RAPID COMMUNICATION

HUMAN PAPILLOMAVIRUS TYPE 16 TRANSFORMATION OF RAT 3Y1 CELLS

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The molecularly cloned, prototype human papillomavirus (HPV) 16 DNA has a single-base deletion in the El gene. We reconstructed a putative non-defective HPV 16 genome with an uninterrupted El gene, and examined its biological functions. The reconstructed HPV 16 DNA formed foci of morphologically transformed cells in transfected rat 3Y1 cell cultures (an immortalized normal cell line). The transformed rat cells contained transcriptionally active HPV 16 DNA, which appeared to be integrated within the cell DNA, and formed colonies in soft agar.

Key words: Human papillomavirus type 16 — Transformation — Rat 3Y1 cells

Human papillomavirus (HPV) type 16 is suspected to be one of the agents causing cervical carcinomas, since its DNA has been frequently found in close association with these cancers. The in vitro transforming capacity of HPV 16 needs to be demonstrated and cells allowing the expression of HPV 16 genes must be available for studies on the functions of HPV 16 genes in transformation and on the possible oncogenicity of HPV 16 in humans. One of such attempts to obtain an appropriate system has been successful with the recombinant DNA consisting of pSV2-neo and two tandem repeats of the prototype HPV 16 DNA. The recombinant malignantly transformed mouse NIH 3T3 cells, and the transformed cells contained transcriptionally active HPV 16 DNA integrated within the cell DNA. Furthermore, a genomic DNA sample from a cervical cancer containing HPV 16 DNA has been shown to transform NIH 3T3 cells.

The prototype HPV 16 DNA, whose total nucleotide sequence has been determined, is probably defective, because the El gene, which is the single largest open reading frame among the putative early genes in other papillomaviruses, is comprised of two separate reading frames in the HPV 16 genome. We have found that the El gene of a defective HPV 16 clone (022) from a cervical carcinoma has three guanines in place of the two at nucleotides 1,137 and 1,138 (numbering on the sense strand of the prototype in the El gene of the prototype HPV 16 DNA. Thus, proper addition of a guanine to the prototype DNA is expected to shift the reading frames and make the El gene a single continuous unit.

To investigate the biological functions of the presumably nondefective HPV 16, we reconstructed a HPV 16 genome with an uninterrupted El gene, using the two recombinant plasmids, pHVP16D and p022. Plasmid pHVP16D contains pBR322 DNA and the prototype HPV 16 DNA ligated at their BamHI sites (the original pBR322-HPV 16 recombinant, kindly supplied by Dr. Harald zur Hausen, Deutsches Krebsforschungszentrum, Heidelberg, Federal Republic of Germany, contains one prototype HPV 16 DNA molecule and two pBR322 molecules, one of which was removed to make pHVP16D), and p022 consists of pBR322 and 022 (a defective HPV 16 DNA), ligated at their HindIII sites. The plasmids were digested with appropriate restriction endonucleases, and the fragments 1-4 (Fig. 1) needed for reconstruction were purified by electrophoresis. After three cycles of ligation-purification, we obtained recombinant plasmid pHVP16K comprised of pHVP16D and reconstructed pHVP16 (HPV 16K) in which the NcoI-AccI segment (nucleotides 864 to 1,168) of the prototype was replaced by the corresponding segment of 022. The areas around all the junctions and the site of mutation were sequenced by the M13-dideoxy method for confirmation. The genomic
The organization of the reconstructed HPV 16K DNA is shown in Fig. 1. Transforming activity was tested in Fischer rat 3Y1 cells, an immortalized normal cell line highly susceptible to contact inhibition and transformable by mouse polyoma virus, SV40, and BK virus. Three types of the reconstructed DNAs; linear HPV 16K DNA isolated from pHPV16K, circularized (ligated) HPV 16K DNA (isolated from pHPV16K), and newly constructed recombinant plasmid pSVneo-HPV 16K2 (pSV2-neo) ligated with head-to-tail dimer of HPV 16K DNA; the construct is identical with that used for the prototype HPV 16 DNA by Yasumoto et al. Since one base (guanine) was inserted into the prototype HPV 16 DNA between nucleotides 1,137 and 1,138 to make HPV 16K (7,905 base-pairs), one must be added to each nucleotide number of 1,138 or more for numbering the reconstructed HPV 16K DNA. Segments 1, 2, and 4 are from the prototype HPV 16 DNA and segment 3 is from a defective HPV 16 clone, 022. The recombinant DNA, pHPV16K, contains a pBR322 insert at BamHI site. The open reading frames were reconstructed from Seedorf et al. and Matsukura et al.

