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Correlation between Tumorigenicity and Expression Levels or Splicing Patterns of Transcripts of the Human Papillomavirus Type 16 E6 Gene

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For determination of the correlation between tumorigenicity and the expression levels or splicing patterns of E6 mRNAs of the human papillomavirus type 16 in established cells, a vector containing the intact E6 open reading frame which expresses both unspliced and spliced mRNAs, one expressing only unspliced E6 mRNA, and one expressing both unspliced and spliced mRNAs but producing only truncated E6 proteins were constructed. In transformation assays and analyses of E6 mRNAs, a higher expression level of unspliced E6 mRNA was found to be closely associated with tumorigenicity. Furthermore, it was also related with anchorage-independent growth and a decreased serum requirement of the cells.

Key words: Human papillomavirus type 16 E6 — Tumorigenicity — Decreased serum requirement — Splicing pattern

Infections with certain human papillomaviruses (HPVs) have been demonstrated to be risk factors for development of human anogenital carcinomas. HPVs can be classified into 'low risk' and 'high risk' types on the basis of their association with clinical lesions. High risk types, such as HPV16 and 18, have been detected in 90% of cervical and other genital cancers, whereas low risk types, such as HPV6 and 11, are generally associated with benign lesions, such as condyloma accuminata.[1,2]

In almost all cervical cancers, HPV DNA is integrated into the host chromosomal DNA, and the E6 and E7 open reading frames (ORFs) are retained and expressed, suggesting that expressions of the E6 and E7 genes are important in HPV-associated carcinogenesis.[3,4] There is much evidence that the E6 and E7 ORFs can immortalize normal human foreskin keratinocytes and cervical epithelial cells in culture.[5,6] E6 and E7 can also immortalize primary rat embryo fibroblasts (REFs), although some cellular changes such as overexpression of the K-*ras* gene are needed for transformation.[7-9] In addition, the E6 and E7 genes can transform established rodent cell lines, such as 3Y1,[10] NIH3T3,[11] and Ψ2.[12,13] Thus, E6 and E7 play key roles in HPV-associated carcinogenesis.

The E6 proteins of HPVs are approximately 150 amino acids long and are localized in the nucleus[14] or in the cytoplasm.[15] They contain multiple Cys-X-X-Cys motifs, which are characteristic of zinc finger domains and bind zinc *in vitro*.[16] They also transactivate several heterologous promoters, such as adenovirus E2.[17] Previous studies have shown that E6 proteins of high risk types are able to bind wild type p53[18] and mediate p53 degradation *in vitro* through a ubiquitin-mediated mechanism.[19] The E6 and E7 genes of the high risk types are expressed via structurally bi-cistronic, differentially spliced RNAs, while those of the low risk types are expressed by a single unspliced E6 mRNA and by mono-cistronic E7 mRNA initiated within the E6 ORF. The high risk types produce the full length E6, truncated E6*, and E7 proteins, while the low risk types produce only full length E6 and E7 proteins.[20] E6* proteins of the high risk types have been detected in cervical carcinoma cells transplanted into nude mice.[21]

In previous studies using mouse Ψ2 cells, we showed that the tumorigenic activity is mainly due to the E6 ORF, while the E7 ORF governs the ability to form colonies in soft agar.[12] However, the vector (pZE67B) that we used in previous studies on E6 expression contained both the 5′ half of the E7 ORF and the 3′ half of the LCR (long control region) besides the E6 ORF. Accordingly, the possibility that the observed tumorigenicity might require not only the E6 gene product but also the presumed truncated E7 polypeptide was not excluded. Furthermore, these previous studies did not show whether the full length protein or a truncated one from the E6 ORF determined the tumorigenicity. For examination of these problems, in this study we constructed three new vectors. One contained the E6 ORF but virtually none of its flanking sequence. The second was the same as the first, but carried a splice-donor site mutation so as to express only unspliced E6 mRNA. The third was derived from the first, but produced only truncated

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E6* proteins. As reported here, results using these vectors indicated that expression of the unspliced mRNA or full length E6 was sufficient for tumorigenesis and that the tumorigenicity was correlated with the level of the unspliced mRNA.

MATERIALS AND METHODS

Cells Mouse Ψ2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) under 7% CO_2 in air at 37°C.

