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〔原著〕 TRANSFORMATION OF MOUSE C3H/10T1/2 CELLS INDUCED BY HUMAN PAPILLOMAVIRUS TYPE 16 DNA

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Summarry

We isolated a morphologically transformed cell line from immortalized mouse C3H/10 T1/2 cells which were transfected with the plasmid containing one copy of human papillomavirus type 16 (HPV16) whole genome DNA cloned in pUC19. The transformants could grow in a soft agar medium only when 12-*o*-tetradecanoylphorbol-13-acetate (TPA), dexamethasone, or a combination of estradiol and progesterone was added to the medium. The transformant expressed virus-specific mRNAs similar to those found in premalignant tissues, accompanied by the over expression of the c-Ha-*ras* gene.

Key words: HPV16; Mouse C3H/10T1/2 cells; Transformed cells; TPA

Abbreviation: HPV: Human Papillomavirus, TPA: 12-o-tetradecanoyl phorbol-13-acetate, ORF: Open Reading Frame

I. Introduction

Our understanding of the oncogenic properties of the human papillomavirus (HPV) genome has been significantly advanced by the finding of evidence that human papillomavirus type 16(HPV16) genome DNA is able to transform rodent cells *in vitro*. The transforming activity of HPV16 was first demonstrated by transfecting molecularly cloned viral whole genome DNA or cellular DNA of cervical cancer containing HPV16 DNA into NIH-3T3 cells[1,2]. The E6 and E7 open reading frames (ORFs) of HPV16 genome DNA are constantly retained intact and expressed in the cell lines derived from cervical carcinomas[3-9], and the E7 ORF is demonstrated to encode a transforming gene for rodent cells[10]. In human fibroblasts and keratinocytes, HPV16 genome can extend their life span, but cannot transform these cells in culture[11,12]. These findings suggest that HPV16 DNA is involved in a crucial step of multi-step carcinogenesis, and requires additional co-factors to transform cells completely.

It has been demonstrated that HPV 16 DNA can cooperate with activated-*ras* in trnsforming primary rodent cells[13]. As other factors, glucocorticoids are reported to enhance colony formation of primary rat kidney cells which were transfected with a combination of HPV16 DNA and activated Ha-*ras* oncogene[14].

We roport here the isolation and characterization of morphological transformed cell lines from mouse C3H/10T1/2 cells transfected with HPV16 DNA. The transformants require additional fac-

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tors to form colonies in soft agar, suggesting that some co-factors are involved in a sequential process leading to malignant transformation by HPV genome.

II. Materials and Methods

Cells and culture:

We selected mouse C3H/10T1/2 cells[15] for our transformation studies because these cells are well characterized, immortalized, and non tumorigenic, and have been used frequently in experiments on transformation by radiation, carcinogens, and oncogenes[16-18]. The cells were cultured in Eagle's basal medium supplemented with 2.5% fetal calf serum.

Recombinant HPV16DNA and transfection

The C3H/10T1/2 cells were transfected with plasmid pHPV16 DNA, which contains one copy of HPV16 whole genome DNA[19] cloned at the *Bam*HI site in pUC19, by the calcium phosphate coprecipitation method[20].

DNA and RNA analysis:

Cellular DNA was extracted and digested with restriction endonucleases, *Xho*I and *Bam*HI. The digested DNA was separated on a 0.5% agarose gel, and analyzed by Southern blotting as described previously[21,22].

RNA from the transformed cells was analyzed by Northern blotting, as described previously[6]. The probes were the 1.35 Kbp *Eco*RI/*Pst*I fragment (nt. 7453-875) containing the noncoding region and E6 and E7 ORFs, and the 1.1 kbp *Pst*I fragment (nt. 3692-4755) containing the early poly A signal of HPV16 DNA. The expression of c-Ha-*ras* proto-oncogenes in XPW16 cells was examined by Northern blot analysis using mouse c-Ha-*ras* DNA probe[23].

