Intratypic changes of the E1 gene and the long control region affect ori function of human papillomavirus type 18 variants

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A persistent infection with high-risk human papillomavirus (HPV) constitutes the main aetiological factor for cervical cancer development. HPV16 and 18 are the most prevalent types found in cervical cancer worldwide. It has been proposed that HPV intratypic variations may result in differences in biological behaviour. Three different HPV18 variants belonging to the Asian Amerindian (AsAi), European (E) and African (Af) branches have been associated with specific histological types of cervical cancer with different relative prognoses, suggesting that HPV18 genomic variations might participate in disease evolution. The E1 viral protein plays a critical role in controlling viral replication and load, requiring interaction with the E2 protein to bind to the long control region (LCR). In this work, we analysed if intratypic variations in the LCR and E1 and E2 genes of HPV18 impact ori replication. While the changes found in E2 genes of the tested variants were irrelevant in replication, we found that variations in E1 and LCR in fact affect ori function. It was demonstrated that nucleotide differences in the LCR variants impact ori function. Nevertheless, HPV18 E1 Af gene was mainly involved in the highest ori replication, compared with the E and AsAi E1 variants. Immunofluorescence analysis showed increased levels of Af E1 in the nucleus, correlating with the enhanced ori function. Site-directed mutagenesis revealed that at least two positions in the N-terminal domain of E1 could impact its nuclear accumulation.

INTRODUCTION

Human papillomaviruses (HPVs) are classified as high- or low-risk according to their oncogenic potential. High-risk HPV types (HR-HPV) are those found in cervical cancer, with HPV types 16 and 18 accounting for almost 70% of all cervical cancer cases (zur Hausen, 2002). It is well known that only a small number of cervical lesions infected with HR-HPVs evolve into high-grade lesions or cervical cancer (Bosch et al., 2002). Many naturally occurring HPV intratypic variants have been identified; furthermore, molecular and epidemiological data suggest that variants of the same type are biologically distinct and may confer different pathogenic risks (Berumen et al., 2001; De la Cruz-Hernández et al., 2005; Lizano et al., 1997, 2006). In a previous work examining a Mexican population, three different HPV18 variants were identified, Asian Amerindian (AsAi), European (E) and African (Af), which have been associated with specific histological types of cervical cancer (Lizano et al., 1997). HPV replication has two stages in the natural history of infection: a persistent stage, which is characterized by a low copy number of viral episomes, occurring mainly in the basal layer without generating progeny virions (Hubert et al., 1999; Hubert, 2005); and a productive stage, where viral genomes are amplified in high copy numbers, later genes are expressed and progeny virions are produced (Hubert, 2005). E1 and E2 viral proteins are essential for HPV replication. E1 is the most conserved protein among papillomaviruses (Koonin, 1993), harbouring a C-terminal domain involved in ATP-dependent DNA helicase activity (Abbate et al., 2004; Wilson et al., 2002). The N-terminal domain is the most variable region of E1 within all papillomaviruses (Abbate et al., 2004); nevertheless, it contains two conserved nuclear localization signals (NLS) and a nuclear export signal (NES) (Fradet-Turcotte et al., 2010). It has been reported that the E1 N-terminal region is essential for viral episome maintenance in undifferentiated primary human keratinocytes due to its interaction with the cellular protein p80, which co-localizes with E1 at the nucleus (Côté-Martín et al., 2008). The long control region (LCR) contains the origin of replication, ori, harbouring a single E1- and three E2-binding sites (Demeret et al., 1998). The keratinocyte enhancer (KE) domain contained in the LCR has been described as being directly involved in...
modulating ori function (Hubert et al., 1999). The E2 protein plays an important role in viral replication, forming E1–E2 complexes, which help E1 binding to the ori (Abbate et al., 2004). The aim of this study was to determine whether nucleotide variations in the LCR and/or E1 or E2 genes of HPV18 intratype variants could affect viral replication. Our results demonstrate that nucleotide variations in these sequences affect replication, with the highest impact for E1-Af.