Plasmids The pZipNeoSV(X)1 vector,[22] shown previously to be a eukaryotic retroviral expression vector and to contain long terminal repeats (enhancer-promoter) of murine leukemia virus, was used. As it also possesses the neomycin-resistance gene, cells containing it can be selected with the neomycin analog G418. pZE6 1766[23] and pZE67BN contain 450 bp (nt. 104–556), and 600 bp (nt. 282–884) fragments, respectively, of the HPV16 early region (Fig. 1). pZE6 1768S.D., derived from pZE6 1766, has one point mutation in the splice donor sequence (nt. 227; from G to A) within the E6 ORF (nt. 104–556). pZE6 1880, derived from pZE6 1766, contains the TTL insert (translational termination linker, TTAGTTAACTAA) at nt. 253 of the E6 ORF, pZE6 1766, pZE6 1768S.D., and pZE6 1880 are the same vectors as pZE6CDS, pZE6C·SD, and pZE6TTL, respectively (Kiyono *et al.*, in preparation).

DNA transfection Ψ2 Cells (5×10^5/100 mm dish) were transfected with 5 μg of various plasmid DNAs by a modification of the calcium phosphate precipitation method. Cells containing these plasmid DNAs were selected with 500 μg/ml of G418. G418-resistant colonies appeared about 2 weeks after transfection. The several hundred clones obtained were pooled and used for transformation assays and analyses of E6 mRNA expression.

Northern (RNA) blot analysis Cellular RNA of each sample was isolated with 5.5 *M* guanidium isothiocyanate and then centrifuged through a CsCl (5.7 *M*) cushion.[24] PolyA$^+$ mRNAs of each sample were selected from 40 μg of total RNA using Oligotex-dT (Nippon Roche, Kamakura), and separated in 1% agarose gel. Then they were blotted onto a nylon filter in 20×standard saline citrate (SSC; 1×SSC is 0.15 *M* NaCl plus 0.015 *M* sodium citrate) and hybridized with HPV16 E6 probes and human β-actin[25] probe ^{32}P-labeled with a multiprime labeling system (Amersham Corp.) in 4×SSC containing 5×Denhardt's solution (0.1% bovine serum albumin, 0.1% Ficoll 400 and 0.1% polyvinylpyrrolidone), 0.2% sodium dodecyl sulfate (SDS), 50 mg of denatured herring sperm DNA per ml, and 50% formamide at 37°C for 24 h. The filters were washed four times each with 2×SSC-0.1% SDS at room temperature for 15 min and 0.1%×SSC-0.1% SDS at 50°C for 15 min and then autoradiographed. The relative amounts of mRNAs were quantitated with a BAS 2000 Bioimage analyzer (Fuji Film, Tokyo).

Probes A 450 bp fragment of HPV16 E6 ORF (nt. 104–556) from pUC19 E6 1766, was obtained by digestion with *Bam*HI. As specific probes of HPV16 E6 mRNA, two cDNA fragments (probe A, nt. 104–258, a *Bam*HI-*Nsi*I fragment, and probe B, nt. 303–402, a 100-base *Mse*I-*Mse*I fragment) within the E6 ORF were used (Fig. 2).

Reverse transcriptase-polymerase chain reaction (RT-PCR) RT-PCR of the E6 mRNAs was carried out as follows. For synthesizing the first strand cDNA, 5 μg of total RNA was mixed in a total volume of 20 μl of reaction mixture containing reverse transcription buffer (BRL, Gaithersburg, MD), 25 units of RNasin (Takara, Tokyo), 1 m*M* each of dNTPs, 35 pmol of poly(dT) primer and 200 units of cloned murine leukemia virus reverse transcriptase (BRL). The reaction was allowed to proceed for 50 min at 42°C, and then the reverse transcriptase was inactivated by heating at 95°C for 5 min. Subsequently, cDNA was amplified by adding 80 μl of solution containing 10 pmol of each PCR primer, amplification buffer and 2 units of *Taq* polymerase (Perkin Elmer Cetus, Norwalk, CT) and subjected to 35 PCR amplification cycles, beginning with DNA denaturation at 94°C for 1 min, followed by a primer annealing step at 55°C for 1 min and an elongation step at 72°C for 2 min. The final elongation step was prolonged to 7 min. One-tenth of the amplified DNA was fractionated by electrophoresis in 6% polyacrylamide gel. Then the electrophoresed DNA was blotted onto a nylon filter and hybridized with E6 probe A ^{32}P-labeled as described above, and autoradiography was performed for 2 h. The primers used for PCR were primer 1 (nt. 147–162, TATGCACAGAGCTGCA) and primer 2 (nt. 537–556, CAGCTGGGTTTCTCTACGTG). The following DNA fragment sizes were expected from RT-PCR analysis: unspliced type, 410 bp, spliced types, 111 bp and 227 bp. RNA extracted from SiHa cells was used in analyses as a positive control.

