ vector itself (Fig. 4). Thus, we hypothesize that *mtc3* and perhaps *mtc2* are responsible for the low plasmid copy number of the HPV16 genome in yeast.

Each of the three *mtc* elements rescued the stability of a plasmid lacking a centromere to various extents, as indicated by the restored colony growth even after growth in the absence of selection (Fig. 5). Whereas pΔYac (ARS$^+$ CEN$^+$) had a modest loss rate in our hands of 1.8 ± 0.2% per cell generation, pPA94 (ARS$^+$ CEN$^-$), lacking a centromere, resulted in a plasmid loss rate of 15.8 ± 2.7% per cell generation. The addition of HPV16 fragments lowered the loss rate of pPA94 as follows: *mtc1*: 3.9 ± 2.7% per cell generation; *mtc2*: 2.1 ± 0.6% per cell generation; and *mtc3*: 2.0 ± 0.9% per cell generation.

A 1-kbp region of HPV16 contains both *rep* and *mtc* elements. The two plasmid libraries described above allowed us to map regions of the HPV16 genome that contain either *rep* or *mtc* elements. We also generated a third plasmid library to identify the minimal region of the HPV16 genome that contains both *rep* and *mtc* function. For this purpose, we constructed pBH104, a bacterial/yeast shuttle vector containing the *URA3* gene (selective marker) but lacking both yeast ARS and CEN elements. Into this vector linearized with SphI, we ligated 0.5- to 4.0-kbp size-selected, subgenomic fragments of