Colony formation in soft agar:

Cells were plated in 0.35% soft agar containing 10% fetal calf serum with or without addition of

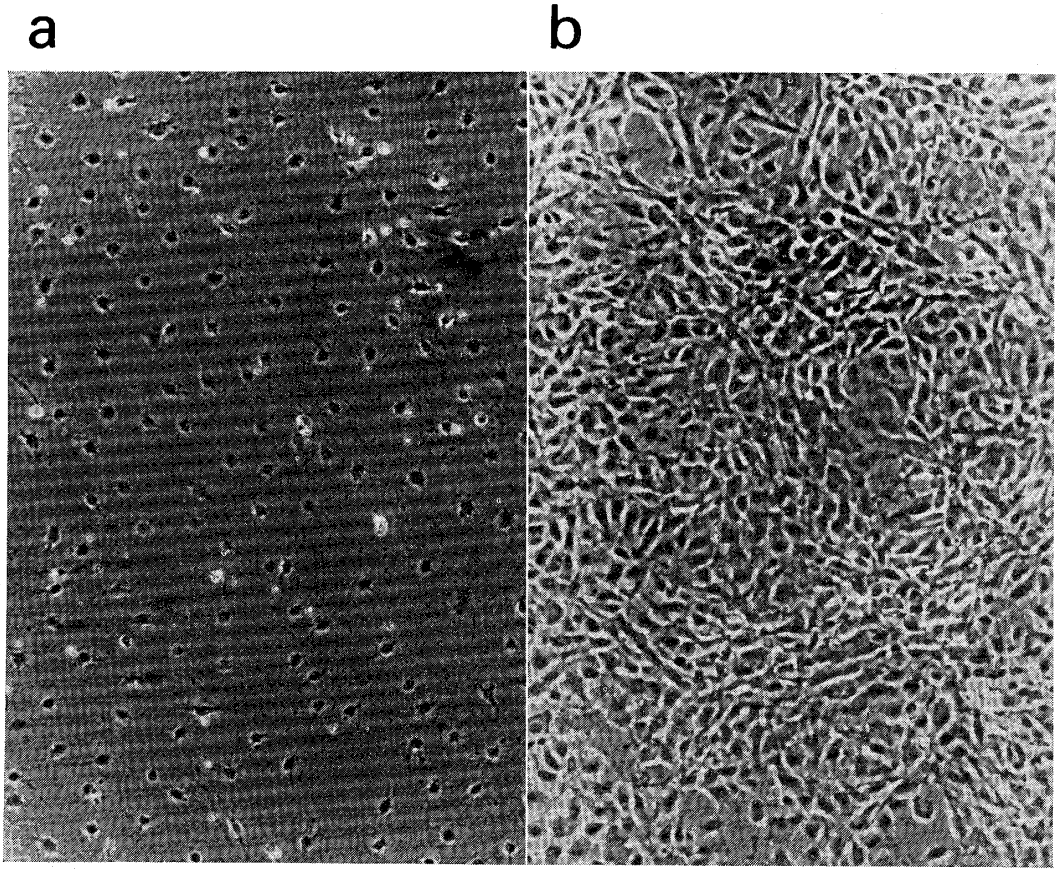

Fig. 1. Morphological appearance of C3H/10T1/2 cells transfected with pUC19 DNA (a) and HPV16 DNA-induced transformant XPW16 cells (b). These photographs were taken under a phase contrast microscope at ×200 magnification.

