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Tumorigenic Transformation of Primary Rat Embryonal Fibroblasts by Human Papillomavirus Type 8 E7 Gene in Collaboration with the Activated H-*ras* Gene

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Particular types of human papillomavirus (HPV) are associated with skin cancer of epidermodysplasia verruciformis (EV) patients. Here, we show, for the first time, that the E7 gene of EV-associated HPV8 possesses a potential oncogenic transforming ability. The HPV8 E7 open reading frame (ORF) and the HPV16 E7 ORF were cloned under the SV40 promoter/enhancer to construct recombinant plasmids pcD2-8E7 and pcD2-16E7, respectively. Transfection of primary rat embryonal fibroblasts having an activated H-*ras* gene revealed that pcD2-8E7 as well as pcD2-16E7 induced transformation of cells in G418-resistant colonies at an efficiency of 12.3% and 42.9%, respectively. The resulting transformed cell lines induced by pcD2-8E7 and activated H-*ras* were tumorigenic when injected into syngeneic immunocompetent rats. The potential tumorigenicity of HPV8 E7 seemed to be higher than that of HPV16 E7.

Key words: Human papillomavirus type 8 — E7 region — Activated H-*ras* gene — Transformation

Particular subgroups of human papillomavirus (HPV) have been reported to associate with various human cutaneous and mucous tumors. HPV16, 18, 31, 33 and 52 DNAs[1–4)] are often detected in the premalignant lesions and cancers of the uterine cervix, whereas HPV5, 8, 14, 20 and 47[5–7)] are detected in skin carcinomas with epidermodysplasia verruciformis (EV). EV is a life-long disease characterized by disseminated, flat wart-like lesions that develop into squamous cell carcinoma in 30 to 50% of the afflicted patients.[8)] HPV5 and HPV8 have also been detected in the skin cancers of immunosuppressed patients.[9)] Infection with these HPVs seems to imply a high risk for malignant conversion in the course of EV. When placed under a strong heterologous promoter, the E7 open reading frame (ORF) of the HPVs associated with cervical carcinoma (HPV16, 18, 31 and 33) are able to cooperate with activated *ras*[10, 11)] or *fos*[12)] oncogene to transform primary baby rat kidney or baby mouse kidney epithelial cells. The HPV16 E6E7 region can immortalize human keratinocytes.[13)] Using C127 mouse fibroblasts as a test system, Watts *et al.*[14)] described the transforming activity of HPV5 and HPV1. LTR-linked E6 ORF of HPV8[15)] and HPV47[16)] induced morphological transformation of murine and rat cell lines. On the other hand, so far the E7 regions of HPV5, 8 and 47 have been found to be inactive in transforming experiments.[16, 17)]

To test whether the E7 ORF of HPV8 was functional in a cooperative transformation with activated H-*ras* gene (pEJ6.6), we constructed the E7 expression plasmids as follows. HPV8 sequence[18)] (5688 base pairs (bp) from *Eco*RI site 7077 to *Bam*HI site 5111 nucleotide position (np)) cloned between the *Bam*HI and *Eco*RI sites of pBR322[6)] was cut with *Hae*III (586 np) and *Acc*I (1026 np), giving a 440 bp fragment containing E7 ORF (653 to 961 np). Similarly, a 374-bp fragment from *Taq*I (555 np) to *Pst*I (879 np) of HPV16[19)] was purified from pSV2HPV16M.[1)] After deletion of the 5′ end of the viral fragment by using *Exo*III to remove most of the E6 ORF, the viral fragment was blunted by T4 DNA polymerase. Obtained viral fragments containing HPV8 E7 ORF (614±5 to 1026 np) and HPV16 E7 ORF (549 np to 879 np) were separately cloned into the expression vector pcD2-Y, where E7 ORF was placed under the SV40 enhancer/promoter. The 5′ end of the cloned HPV8 E7 was elucidated from the mobility in agarose gel electrophoresis and the 5′ end of the cloned HPV16 E7 was determined by nucleotide sequencing. These plasmids were designated pcD2-8E7 and pcD2-16E7, respectively. The pcD2-Y vector, kindly provided by Drs. Claudia Chen and Hiroto Okayama (NIH, USA), has a single *Kpn*I site instead of the cDNA insert of pcD2-X[20)] between the SV40 enhancer/promoter and the splicing, poly(A) signal sequences. The vector carries a neomycin-resistance gene driven by the SV40 promoter. Cultures of primary rat embryonal fibroblasts (REF) were prepared from 15- to 16-day-old rat embryo and frozen until use. Cells were cultured in Dulbecco's modified Eagle's medium (DME) containing 5% fetal

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Table I. Transforming Efficiency of Rat Embryonal Fibroblasts (REF) after Transfection with Human Papillomavirus Type 8 and Type 16 E7 with or without EJras