RESULTS

HPV18 E1 variations reside in the N-terminal region

E1 genes of HPV18 variants (AsAi, E and Af) were sequenced. Nucleotide sequences shared a 99% of similarity with already reported HPV18 isolates (GenBank accession no. EF202155.1, for the Af variant; GenBank accession no. GQ180786.1, for the E variant). Table 1 lists the nucleotide and predicted amino acid changes found in different regions of the E1 variants. Few changes affecting amino acids were present in the Af or E variants compared to the reference AsAi. Nevertheless, variations were mainly located at the N-terminal region, which is essential for E1 nuclear localization.

Viral ori function is dependent on E1 and E2

Plasmid pORI replication was assessed in the presence and absence of E1 and E2 expressing plasmids, Fig. S1 (available in JGV Online). Semi-quantitative RT-PCR assay showed similar expression levels for E1 and E2 in each transfection, Fig. S2. No replication was found with pORI alone or pORI/E2 and minimal replication occurred with pORI/E1, compared when both E1 and E2 were present, where there was an approximately 24-fold amplification. The results confirm that pORI replication is dependent on the E1 and E2 genes. In these experiments it was ensured that the amount of input plasmid was uniform. It was demonstrated that input dam-methylated DNA was comparable in all experiments, after digesting unmethylated DNA with MboI enzyme, Fig. S3. Therefore, differences observed in replication should be due to the biological effect of variations in the tested genes.

Variations in the LCR affect ori function

The E1 and E2 DNA-binding domains (DBDs) are conserved within papillomaviruses, as well as the respective LCR-binding consensus sequences (Auster & Joshua-Tor, 2004; Hegde & Androphy, 1998; Titolo et al., 2003). Moreover, it has been previously suggested that viral proteins E1 and E2 may support the replication of homologous and heterologous papillomaviruses (Chiang et al., 1992). In a previous study, variations in the LCR of HPV18 E and Af sequences were reported (López-Saavedra et al., 2009), particularly in the KE region, known to be involved in viral ori replication (Hubert, 2005). Hence, it was of our interest to analyse whether LCR variations could impact ori replication.

Plasmids expressing E1 and E2 (p40-E1AsAi and pCG-E2AsAi) were co-transfected with each of the LCR variants (pORI-AsAi, -E or -Af) as the only variable. Fig. 1(a) shows that pORI-AsAi had the highest ori function since pORI-Af and pORI-Eu were amplified to almost half of the number of copies of pORI-AsAi (P<0.05). A similar trend was observed for transfected C33 and HaCaT cell lines, where pORI-AsAi had the highest ori function, although some variations in pORI-Eu and pORI-Af replication occurred within cell lines, Fig. 1(b) and (c).

Variations in the E1 gene affect ori function

Next, we sought to determine whether the changes in E2 variant genes could modify ori function. Co-transfections of p40-E1AsAi and pORI-AsAi plasmids with those expressing E2 variants (AsAi, E or Af) as the only variable were performed. As shown in Fig. 2(a), no significant changes in pORI-AsAi amplification were found, related to the tested E2 variants in HEK-293. The same effect was observed for transfected C33 and HaCaT cells lines, Fig. 2(b) and (c). Therefore, the analysed E2 variations do not affect ori function.

To evaluate the effect of variations in E1, pCG-E2AsAi and pORI-AsAi were co-transfected in HEK-293 cell line, with plasmids expressing the different E1 variant genes (AsAi, E or Af) as the only variable. As observed in Fig. 3(a), the presence of E1-Af caused a significantly higher pORI-AsAi amplification than that observed with E1-AsAi or E1-E (P<0.05). This result shows that E1-Af, is able to augment the previously demonstrated strong ori function of pORI-AsAi, in comparison to E1-AsAi, Fig. 1. This trend was reproduced in other cell lines, as demonstrated when HaCat keratinocytes and cervical cancer-derived C33, were tested. As shown in Fig. 3(b) and (c), E1-Af promotes the highest replication level of pORI-AsAi in those cellular contexts.