Table 1. Growth properties and anchorage-independent growth of C3H/10T1/2 and XPW16 cells

cell line	Growth in low concentrated FCS (2.5%) medium		Colony formation in 0.35% agar medium with 10% FCS[c]					
	Population doubling time[a]	Saturation density[b]	Plain	TPA	dexametha-sone	estradiol	proge-sterone	estradiol & progesterone
	(h.)	(×10)						
C3H/10T1/2	68	3.3	0	0	0	0	0	0
XPW16	25	15.2	0	582	57	0	0	68
GT1[d]	NT[e]	NT	0	609	54	0	0	73

[a] Population doubling times of cells were calculated over a 100 h period corresponding to the logarithmic phase of growth (assayed in duplicate).
[b] Saturation densities represent the maximum cell densities obtained from confluent 35 mm dish cultures (assayed in duplicate).
[c] Triplicated 60-mm dishes were seeded with 1×10^4 cells in a 0.35% agar medium without or with 100ng/ml TPA, 1μg/ml dexamethasone, 1μg/ml estradiol, 1μg/ml progesterone, and a combination of 1μg/ml progesterone and 1μg/ml estradiol, respectively.
[d] GT1 cells were isolated from a colony formed in soft agar with TPA.
[e] NT; not tested.

100 ng/ml of 12-*o*-tetradecanoylophorbol-13-acetate (TPA), 1 μg/ml of dexamethasone, and a combination of 1 μg/ml estradiol and 1 μg/ml estradiol and 1 μg/ml progesterone, respectively.

III. Results

Transformation of C3H/10T1/2 cells by HPV16 DNA:

Morphologically transformed foci appeared in C3H/10T1/2 cells at 5 to 6 weeks after transfection with pHPV16 DNA. No focus was developed in cells transfected with pUC19 DNA (Fig. 1). Five foci were picked up with rings and they were morphologically identical. One of them, named XPW16 cell line, which was a typical spindle shaped transformant, was used for this study. The cells grew 2 to 3 times faster than did C3H/10T1/2 cells and their saturation density was 3 to 4 times higher than that of C3H 10T1/2 cells (Table 1).

Detection of HPV16 DNA and mRNAs in XPW16 cells:

Southern blot analysis reveals the presence of HPV16 DNA in the XPW16 cells. The results of undigestion of cellular DNA show that HPV16 DNA is present in a high molecular weight DNA band without any bands corresponding to forms I, II, and III, indicating integration into cellular DNA (Fig. 2a). This was also supported by digestion of *XhoI*, which does not cleave pHPV16 DNA. The results of digestion with *Bam*HI show the presence of full-length 7.9 kbp HPV16 DNA in these cells, indicating the intact viral genome. The 7.9 kbp band was confirmed to contain approximately 30 copies of HPV16 whole genome DNA.

To analyze transcripts of the integrated viral genomes, RNA from XPW16 cells was analyzed by Northern blotting. As shown in Fig. 2b, the RNA transcripts detected were 4.2, 3.2, 2.2, 1.6, and 1.4 kb. The 1.4 kb transcript was most intensely detected with the 1.1 kb *Pst*I probe. The 3.2 kb transcript was detected more weakly using the *Pst*I probe compared with the case of the *Eco*RI/*Pst*I probe. These results indicate that the E6 and E7 ORFs of HPV16 DNA are expressed XPW16 cells and virus-specific mRNAs should terminate at the virus' own polyA site. Furthermore, the sizes and hybridization patterns of transcripts were very similar to those in dysplasias as reported previously[7].

Expression of c-Ha-ras proto-oncogenes in XPW16 cells:

The expression of c-Ha-*ras* proto-oncogenes was examined by Northern blot analysis as shown in