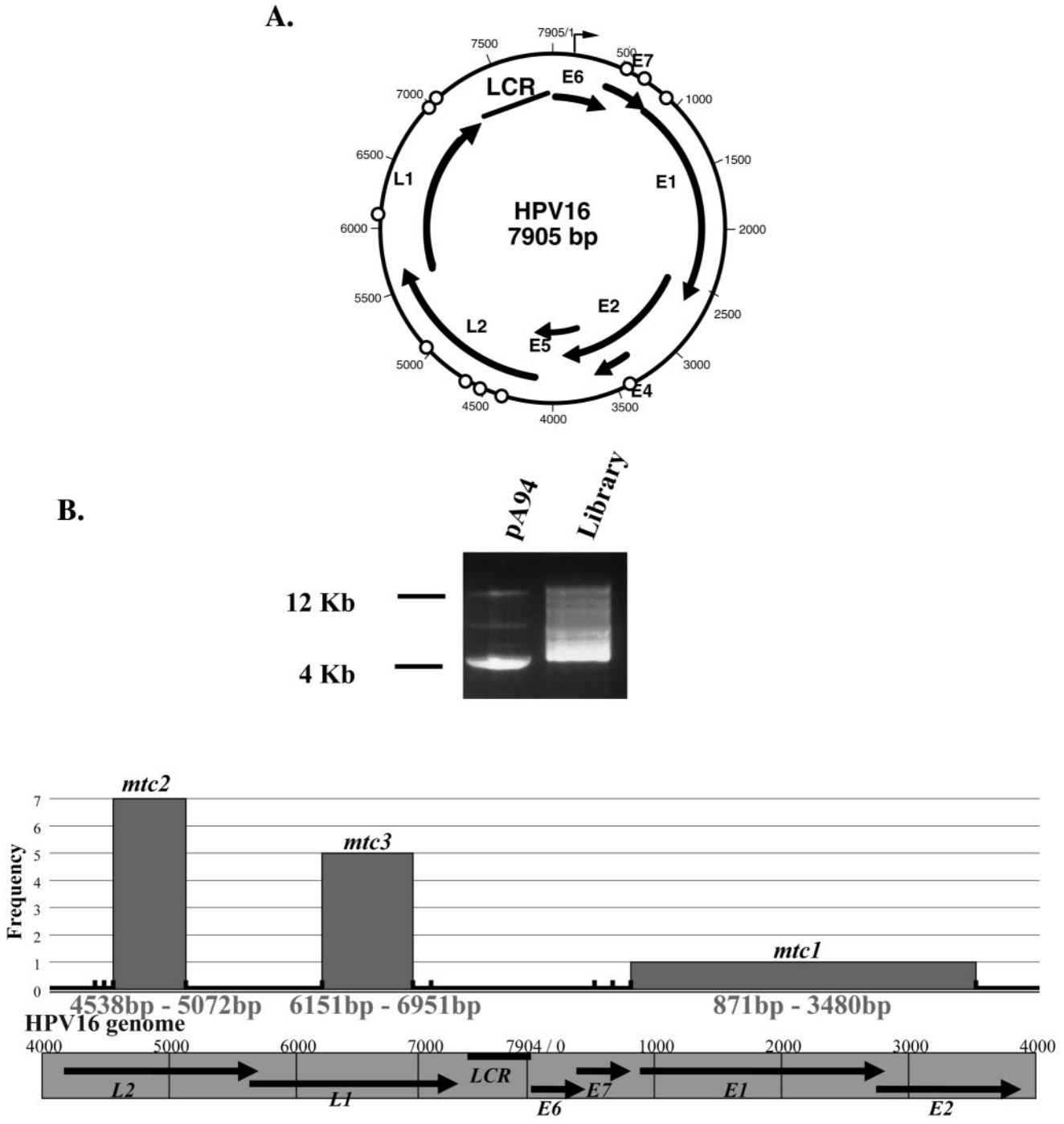

FIG. 3. Mapping of *mtc* elements in HPV16. (A) Shown are the locations of restriction sites for Sau3AI (circles) in the HPV16 genome. (B) Shown at the top is a picture of an ethidium bromide-stained agarose gel on which are plasmid DNAs isolated from bacteria transformed with pPA94 or the plasmid library containing Sau3AI fragments of HPV16 inserted into pPA94. Shown at the bottom is the HPV16 genome linearized at nucleotide 4000. Arrows indicate the positions of the translational open reading frames, and the bar indicates the location of the LCR. The histogram above shows the frequency of recovery of each of the subgenomic HPV16 fragments which contain *mtc* function. The individual designations of three *mtcs* are provided above each bar. Hatchmarks indicate the positions of Sau3AI sites.

HPV16 that had been generated by partial digestion with NlaIII. The ligated DNAs were transformed into *E. coli* and amplified to generate an amplified library of plasmids. This amplified library was then transformed into the yeast strain YPH500 and grown under selection on uracil-minus plates and subcultured more than three weeks prior to analysis. Low-molecular-weight DNAs were isolated from the individual yeast transformants and used to transform *E. coli*, which was

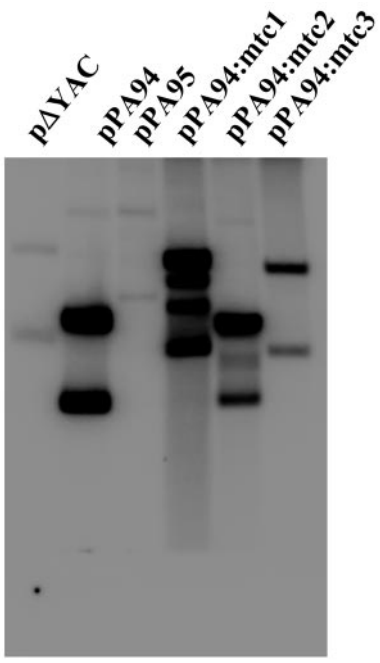

FIG. 4. Comparison of copy numbers of various plasmid replicons in yeast. Shown is a Southern analysis of low-molecular-weight DNAs isolated from yeast transformed with pΔYAC (lane 1, ARS^+/CEN^+), pPA94 (lane 2, ARS^+/CEN^-), pPA95 (lane 3, full-length HPV16 genome in pPA94), pPA94:mtc1 (lane 4), pPA94:mtc2 (lane 5), and pPA94:mtc3 (lane 6). The Southern blot was probed with radioactively labeled pPA94.

then selected on ampicillin to identify colonies harboring the bacterial shuttle vector.

Plasmid DNAs were isolated from *E. coli*, confirming the plasmid replication competence of the individual clones in yeast, and sequenced to learn the region of the HPV16 insert. The sizes of plasmid DNAs were also determined on agarose gels. In contrast to the prior libraries used in this study, the majority of the clones contained multiple, noncontiguous NlaIII fragments, owing to suboptimal ratios of insert to vector used in the ligation. Consequently, only those clones that had single inserts as judged by the presence of contiguous HPV16 sequence were analyzed further. Interestingly, the four single-insert clones that were rescued back into bacteria all contained single NlaIII subgenomic fragments from the same overlapping region of the HPV16 genome (Fig. 6). We conclude that the common region, from HPV16 nucleotides 6732 to 7793, contains both *rep* and *mtc* elements. This coincides exactly with the region that we had identified to contain the *rep* element (Fig. 1B) and to overlap a region containing one of the more efficient *mtc* elements, *mtc3* (Fig. 3B).