We next tested the effect of variant homologous elements by the analysis of the ori function of each variant in the presence of the corresponding E2 and E1 variant genes in HEK-293. In this context, the highest effect was observed for pORI-Af-related amplification as shown in Fig. 4(a), even it previously appeared weaker than pORI-AsAi in the presence of E1- and E2-AsAi, Fig. 1. This effect was reproduced in the context of C33 and HaCaT cell lines, Fig. 4(b) and (c), respectively.

The observed effect on ori function was not due to slight differences in E1 concentration, as observed through transfections of HEK-293, C33 and HaCaT cell lines with p40-E1-Af in concentration gradient. Fig. S4(a), (b) and (c) show that E1-Af affect ori replication in a dose-dependent manner, where >0.8 µg p40-E1-Af plasmid overload E1 enzymic function in all cell lines. In the present study an amount of 1.75 µg p40-E1 was used for all transfections.
Nuclear localization of E1 variants

Nuclear localization of E1 is essential for its helicase function. Therefore, it is possible that E1 variations in the N-terminal domain, Table 1, could impact on protein localization explaining the observed differences related to ori replication.

To compare E1 nuclear localization among variants, immunofluorescence analysis was performed on C33 cells transfected with plasmids containing E1 variants tagged with HA. As shown in Fig. 5(a), a stronger HA signal was observed in the nucleus of cells transfected with E1-Af, compared to those with E1-AsAi, with only a subtle difference for E1-Eu. E1 real-time PCR assays were performed, confirming that there were no significant differences in RNA expression and neither in DNA-copy number of the introduced pE1-HA. This trend was reproducible when E1, E2 and LCR of each variant were transfected altogether, Fig. 5(b).

Three of the E1-Af nucleotide variations result in amino acid substitutions, related to the reference E1-AsAi. The participation of these variations in the observed nuclear accumulation was evaluated. Through direct mutagenesis the respective changes at positions 1256, 1378 and 1470 from the Af variant were introduced one by one into the E1-AsAi variant. With single mutations, nuclear signal intensity slightly increased in relation to that previously observed for E1-AsAi, Fig. 5(c). Double mutations were also tested and surprisingly, the changes at positions 1256 and 1470 resulted in greater E1 nuclear accumulation, similar to that obtained for E1-Af, Fig. 5(c).

### Table 1. Comparison of E1 nt and amino acid sequences of the HPV18 variants

Nucleotide and predicted amino acid changes, compared to the reference AsAi, are shown. The negative sign (−) indicate no change at the respective nucleotide or amino acid position. Changes affecting amino acid sequence are located in N- and C-terminal domains.

<table>
<thead>
<tr>
<th>Position</th>
<th>N-terminal: NLS and NES</th>
<th>DNA-binding domain</th>
<th>C-terminal: helicase/ATPase and E2-binding site</th>
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<td>E1-AsAi</td>
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<td>E1-E</td>
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<tr>
<td></td>
<td>Nucleotide</td>
<td>Amino acid</td>
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<td>A Ser 33</td>
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</tr>
<tr>
<td></td>
<td>1087</td>
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DISCUSSION

Several lines of evidence suggest that HPV intratype variations may affect viral biological behaviour potentially altering the prognosis of HPV infections. To examine this possibility further, it is important to identify whether intratype variations may affect viral functions. The identification...

Fig. 1. HPV18 LCR variations affect pORI replication. (a) Plasmids with E1 and E2 of AsAi origin were maintained constant, while the variable was the pORI plasmid. The pORI-AsAi has the highest replication level, almost twofold compared with that of E and Af variants, \( *P = 0.036 \). (b) A similar trend was observed for transfected C33 cell line, \( *P = 0.0023 \) and \( **P = 0.0215 \). (c) In HaCaT cell line a significant difference was observed only for AsAi and E variant, \( *P = 0.00027 \).

Fig. 2. Variations in the HPV18 E2 gene do not significantly affect replication. Transfections were performed where the variable was the E2-variant gene, while E1-AsAi and pORI-AsAi were maintained constant. No significant changes in pORI replication were observed, with similar data obtained with transfected HEK-293 (a), C33 (b) and HaCaT cell line (c).
Fig. 3. E1 variations of HPV18 affect replication in different cell lines. (a) E1-Af promotes the highest plasmid amplification level, compared with E1-E and E1-AsAi in HEK-293 cell line (a), being nearly twice that observed for the AsAi, *P=0.01; (b) C33 cell line, *P=0.0091, **P=0.0171; and (c) HaCaT cell line, with differences for almost threefold related to both, E1-E *P=0.0103 and E1-AsAi **P=0.0188.

