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Growth Dependence of Human Papillomavirus 16 DNA-positive Cervical Cancer Cell Lines and Human Papillomavirus 16-Transformed Human and Rat Cells on the Viral Oncoproteins

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The dependence on human papillomavirus (HPV) oncoproteins of the growth of cervical cancer cell lines [C4-1, HeLa (both containing HPV 18 DNA), CaSki and SiHa (both containing HPV 16 DNA)], HPV 16-transformed human embryonic kidney cells, and HPV 16-transformed rat brain and 3Y1 cells was examined by using antisense RNA approaches. The cells were transfected with plasmids expressing RNA antisense to the HPV 16 or 18 open reading frames E6E7, together with plasmids expressing the hygromycin B resistance gene, and drug-resistant colonies were scored three weeks later. In all the human cell lines, the efficiency of colony formation was lowered by RNA antisense to the resident HPV type. Some of the rat cell lines responded to the antisense plasmids, but some did not. From a nonresponding rat tumor line (3Y1HP-1T), cell clones with various levels of E7 protein were isolated after transfection with the antisense plasmid, and were examined for anchorage-independent growth in soft agar. The colonies formed by the clones with lower E7 levels tended to be smaller and fewer than those formed by the clones with higher E7 levels. These findings strongly suggest that some of the transformed or cancer phenotypes of cells *in vitro* are dependent, even after extensive passages and malignant changes, on expression of the oncoproteins of the resident HPV.

Key words: HPV 16 — Oncoprotein — Cell growth — Antisense RNA — Cancer cell

Human papillomaviruses type 16 and 18 (HPV 16/18) encode two oncogenes, E6 and E7, capable of synergetically transforming rat cells[1] and immortalizing (or extending the life-span of) human cells.[2–4] The finding that E6E7 genes are frequently expressed in cervical cancer cells represents strong, though inconclusive, evidence that these HPVs, probably in conjunction with yet unidentified agents, can cause cancer in humans.[5] In view of the immortalizing effects of the E6E7 genes on human keratinocytes *in vitro*,[3,4] it appears very likely that these two genes play a decisive role in the initial stage of long-term carcinogenesis.

Although it is yet to be investigated whether or not expression of the viral genes is required for carcinogenic changes of immortalized cells, the growth of some lines of cancer cells *in vitro* has been shown to be dependent on the HPV 18 oncoproteins.[6–8] von Knebel Doeberitz *et al.*[6] have shown, using glucocorticoid and plasmids expressing antisense RNA, that expression of the E6E7 genes modulates growth of C4-1 cervical cancer cells containing HPV 18 DNA. Subsequently, the growth of HeLa cells has been shown by an antisense approach to depend on the viral oncoproteins.[7] These findings, together with a study on chromosomal integration of HPV DNA,[8] strongly suggest that expression of E6E7 is required for maintenance of the cancer cell phenotypes. In this study we attempted to generalize the above observations by extending them to other cervical cancer cell lines containing HPV 16 DNA (CaSki and SiHa) and HPV 16-transformed human and rat cells, using an antisense colony assay.

MATERIALS AND METHODS

Plasmids Expression vector pSRα-0[9] was used as a backbone vector for construction of the plasmids to express antisense RNA. Plasmids pSV2*neo*[10] and pChmBpl (constructed by Dr. Izumu Saito), a derivative of pCHD2L,[11] contain neomycin (G418) resistance and hygromycin resistance genes, respectively, under the control of the SV40 promoter. Plasmid pSRα-HP16E6E7[2] contains HPV 16 E6/E7 genes in a sense orientation.

Cells The cell lines used in this study are listed in Table I. They include four established cell lines from human cervical cancer, which have been passaged *in vitro* for years and well characterized as regards the resident HPV DNA types,[12–15] four HPV 16-transformed human embryonic kidney (HEK) cell lines, and six HPV 16-transformed rat cell lines.[1,16,17] The HEK cell lines were prepared for this study, as described previously for human primary fibroblasts,[2] by cotransfection of primary HEK cells (in 50-mm dishes) with 10 μg of pSRα-HP16E6E7 and 5 μg of pSV2*neo*. The transfected cells were cultured in the presence of G418 (400 μg/ml) and the cells that survived continued to grow for more than 50 to 100 population doublings beyond the normal life span. These late passage cells were morphologically distinguishable from the normal HEK cells and formed