Analysis of potential ARS elements. ARS-like sequences in HPV16 were identified using two different approaches. First, an ARS-consensus analysis using a web-based program, fuzznuc, was performed. This analysis predicts ARS-like sequences but does not predict their functionality. Using this method, a total of eight ARS-like sequences were identified throughout the genome which were at most 1 nucleotide divergent from the consensus (Fig. 7A and 7B). A comparison of these sequences with a previous detailed mutagenic ARS analysis (32) revealed that only one of the eight ARS elements (nucleotides 1640 to 1650) is predicted to have any function, and that being at most twenty-five percent of wild-type function (Fig. 7A).

A separate analysis was performed using the Oriscan software, which predicts ARS function. This approach identified only two detectable ARS-like elements (nucleotides 1639 to 1655, ATGTTTTACATTATGTC, and nucleotides 3736 to 3752, ATTGTTTATATTTACAC), both of which had probability scores less than 0.1, indicating that they are very unlikely to be functional. The identification of the potential ARS element between nucleotides 1639 and 1655 was consistent between the two approaches, and the predicted low level of activity is supported by the previous mutagenic evidence (32).

DISCUSSION

In this study we made use of powerful selection schemes to identify *cis* elements in the human papillomavirus type 16 genome that support its stable plasmid replication in yeast. A single element within the HPV16 genome, termed the *rep* element, that could substitute for a yeast ARS was located to a 1,000-bp region overlapping the 3′ end of the L1 ORF and the 5′ portion of the LCR. This *rep* element is positioned close to but separable from the E1-dependent replication origin, *E1 ori*, which is positioned at the very 3′ end of the LCR. Consistent with this fact, the *rep* element substituted for a yeast ARS in the absence of any HPV genes, including E1, indicating that, like the full-length genome (2), this element supports plasmid replication in yeast in the absence of the viral E1 DNA helicase.

In a separate study, in which we investigated the capacity of HPV16 DNA to replicate in highly transformed human cell lines independently of E1, a subgenomic DNA fragment that closely overlaps this region of the HPV16 genome replicates in short-term replication assays (K. Kim and P. F. Lambert, unpublished observations). This raises the possibility that sequences within this region of the viral genome can function to support replication in many types of eukaryotic cells. Further studies are necessary to learn whether the same, different, or partially overlapping sets of DNA sequences within this region contribute to replication in both yeast and mammalian cells.

Three separable regions within the HPV16 genome were found to substitute for a yeast CEN element. Two of these elements, *mtc2* and *mtc3*, were identified in multiple independent clones that were rescued from yeast following outgrowth, indicating that they are efficient in supporting stable plasmid inheritance; whereas *mtc1* was represented only once among the rescued clones. As predicted, each of the *mtc* elements restored plasmid stability to a plasmid lacking a centromere (Fig. 5). *mtc3* and less so, *mtc2*, caused the ARS^+CEN^- shuttle vector to replicate at a low copy number in yeast, similar to that seen with yeast plasmids containing a CEN element, as well as with the intact HPV16 genome in yeast. It is not yet clear what sequence elements in *mtc2* and *mtc3* might confer this property or whether it has relevance to low-copy replication observed in human cells.

Because *rep* and *mtc* elements can substitute for ARS and CEN elements, we analyzed the HPV16 DNA sequence for sequence motifs that are thought to contribute to the function

