Human Papillomavirus Type 16 Recombinant DNA Is Maintained as an Autonomously Replicating Episome in Monkey Kidney Cells

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SUMMARY

Human papillomavirus type 16 (HPV16) DNA cloned in the expression vector pSV2-neo has been shown to be maintained in transfected monkey kidney cells as an autonomously replicating episome at a level of 2 to 10 copies per cell. Integration of pSV2-neo/HPV16 DNA with the host genome occurred in transfected mouse fibroblasts. Neither type of cell appeared to be phenotypically transformed.

Papillomaviruses are small (50 to 55 nm diam.) icosahedral viruses containing double-stranded DNA. They are found in many animal species, causing benign epithelial tumours (warts) which in some cases may become malignant (Lancaster & Olson, 1982). Human papillomaviruses (HPV) include the only DNA viruses known to cause tumours in man. Although warts are benign, evidence has accumulated strongly associating HPV with malignancies of the genital tract (Singer et al., 1984). Despite the potential importance of HPV, little is known of their biology owing to the lack of a cell line in which they can be propagated (Butel, 1972). DNA from bovine papillomavirus (BPV) and some other papillomaviruses has been shown to transform cultured mouse fibroblasts while replicating autonomously as an episome (Groff et al., 1983; Howley, 1983; Watts et al., 1983). However, no instance of transformation of cultured cells by HPV DNA has been reported.

Replication of HPV DNA in vivo occurs in keratinizing cells. Infection of cultured human keratinocytes with purified HPV1 particles has been shown to result in the establishment of viral DNA as a stable, autonomously replicating episome, but not in the production of virus particles (LaPorta & Taichman, 1982). Attempts to obtain replication of plasmid-cloned HPV DNA by transfection of cultured human keratinocytes have not been successful and it was suggested that the low level of viral transcription detected was a possible limiting factor (Burnett & Gallimore, 1983). There have been no reports of the replication of HPV DNA in other cells using infectious virus particles or purified DNA.

To try to overcome these problems HPV16 DNA has been cloned into the BamHI site of the expression vector pSV2-neo (Fig. 1) (Southern & Berg, 1982). This vector contains a bacterial gene conferring resistance to the neomycin–kanamycin group of antibiotics, together with the early promoter region and mRNA processing sequences of simian virus (SV) 40. The SV40 sequences allow the expression of the neo gene in eukaryotic cells, conferring resistance to the aminoglycoside G418 (Geneticin; Gibco) which is otherwise lethal, so providing a method for selecting successfully transfected cells. There is a single BamHI site in the SV40 mRNA processing sequences and any DNA cloned at this site will be transcribed under the control of the SV40 promoter. This gives a simple way of testing the hypothesis that HPV DNA replication in cultured cells is limited by the level of transcription. The vector cannot replicate by itself, since it lacks the SV40 sequences needed for the synthesis of T-antigen, which is necessary for SV40 replication (Acheson, 1981). Southern & Berg (1982) have found that, with the exception of cos cells, each of the cell lines they examined after transfection with pSV2-neo DNA, including normal monkey kidney (CV1) cells, contained vector DNA integrated with the host genome. Cos cells are a strain of monkey cells containing defective SV40 DNA that produces large T antigen constitutively, but cannot replicate. Examination of DNA from BSC1 cells transfected with pSV2-neo DNA (data not shown) has confirmed that the vector DNA exists in...
Short communication

Fig. 1. The structure of the expression vector, pSV2-neo is 5.6 kb in size and is made up of DNA from different sources. The solid region represents pBR322 DNA, including the origin of replication (Ori) and the ampicillinase (amp) gene. The clear regions represent SV40 DNA, consisting of the early promoter and origin of replication at the 5' end of the neo gene and at the 3' end the small t intron, early polyadenylation site and late termination sequence (Mulligan & Berg, 1980). The dotted region represents the bacterial neo gene, 1.4 kb in length, including a prokaryotic promoter. The protein product of the neo gene, the bacterial phosphotransferase APH(3')II, confers resistance to the neomycin-kanamycin group of antibiotics. The amp and neo genes are both expressed in E. coli, conferring resistance to ampicillin and neomycin. The neo gene is also expressed in eukaryotic cells under the control of the SV40 promoter, conferring resistance to the aminoglycoside G418. Foreign DNA may be integrated with vector DNA at the BamHI or EcoRI sites without affecting the expression of the neo gene in eukaryotic cells. Such a recombinant will also replicate in E. coli and express the neo and the amp genes (Southern & Berg, 1982).

these cells solely in an integrated form. HPV16 has been chosen for this study because of its association with malignancy (carcinoma of the uterine cervix) which is currently being studied in this laboratory.