Anchorage independence of growth Samples of 1×10^4 cells were seeded into 60 mm dishes with 0.33% noble agar in DMEM supplemented with 10% FBS on top of 0.5% base agar in DMEM supplemented with 10% calf serum. Colonies larger than 0.125 mm were counted after incubation for 2 weeks.

Tumorigenicity assay Female BALB/c, (nu/nu) mice of 4–6 weeks old were used. The tumorigenic potential was assayed by injecting 2×10^5 cells subcutaneously into ten or more nude mice, except in the case of Ψ2-pZE6 1766-Tu, and measuring the sizes of the resulting tumors every 7 days for 4 weeks.

Serum requirement The growth rates of cells containing various plasmids were examined in DMEM supple-

mented with 5% FBS or 0.2% FBS. After preliminary experiments, cells containing various plasmids were seeded into 60 mm dishes at a suitable concentration to obtain 2×10^4 plated cells on the next day. Media were replaced every other day. All cells were observed morphologically before trypsinization and counting in a hemocytometer every 24 h.

RESULTS

Differently spliced E6 mRNAs are expressed in mouse Ψ2 cells containing pZE6 1766, pZE6 1768S.D., and pZE6 1880 To determine whether the E6 ORF alone is really associated with tumorigenicity, we constructed three new E6 expression vectors, pZE6 1766, pZE6 1768S.D., and pZE6 1880, which contain neither the E7 ORF nor the LCR (Fig. 1). pZE6 1766 contains the intact E6 ORF (nt. 104–nt. 556). pZE6 1768S.D., derived from pZE6 1766, has one point mutation in the splice donor sequence (nt. 227; from G to A) within the E6 ORF, resulting in inactivation of the 5′-splice site and production of only the full length E6 protein. pZE6 1880, derived from pZE6 1766, contains an insertion of TTL at nt. 253 which is between the splice donor site and acceptor sites of the E6 ORF, resulting in production of only truncated E6* proteins.

These plasmids were transfected into mouse Ψ2 cells, and the cells containing these plasmid DNAs were selected with G418. The several hundred G418-resistant clones obtained from each of three different vector-transfected cells were pooled, named Ψ2-pZE6 1766, Ψ2-pZE6 1768S.D., and Ψ2-pZE6 1880 cells, respectively, and used for transformation assays and analyses of E6 mRNAs.

Northern blot hybridization was carried out with two cDNA fragments within the E6 ORF as probes (probe A, nt. 104–258, probe B, nt. 303–402) (Fig. 2). The former

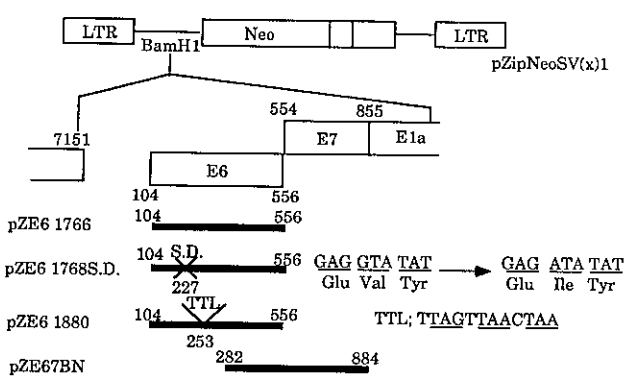

Fig. 1. Construction of recombinant plasmid DNAs containing various subgenomic fragments of the HPV 16 E6 and E7 regions.