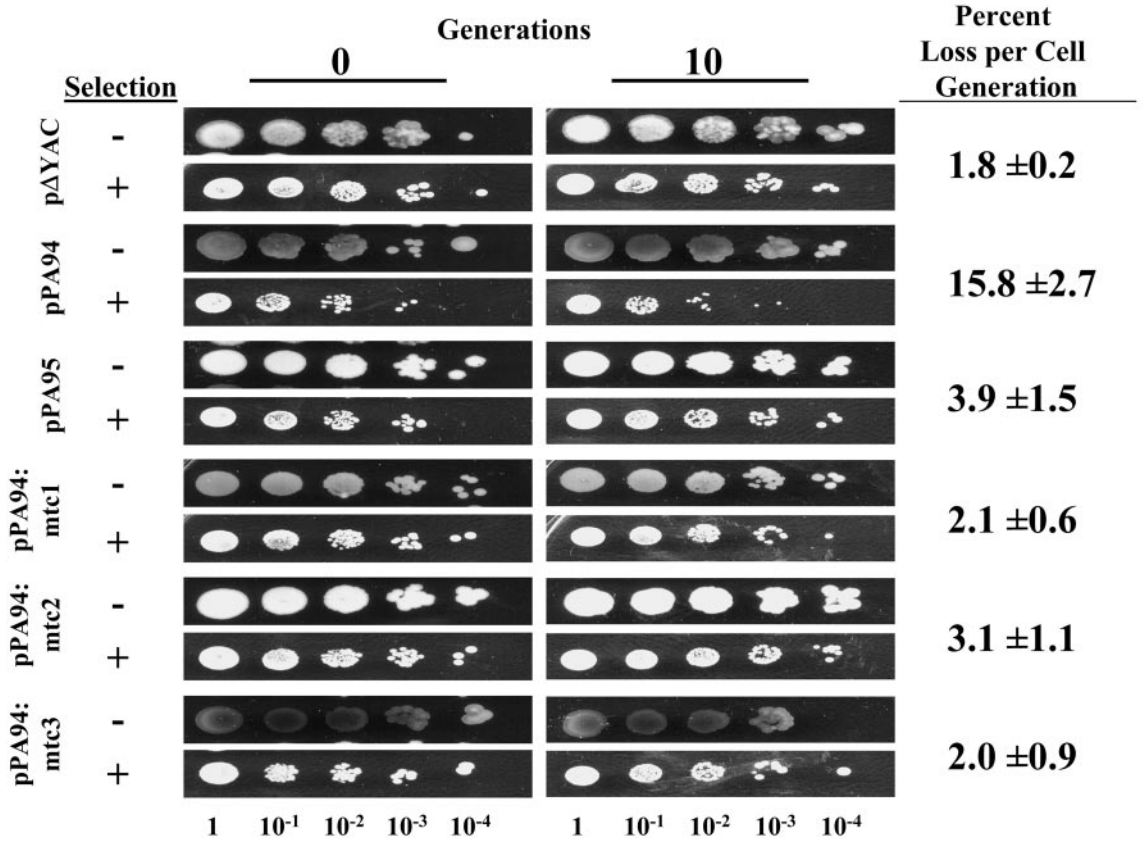

FIG. 5. Plasmid stability. Yeast cells harboring episomal copies of pΔYac (ARS^+ CEN^+), pPA94 (ARS^+ CEN^-), pPA95 (ARS^+ CEN^-: HPV16), pPA94:mtc1, pPA94:mtc2, or pPA94:mtc3 were grown in the absence of nutritional selection to determine their mitotic stability. Cultures were initially grown in selective medium to mid-log phase and then diluted to an optical density at 600 nm of 0.1 into nonselective medium (with tryptophan). These cultures were grown from 0 to 10 cell generations and then diluted to an optical density at 600 nm of 0.1 and further serially diluted from 1 to 10^{-5} as indicated. Five-μl aliquots of each dilution were spotted to selective (+) and nonselective (−) medium. The result is a visual comparison which indicates the degree of plasmid stability. In order to determine the percentage of cells retaining the plasmid, equal amounts of an appropriate dilution of the cultures were plated on selective and nonselective medium, and colonies were counted after 3 days of growth. The average rate of loss per cell generation for each plasmid was calculated in triplicate and is indicated in the rightmost column.