Recombinant pSV2-neo/HPV16 DNA was used to transfect NIH 3T3 mouse fibroblasts and BSC1 monkey kidney cells using the calcium phosphate co-precipitation technique (Graham & Van der Eb, 1973) without carrier DNA, as described by Gorman et al. (1983). Successfully transfected cells were selected by treatment with G418 at a concentration of 400 μg/ml (Southern & Berg, 1982). Isolated colonies of resistant cells were removed using sterile cloning rings after 7 to 14 days and subcultured in the presence of 100 μg/ml G418 for at least 25 generations before total DNA was extracted (Gross-Bellard et al., 1973) for analysis. High molecular weight (HMW) DNA and low molecular weight (LMW) DNA were separated by differential precipitation as previously described (Heritage et al., 1981) and 10 μg samples, either digested with restriction enzymes or left undigested, were analysed by agarose gel electrophoresis. After partial depurination (Wahl et al., 1979) the DNA was transferred to nitrocellulose (Southern, 1975) and hybridized under optimum conditions with plasmid-purified HPV16 DNA labelled by nick-translation in the presence of 32p-nucleoside triphosphates (Amersham) (Rigby et al., 1977). The filters were washed under stringent conditions and exposed to Fuji RX X-ray film at −70 °C for 6 days with Hanimek Mach 2 intensifying screens.

Fig. 2(a) shows an analysis of HMW DNA and LMW DNA extracted from two clones of transfected BSC1 cells. Comparison with purified pSV2-neo/HPV16 control DNA in the same gel (lane 10) shows that undigested cellular DNA from each clone (lanes 1, 3, 5 and 7) contains two DNA species representing open circular (OC) and covalently-closed circular (CCC) forms of pSV2-neo/HPV16 recombinant DNA. However, the two bands have migrated further than the corresponding forms of the control DNA. This indicates that pSV2-neo/HPV16 recombinant DNA exists in a deleted form, free of cellular DNA. That the DNA has undergone deletion is confirmed by comparison of cellular DNA digested with BamHI (lanes 2, 4, 6 and 8) with similarly treated pBR322/HPV16 control DNA (lane 9). BamHI excises the entire HPV16
genome from the pBR322 plasmid and the pSV2-neo vector to give two linear DNA species in each case. Each of the cellular DNA samples shows two bands, of which the upper band comigrates with linear HPV16 DNA, but the lower band migrates further than linear pBR322 DNA (4.3 × 10^3 base pairs) (4.3 kb). This shows that the deletion is in the vector DNA (normally 5.6 kb) rather than in the HPV16 DNA insert. Two other clones of transfected BSC1 cells (data not shown) exhibited episomal replication of pSV2-neo/HPV16 DNA, with deletions similar in size to those shown.

DNA from clone C, digested with BamHI (lanes 6 and 8) shows additional bands, one of which (lane 6) comigrates with OC pSV2-neo/HPV16 DNA and is a result of incomplete digestion with BamHI. However, the remaining supernumerary bands do not comigrate with any undigested band (lane 10), suggesting that some integration with cellular DNA has occurred. However, it is possible that this represents a rearranged circular oligomer of pSV2-neo/HPV16 DNA, since the same pattern is shown in HMW DNA and LMW DNA. Some support for the latter possibility is given by the pattern shown by the undigested samples from the same clone (lanes 5 and 7). These samples show an additional band that migrates just behind the OC form. This band is possibly the CCC form of a dimer of pSV2-neo/HPV16 DNA.

In order to be able to detect host DNA/pSV2-neo DNA junction fragments that could arise from restriction enzyme digestion if pSV2-neo/HPV16 DNA were integrated at a site within the vector, labelled recombinant pSV2-neo/HPV16 DNA was used as a probe in the experiments described here. The use as a probe of 32P-labelled HPVI6 DNA purified free of plasmid DNA (data not shown) has confirmed the existence of full-length HPV16 DNA in these cells.