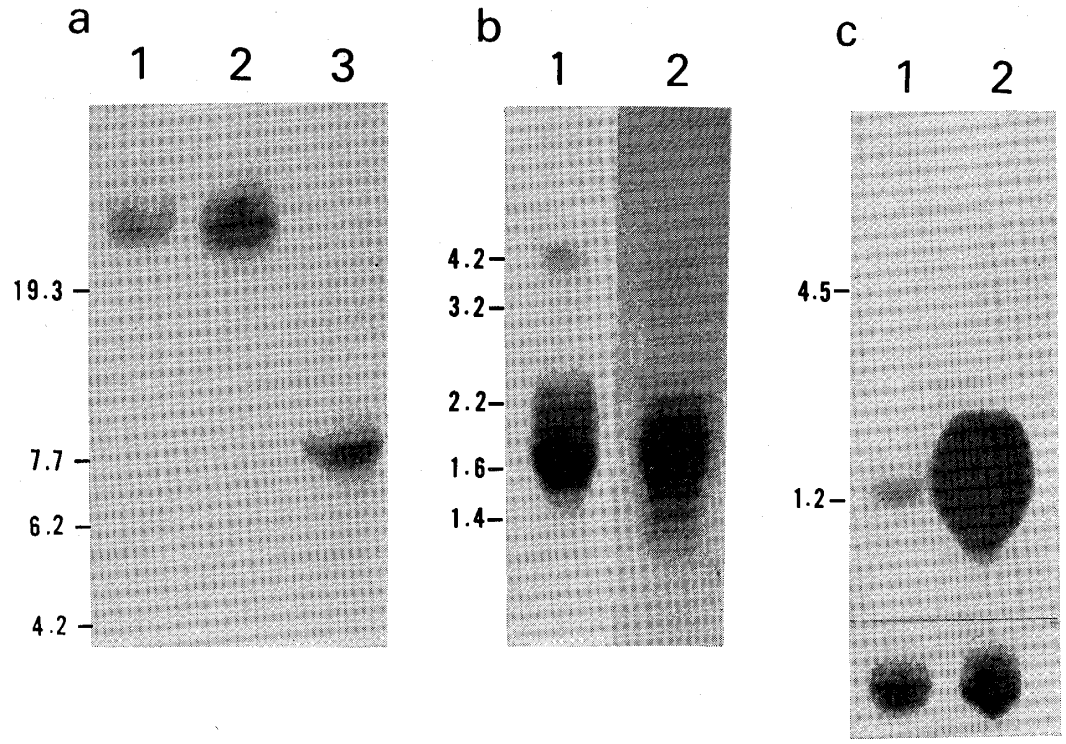

Fig. 2. Detection of HPV16 DNA, and expression of virus specific mRNA and c-Ha-*ras* in XPW16 cells. (a) Cellular DNA (5 μg) was hybridized to HPV 16 whole genome. lane 1, undigest; lane 2, digested with *Xho*I; lane 3, digested with *Bam*HI. (b) Total cellular RNA (12 μg) of XPW16 cells was hybridized to the 1.35 kbp *Eco*RI/*Pst*I fragment (lane 1) and the 1.1 kbp *Pst*I fragment (lane 2) of HPV16 DNA. (c) Total cellular RNA (12 μg) of normal C3H/10T1/2 cells (lane 1) and the transformant XPW16 cells (lane 2) was hybridized to mouse c-Ha-*ras* DNA probe. An arrowhead indicates beta-actin RNA. Bars indicate the positions of fragments of lambda phage DNA digested with *Eco*T141 (a), and the transcripts hybridised to the probes (b and c). The accompanying numbers represent the sizes of transcripts in kilobases.

Fig. 2c. A major 1.2 kb band and a faint 4.5 kb band were detected in XPW16 cells as reported[24] and apparently enhanced compared with those in wild C 3 H/10 T 1/2 cells.

Anchorage independent growth of XPW16 cells:

When C 3 H/10 T 1/2 cells transfected with pUC 19 DNA and transformed XPW16 cells were plated in 0.35 % soft agar, any colonies were not observed (Table 1). However, small colonies (about 100 to 200 cells) were detected at approximately 6 % when 100 ng/ml of 12-*o*-tetradecanoylphorbol-13-acetate (TPA) was added to the medium. Addition of dexamethasone and a combination of estradiol and progesterone was also slightly effective (Fig. 3). Colonies appeared after about 4 days, continued to grow slowly during 4 to 6 weeks, and became small ones which contained about 32 to 64 cells. The reversibility of TPA-induced anchorage-independence was tested by isolating a colony (GT 1) grown in soft agar with TPA. The GT 1 cells could not grow in soft agar, but could grow in soft agar with TPA. These results reveal that the morphologically transformed XPW16 cells require a certain co-factor to grow in soft agar.