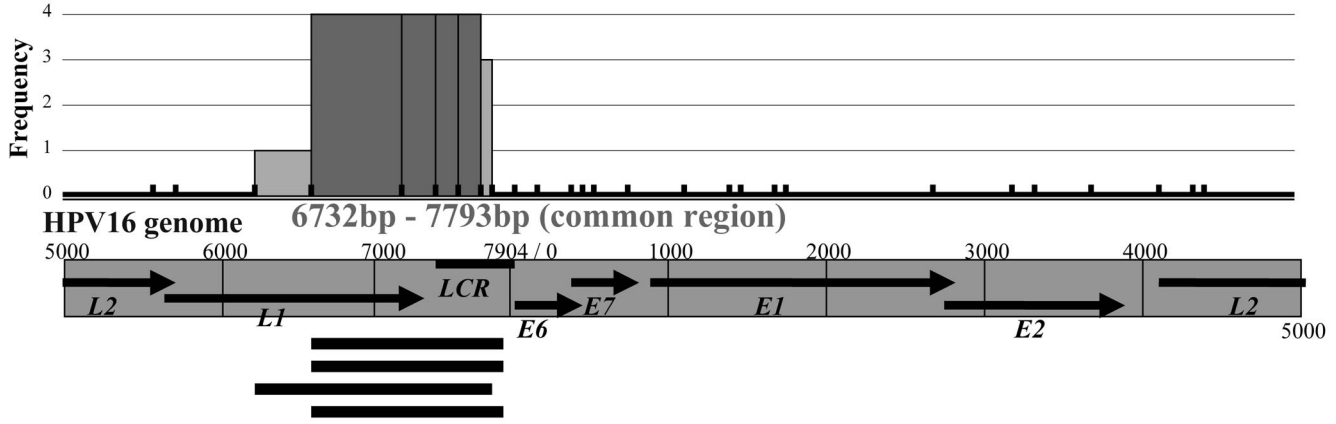

FIG. 6. Identification of regions of HPV16 containing both *rep* and *mtc* function. Shown is the HPV16 genome linearized at nucleotide 5000. Arrows indicate the positions of the translational open reading frames, and the bar indicates the location of the LCR. Shown below are the subgenomic HPV16 fragments identified from individual clones (see text). The histogram above shows the frequency of recovery of each of the subgenomic HPV16 fragments which contain *rep* and *mtc* functions. The darker area indicates the minimal overlapping region. Hatchmarks and vertical lines indicate the positions of NlaIII sites.

A.

CDE I-like elements in HPV16		CDE III-like elements in HPV16	
Consensus:	RCACRTG	Consensus:	WTGWTTTCC
nt 963-969	TCAGATG	nt 5885-5894	TGGTTTTCC
1062-1068	GCACATG *	6506-6515	TTATTTTCC
2856-2862	ACACATG *	7563-7572	CTGTTTTCC
5290-5296	TCACATG		
5589-5595	TTACATG		

ARS CONSENSUS:	WTTTATRTTTW	PREDICTED FUNCTIONALITY
nt 249-259	ATTTATGTATA	-
1640-1650	GTTTATATTTA	+/-
2190-2200	TGTTATGTTTT	-
3838-3848	ATTTATGTCTA	-
4165-4175	TTTTTTATTTT	-
4177-4187	ATATATATTTT	-
6376-6386	TTTTTTATTTA	-
7402-7412	TTTTATATATA	-

B.