To confirm the extrachromosomal state of the recombinant pSV2-neo/HPV16 DNA, 10 μg of Hirt supernatant DNA (Hirt, 1967) was used to transform competent *Escherichia coli* cells (E. coli, DH-1 strain; Hanahan, 1983). Transformed colonies were screened by transferring to nitrocellulose and hybridizing *in situ* (Maniatis et al., 1982) with plasmid-free HPV16 DNA labelled with 32P by nick translation (Rigby et al., 1977). A total of 450 transformants was obtained using neomycin at 10 μg/ml for selection, but none was detected using ampicillin for selection. Circular plasmid DNA (pAT153; Twigg & Sherratt, 1980) transformed a sample of the same batch of competent DH-1 with an efficiency of 6 × 10^7 transformants/μg DNA. The colonies obtained after transformation with the Hirt supernatant DNA were very slow-growing and yielded very little plasmid DNA. This indicates that the deleted portion of the recombinant pSV2-neo/HPV16 DNA includes at least part of the β-lactamase gene and also part of the origin of replication of pBR322, resulting in the slow growth of the Hirt isolates.

Transfection of plasmid-cloned BPV DNA into mouse C127 cells or FR3T3 rat fibroblasts has been shown to result in deletions that involve different stretches of DNA, but always occurred in regions not subject to selection pressure: bacterial sequences or BPV sequences not required for replication or transformation (Binétruy et al., 1982). Reducing the size of the plasmid prevented the occurrence of deletions, suggesting that distance from a region subject to selection pressure is an important factor in determining whether or not a particular stretch of DNA is deleted (Meneguzzi et al., 1984). In this context it should be noted that pSV2-neo/HPV16 is a large plasmid, 13 kb in size, containing a substantial region of bacterial DNA (2.3 kb) that is not subject to selection pressure in mammalian cells.

The pattern obtained with DNA from three separate clones of transfected NIH 3T3 mouse fibroblasts is more complex (Fig. 2b). BamHI-digested DNA from two clones is shown (lanes 3 and 6). Both samples give two strongly hybridized bands corresponding to linear HPV16 DNA and linear pSV2-neo DNA and a number of weakly hybridized bands, suggesting that the pSV2-neo/HPV16 recombinant DNA is tandemly integrated with cellular DNA. Undigested DNA from all three clones (lanes 1, 4 and 7) gives an inconclusive pattern that is similar in each case. The possibility of rearrangement of circular oligomers cannot be excluded, but it would have to be extensive to produce the number of bands, many of high mol. wt., seen after restriction with BamHI. It seems unlikely that rearrangement to such an extent would leave the SV40 mRNA processing sequences and neo gene intact, as they have to be to confer resistance to G418. It is also important to note that even the high mol. wt. supernumerary bands are only weakly hybridized. This would be expected for junction fragments of host DNA and viral or plasmid
Fig. 2. Autoradiographs of DNA samples extracted from BSC1 monkey kidney cells and NIH 3T3 mouse fibroblast cells transfected with pSV2-neo/HPV16 recombinant DNA. Ten-microgram samples of DNA extracted from transfected cells were digested with BamHI or EcoRV under conditions specified by the supplier (Boehringer Mannheim) or left undigested. EcoRV has no recognition site in pSV2-neo/HPV16 recombinant DNA and was used to reduce the viscosity of host DNA. HPV16 DNA is integrated with pSV2-neo DNA at the BamHI site, so treatment with this enzyme will release HPV16 DNA from the vector. DNA was analysed by electrophoresis in 0.8% agarose gels, transferred to nitrocellulose and hybridized under optimum conditions (Tm - 25 °C) with pSV2-neo/HPV16 DNA labelled with 32P by nick translation to a sp. act. of 1.5 (± 0.5) × 109 d.p.m./μg. After having been washed under stringent conditions (Tm - 10 °C) the nitrocellulose filters were exposed to Fuji RX X-ray film with a Hanimex Mach 2 intensifying screen for 6 days at -70 °C. (a) BSC1 monkey kidney cells. The pattern obtained from cellular DNA digested with BamHI is shown in lanes 2, 4, 6 and 8. Lanes 2 and 4 respectively represent HMW DNA and LMW DNA from clone B. Lanes 6 and 8 represent the...
DNA, which would be present at comparatively low copy numbers. The range in size shown by
the bands suggests that integration occurs at more than one site.