recognizes both spliced and unspliced E6 mRNAs while the latter recognizes only unspliced mRNA. With probe A, the total E6 mRNA expression level in Ψ2-pZE6 1766 cells was as much as that in Ψ2-pZE6 1768S.D. cells, but the splicing patterns of the two differed. With probe B, the mRNA expression level in Ψ2-pZE6 1768S.D. cells was more than ten times that in Ψ2-pZE6 1766 cells. The amount of unspliced mRNA was less than 10% of the total E6 mRNA expressed in Ψ2-pZE6 1766 cells containing the intact E6 ORF. This result was consistent with that reported by Desaintes *et al.*[17] The β-actin mRNA[25] expression levels of the samples were similar (data not shown). The E6 mRNAs species in various cells were examined further by RT-PCR. Results showed that only unspliced E6 mRNA was expressed in Ψ2-pZE6 1768S.D. cells, whereas both unspliced and spliced E6 mRNAs were expressed in Ψ2-pZE6 1766 and Ψ2-pZE6 1880 cells, although for precise measurements of mRNA expression levels. Northern blot hybridization is generally superior to RT-PCR. Thus, the results obtained from Northern blot hybridization and RT-PCR indicated that the total E6 mRNA expression levels of these cells were the same, but that only unspliced mRNA was expressed in Ψ2-pZE6 1768S.D. cells, whereas spliced E6* mRNAs were expressed at a high level in Ψ2-pZE6 1766 and Ψ2-pZE6 1880 cells.

Unspliced mRNA expression level is closely associated with tumorigenicity The saturation density of Ψ2-pZE6 1766 cells was similar to those of Ψ2-pZipNeo and pZE6 1880 cells. Ψ2-pZE6 1768S.D. cells were slightly smaller than Ψ2-pZipNeo or Ψ2-pZE6 1766 cells. The saturation density of Ψ2-pZE67BN cells, which expressed only the E7 protein was greater than that of the other cells (data not shown). There were no distinct morphological differences in the cells, except that Ψ2-pZE67BN cells were slightly more spindle-shaped than the others (data not shown).

With respect to anchorage-independence and tumorigenicity, Ψ2-pZipNeo and Ψ2-pZE6 1880 cells showed neither colony-forming activity in soft agar ($<0.01\%$, $<0.01\%$, respectively) nor tumorigenic activity (0/12, 0/11, respectively) (Tables I and II, and Fig. 3). Ψ2-pZE67BN cells formed colonies in soft agar with high efficiency (18–33%), but were not tumorigenic (0/14). Ψ2-pZE6 1766 cells showed colony-forming activity with low efficiency (0.21–0.75%), and weak tumorigenic activity (2/13), while Ψ2-pZE6 1768S.D. cells showed an intermediate level of colony-forming activity (2.2–3.9%) and strong tumorigenic activity (13/13). Reinjection of the same number of Ψ2-pZE6 1766 cells reconstituted from a single tumor, named Ψ2-pZE6 1766-Tu cells, induced tumors after 3 weeks in all three nude mice tested. By Northern blot analysis, Ψ2-pZE6 1766-Tu cells were shown to express several times more unspliced mRNA