Fig. 4. The HPV18 Af variant has the highest replication level in different cell lines. pORI replication in the presence of homologous E1 and E2 from each variant were tested. (a) The Af variant had the strongest pORI replication level in HEK-293. *P=0.0413, **P=0.0235; (b) transfected C33 cell line, with approximately fourfold pORI replication level for the Af variant than that observed for E and AsAi variants, *P=0.0058 and **P=0.0026, respectively; and (c) HaCaT cell line, with approximately threefold pORI replication level for the Af variant, *P=0.00129 and **P=0.00120.
of the potential effects of HR-HPV intratype variations could help us to understand the evolution of HPV infections.

Different biological behaviours have previously been proposed for HPV18 variants, where an Af variant has been associated with lesions with a better relative prognosis.

Fig. 5. Differences in nuclear amounts of E1 variants in C33 cell line. (a) Cells were separately transfected with the three HA-tagged E1 variants. E1 recombinant proteins were identified with anti-HA antibody; nuclei were stained blue with DAPI. HA-E1-Af shows the highest signal in the nucleus; pcDNA 3.1-C, empty vector. E1 real-time PCR was performed in transfectants; \( \Delta \Delta C_t \) showed no difference in expression. (b) A comparable E1 signal was observed when E1 was transfected with both E2 and LCR from each variant. Copy number of the input plasmid was analysed by real-time PCR. (c) Analysis of mutations at nucleotide positions 1256, 1378, 1470 performed on E1-AsAi sequence. The effect on E1 nuclear signal was analysed in single and double mutants.
compared to those harbouring E and AsAi variants. (De la Cruz-Hernández et al., 2005; Contreras-Paredes, et al., 2009; Lizano et al., 1997, 2006).

In the present study, it was examined whether intratype variations of E1, E2 or the LCR may affect viral ori function. We found that E1 intratype variations significantly impact ori function; the highest ori activity was found in the context of E1 Af variant.

Variations in the HPV18 LCR also affect ori function since a significant higher pORI amplification was obtained with LCR-AsAi compared with the other LCR variants tested. This effect may be related to the changes found in the KE region as it has been reported that mutations in this domain affect HPV replication (Hubert et al., 1999).

Even HPV18 E2 variant genes exhibit various changes within their sequences compared with the reference AsAi E2 gene, those changes did not affect ori function. It is noteworthy that variations in E2 are not located in key amino acids important for DNA binding or E1 protein interaction. It is possible that the E2 changes found in the
tested isolates do not alter E2 protein conformation in such a way as to affect its function in replication. As previously reported, variations in the HPV18 E2 gene did not affect LCR transcriptional activity (López-Saavedra et al., 2009), also suggesting that E2 DNA-binding performance is similar in these variants.

Interesting results were obtained with regard to E1 variants. The presence of Af and E E1 variants increased replication compared with E1-AsAi. Variations reside in the N-terminal region of the E1 predicted proteins, where nuclear localization (NLS) and export (NES) signals are located. On the other hand, it has been shown that changes in N-terminal, particularly in the amphipathic helix may alter replication (Morin et al., 2011).

After examining the importance of variations in each of the elements responsible for HPV replication, we then analysed the effect within a unique variant context, namely attesting the E1, E2 and LCRs of the same variants. It was demonstrated that replication variability was mainly due to changes in E1, even in the presence of homologous elements. This finding could be reproduced in distinct cellular contexts, since E1-Af also had the highest impact in ori function in C33 and HaCaT cell lines as in HEK-293.