Transfected clones of BSC1 cells and of NIH 3T3 cells were cultured over a number of
passages in medium containing a reduced level of serum (1%). The growth rate in each case was
low and comparable with that of control cells. Furthermore the morphology in each case was
similar to that of the parental cells and the transfected cells showed no tendency to pile up in
medium containing normal levels of serum (5% to 10%). This indicates that neither type of
transfected cell is transformed. However, Meneguzzi et al. (1984) showed that plasmid-cloned
BPV DNA carrying the neo gene did not transform rat FR3T3 fibroblasts immediately. Clones
selected for resistance to G418 showed spontaneous formation of transformed foci after 20 to 30
generations, with the frequency of transformation increasing as the cells were passaged,
indicating a stepwise mechanism of transformation. It is possible that the transfected clones
described here have not been passaged for a sufficient length of time to exhibit detectable levels
of transformation. Alternatively, it is possible that different viral functions are involved in
transformation and replication, as they are with BPV1 (Nakabayashi et al., 1983) and that
linearizing HPV16 DNA at the BamHI site disrupts the transforming function. However, this is
unlikely as the BamHI site occurs in the late region (open reading frame L1: K. Seedorf & W.
Röwekamp, personal communication) which is presumed to code for structural proteins.

It is interesting that pSV2-neo/HPV16 recombinant DNA replicates in monkey kidney cells,
but apparently not in mouse fibroblasts. Monkey cells are permissive for the replication of SV40
viral DNA, but in mouse cells integration of SV40 DNA with cellular DNA occurs, usually
resulting in transformation of the cell. Transcription of pSV2-neo/HPV16 DNA is dependent on
the SV40 early promoter, the promoter responsible for the production of T-antigen that is
required for replication of SV40 DNA (Acheson, 1981). It would seem likely that this promoter
would function more efficiently in permissive cells than in non-permissive cells.

Episomal recombinant DNA has also been detected during preliminary experiments with
BSC1 cells transfected with pSV2-neo/HPV1a DNA (data not shown). However, the episomal
state appears to be less stable than is the case with pSV2-neo/HPV16 DNA. In one clone tested,
the transfecting DNA appeared to be integrated in the early stages of cell culture, to become
episomal and eventually to revert to the integrated state in later stages. Two other clones did not
show this early integration and have yet to be tested at later stages. So, although pSV2-
neo/HPV16 DNA has remained in an episomal form for over 40 cell generations, it cannot be
ruled out that integration with the host genome will occur after prolonged cell culture. It is
nonetheless interesting that the recombinant DNA is episomal and although the significance of
this remains to be determined, it does invite comparison with the episomal state of recombinant
BPV1 DNA and its consequences for the cell (Howley, 1983). The results presented here and
preliminary data obtained using pSV2-neo/HPV1a DNA support the hypothesis that the
apparent inability of plasmid-cloned HPV DNA to replicate in cultured cells is a consequence of
the low level of viral transcription in such conditions.

Studies of the transcription of HPV DNA in cultured cells have been made more difficult by
the integration of plasmid-cloned DNA with the host DNA and by low levels of production of

respective DNA fractions obtained from clone C. Undigested samples are shown in lanes 1 (clone B,
HMW DNA), 3 (clone B, LMW DNA), 5 (clone C, HMW DNA) and 7 (clone C, LMW DNA). Lane 9
shows the result of digesting with BamHI, a reconstruction consisting of 10 μg of HMW DNA and 100
pg of purified pBR322-HPV16 recombinant DNA. Lane 10 shows the OC (top band) and CCC (bottom
band) forms of purified pSV2-neo/HPV16 recombinant DNA. Lane M shows size markers obtained by
digesting purified pSV2-neo/HPV16 recombinant DNA with EcoRI. The size of each band is marked
at the left (kb) with the size of the linear pBR322 DNA (4.3 kb) and that of linear viral DNA (7.6 kb)
present in lane 9. The number of copies of recombinant DNA present in transfected BSC1 cells is
estimated at 2 to 10/cell by comparing the intensity of the bands obtained with that of the bands given
by the control DNA (lane 9). (b) NIH 3T3 mouse fibroblast cells. Lanes 3 and 6 respectively show the
result of digesting with BamHI. DNA samples from clones A and D. Lanes 2 and 5 respectively show
the corresponding samples treated with EcoRV. The DNA in lanes 1, 4 and 7 was left undigested. Lane
8 shows the result of digesting with BamHI, a reconstruction consisting of 10 μg of HMW DNA and 100
pg of pBR322-HPV16 recombinant DNA. Lane 9 shows size markers as in (a), lane M. Lane 10 shows
the OC (top band) and CCC (bottom band) forms of purified pSV2-neo/HPV16 DNA.
mRNA. The availability of a system allowing the maintenance of plasmid-cloned HPV DNA in an episomal state by the induction of high levels of transcription will facilitate such studies. Such a system will also enable studies of HPV DNA replication to be carried out and make it possible to determine if replication is a necessary or sufficient requirement for transformation of cultured cells. This in turn may shed some light on the role of HPV in oncogenesis.

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