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denser colonies than the normal HEK cells. The transformed HEK cell lines were shown to contain transcripts for the E6E7 open reading frames (ORFs) by the Northern blot method (data not shown). Two rat cell lines, RBE6E7g and RBE6E7k, derived from primary Fischer rat brain cells[17] and four rat 3Y1 cell lines have been characterized previously.[1, 16]

Construction of antisense plasmids Two plasmids expressing antisense RNA were constructed by inserting the 851 bp HPV 16 DNA *Dde*I-*Pst*I fragment (nt 25 to 875)[18] or the 1,049 bp HPV 18 DNA *Rsa*I-*Rsa*I fragment (nt 7740 to 931),[19] each containing the E6E7 ORFs intact and with added *Hin*dIII linkers, into pSRα-0 at the *Hin*dIII site in an antisense orientation. The two expression plasmids containing the HPV 16/18 fragments were designated pSRα-anti16E6E7 and pSRα-anti18E6E7, respectively.

Transfection The calcium coprecipitation method[20] was used for transfection.

Detection of HPV E7 protein For a protein (Western) blot assay, the E7 protein was immunoprecipitated from cytoplasmic fractions with a rabbit antiserum, anti-*lac*-E7[21] or anti-*lac*-18E7,[22] and was electrophoresed in SDS-15% polyacrylamide gels. The protein bands were blotted onto nitrocellulose membranes and visualized by staining with anti-*lac*-E7 or anti-*lac*-18E7 and Konica Immunostain HRP (Konica Corp, Tokyo). For an immunoprecipitation assay of selected rat 3Y1HP-1T cell clones, the HPV16 E7 protein was pulse-labeled with [^{35}S]methionine-cysteine for 1 h, then immunoprecipitated with anti-*lac*-E7 from the cytoplasmic fraction, electrophoresed and processed as described previously.[21]

Antisense colony assay The antisense plasmid (20 μg) was cotransfected with 5 μg of pChmBpl expressing the hygromycin resistance gene into a subconfluent cell culture (50-mm dish). Duplicate transfected cultures were replated 24 h later, each into two 90-mm dishes (four dishes in total) at a split ratio giving less than 200 colonies per dish at the time of reading, and thereafter maintained in the presence of hygromycin B (100 to 200 μg/ml) with refeeding every 5 days for 3 weeks. Then, the cultures were fixed with methanol and stained with 5% Giemsa for colony scoring.

Assay for anchorage-independent growth Cells (10^3 or 3×10^3 per 50-mm dish) were seeded in a 0.4% agarose medium and cultured for 3 weeks.

RESULTS

We examined the cell lines listed in Table I by Western blot assay to confirm the presence of the steady-state HPV oncoproteins. Although the level of E7 protein was different from cell line to cell line, all of the cell lines contained the E7 protein and a representative set of the Western blot assays is shown in Fig. 1. In the HPV 16-positive cell lines in Table I, the E6 oncoprotein was not detectable by Western blot assay with anti-E6 mouse monoclonal antibody MAb618,[23] which could readily stain the E6 protein bands from the transiently expressing COS-1 cells (data not shown). We used human and rat cell lines containing the E6E7 ORFs intact (Table I), and expressing at least the E7 oncoprotein (Fig. 1), to test the effect of RNA antisense to the E6E7 ORFs on cell growth.

Table II summarizes the numbers of hygromycin-resistant colonies of the human cell lines (Table I) after cotransfection with the antisense plasmid and the plasmid expressing a dominant selection marker, and Fig. 2A shows representative cultures with the drug-resistant colonies. The HPV 18-positive C4-1 cells formed colonies after transfection with pSRα-anti18E6E7 half

Table I. Cervical Cancer Cell Lines and HPV 16-Transformed Cell Lines

Cell line	Origin	HPV DNA retained or transforming	References
C4-1	Human cervical cancer	HPV 18	12, 13, 14, 15
HeLa	Human cervical cancer	HPV 18	12, 13, 14, 15
CaSki	Human cervical cancer	HPV 16	13, 14
SiHa	Human cervival cancer	HPV 16	13, 14
HEK6492	Human embryonic kidney cell	HPV 16 E6E7	
HEK6850	Human embryonic kidney cell	HPV 16 E6E7	
HEK9330	Human embryonic kidney cell	HPV 16 E6E7	
HEK9350	Human embryonic kidney cell	HPV 16 E6E7	
RBE6E7g	Primary rat brain cell	HPV 16 E6E7	17
RBE6E7k	Primary rat brain cell	HPV 16 E6E7	17
3Y1HP-1	Rat 3Y1 cell line	HPV 16 whole	16
3Y1HP-1T	Rat transplantable tumor	HPV 16 whole	
3Y158147	Rat 3Y1 cell line	HPV 16 E6E7	1
3Y158148	Rat 3Y1 cell line	HPV 16 E6E7	1