We then analysed whether differences in replication level could be due to a disparity in variant E1 nuclear levels. Immunofluorescence results showed that E1-AsAi protein was moderately detected in the nucleus, while E1-Af had the strongest nuclear signal, correlating with the enhanced ori function. This trend was similar when immunofluorescence was performed in cells transfected with E1, E2 and the LCR of each variant. When Af nucleotide changes at positions 1256, 1378 and 1470 were introduced in the AsAi E1 gene, E1 nuclear intensity augmented, particularly with the double mutant 1256–1470. The respective predicted amino acids (positions 115, 157 and 186) could be part of a domain that impact E1 nuclear stability. Nevertheless, even that mRNA E1 levels were similar; it is possible that the increased levels of Af E1 in the nucleus is due to higher steady-state levels of the protein.

On the other hand, even replication differences are clear, E E1 showed only a subtle difference in nuclear signal related to the Af E1. Therefore, even nuclear accumulation may have a main role in ori replication, other unknown mechanisms could also be participating. E1 was not detected in any case at the cytoplasm, probably evidencing that this protein is mainly stable in the nucleus.

The present data demonstrate that changes in the E1 gene affect ori function, and differences in replication among variants could be explained partially by its nuclear accumulation, but mechanisms such as post-translational modifications, enzymic cleavage, as other unknown mechanism could play an important role in this biological effect. However, our results suggest that E1 variations could impact the natural history of HPV infection, mainly affecting viral episome copy number.

In previous reports, we proposed that the HPV18 Af variant could exhibit a less aggressive behaviour (López-Saavedra et al., 2009; De la Cruz-Hernández, et al., 2005) based on data obtained through the analysis of the LCR activity and E6 oncogene function. The results obtained in this work show that the Af E1 has the highest impact on replication. Other authors proposed that a high viral load is a potential risk factor for the development of high-grade cervical lesions (Broccolo et al., 2009; Flores et al., 2006, Xu et al., 2009; Wanram et al., 2009), someway contrasting with our data. The HPV18 Af variant has a lower oncogenicity for certain so it could not be discarded that a high number of HPV copies at initial infection, due to an overregulation of E1 function, could consequently be linked to a high expression of the E1 and E2 proteins. Moreover, it has been demonstrated that the E2 protein controls viral early gene expression and induces apoptosis (Demeret et al., 2003). Therefore, an HPV variant promoting an increase in viral replication could have a diminished transforming potential. Furthermore, Fradet-Turcotte et al. (2011) demonstrated that the nuclear export of E1 is required for the maintenance of the viral genome in undifferentiated keratinocytes but not for its amplification in differentiated cells, suggesting that the increased levels of E1 in the nucleus could create a favourable environment for viral DNA amplification by blocking cells in the S-phase. This blockage would induce DNA damage response by ATM-dependent pathway, promoting DNA repair or leading to apoptosis.

To closer define the impact of HPV intratype variations on the oncogenic potential, it would be necessary to explore the biological implication of other viral interactions with cellular targets.

METHODS

Plasmid construction. The pCG-E2 plasmids contain versions of the HPV18 E2 variant genes (E, AsAi and Af) cloned into pCG vector. Plasmids were named pCG-E2AsAi, pCG-E2E and pCG-E2Af according to each HPV18 variant type. Variants of the HPV18 LCRs (E, AsAi and Af) were cloned into the pGL2-basic reporter vector (Promega), resulting in the pORI-AsAi, pORI-E and pORI-Af plasmids. The nucleotide sequences of the E genes and LCRs have been previously reported (López-Saavedra et al., 2009). The E1 AsAi gene was PCR amplified from pBR322-HPV18 (a kind gift from Professor H. zur Hausen, Deutsches Krebsforschungs zentrum, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany), which contains the entire genome of the HPV18 AsAi variant, using the following primers: forward: 5′-GGGGGATCCATGGCTGATCCAG-3′ (nt 914–926) and reverse: 5′-GGGAAGCTTCTAATCTGGACCA-3′. The resulting PCR product was cloned into the pXJ40 FT vector (p40), described by Xiao et al. (1991), to obtain p40-E1AsAi.