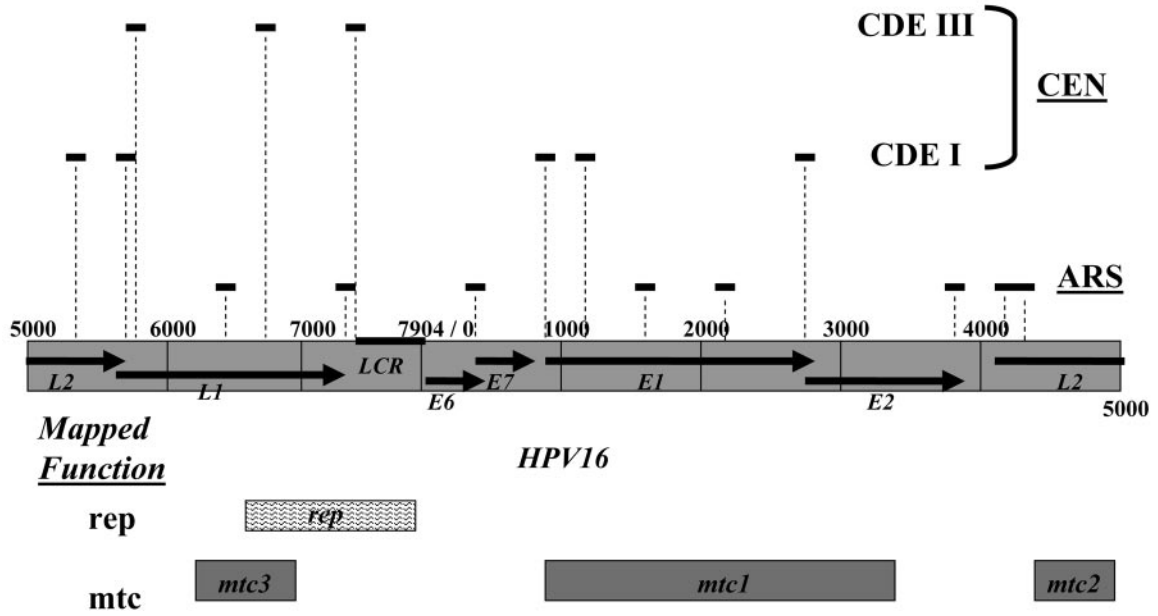

FIG. 7. Identification of sequences in HPV16 bearing homology or similarity to ARS and CEN elements. (A) Shown are the sequences of the CDE I and CDE III and ARS-like elements in the HPV16 genome. The nucleotide positions of these sequences are indicated to the left. Mismatches with the consensus sequences are underlined. Perfect matches are indicated with an asterisk. W = A or T, R = A or G. (B) Shown is the HPV16 genome linearized at nucleotide 5000. Arrows indicate the positions of the translational open reading frames, and the bar indicates the location of the LCR. Shown below are the subgenomic HPV16 fragments containing *rep* or *mtc* elements. Shown above are the approximate locations of ARS and CEN-like sequences in the HPV16 genome that were listed in part A.

of ARS and CEN elements. Eight imperfect matches to the ARS consensus sequence, [A/T]TTTAT[A/G]TTT[A/T], were identified; each was mispaired by one nucleotide from the consensus (Fig. 7A). Only the ARS-like sequence within the E1 ORF (nucleotides 1640 to 1650) was predicted to have any activity, that being extremely weak compared to ARS consensus variants (32) (Fig. 7A). The Oriscan results concurred that none of the identified ARS-like elements could be predicted to have significant activity. Further confirmation was that the functional ARS screens (Fig. 1 and 6) only recovered the region spanning the L1 ORF (nucleotides 6732 to 7793) and not the weak ARS predicted in the E1 ORF (nucleotides 1639 to 1655). Thus, sequence similarity between *rep* and ARS elements is insufficient to predict the function of *rep*.

In yeast, not only ARS element clusters but also DNA unwinding elements and AT-rich sequences influence the efficiency of replication (19). Therefore, a reasonable interpretation is that the region of the viral genome that contains the *rep*

element provides replicative function owing to the presence of additional undefined sequence elements. Further dissection of the *rep* element will be required to identify those relevant DNA sequences and assess of their potential role in the viral life cycle. One possibility is that the replicative function residing within the L1 ORF (nucleotides 6732 to 7793) provides low-copy replication and functions in an adjunct fashion to the E1-dependent origin, which resides at the 3′ end of the LCR. It has been argued that papillomaviruses can exist in a latent infective state. Under such conditions, one might predict that the E1-independent origin could provide the virus with an ability to persist as a nuclear plasmid in the absence of viral gene expression. Testing for such a role would require a laboratory model for latency, which today does not exist.

The positions of the *mtc* elements in the HPV16 genome are not well explained by the locations of sequence homology to CEN elements CDE I and CDE III (Fig. 7A). Only the region of the HPV16 genome harboring *mtc1* contained two perfect matches, both to CDE I, in addition to one imperfect match, also to CDE I (Fig. 7B). Yet we infer that this is the weakest of the *mtc* elements because it was identified in only one of the rescued clones following selection. *mtc2* and *mtc3*, the elements more frequently identified in the selection scheme, have no consensus CDEs, though the region of the viral genome that harbors *mtc3* does contain one single mismatched CDE III element (Fig. 7B). These results suggest that *mtc* elements may be functioning to support plasmid maintenance by a mechanism that is dissimilar to centromere function. Further dissection of the sequences that confer *mtc* function and analysis of their role in the viral life cycle is currently under way. It remains unclear whether these *mtc* elements function in mammalian cells and, if so, act independently of the E2-mediated plasmid maintenance activity or act to augment it.

