Human Papillomavirus DNA Replication Mediated by Simian Virus 40 T Antigen in trans

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SUMMARY

The putative E1 gene product of papillomaviruses is thought to be involved in the initiation of viral replication, as large-T antigen (T antigen) is in the case of polyomaviruses. Mouse cell lines cloned after transformation by a plasmid consisting of the simian virus 40 (SV40) early region and the complete genome of human papillomavirus type 16 (HPV16) maintained episomal plasmid DNA. In contrast, the DNAs of either SV40 or HPV16, when employed separately in transfection experiments, were consistently integrated into the host DNA. To test the hypothesis that SV40 T antigen might be involved in the replication of the hybrid plasmids, HPV16 DNA was used in a transient replication assay for transfection of either CV-1 or COS-7 cells. The HPV16 DNA replicated to a high copy number in the T antigen-producing COS-7 cells, but failed to replicate in the CV-1 cells. To define the HPV16 sequences that were essential for the plasmid maintenance in SV40 T antigen-producing cells, restriction fragments of HPV16 were analysed for their replication capacity in COS-7 cells. Here it is reported that the presence of the coding region of the putative E1 gene product of HPV16 together with the 5' transcriptional control elements is essential and is sufficient to support plasmid replication mediated by SV40 T antigen in trans.

INTRODUCTION

The papovavirus family is subdivided into two genera, papillomaviruses and polyomaviruses. Papillomaviruses differ from polyomaviruses in their genome organization, and the genomes display a completely different behaviour in cells transformed in vitro and in latently infected cells. The DNA of simian virus 40 (SV40) consistently integrates into the DNA of transformed cells (Sambrook et al., 1968; Botchan et al., 1976; Ketner & Kelly, 1976), whereas the DNA of papillomaviruses is maintained as autonomously replicating molecules in transformed rodent cells (Lancaster, 1981; Law et al., 1981) and in non-transformed human keratinocytes (La Porta & Taichman, 1982).

Despite these differences, homologous regions between the large-T proteins (T antigens) of polyomaviruses and the putative gene product of the open reading frame (ORF) E1 of papillomaviruses have been observed (Clertant & Seif, 1984). Based on these homologies, a common function has recently been proposed for polyomavirus T antigen and papillomavirus E1 protein (Clertant & Seif, 1984). The E1 gene product is thought to be essential for the plasmid maintenance of the bovine papillomavirus type 1 (BPV-1) genome in transformed cells (Lusky & Botchan, 1984, 1985; Sarver et al., 1984), and one of the multiple functions of polyomavirus T antigen is its requirement for the initiation of viral DNA replication (Acheson, 1980).

In the present study, experiments were performed to test whether SV40 T antigen was able to substitute for papillomavirus functions in a biologically well-defined tissue culture system. The behaviour of the DNA of human papillomavirus type 16 (HPV16), which had recently been molecularly cloned (Dürst et al., 1983) and sequenced (Seedorf et al., 1985), was studied in cells either containing or lacking T antigen. It was shown that the HPV16 DNA was able to replicate transiently in SV40 T antigen-producing COS-7 cells (which are CV-1 cells obtained by
transformation and stable integration with origin-defective SV40 DNA; Gluzman, 1981) while no replication was observed in the parental CV-1 cells. Deletion mutants of HPV16 were tested for their ability to replicate in COS-7 cells, and the HPV16 DNA sequences essential for the extrachromosomal maintenance of HPV16 in COS-7 cells were identified. Additionally, the data suggest that efficient replication of HPV16 depends on the presence of trans-acting factor(s) present in SV40 T antigen-containing cells.

METHODS

Cells. C127 mouse cells were grown in Eagle’s basal medium (BME) containing 5% foetal calf serum (FCS). To isolate morphologically transformed C127 cell clones, 2 × 10⁶ cells per 6 cm Petri dish were plated in medium supplemented with 0.5% FCS. While the normal cells ceased to grow under these conditions, transformed single colonies were obtained 2 weeks after transfection with plasmid DNA containing the early region of SV40.

The antibiotic Geneticin (G418) was purchased from Gibco. For G418 selection, the C127 cells were replated 48 h after transfection, at a density of 0.5 × 10⁶ cells/plate. Twenty-four h later, 800 μg/ml G418 was added. Resistant colonies became visible after 10 to 12 days.

The other cell lines used in transient replication assays were grown as follows: CV-1 in BME (plus 10% FCS); COS-7 in BME (plus 10% FCS).

DNA transfection into eukaryotic cells. To obtain stable transformed cell clones, plasmid DNA was introduced into C127 cells by the calcium phosphate transfection method (Graham & van der Eb, 1973). For cloning, single colonies were isolated using microscopical control with Pasteur pipettes and seeded in Petri dishes containing BME supplemented with 10% FCS.