Recombinant pE1-1-5TF plasmids containing E1-AsAi, E1-E or E1-Af variants were constructed from pcDNA 3.3 TOPO vector. E1 variant genes were PCR-amplified from the p40-10 E vectors using the following primers: forward, 5′-GCCATGGTGCCTAGTGACGATGACGATAAGATGGCTGATCCAGAA-3′; reverse, 5′-CTATCTGGACCATGTCCTTTCAAA-3′.

The obtained product contained a haemagglutinin domain at the E1 N terminus. The plasmids were constructed with the pcDNA
3.3-TOPO TA Cloning kit (Invitrogen) and named pE1-HA-AsAi, pE1-HA-E and pE1-HA-Af. HA-E1 recombinant genes were confirmed by sequencing.

Multi-site-directed mutagenesis assays. Plasmids expressing E1 E (p40-E1E) or E1 Af (p40-E1Af) were obtained through direct mutagenesis of p40-E1AsAi (QuickChange Multi-Site-Directed Mutagenesis kit; Stratagene) with the primers shown in Tables S1 and S2. Mutants were confirmed by direct sequencing (see Figs S5 and S6). Single and double mutations at positions 1256, 1370 and 1470 were introduced in the plasmid pE1-HA AsAi, to obtain three AsAi mutants with an Af site (1256, 1378 or 1470) and three AsAi mutants with two Af sites (1256–1378, 1256–1470 and 1378–1470). The primers used were the same as those shown in Tables S1 and S2. Mutants were confirmed by direct sequencing (see Figs S7 and S8).

E1 gene sequences. E1 nt sequences were obtained from overlapping PCR products from pre-malignant lesions of the cervix containing HPV18 E or Af variants (Lizano et al., 2006). Three samples for each variant were sequenced. The obtained sequence of the E1-AsAi gene from plasmid pBR322-HPV18 was confirmed using the published HPV18 AsAi sequence (HPV Sequence Database, http://www.stdgen.lanl.gov). Direct sequencing was performed from PCR products using the Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). Primers used to amplify the E1 genes are showing in Table S3.

Cell culture and transfection. HEK-293, C33 and HaCat cells were grown in Dulbecco’s modified Eagle’s medium F12 supplemented with 10 % FBS. Then, 2 × 10^5 cells were plated in six-well plates (30 mm² each well) and transfected with Lipoextamine reagent and Plus reagent (Invitrogen) 24 h later. Co-transfections with the E1 (1750 ng), E2 (55 ng) and LCR plasmids (12 ng) were performed. All transfections were carried out in triplicate in at least three independent experiments.

RNA extraction and expression analysis. To determine E1 and E2 expression, total RNA was extracted from transfected cells with TRIzol (Invitrogen Life Technologies) according to the manufacturer’s instructions. Samples were treated with 1 U DNase I (Gibco-BRL). The amount of RNA was determined by UV spectrophotometry, and the RNA quality was confirmed using 2% agarose gels. For cDNA preparation, 2 mg total RNA was reverse-transcribed with random hexamers using the SuperScript first-strand synthesis system for RT-PCR (Invitrogen Life Technologies). cDNA was PCR-amplified for E1 and E2-expression analysis.

Immunofluorescence. Duplicate assays were performed in C33 cells transfected with 800 ng of pE1-HA plasmids. At 24 h post-transfection one sample was fixed for 20 min at room temperature in 4% paraformaldehyde. Then, cells were rinsed with PBS, and permeabilized for 5 min at room temperature with 0.2% Triton X-100. Then, cells were incubated with 0.1 M glycine in PBS to quench free aldehyde groups. Primary antibody anti-HA (HA-probe (Y-11): sc-805, Santa Cruz Biotechnology) was incubated in PBS for 1 h at room temperature. After washing with PBS (3 ×, 10 min each), cells were incubated for 30 min at room temperature with anti-goat Alexa-488 secondary antibody diluted in PBS. Finally, cells were washed with PBS (3 ×, 10 min each), counterstained and mounted with Prolong Gold Antifade Reagent (Invitrogen). The second sample was used for real-time RT-PCR and real-time PCR E1 assays.

Confocal microscopy. Images were collected with an LSM710-DUO confocal laser system couple to an AxioObserver inverted scanning microscope (Zeiss) and were analysed using LSM Image Browser version 3.2.0.70 (Zeiss). Images were acquired with a ×40 objective (E1-AsAi, E and Af) and ×60 for the empty vector control.