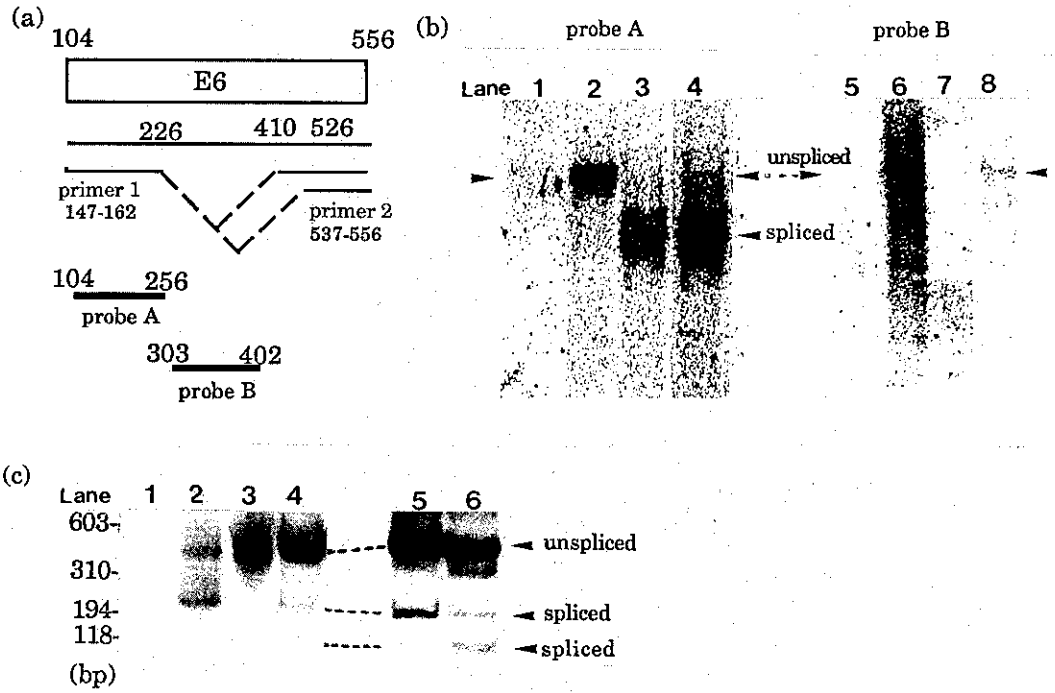

Fig. 2. (a) Splicing pattern of E6 mRNA and specific probes for E6 mRNA of HPV16. (b) Northern blot hybridization analysis of expressions of E6 mRNAs in Ψ2-pZipNeo (lanes 1 and 5), Ψ2-pZE6 1768S.D. (lanes 2 and 6), Ψ2-pZE6 1766 (lanes 3 and 7), and Ψ2-pZE6 1766-Tu cells (lanes 4 and 8) determined with probe A and probe B. PolyA mRNAs of each sample were selected from 40 μg of total RNA, separated in 1% agarose gel, transferred to a nylon filter, and hybridized with probe A or probe B radiolabeled by the multiprime DNA labeling method. (c) RT-PCR analysis of expressions of E6 mRNAs in Ψ2-pZipNeo (lane 1), Ψ2-pZE6 1766 (lane 2), Ψ2-pZE6 1768S.D. (lane 3), Ψ2-pZE6 1880 (lane 5), and SiHa cells (lanes 4 and 6). Primer 1 (TATGCACAGAGCTGCA) and primer 2 (CAGCTGGGTTTCTCTACGTG) were used for PCR. The following DNA bands were expected from RT-PCR analysis: unspliced type, 410 bp, spliced types, 111 bp and 227 bp.

Table I. Colony-forming Abilities in Soft Agar of Cells with HPV 16 E6 or E7

Cells	Colony formation in soft agar (%)[a]		
	exp. 1	exp. 2	exp. 3
Ψ2-pZipNeo	<0.01	<0.01	<0.01
Ψ2-pZE6 1766	0.75	0.28	0.21
Ψ2-pZE6 1768S.D.	—	2.23	3.88
Ψ2-pZE6 1880	—	<0.01	—
Ψ2-pZE67BN	32.7	23.5	17.85

a) Samples of 1×10^4 cells were inoculated into 0.33% top agar in DMEM supplemented with 10% FBS, and layered onto 0.5% base agar in DMEM supplemented with 10% calf serum. After incubation for 2 weeks, colonies larger than 0.125 mm in diameter were counted.

Table II. Tumorigenicity in Nude Mice

Cells	Latency[a] (days)	Efficiency[b]	Tumor size in nude mice[c] (cm^3)
Ψ2-pZipNeo	>28	0/12	0
Ψ2-pZE6 1766	20–24	2/13	0.27–1.8 (mean 1.0)
Ψ2-pZE6 1768S.D.	10–17	13/13	0.7–33.5 (mean 12.5)
Ψ2-pZE6 1880	>28	0/11	0
Ψ2-pZE67BN	>28	0/14	0
Ψ2-pZE6 1766-Tu	15–21	3/3	0.9–4.2 (mean 2.6)

a) The minimal time to produce apparent tumors of at least 5 mm in diameter was taken as the latency period.
b) Number of tumors/number of nude mice injected.
c) The tumor size was determined 4 weeks after subcutaneous injection of cells.

than Ψ2-pZE6 1766 cells in culture (Fig. 2b). Therefore, we conclude that the unspliced mRNA expression levels of the three kinds of E6 mRNAs were closely associated with their tumorigenicities. Interestingly, Ψ2-pZE6 1768S.D. cells showed higher colony-forming ability than Ψ2-pZE6 1766 and Ψ2-pZE6 1880 cells. These results suggested that the unspliced E6 mRNA expression level may be associated with colony-forming ability besides

tumorigenicity, although the E7 gene has the highest colony-forming ability.