Fig. 1. The physical map and open reading frames of the reconstructed HPV 16K DNA. Nucleotide numbering and restriction endonuclease cleavage sites (indicated by arrowheads; one NcoI site, two KpnI sites, two HaeII sites, one BamHI site, and 13 AccI sites) are based on the total sequence of prototype HPV 16 DNA (7,904 base-pairs) determined by Seedorf et al. Since one base (guanine) was inserted into the prototype HPV 16 DNA between nucleotides 1,137 and 1,138 to make HPV 16K (7,905 base-pairs), one must be added to each nucleotide number of 1,138 or more for numbering the reconstructed HPV 16K DNA. Segments 1, 2, and 4 are from the prototype HPV 16 DNA and segment 3 is from a defective HPV 16 clone, 022. The recombinant DNA, pHPV16K, contains a pBR322 insert at BamHI site. The open reading frames were reconstructed from Seedorf et al. and Matsukura et al.

The three types (linear, circular, and recombinant) of HPV 16K DNA produced foci of morphologically transformed cells (Fig. 2) detectable from the third week after transfection to the rat 3Y1 cultures. The foci were composed of densely packed, round cells morphologically similar to those transformed by SV40 or BK virus. The efficiency of focus formation by HPV 16 DNA was I to 14 foci (counted approximately 40 days after transfection) per microgram of DNA. Under similar conditions, the efficiencies of focus formation by SV40 and BK virus (transforming mutants) DNAs were 200 to 500 foci and 20 to 150 foci (counted 25 days after transfection) per microgram of DNA, respectively. The transforming capacity of HPV 16 DNA seems to be lower than those of the polyoma virus DNAs.

Foci of transformed cells were picked up and examined for the presence of HPV 16 DNA and HPV 16-specific mRNA by the blot-hybridization methods. Total cellular DNA was extracted by treatment of cells with SDS and proteinase K, followed by phenol extraction. The cells from three foci, 3Y1HP-1 and 3Y1HP-6 transformed by linear HPV 16K DNA and 3Y1HP-2 transformed by circular HPV 16K DNA, were found to contain DNA hybridizable to the 3P-labeled HPV 16 DNA (Fig. 3a). The three contained no monomeric form I DNA (lanes 1, 4 and 7). Comparison of DNA before and after digestion with HindIII (no cut enzyme for HPV 16 DNA) indicates that HPV 16 DNA in these cells was not circular (lanes 1, 2, 4, 5, 7 and 8). Furthermore, analysis by two-dimensional electrophoresis (in 0.4% and 1% agarose), which can discriminate between linear and circular DNAs, indicated that HPV 16 in the transformed cells was exclusively linear (data not shown). Digestion with BamHI yielded a band of form III HPV 16 DNA with 3Y1HP-2 DNA (lane 6), but not with 3Y1HP-6 DNA (lane 9). Apparently, HPV 16 DNA in 3Y1HP-6 cells (which were transformed by linear HPV 16 DNA) lacked the BamHI site. Comparison of BamHI digestion (lane 6) and NcoI

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Fig. 2. HPV 16-transformed rat 3Y1 cells. (a) Foci of transformed cells (100-mm dish). Mock-infected (left) and transfected (right) cultures were fixed with methanol and stained with 5% Giemsa, 37 days after infection. The culture shown here (right) had been transfected with pSVneo-HPV16K2 and contains approximately 30 foci of morphologically transformed cells. Each focus was examined under a microscope. (b) Focus of transformed cells (magnification; 130×). The transfected culture was fixed and stained 42 days after transfection. Normal cells are seen in the left part. (c) Focus of transformed cells (magnification; 65×). This photomicrograph was taken under a phase-contrast microscope 26 days after transfection. Normal cells are seen in the left part.