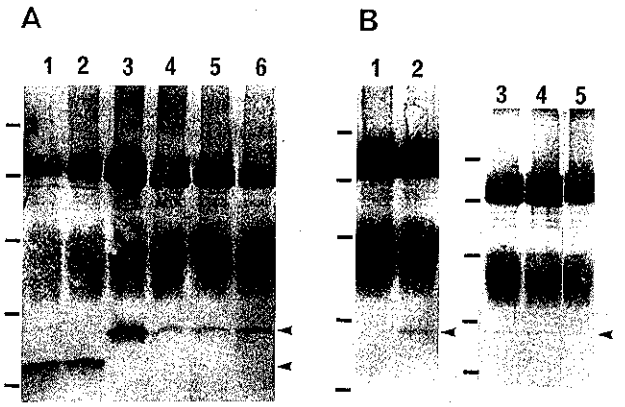

Fig. 1. Western blot analyses of HPV 16/18 E7 proteins in human cervical cancer cell lines, and HPV 16-transformed HEK and rat cells. The E7 protein was immunoprecipitated with anti-*lac*-E7 or anti-*lac*-18E7 from cytoplasmic fractions (of three 9.0-cm dish cultures), electrophoresed in polyacrylamide gels, blotted on nitrocellulose membranes, and visualized. The positions of HPV 18 and HPV 16 E7 are indicated by arrowheads. Bars on the left indicate the positions of molecular size markers of 15, 20, 28, 47, and 74 kDa (from the bottom). (A) E7 protein from human cells; C4-1 (lane 1), HeLa (lane 2), CaSki (lane 3), SiHa (lane 4), HEK6492 (lane 5), and HEK9330 (lane 6). (B) E7 protein from rat cells; RBE6E7g (lane 1), RBE6E7k (lane 2), 3Y1HP-1 (lane 3), 3Y1HP-1T (lane 4), and 3Y158147 (lane 5).

Table II. Colony Formation by Hygromycin-resistant Human Cells after Cotransfection with Plasmid Encoding a Dominant Selection Marker Gene and Plasmid Encoding RNA Antisense to HPV 16 or 18 E6E7 ORFs

Cell line	Expt. No.	Colony No./dish ± SD[a]		
		anti16E6E7[b]	anti18E6E7[c]	backbone[d]
C4-1	1	110±13	58±5.2	98±5.2
	2	45±5.4	19±4.6	
	3	53±4.6	35±3.2	
HeLa	1	40±15	19±5.5	33±8.3
	2	102±11	14±4.1	
	3	161±18	70±8.3	
CaSki	1	27±2.3	59±12	52±4.5
	2	10±2.0	24±3.5	
	3	6.5±1.1	25±8.4	
SiHa	1	6.3±2.4	14±2.8	
HEK6492	1	32±3.0	70±3.0	75±4.2
	2	21±1.8	53±7.5	
	3	23±3.8	64±8.3	
HEK6850	1	6.0±2.6	18±3.5	13±2.0
	2	5.3±1.1	12±3.3	
HEK9330	1	1.5±0.9	7.5±1.5	7.5±1.9
	2	4.0±2.9	24±5.3	
HEK9350	1	23±3.3	38±8.0	46±5.2
	2	5.8±1.9	18±7.5	
HEK	1	16±0.5	15±0.5	13±3.5

a) Mean of four 90-mm dishes ± standard deviation (SD).
b) Transfection with pSRα-anti16E6E7.
c) Transfection with pSRα-anti18E6E7.
d) Transfection with pSRα-0, a backbone vector for pSRα expression plasmids.

as efficiently as after transfection with pSRα-anti16E6E7 or with backbone vector pSRα-0. These results are essentially the same as those obtained by von Knebel Doeberitz *et al.*[6] in a similar antisense colony assay, indicating that the growth of C4-1 is dependent on expression of the HPV 18 oncoproteins. Like C4-1, other cervical cancer cell lines HeLa, CaSki, and SiHa were found to be similarly sensitive to colony inhibition by the respective antisense plasmid. The drug-resistant colonies formed after transfection with the plasmid for RNA antisense to the resident HPV type were fewer and sometimes smaller than those formed after transfection with the plasmid for RNA antisense to the heterologous HPV type. It is unlikely that the observed antisense effects on colony formation were produced through inhibition of the HPV oncoprotein-mediated transactivation of the promoter controlling the dominant selection marker gene, because, unlike the adenovirus E2 promoter, the SV40 promoter was not transactivated by either the HPV 16 E6 or E7 gene in the chloramphenicol acetyltransferase assay (data not shown).