IV. Discussion

In this study, we found that HPV16 DNA stimulates immoratlized mouse C 3 H/10 T 1/2 cells to grow beyond their saturation densities in monolayer cultures with apparent morphological changes after a relative long latent period. However, the

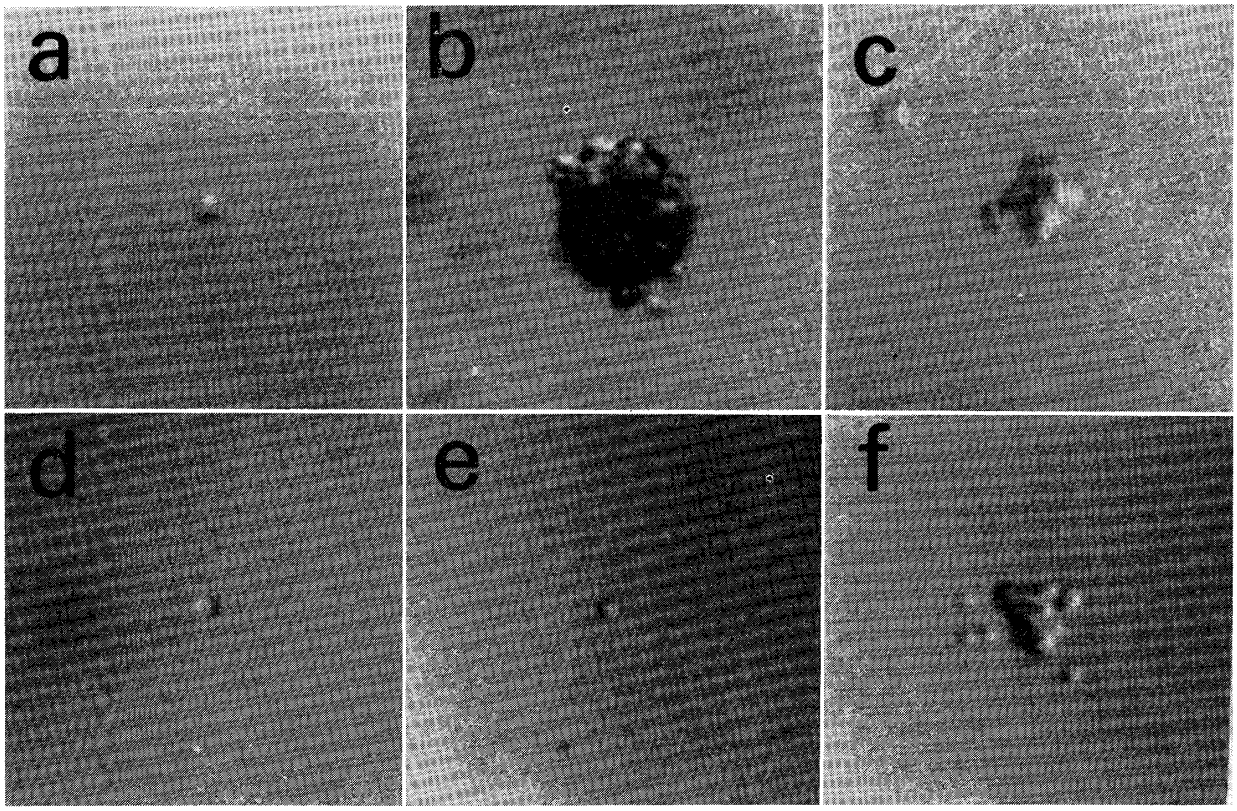

Fig. 3. Anchorage-independent growth of XPW16 cells. The photographs were taken 6 weeks after seeding XPW16 cells in a 0.35% agar medium addition of no agent (a), 100 ng/ml TPA (b), 1.0 μg/ml dexamethasone (c), 1.0 μg/ml estradiol (d), 1.0 μg/ml progesterone (e), and a combination of 1.0 μg/ml estradiol and 1.0 μg/ml progesterone (f).

transformed cells were incapable of forming any colonies in soft agar. The transforming activity of HPV16 DNA was not so efficient for C3H/10T1/2 cells as for other immortalized rodent cells[1,8]. These results suggest that morphological changes and anchorage independent growth should be different events in a multi-step transforming process, and that the HPV16 gene and host cell interaction is required for transformation.