Fig. 3. Viral DNA and mRNA in HPV 16-transformed rat 3Y1 cells. (a) Southern blot analysis of DNA from transformed cells. Total cellular DNAs were from 3Y1HP-1 and 3Y1HP-6 cells, transformed with linear HPV 16K DNA, and from 3Y1HP-2 cells, transformed with circularized HPV 16K DNA. Undigested and digested DNAs (10 µg per slot for 3Y1HP-1 and 5 µg per slot for 3Y1HP-2 and 3Y1HP-6) were electrophoresed in a vertical 1% agarose gel in E buffer at 26V for 13 hr, transferred to a nitrocellulose membrane filter, and allowed to hybridize to nick-translated HPV 16 DNA (32P-labeled; 2×10⁶ cpm/µg DNA). The copy number of HPV 16 genome per cell was approximately 4 for 3Y1HP-1 and 10 for 3Y1HP-2 and 3Y1HP-6, as estimated from a comparison with serially diluted reference DNA (data not shown). Cells picked up from the area outside of the foci in the transfected culture were negative for HPV 16 DNA (data not shown). Lane 1, undigested HP-1 DNA; lane 2, HindIII-digested HP-1 DNA; lane 3, BamHI-digested HP-1 DNA; lane 4, undigested HP-2 DNA; lane 5, HindIII-digested HP-2 DNA; lane 6, BamHI-digested HP-2 DNA; lane 7, undigested HP-6 DNA; lane 8, HindIII-digested HP-6 DNA; lane 9, BamHI-digested HP-6 DNA. I, II, and III refer to the positions of forms I, II, and III, respectively, of ca. 8,000-bp DNA run in parallel. (b) Northern blot analysis of RNA from transformed cells. Total cellular RNAs enriched with poly(A)⁺ RNA were from 3Y1HP-1, 3Y1HP-2, 3Y1HP-105 (transformed by pSVneo-HPV16K2), and normal 3Y1 cells. RNA (20 µg per slot) was denatured with glyoxal and dimethyl sulfoxide, electrophoresed in 1% agarose gel in 10 mM sodium phosphate buffer, pH 7.0, at 45V for 9 hr, and transferred to a nitrocellulose membrane filter. RNA on a filter was allowed to hybridize to nick-translated HPV 16 DNA. Lane 10, HP-1 RNA; lane 11, HP-2 RNA; lane 12, HP-105 RNA; lane 13 normal, 3Y1 RNA. Bars indicate the positions of 18S and 28S RNAs run in parallel.
digestion (data not shown) showed that the linear HPV 16 DNA in 3Y1HP-2 seemed to be composed of head-to-tail tandem repeats. All these data are similar to those obtained with DNA from the malignant carcinoma cells, in which the great majority of HPV 16 genomes are integrated within cell DNA, suggesting that HPV 16 DNA in the transformed rat cells is also integrated within cell DNA. Analyses are in progress to determine the sites of integration. Total cellular RNA from the transformed rat cells, extracted by the guanidium thiocyanate method and enriched with poly(A)+ RNA by oligo(dT)-cellulose chromatography, was found to contain HPV 16-specific mRNA, which was much more abundant in 3Y1HP-1 cells than in 3Y1HP-2 and 3Y1HP-105 (transformed by pSVneo-HPV16K2) cells (Fig. 3b). The HPV 16 DNA in the transformed rat cells was transcriptionally active.

Malignancy of the HPV 16-transformed cells was tested by examining growth in soft agar medium and nude mice (Balb/c-nu/nu). Unlike normal 3Y1 cells, the transformed cells formed colonies in 0.4% agarose (Fig. 4). The 3Y1HP-2 and 3Y1HP-6 colonies were smaller and fewer than those of 3Y1HP-1. As regards anchorage-independent growth, the 3Y1HP-2 and 3Y1HP-6 cells seemed to be less malignant than the 3Y1HP-1. When $5 \times 10^6$ cells per mouse were injected subcutaneously into 4-week-old nude mice, neither normal 3Y1 nor the transformed 3Y1 (HP-1 and HP-2) cells produced tumors during the observation period of 10 weeks. (In the 4th and 5th weeks one mouse that had received HP-1 cells seemed to have a tiny tumor, which regressed later.) The relation of HPV 16 gene functions to the malignancy of transformed rat cells remains to be investigated.

In this study we reconstructed an HPV 16 genome with an uninterrupted E1 gene and found rat 3Y1 cells to be transformable by the reconstructed DNA. One of the reasons for the use of nondefective HPV 16 DNA was to test whether the uninterrupted E1 gene affects the state of viral DNA in the transformed cells. Interestingly, episomal HPV 16 DNA was undetectable in the cells transformed by

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Fig. 4. Anchorage-independent growth of HPV 16-transformed rat 3Y1 cells. In each 50-mm dish, $3 \times 10^4$ cells were cultured in 0.4% agarose medium for 4 weeks before photographs were taken. (a) Normal 3Y1 cells. (b) HPV 16-transformed (3Y1HP-1) cells.
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the presumably nondefective HPV 16 DNA. In this respect HPV 16 seems to be different from bovine papillomavirus (BPV) type 1, whose DNA persists as extrachromosomal circular forms in the transformed mouse C127 cells,\(^{27}\) under the control of its El gene.\(^{28,29}\) HPV 16 genomes in malignant cancer cells are mostly or exclusively integrated within cell DNA,\(^{3-5}\) whereas those in benign tumors are found as free, episomal forms.\(^{39}\) Although it is unclear at present whether or not the pathogenicity of HPV 16 is related to the state of viral DNA characteristic of HPV 16-transformed cells or cancer cells, it is possible that, unlike the El gene of BPV 1, the HPV 16 El gene does not maintain viral DNA in an episomal state in the transformed cells.

Recently, defective HPV 16 genomes, the fragment containing the E6 and E7 genes,\(^{15}\) DNA,\(^{15}\) and the prototype HPV 16 DNA (defective in the El gene),\(^{10}\) were found to transform rat 3Y1 cells (unpublished data). Analyses to identify the functions of each HPV 16 gene in transformation are in progress. The HPV 16K DNA and rat cells seem to be a suitable system for such studies.

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