The antisense colony assay also showed that, whereas the growth of normal HEK cells was unaffected (Table II), the four freshly transformed HEK cell lines were susceptible to inhibition of colony formation by the antisense plasmid. Thus, the data (Table II and Fig. 2A) indicate that, despite the varied levels of the steady-state E7 protein (Fig. 1), the human cancer cell lines and the HPV 16-transformed HEK cells grew dependently on expression of the resident HPV oncoproteins, at least to the degree shown by the C4-1 cells.[6]

Unlike the human cell lines, some of the HPV 16-transformed rat cell lines were insensitive and some were sensitive to colony inhibition in the antisense colony assay (Table III and Fig. 2B). Two rat cell lines RBE6E7g and RBE6E7k derived from primary rat brain (glia) cells and one line 3Y1HP-1 and its malignant derivative 3Y1HP-1T (rat tumor cell line, described below), as well as normal 3Y1 cells, formed hygromycin-resistant colonies equally well after transfection with the plasmids expressing RNA antisense to HPV 16 and HPV 18 E6E7 ORFs. On the other hand, two 3Y1 cell lines 58147 and 58148 were susceptible, like the human cell lines, to colony inhibition by transfection with the antisense plasmid. The susceptibility of 3Y158147 was unchanged even after 27 passages at a split ratio of 1:30.

Because anchorage-independent growth of the HPV 16-transformed rodent cells is known to be influenced by the