Plasmid DNA transfected[a]	Number of morphologically transformed colonies/ Number of total G418-resistant colonies	
	Exp. 1	Exp. 2
pcD2-Y	0/24 (0%)	0/28 (0%)
pcD2-Y+pEJ6.6	0/24 (0%)	0/20 (0%)
pcD2-8E7	0/26 (0%)	0/26 (0%)
pcD2-8E7+pEJ6.6	3/20 (15.0%)	3/31 (9.7%)
pcD2-16E7	9/27 (0%)	0/24 (0%)
pcD2-16E7+pEJ6.6	17/39 (43.6%)	19/45 (42.2%)

a) REF was transfected with 2 μg of pcD2-Y, pcD2-8E7 or pcD2-16E7 with or without pEJ6.6 DNA (8.0 μg), split and cultured in the presence of G418 as described in the text. The total amount of input DNAs was adjusted to 18 μg with herring testis DNA.

bovine serum (FBS). REF were seeded at 2×10^5 in 6 cm dishes, cultured for 24 h and then transfected with 2 μg of pcD2-Y, pcD2-8E7 or pcD2-16E7 with or without 8 μg of pEJ6.6 by the calcium phosphate coprecipitation method.[21] Transfected cells were split and cultured in the DME with 5% FBS and 350 μg/ml of G418. Two weeks after transfection, colonies were either isolated with cloning rings to establish cell lines or fixed with methanol and stained with Giemsa. As shown in Table I, both pcD2-8E7 and pcD2-16E7 plasmids cooperated with pEJ6.6 to give transformed colonies. Neither pcD2-Y alone nor pEJ6.6 alone produced transformed colonies. The number of colonies obtained with pcD2-8E7 plus pEJ6.6 was fewer than that with pcD2-16E7 plus pEJ6.6, suggesting that HPV8 E7 ORF possesses a lower transforming potential than the E7 ORF of HPV16.