For the transient replication assay, the plasmid DNAs were introduced into CV-1 and COS-7 cells by the DEAE-dextran technique (McCutchan & Pagano, 1968).

Extraction and analysis of DNA. Isolation of high molecular weight DNA from C127 clonal cell lines was performed according to standard procedures (Gross-Bellard et al., 1973). After digestion with restriction enzymes (in buffers recommended by the suppliers), the DNA was fractionated by electrophoresis on 1% agarose gels (10 μg DNA/lane), transferred to nitrocellulose filter paper (Southern, 1975) and hybridized to 1 × 10⁶ c.p.m./ml 32P-labelled nick-translated (Rigby et al., 1977) cloned DNA. After washing, the filters were exposed with XAR-5 X-ray films (Kodak) with intensifying screens.

Transient plasmid replication in COS-7 and CV-1 cells was assessed by extraction of low molecular weight DNA (Hirt, 1967) from transfected cells, usually at 6 h, 24 h, 48 h, 72 h and 96 h after transfection. The purified DNA from Hirt supernatants was subjected to agarose gel electrophoresis directly or after DpnI digestion to remove the input plasmid (Vovis & Lacks, 1977) and was analysed by Southern blot analysis.

DNA manipulation. Plasmid pHV16, containing the full-length genome of HPV16 cloned at its single BamHI site in pBR322 (Durst et al., 1983) was used as the source for all plasmid constructions. The HPV16 DNA was removed from the pBR322 moiety by digestion of pHV16 with BamHI and subsequent gel purification and was cloned into the single BamHI site of pSV1, a plasmid containing the complete early region of SV40 (0.73 to 0.15 map units) in pBR322 (Benoist & Chambon, 1980). The resulting plasmid was designated pSVHPV16.

The cloning of the PstI fragments of HPV16 in pBR322 and of the HaeIII A fragment of HPV16 in pSP62-PL is described elsewhere (Lehn et al., 1985; Lehn & Sauer, 1985).

The different deletion mutants of HPV16, cloned in pBR322 (see Fig. 5) were obtained as follows. Plasmid pH16-AB lacked an Apal/BamHI fragment from the late region and still contained the complete early region with all known regulatory sequences. Mutants pH16-P1 and pH16-P2 were obtained by ligating the PstI A and PstI B fragments of HPV16 (which are adjacent to each other on the viral genome, see Fig. 4) into the PstI site of pBR322; in pH16-P1 the two viral fragments were located in the same direction as in the wild-type genome, whereas in pH16-P2 the fragments showed head-to-head orientation (indicated by arrows in Fig. 5). Mutant pH16-HB represented the large HindIII/BamHI head-to-head fragment of HPV16 cloned into the large BamHI/NruI fragment of pBR322 (this fragment lacked the small BamHI/NruI fragment from nucleotides 375 to 974 within the tetracycline resistance gene of pBR322). In this deletion mutant, the ORFs E2, E4, E5 of HPV16 are affected. Another HPV16 DNA segment (PvuII/BamHI fragment) was also cloned into the large BamHI/NruI fragment of pBR322; this plasmid designated pH16-PB lacked the putative transcriptional regulatory sequences of HPV16 at the 5' end of the early region. Plasmids were propagated in the dam + strain HB101 of Escherichia coli. Plasmid DNA was prepared according to Ish-Horowicz & Burke (1981).

RESULTS AND DISCUSSION

Physical state of the HPV16–SV40 chimera in clonal mouse cell lines

A hybrid plasmid (pSVHPV16) containing the early region of SV40 and the complete genome of HPV16 in both possible polarities was transfected into C127 mouse cells. To select cells
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Fig. 1. Southern blot analysis of DNA from two (out of ten) clonal C127 cell lines (clones 1 and 2) transformed with plasmid pSVHPV16 consisting of the complete genome of HPV16 and the early region of SV40. Total cellular DNA samples from SV40 T antigen-producing transformed clones were separated on a 1% agarose gel (10 µg DNA per lane), transferred to nitrocellulose filters and hybridized under stringent conditions to 32P-labelled nick-translated pSVHPV16 DNA (3 × 10^6 c.p.m./µg). Filters were washed and exposed to X-ray films for 3 days. (a) 150 pg KpnI-digested pSVHPV16 DNA; (b, e) untreated cellular DNA; (c, f) SstI-digested cellular DNA (SstI is a no-cut enzyme for pSVHPV16); (d, g) KpnI-digested cellular DNA.

displaying a transformed phenotype, transfected cells were plated under selective growth conditions (colony formation at low cell density in low serum). In ten individual transformed clonal cell lines, all expressing T antigen, the chimeric plasmids were maintained as extrachromosomal molecules (Fig. 1). The total DNA from these clones either undigested (Fig. 1 b, e) or treated with the no-cut enzyme SstI (Fig. 1 c, f) yielded only unintegrated molecules although SV40 DNA itself fails to replicate in mouse cells (Topp et al., 1980). Digestion with the multi-cut enzyme KpnI produced the original restriction fragments (Fig. 1 a, d, g) which indicates that plasmids had stably maintained their configuration.