Decreased serum requirement is caused by the E6 gene

The ability to grow medium with a reduced serum concentration is reported to be a characteristic of transformed cells.[26] To determine whether the E6 gene is related to the reduction of the serum requirement, we measured the growth rates of cells containing various expression vectors in normal (5%) or low (0.2%) serum media (Fig. 4). In medium containing 5% FBS, all cells grew promptly, soon became confluent and stopped growing. Mouse Ψ2 cells containing the E6 or E7 ORF showed slightly better growth than Ψ2-pZipNeo cells. But with 0.2% FBS, mouse Ψ2 cells expressing unspliced E6 mRNAs, especially Ψ2-pZE6 1768S.D. cells, grew steadily, whereas Ψ2-pZipNeo, Ψ2-pZE6 1880, and Ψ2-pZE67BN cells first grew slowly and then their cell numbers decreased, and the cells exfoliated completely after culture for 4 or 3 days. Thus the E6 gene (especially the unspliced E6 gene), but not the E7 gene, was also associated with cell growth in low serum medium.

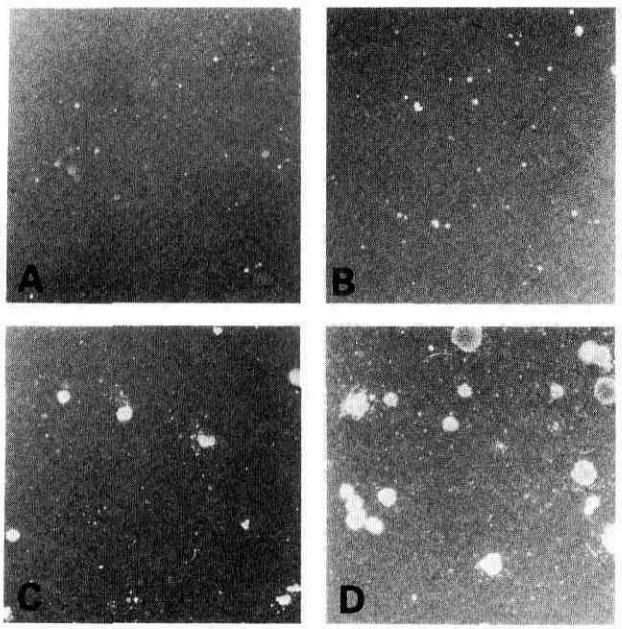

Fig. 3. Colony formations in soft agar of Ψ2 cells containing various plasmids. Ψ2-pZipNeo (A), Ψ2-pZE6 1766 (B), Ψ2-pZE6 1768S.D. (C), Ψ2-pZE67BN (D).

DISCUSSION

Previously, we reported that an expression vector pZE67B that contains the 3′ half of LCR, the entire E6 ORF, and the 5′ half of the E7 ORF had tumorigenicity. This finding suggested that tumorigenicity might be caused by the E6 ORF, but it was possible that the truncated E7 protein might be involved in the tumorigenicity. In this study, to eliminate this possibility, we constructed various vectors containing only the E6 ORF and examined their tumorigenicities. Results showed that the E6 ORF alone conferred tumorigenicity and ability to grow in low serum medium. Furthermore, to determine whether the unspliced or spliced E6 mRNA is involved in tumorigenicity and other transforming activities, we examined the transforming activities of various E6 expression vectors. We found that unspliced E6 mRNA, but not spliced mRNAs, was responsible for these transforming activities.