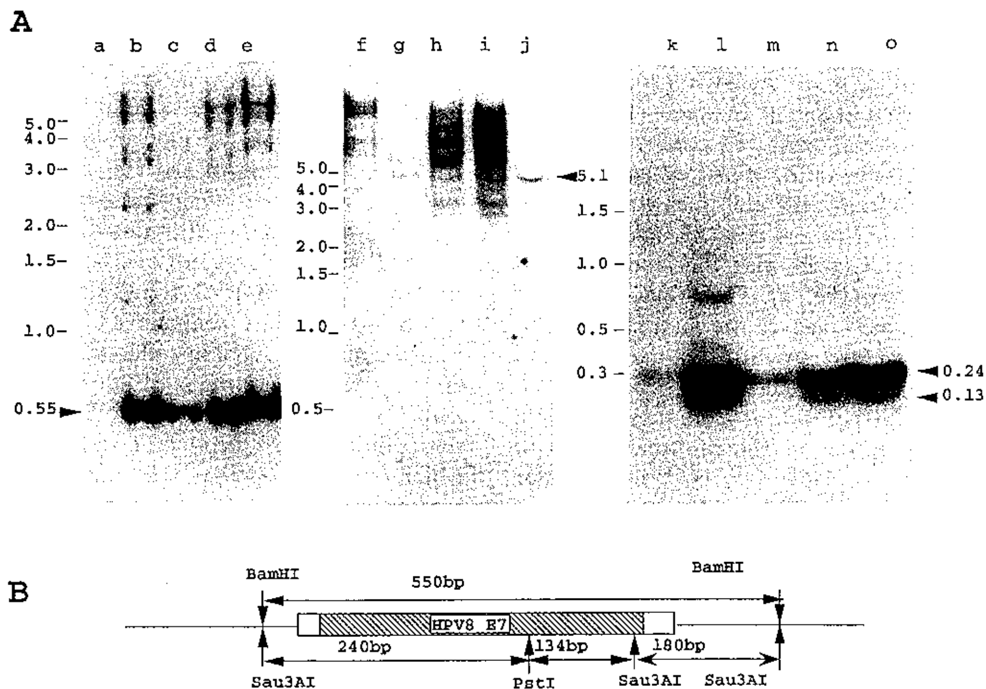

Fig. 1. Southern blot hybridization of cellular DNA from 8RE cell lines with ^{32}P-labeled HPV8 E7 DNA as a probe. A. High-molecular-weight cellular DNA (20 μg) purified from transformed cells (8RE1, 8RE2, 8RE3 and 8RE4) was cleaved with *Bam*HI (lanes a to e), *Hin*dIII (lanes f to j) or *Pst*I and *Sau*3AI (lanes k to o). Herring testis DNA (20 μg) and 1.7×10^{-5} μg of pcD2-8E7 (equivalent to one copy per cell) were also cleaved with *Bam*HI (a), *Hin*dIII (j) or *Pst*I and *Sau*3AI (k) and used as references (lanes a, j, k). The digested DNA was electrophoresed on 1% agarose gels and transferred to GeneScreen Plus (NEN) according to Southern's technique.[26] Hybridizations were performed in 5×SSC, 5×Denhardt's solution, 0.5% SDS, 100 μg/ml of denatured herring testis DNA and 5 ng/ml of ^{32}P-labeled HPV-8 E7 fragment prepared by the random priming method[27] at 65°C for 18 h. The filter was washed in 2×SSC and 0.1% SDS at room temperature for 30 min, in the same buffer at 60°C for 30 min, and then in 0.1×SSC and 0.1% SDS at 55°C for 30 min and exposed to a Fuji RX X-ray film with intensifying screens. B. Restriction map of the HPV8 E7 insert in pcD2-8E7. Restriction sites of *Bam*HI, *Pst*I and *Sau*3AI are shown on the HPV8 E7 sequence (open box) and on the flanking plasmid sequence (solid bar). The 550 bp *Bam*HI E7 fragment was used as a hybridization probe. The hatched box indicates the HPV8 E7 coding region (653 np to 961 np).

Table II. Tumorigenicity of the Transformed Cell Lines in Syngeneic Newborn Rats

	Cell line	Number of rats injected	Number of cells injected	Number of rats with tumor/ Total number of rats							Mean survival days
				0	5	9	13	16	22	27 (days)	
Exp. 1[a]	8RE2	5	5×10^5	0/5	5/5	5/5					9.8
	16RE1	5	5×10^5	0/5	5/5	5/5	5/5	5/5	5/5	5/5	27.0
Exp. 2	8RE4	5	5×10^5	0/5	5/5						5.0
	16RE2	5	5×10^5	0/5	5/5						13.0
Exp. 3	8RE1	4	5×10^5	0/4	4/4	4/4	4/4				14.5
	8RE3	5	5×10^5	0/5	5/5	5/5	5/5				14.8

a) In each experiment, transformed cells were injected subcutaneously into the flanks of five newborn sibling rats (24–48 h old).

Transformed cell lines induced by pcD2-8E7 with pEJ6.6 (8RE1, 8RE2, 8RE3 and 8RE4) and by pcD2-16E7 with pEJ6.6 (16RE1 and 16RE2) were established and characterized. The cells of the 8RE cell line were morphologically indistinguishable from those of 16RE1 and 16RE2 lines. Cell lines of the 8RE group formed colonies in 0.3% soft agar at almost the same efficiency as 16RE cell lines (data not shown). To confirm the presence of the HPV8 sequence in the transformed cell lines, cellular DNA isolated from 8RE cell lines was digested with *Bam*HI, *Hin*dIII or *Pst*I and *Sau*3AI, and analyzed by Southern blot hybridization with the labeled HPV8 E7 fragment as a probe (Fig. 1). Digestion of cellular DNA with *Bam*HI, which cleaves the flanking plasmid sequences (Fig. 1B), yielded a hybridized 0.55 kb band (Fig. 1A, lanes a to e). When cellular DNA was cleaved with *Hin*dIII, which cut pcD2-Y at a single site, HPV8 E7 sequences were detected in the multiple bands of larger molecules (Fig. 1A, lanes f to i). When cellular DNAs were digested with *Pst*I and *Sau*3AI, each of which cleaves the cloned HPV8 E7 sequence at a single site (Fig. 1B), HPV8-specific bands of 0.24 kb and 0.13 kb were observed (Fig. 1A, lanes k to o). The 0.13 kb band of 8RE2 became more obvious after a longer exposure of the blot (data not shown). This suggests that each of the 8RE cell lines contains multiple copies of HPV8 E7 sequences which are integrated colinearly in multiple sites of the chromosome.

In order to test the tumorigenic potential of the established transformed cell lines, 5×10^5 of the transformed cells were injected subcutaneously into the flanks of syngeneic newborn rats (Fischer F-344). Tumors formed at the site of injection within five days after injection in all cases (Table II). Rats bearing tumors induced by HPV8 E7 and *ras* transformants could not live longer than rats with tumors induced by HPV16 E7 and *ras* transformants. This suggests that the potential tumorigenicity of the HPV8 E7 may be higher than that of the HPV16 E7, in spite of the fact that HPV8 E7 showed lower transforming efficiency in collaboration with the activated H-*ras* gene. Although HPV8 E7 has no transforming activity in rodent cell lines, our results demonstrated that the HPV8 E7 ORF, when placed under the SV40 promoter/enhancer, showed an oncogenic transforming potential in collaboration with the activated H-*ras* gene. Oncoproteins of adenovirus E1A, SV40 LT and HPV16 E7 bind to the cellular retinoblastoma susceptibility gene product (RB), and viral mutations which result in the loss of RB binding also lose transforming activity.[22–24] This suggests that RB plays a key role in the control of cell proliferation. However, the binding ability of HPV8 E7 protein to RB was hardly detectable or was at a very minor level,[25] suggesting that unknown functions of the HPV8 E7 gene product other than RB binding may be involved.

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