In contrast, the pSV1 plasmid containing only the early region of SV40 was integrated in four transformed C127 clonal cell lines analysed (Fig. 2a to f). This is in agreement with published data (for review, see Topp et al., 1980).
Fig. 2. Detection of SV40 DNA and HPV16 DNA in clonal C127 cell lines. (a to f) DNA from two (out of four) clones (Cl11 and Cl14) transformed with the pSV1 plasmid containing the early region of SV40 (Benoist & Chambon, 1980): (a, d) untreated DNA; (b, e) SstI-cleaved DNA; (c, f) PstI-cleaved DNA. (g to m) DNA from two (out of eight) G418-resistant C127 clones obtained after co-transfection of pHPV16 and pAG60: (g, k) untreated DNA; (h, l) SstI-cleaved DNA; (i, m) PvuII/ApaI-cleaved DNA. After transfer, the nitrocellulose filters were hybridized to 32P-labelled pSV1 DNA (a to f) or 32P-labelled HPV16 DNA (g to m) from which the pBR322 moiety had been removed before nick translation. Exposure of X-ray films was for 4 days.

Attempts to transform C127 cells with HPV16 DNA failed; however, exclusively integrated HPV16 DNA was found in clonal C127 cell lines (Fig. 2g to m) which were obtained after co-transfection of pHV16 and a plasmid (pAG60) containing a dominant selectable marker gene (kanamycin resistance gene of Tn5). In the undigested DNA preparations from G418-resistant clones, the HPV16 sequences migrated together with the high molecular weight fractions of DNA (Fig. 2g, k). After digestion with the no-cut enzyme SstI, the migration properties of the viral DNA sequences were altered in relation to the undigested samples (Fig. 2h, l). These data document integration of the HPV16 DNA into the host DNA.
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CV-1

(a) (b) (c) (d) (e)

COS-7

(f) (g) (h) (i) (k)

Fig. 3. HPV16 transient replication assay in CV-1 and COS-7 cells. Low molecular weight DNA prepared according to Hirt (1967) from CV-1 (a to e) and COS-7 (f to k) cells was isolated at several times after transfection with pHPV16 and analysed in Southern blots with radiolabelled pHPV16 DNA (1 x 10^6 c.p.m./ml): 6 h (a, f); 24 h (b, g); 48 h (c, h); 72 h (d, i); 96 h (e, k). The DNAs in (i) and (k) were digested with DpnI prior to gel electrophoresis; therefore, the form I DNA in these lanes represented newly synthesized DNA owing to its DpnI resistance (Vovis & Lacks, 1977). The filter was exposed for 2 days.

Fate of HPV16 DNA after transfection of monkey cells

Two cell lines were employed to test whether SV40 T antigen played a role in the replication of the SV40–HPV16 hybrid plasmids. African green monkey kidney cell line CV-1 and COS-7 cells (which is a CV-1 derivative expressing SV40 T antigen constitutively; Gluzman, 1981) were transfected with the cloned DNA of HPV16. The HPV16 DNA was found to replicate to a high copy number in the T antigen-producing cells, but failed to replicate in the parental CV-1 cells (Fig. 3). The efficiency of replication of HPV16 DNA in COS-7 cells was comparable to that of plasmids containing the SV40 replication origin (data not shown). The de novo synthesis of HPV16 DNA in COS-7 cells was confirmed by the removal of the input plasmid using the sensitive DpnI assay (Vovis & Lacks, 1977); the plasmid pHPV16 was isolated from a dam^+ strain of E. coli (HB101) and was efficiently digested by DpnI, whereas the newly synthesized DNA molecules were resistant to DpnI digestion (Fig. 3).

Behaviour of cloned subgenomic fragments of HPV16 after transfection of CV-1 and COS-7 cells

To identify the part(s) of the HPV16 genome that are essential for plasmid maintenance in the T antigen-producing cell lines, Psrl DNA subfragments of HPV16 were cloned in pBR322 and
tested for their ability to replicate transiently in the CV-1/COS-7 cell system. All PstI fragments and the HaeIII A fragment failed to replicate, both in CV-1 and in COS-7 cells (Fig. 4). This indicates that the presence of these small DNA fragments of HPV16 (including the segment with the putative HPV16 replication origin) is not sufficient to support plasmid replication in SV40 T antigen-producing cells.