ACKNOWLEDGMENTS

We thank Paul Ahlquist for valuable discussion and critique of this work. We also thank members of the Lambert laboratory for helpful discussions and comments. We thank Adam Breier and Nicolas Cozzarelli (U.C. Berkeley) for sharing the Oriscan software.

This work was supported by NIH grants CA22443 and CA07175 to P.F.L. and CA100736 to P.C.A. K.K. is a Cremer Scholar.

REFERENCES

1. **Ai, W., J. Narahari, and A. Roman.** 2000. Yin yang 1 negatively regulates the differentiation-specific E1 promoter of human papillomavirus type 6. J. Virol. **74:**5198–5205.
2. **Angeletti, P. C., K. Kim, F. J. Fernandes, and P. F. Lambert.** 2002. Stable replication of papillomavirus genomes in *Saccharomyces cerevisiae*. J. Virol. **76:**3350–3358.
3. **Bastien, N., and A. A. McBride.** 2000. Interaction of the papillomavirus E2 protein with mitotic chromosomes. Virology **270:**124–134.
4. **Breier, A. M., S. Chatterji, and N. R. Cozzarelli.** 2004. Prediction of Saccharomyces cerevisiae replication origins. Genome Biol. **5:**R22.
5. **Clarke, L., and J. Carbon.** 1980. Isolation of a yeast centromere and construction of functional small circular chromosomes. Nature **287:**504–509.
6. **Dvoretzky, I., R. Shober, S. K. Chattopadhyay, and D. R. Lowy.** 1980. A quantitative in vitro focus assay for bovine papilloma virus. Virology **103:** 369–375.
7. **Fouts, E. T., X. Yu, E. H. Egelman, and M. R. Botchan.** 1999. Biochemical and electron microscopic image analysis of the hexameric E1 helicase. J. Biol. Chem. **274:**4447–4458.
8. **Gopalakrishnan, V., and S. A. Khan.** 1994. E1 protein of human papillomavirus type 1a is sufficient for initiation of viral DNA replication. Proc. Natl. Acad. Sci. USA **91:**9597–9601.
9. **Howley, P. M.** 1983. The molecular biology of papillomavirus transformation. Warner-Lambert Parke-Davis Award Lecture. American J. Pathol. **113:**414–421.
10. **Hsiao, C. L., and J. Carbon.** 1981. Characterization of a yeast replication origin (ars2) and construction of stable minichromosomes containing cloned yeast centromere DNA (CEN3). Gene **15:**157–166.
11. **Hsiao, C. L., and J. Carbon.** 1981. Direct selection procedure for the isolation of functional centromeric DNA. Proc. Natl. Acad. Sci. USA **78:**3760–3764.
12. **Kim, K., and P. F. Lambert.** 2002. E1 protein of bovine papillomavirus 1 is not required for the maintenance of viral plasmid DNA replication. Virology **293:**10–14.
13. **Kramer, D. J., L. Gauthier, and K. Yankulov.** 2002. Higher-accuracy method for measuring minichromosome stability in Saccharomyces cerevisiae. BioTechniques **32:**1036, 1038, 1040 passim.
14. **Lambert, P. F., N. Dostatni, A. A. McBride, M. Yaniv, P. M. Howley, and B. Arcangioli.** 1989. Functional analysis of the papilloma virus E2 trans-activator in Saccharomyces cerevisiae. Genes Dev. **3:**38–48.
15. **Law, M. F., D. R. Lowy, I. Dvoretzky, and P. M. Howley.** 1981. Mouse cells transformed by bovine papillomavirus contain only extrachromosomal viral DNA sequences. Proc. Natl. Acad. Sci. USA. **78:**2727–2731.
16. **Lehman, C. W., and M. R. Botchan.** 1998. Segregation of viral plasmids depends on tethering to chromosomes and is regulated by phosphorylation. Proc. Natl. Acad. Sci. USA **95:**4338–4343.
17. **Longtine, M. S., S. Enomoto, S. L. Finstad, and J. Berman.** 1992. Yeast telomere repeat sequence (TRS) improves circular plasmid segregation, and TRS plasmid segregation involves the *RAP1* gene product. Mol. Cell. Biol. **12:**1997–2009.