It is very likely that the *ras* gene is involved in the action of the oncogenic HPV16 gene in the transformed cells. Matlashewski *et al.*[13] reported that the transforming gene of HPV16 and activated *ras* gene cooperate to establish and maintain the transformed state in primary rat cells. Involvement of the activated *ras* gene in progession of cervical tumours is also reported[25].

Anchorage-independent growth of HPV16 transformed cells has been accompanied by morphological alterations; therefore it is difficult to know whether each event occurs independently in an early stage of transformation[10]. The morphologically transformed C3H/10T1/2 cells could form colonies in soft agar when co-factors were added. Therefore, the morphological alterations and anchorage-independent growth of the transformant should be different steps in a transforming process.

We could elucidate co-factors responsible for anchorage-independent growth of the XPW16 transformant and they enhanced the expression of HPV 16 specific RNAs. TPA may exert its effect on the transformed phenotype by altering the expression of cellular genes. In bovine papillomavirus type 1 (BPV-1) transformed mouse C127 cells, no significant change in the level of BPV-1 specific mRNA was found, although addition of TPA to soft agar resulted in an increase in colony-forming efficiency, as reported in our previous paper[26]. Matlashewski *et al.*[13] have reported the cooperation of HPV16 DNA with activated-*ras* in transforming primary rodent cells. These reports suggested that co-factors may act through cellular genes, while

some reports have suggested that co-factors may also act through virus genome. Gius and Laiminis[27] reported that the noncoding region of HPV18 contains a TPA-responsive element, and we detected by computer analysis that the noncoding region of HPV16 also contains five sequences of element (nt. 7179-7186, nt. 7312-7319, nt. 7512-7519, nt. 7639-7646, nt. 7656-7663) very similar to the consensus TPA-responsive element which was reported by Angel *et al.*[28]. It is known that HPV16 contains sequences very similar to the consensus glucocorticoid-responsive element sequence[29,14] and that the glucocorticoid-responsive element in HPV 16 also mediates progesterone responsiveness[30]. Recently, Mitrani-Rosenbaum *et al.*[31] have revealed that the sequences similar to the consensus estrogen-responsive element are present in HPV 16 DNA, and that estradiol enhances the viral transcriptions.

Whether these co-factors acted through HPV16 genome or cellular genes have not yet been elucidated. However, these co-farors and their cooperation were needed for the HPV16 induced-premalignant ransformant to acquire more advanced phenotypes.

要　旨

ヒトパピローマウイルス16型 (HPV16) DNA をマウス C3H10T1/2 細胞に導入し形質転換細胞 XPW16 を得た。XPW16 細胞において HPV16 DNA は宿主 DNA に組み込まれ，1細胞あたり約30コピー存在した。HPV16mRNA は4.2kb, 2.2kb, 1.6kb, 1.4kb のサイズでウイルスの polyA を用いており，子宮頸部癌の前癌状態である異形成類似のパターンを示した。XPW16 細胞の成長速度と飽和密度は親株 C3H10T 1/2 細胞の約3倍であり，接触阻害を喪失し，形態的にも形質転換細胞の特徴を有していた。しかし，軟寒天中においてコロニーを形成せず，かつ，estradiol, progesterone それぞれの単独添加によってもコロニーを形成しなかった。他方，12-o-tetradecanoyl phorbol-13-acetate (TPA), dexamethazone それぞれの単独添加，および，estradiol, progesterone の併用によりコロニーを形成した。

以上のことから，XPW16 細胞は前癌病変である異形成に相当する性格を保有し，この細胞系により HPV16 の形質転換には TPA, sex steroid 等の co-factor が重要であることが示唆された。

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