Replication of HPV16 deletion mutants in COS-7 cells

To examine whether a HPV16-coded gene product might be essential for SV40 T antigen-mediated HPV16 DNA replication, several deletion mutants of HPV16 were transfected into COS-7 cells and tested for their replication capacity. The full-length genome and a fragment containing the complete early region with all transcriptional control elements were able to replicate in COS-7 cells (Fig. 5). In addition, mutants pH16-HB and pH16-P1, affecting the E2, E4 and E5 open reading frames, replicated in COS-7 cells (Fig. 5). These data suggest that the part of the early region of HPV16 containing the E6, E7 and E1 ORFs is sufficient for the replication in SV40 T antigen-producing cells, although plasmids pH16-HB and pH16-P1 lacked the polyadenylation signal of the viral genome (Fig. 5). The inability of plasmid pH16-HL coding only for the E6 and E7 ORFs to replicate indicates that the E1 ORF is required for the replication of HPV16 DNA in COS-7 cells. Interestingly, the cloned HPV16 DNA used for all these experiments had the E1 coding region split between two reading frames (Seedorf et al., 1985; see Fig. 4, 5). From the results described in this report, this interruption in the E1 ORF seems to have no functional relevance.

Mutant pH16-PB was unable to replicate despite the presence of the coding region of the E1 ORF. This mutant, however, may lack putative cis-essential plasmid maintenance sequences, and it lacks the HPV16 transcriptional regulatory sequences. The requirement for the functional orientation of these latter sequences was documented by comparison of the behaviour of plasmids pH16-P1 and pH16-P2; both of these contained the PstI fragments A and B of HPV16 (Fig. 4, 5), albeit in a different orientation. In the replication-competent plasmid pH16-P1 the fragments are colinear, as in the HPV16 genome, whereas in the replication-deficient plasmid pH16-P2 the fragments are arranged in opposite directions (Fig. 5).
These data suggest that expression of the HPV16 genome was necessary for HPV16 DNA replication. Indeed, HPV16-specific transcripts were identified in COS-7 cells after transfection with replication-competent mutants, while HPV16 expression was not detectable in COS-7 cells after transfection with pH16-P2 and pH16-PB lacking the functional transcriptional regulatory region (data not shown). The same observation, i.e. the absence of HPV16 transcripts, was made using CV-1 cells transfected with pHPV16 and pH16-AB, which failed to replicate in CV-1 cells. These data demonstrate that in addition to the E1 ORF, the 5′ transcriptional regulatory sequences of HPV16 were necessary for plasmid replication mediated by SV40 T antigen in trans.

The comparison of a segment of this non-coding region of HPV16 (nucleotides 37 to 83), located at the 5′ end of the early region and containing a promoter element, with the SV40 T antigen binding site II (Myers et al., 1981; Bergsma et al., 1982; Di Maio & Nathans, 1982) revealed close similarities with regard to the secondary structures; in each DNA there are two palindromes followed by an AT-rich region (data not shown).

Fig. 5. Structure of HPV16 DNA deletion mutants employed for transient replication in COS-7 cells. At the top, the direction of transcription and the ORFs are indicated according to Seedorf et al. (1985). Below this, the genome of HPV16 (7904 bp) is shown with putative transcriptional control sequences: C, cat-box region; T, TATAAA boxes; A, polyadenylation signals (Seedorf et al., 1985). The HPV16-based constructs used for the transient replication assay are listed below this, with the lines indicating HPV16 sequences included in the constructs. The ORFs affected by the deletion mutants and the replication capacity of the different constructs in COS-7 cells are presented on the right, the plus sign representing mutants replicating in COS-7 cells and the minus sign indicating lack of replication activity. Transfection of CV-1 and COS-7 cells, DNA extraction and Southern blot analysis with radiolabelled pHPV16 DNA were performed as described in the legend to Fig. 3. The de novo synthesis of plasmid DNAs from cells containing replication-competent HPV16 deletion mutants was assessed by their resistance to DpnI. Orientation of the PstI A and PstI B fragments in mutants pH16-P1 and pH16-P2 is indicated by arrows.
Conclusions

It is proposed that SV40 T antigen is involved in the control of the extrachromosomal replication of plasmids carrying the E1 ORF and the transcriptional regulatory sequences of HPV16 by the process known as trans-activation. In the case of BPV-1, the putative E1 gene product seems to be responsible for plasmid maintenance (Lusky & Botchan, 1984, 1985; Sarver et al., 1984), and the putative gene product of the E2 ORF was shown to activate in trans a transcriptional regulatory element located in the non-coding region of the BPV-1 genome (Spalholz et al., 1985; Yang et al., 1985). The SV40 T antigen may substitute in trans in the C127 clonal lines and COS-7 cells for the corresponding HPV16 gene product, representing a trans-acting factor which activates a heterologous transcriptional control element. It is conceivable, on the other hand, that SV40 T antigen may be involved in an indirect fashion in the replication of the HPV16 DNA, by inducing cellular factors which then might provide a replication-permissive environment.

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REFERENCES


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