18. **Mansky, K. C., A. Batiza, and P. F. Lambert.** 1997. Bovine papillomavirus type 1 E1 and simian virus 40 large T antigen share regions of sequence similarity required for multiple functions. J. Virol. **71:**7600–7608.
19. **Miller, C. A., R. M. Umek, and D. Kowalski.** 1999. The inefficient replication origin from yeast ribosomal DNA is naturally impaired in the ARS consensus sequence and in DNA unwinding. Nucleic Acids Res. **27:**3921–3930.
20. **Mohr, I. J., R. Clark, S. Sun, E. J. Androphy, P. MacPherson, and M. R. Botchan.** 1990. Targeting the E1 replication protein to the papillomavirus origin of replication by complex formation with the E2 transactivator. Science **250:**1694–1699.
21. **Newlon, C. S.** 1988. Yeast chromosome replication and segregation. Microbiol. Rev. **52:**568–601.
22. **Ozbun, M. A., and C. Meyers.** 1998. Human papillomavirus type 31b E1 and E2 transcript expression correlates with vegetative viral genome amplification. Virology **248:**218–230.
23. **Patel, S. S., and K. M. Picha.** 2000. Structure and function of hexameric helicases. Annu. Rev. Biochem. **69:**651–697.
24. **Piirsoo, M., E. Ustav, T. Mandel, A. Stenlund, and M. Ustav.** 1996. Cis and trans requirements for stable episomal maintenance of the BPV-1 replicator. EMBO J. **15:**1–11.
25. **Rabson, M. S., C. Yee, Y. C. Yang, and P. M. Howley.** 1986. Bovine papillomavirus type 1 3′ early region transformation and plasmid maintenance functions. J. Virol. **60:**626–634.
26. **Sedman, J., and A. Stenlund.** 1998. The papillomavirus E1 protein forms a DNA-dependent hexameric complex with ATPase and DNA helicase activities. J. Virol. **72:**6893–6897.
27. **Seo, Y. S., F. Muller, M. Lusky, E. Gibbs, H. Y. Kim, B. Phillips, and J. Hurwitz.** 1993. Bovine papilloma virus (BPV)-encoded E2 protein enhances binding of E1 protein to the BPV replication origin. Proc. Natl. Acad. Sci. USA. **90:**2865–2869. (Erratum, **90:**5878.)
28. **Sikorski, R. S., and P. Hieter.** 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics **122:**19–27.
29. **Skiadopoulos, M. H., and A. A. McBride.** 1998. Bovine papillomavirus type 1 genomes and the E2 transactivator protein are closely associated with mitotic chromatin. J. Virol. **72:**2079–2088.
30. **Ustav, M., and A. Stenlund.** 1991. Transient replication of BPV-1 requires two viral polypeptides encoded by the E1 and E2 open reading frames. EMBO J. **10:**449–457.
31. **Ustav, M., E. Ustav, P. Szymanski, and A. Stenlund.** 1991. Identification of the origin of replication of bovine papillomavirus and characterization of the viral origin recognition factor E1. EMBO J. **10:**4321–4329.
32. **Van Houten, J. V., and C. S. Newlon.** 1990. Mutational analysis of the consensus sequence of a replication origin from yeast chromosome III. Mol. Cell. Biol. **10:**3917–3925.
33. **Yang, L., R. Li, I. J. Mohr, R. Clark, and M. R. Botchan.** 1991. Activation of BPV-1 replication in vitro by the transcription factor E2. Nature **353:**628–632.
34. **Yang, L., I. Mohr, E. Fouts, D. A. Lim, M. Nohaile, and M. Botchan.** 1993. The E1 protein of bovine papilloma virus 1 is an ATP-dependent DNA helicase. Proc. Natl. Acad. Sci. USA **90:**5086–5090.
35. **Zakian, V. A., and D. M. Kupfer.** 1982. Replication and segregation of an unstable plasmid in yeast. Plasmid **8:**15–28.
36. **Zanardi, T. A., C. M. Stanley, B. M. Saville, S. M. Spacek, and M. R. Lentz.** 1997. Modulation of bovine papillomavirus DNA replication by phosphorylation of the viral E1 protein. Virology **228:**1–10.
37. **Zhao, K. N., and I. H. Frazer.** 2002. Saccharomyces cerevisiae is permissive for replication of bovine papillomavirus type 1. J. Virol. **76:**12265–12273.