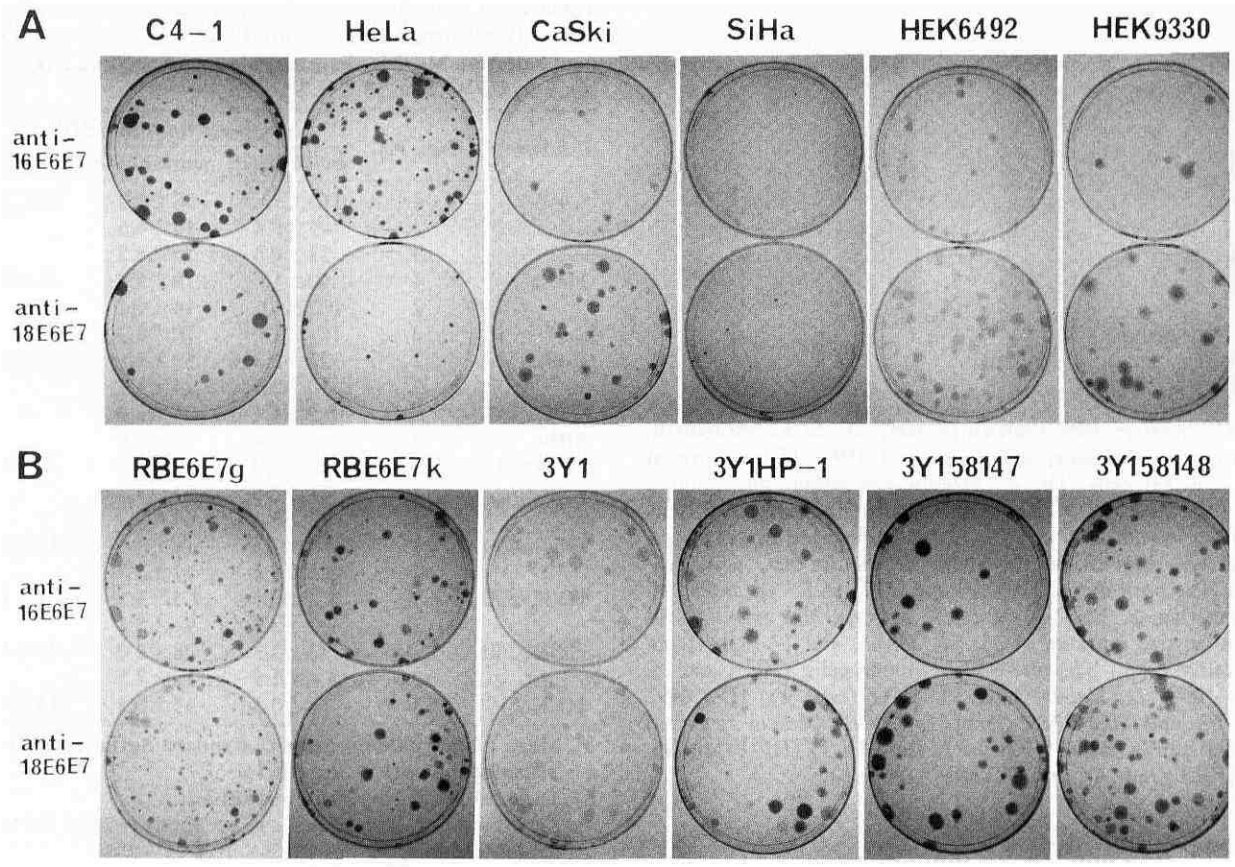

Fig. 2. Antisense colony assay; hygromycin-resistant colonies formed by human cells (A) and rat cells (B) after transfection with plasmids expressing RNA antisense to HPV 16/18 E6E7 ORFs. Cells were transfected with pSRα-anti16E6E7 or pSRα-anti18E6E7 and pChmBpl (encoding the hygromycin resistance gene), were replated at an appropriate concentration in a 90-mm dish, and cultured in the presence of hygromycin. Cell lines shown on the top are described in Table I. Descriptions on the left, anti-16E6E7 and anti-18E6E7, indicate transfection with pSRα-anti16E6E7 and pSRα-anti18E6E7, respectively. SiHa colonies were much smaller than the others in the presence or absence of hygromycin.

E7 gene,[24] we examined such properties of the 3Y1HP-1T cells, which, like their ancestral 3Y1HP-1 cells, were non-responding in the antisense colony assay (Table III and Fig. 2B), but unlike 3Y1HP-1 were capable of forming colonies in a soft agar medium. The 3Y1HP-1T cell line was established from a Fischer rat tumor produced by inoculation of 3Y1HP-1 cells that had been extensively passaged *in vitro* over a year and were morphologically different from the early passage 3Y1HP-1 cells. Whereas the early passage 3Y1HP-1 was not transplantable into nude mice,[16] the late passage 3Y1HP-1 spontaneously became transplantable into newborn Fischer rats. Immunoprecipitation assays showed that the 3Y1HP-1T cells contained more E7 protein per cell than the early passage 3Y1HP-1 cells.

In a preliminary study we tested the response of 3Y1HP-1T cells to the antisense plasmids in terms of anchorage-independent growth. The 3Y1HP-1T cells (in a 30-mm dish) were transfected with pChmBpl (2 μg) and pSRα-antiHP16E6E7 or pSRα-antiHP18E6E7 (8 μg) and were transferred into an agarose medium 24 h later. Hygromycin (200 μg/ml) was added to the medium on days 3 and 8, and the cultures were maintained for 10 days. The cultures that survived after transfection with pSRα-antiHP18E6E7 appeared to contain more large colonies than did those with pSRα-antiHP16E6E7. Thus, the growth of 3Y1HP-1T cells in soft agar appeared to be affected by the antisense plasmid.

We examined the relation of anchorage-independent growth to levels of E7 proteins with individual 3Y1HP-1T cell clones. Hygromycin-resistant cell clones were isolated from 3Y1HP-1T cells (30-mm dish) cotransfected with pChmBpl (2 μg) and pSRα-anti16E6E7 or pSRα-anti18E6E7 (8 μg). The transfected cells were

replated one day later and thereafter maintained in the presence of hygromycin (100 μg/ml) for 11 days. Clones were isolated and tested for the presence of the HPV 16 E7 protein by the Western blot method and for colony formation in soft agar. Among 14 out of 16 clones tested, there was a tendency that the clones yielding a visible E7 band on a Western blot formed larger colonies than did the clones without the visible band.

To confirm the above observation, we characterized G418 (400 μg/ml)-resistant cell clones randomly isolated from 3Y1HP-1T cells (30-mm dish) cotransfected with pSV2*neo* (2 μg) and pSRα-anti16E6E7 or pSRα-0 (18 μg). Fig. 3 shows the immunoprecipitated E7 from some representative clones. The majority of the clones had an E7 level similar to that of clone 16 or clone 20. Clone 19, which was one of the 10 clones isolated and characterized after transfection with pSRα-antiE6E7, showed an E7 level significantly lower than the others. The 3Y1HP-1T

Table III. Colony Formation by Hygromycin-resistant Rat Cells after Cotransfection with Plasmid Encoding RNA Antisense to HPV 16 or 18 E6E7 ORFs and Plasmid Encoding a Dominant Selection Marker Gene