E1 quantitative real-time PCR (qRT-PCR). E1 copy number (from DNA) and transcript levels (from cDNA) were measured in cells tested for E1 confocal microscopy. The quantity of E1 transcript was calculated by ΔΔCt. The primers used to amplified E1 and GAPDH genes were: E1-914-F: 5’-ATGGCTGATCCAGAAGGTACA-3’; E1-1125-R: 5’-TGGAACAATGCCTGTCGTGTC-3’ and GAPDH-F: 5’-TGACACACAACTGTTAGC-3’; GAPDH-R: 5’-GGGATGGACTGTGGCTACATGAG-3’. E1 quantification was performed using a standard curve from pE1-HA with dilution series from 1 × 10^6 to 1 × 10^2 copy number.

cDNA was obtained by GeneAmp (RNA PCR kit; Applied Biosystems). E1 qRT-PCR was performed with SBRY Green (Applied Biosystems). PCR was optimized for 300 nM of reverse and forward E1 and GAPDH primers, on a StepOnePlus real-time PCR system (Applied Biosystems) with the following PCR conditions: 95 °C for 30 s and 60 °C for 1 min for 40 cycles.

DNA harvest. Low molecular mass DNA was extracted 72 h after transfection by using the Hirt method (Morgan & Taylor, 2005; Taylor & Morgan, 2003). Briefly, cells were lysed in 400 μl Hirt solution (0.6% SDS, 10 mM EDTA) and scraped into a 1.5 ml microcentrifuge tube; 100 μl 5 M NaCl was added, and the samples were stored at 4 °C overnight. After centrifugation for 30 min at 4 °C, DNA was extracted once with phenol/chloroform-isooamyl-alcohol and precipitated with 100% ethanol and ammonium acetate (pH 5.2, 3 M). Following centrifugation, the DNA pellet was washed with 70% ethanol, dried and then suspended in 50 μl dH₂O. Plasmid replication was analysed using real-time PCR.

Plasmid replication assay. To test pORI plasmid replication, a 130 bp fragment of the luciferase gene contained in the pGL2-basic plasmid was amplified. This fragment contains two DpnI sites, which are necessary to evaluate de novo replication in HEK-293 cells. The primers used to amplify the luc fragment were: Luc-Sense-5’-TGTTGACATCTCATCTACCTCC-3’ and Luc-Antisense-5’-TCGTCACATCTCATCTACCTCC-3’. Real-time PCR assays were performed using SBRY Green (Applied Biosystems). PCR conditions were optimized to 50 nM Luc-Sense primer and 300 nM Luc-antisense primer. PCRs were performed on an ABI Prism 7700 detection system (Applied Biosystems) with the following PCR conditions: 95 °C for 30 s and 60 °C for 1 min for 40 cycles.

Harvested DNA was digested with either DpnI or Mbol. DpnI can only digest GATC sites when both DNA strands are dam-methylated (dam positive, a bacterial-specific methylation at adenines), whereas Mbol digestions are only possible when both strands are unmethylated at the adenines. Therefore, DpnI will not digest DNA replicated in eukaryotic cells, and Mbol will not digest input DNA (methylated in bacteria). The digestion conditions were previously described (Morgan & Taylor, 2005; Taylor & Morgan, 2003). To detect replicated pORI-(AsAi, E or AF), 25 μl sample was digested with DpnI; to detect input plasmid, 25 μl of sample were digested with Mbol. Exonuclease III digestion was performed for 30 min to reduce the amount of incompletely digested DNA (Taylor & Morgan, 2003). Two microliters of each treated sample was analysed in triplicate using real-time PCR. Quantification was performed using a standard curve from pORI-AsAi (which contains the luciferase fragment to be amplified) with dilution series from 1 × 10³ to 1 × 10⁰ copy numbers.

Statistical analysis. Differences in plasmid replication levels were analysed with Student’s t-test, expressing replication levels as copy number ± 1 σ.


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REFERENCES