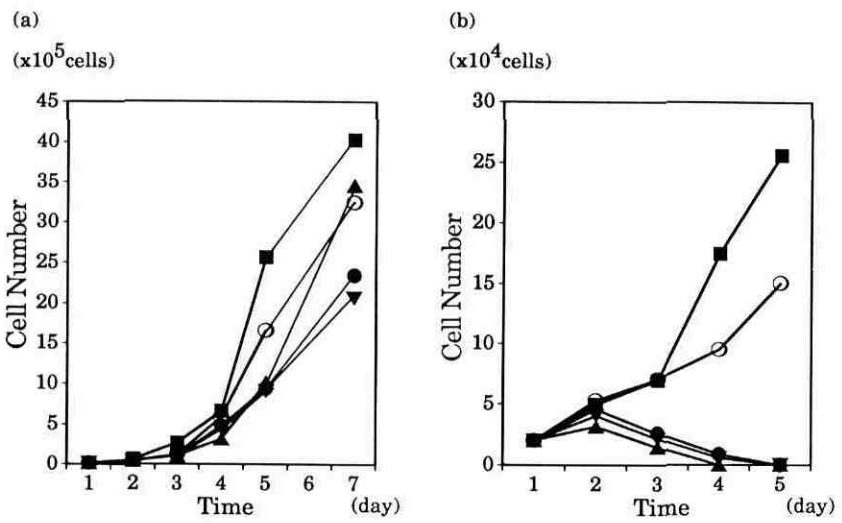

Fig. 4. Growth rates of Ψ2 cells containing various vectors in DMEM supplemented with a normal level (5%) (a), or low level (0.2%) of serum (b). Samples of 2×10^4 cells of each preparation were seeded into 60-mm dishes and cell numbers were scored at 24 h intervals. Ψ2-pZipNeo (●), Ψ2-pZE6 1766 (○), Ψ2-pZE6 1768S.D. (■), Ψ2-pZE6 1880 (▼) and Ψ2-pZE67BN (▲).

pZE6 1766 containing the intact E6 ORF expressed unspliced E6 mRNA responsible for transformation at a very low level in transfectants and exhibited low transforming activity (Fig. 2). We do not know why pZE6 1766 expressed only a little unspliced E6 mRNA, but we suspect that some sequence outside the E6 ORF may contribute to the stability of unspliced E6 mRNA. Our previous E6 expression vector pZE67B, which contains parts of the LCR and the E7 ORF besides the E6 ORF expressed a high level of the unspliced E6 mRNA (data not shown) and was highly tumorigenic.[12]

Although the pZE6 1768S.D. vector, which had strong transforming activity contains one-point mutation within the donor splice site (GAG GTA TAT to GAG ATA TAT, Glu Val Tyr to Glu Ile Tyr), its strong activity might not be induced by this mutation because the sequence around the mutation is the same as those of non-transforming HPV type 11 and 6b (GAG ATA TAT, Glu Ile Tyr and GAG ATT TAT, Glu Ile Tyr, respectively).[27,28] However, we cannot completely exclude the possibility that the high activity resulted from alteration of the E6 protein caused by the mutation.

The spliced forms of E6 mRNA, which may produce E6* proteins, are specific for high risk types of HPV, and therefore have been thought to be essential for malignant transformation.[29] However, Sedman *et al.* reported that the truncated E6* proteins are not associated with transformation,[30] assessed in terms of characteristics such as colony formation in soft agar and immortalization of human keratinocytes. In addition, we showed here that the E6* proteins were not associated with tumorigenicity or ability to grow in low serum medium.

The E6 proteins have been reported to bind to the p53 tumor suppressor gene product and to inhibit its suppressor function.[18,31] Several other cellular proteins such as mdm-2 also bind to p53.[32] These associations have been shown to block p53-mediated transcriptional regulation.[33] Interestingly, overexpression of mdm-2 in mouse NIH3T3 cells resulted in acquisition of tumorigenicity.[34,35] Therefore, inactivation of p53 function may be involved in tumorigenicity. On the other hand, the E6 protein itself has transactivating activity on heterologous promoters.[17] These transactivations, alone or in cooperation with inactivation of p53, may be involved in the tumorigenicity. Further studies are required on the relationship between tumorigenicity and the inactivation of p53 or transactivation by the E6 protein.

We conclude that overexpression of unspliced E6 mRNA is closely associated with characteristics of transformation of cells, such as tumorigenicity and a decreased serum requirement for growth.

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