Cell line	Expt. No.	Colony No./dish ± SD[a]		
		anti16E6E7[b]	anti18E6E7[c]	backbone[d]
RBE6E7g	1	120±10	118±13	
RBE6E7k	1	79±1.5	74±12	
	2	71±7.3	71±5.8	
3Y1HP-1	1	30±6.5	29±1.0	27±6.0
	2	77±6.5	83±2.5	70±2.0
	3	28±8.4	28±5.5	34±3.5
3Y1HP-1T	1	123±2.5	130±10	142±15
3Y158147	1	18±5.0	37±2.5	30±7.5
	2	18±5.5	40±8.6	48±4.5
	3	17±5.0	41±6.5	30±1.5
3Y158148	1	39±9.5	61±0.5	56±4.5
3Y1	1	52±5.5	48±4.1	43±9.9

a) Mean colony number of four 90-mm dishes ± standard deviation (SD).
b) Transfection with pSRα-anti16E6E7.
c) Transfection with pSRα-anti18E6E7.
d) Transfection with pSRα-0, a backbone vector for pSRα expression plasmids.

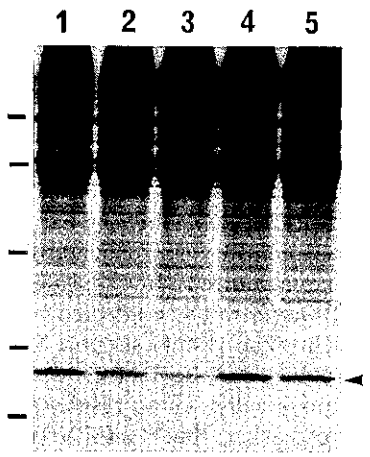

Fig. 3. Immunoprecipitation of HPV 16 E7 protein from HPV 16-transformed rat 3Y1HP-1T cell clones. The ^{35}S-labeled E7 protein was immunoprecipitated with rabbit anti-*lac*-E7 serum from cytoplasmic fractions. Immunocomplexes were electrophoresed in polyacrylamide gels and autoradiographed. Clones 16 (lane 1) and 17 (lane 2) were isolated from 3Y1HP-1T cells after transfection with pSRα-0. Clones 19 (lane 3), 20 (lane 4), and 21 (lane 5) were isolated from 3Y1HP-1T cells after transfection with pSRα-anti16E6E7. The arrowhead denotes the position of HPV 16 E7. Bars (on the left) indicate the positions of molecular size markers of 15, 20, 28, 47, and 74 kDa (from the bottom).

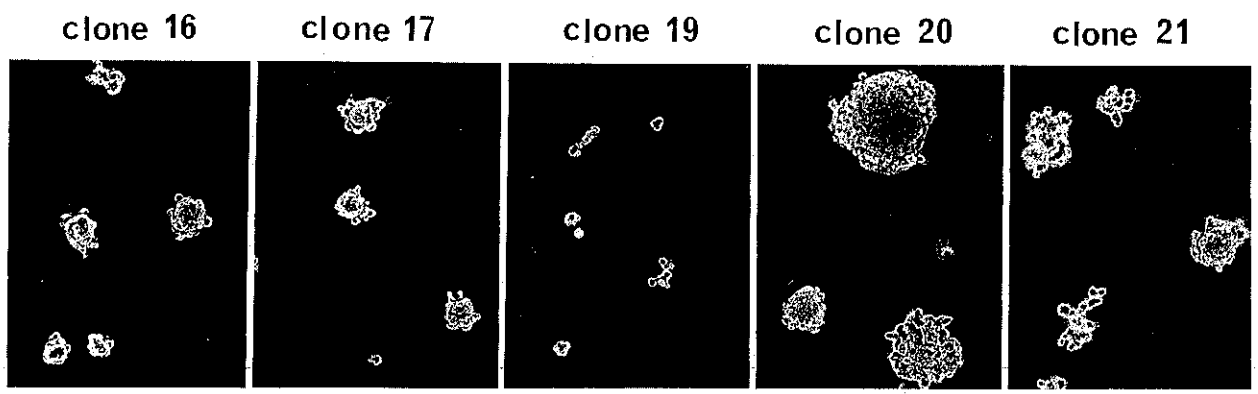

Fig. 4. Anchorage-independent growth of HPV 16-transformed 3Y1HP-1T cell clones. Expression of the HPV 16 E7 protein in each cell clone and the origin of each clone are shown in Fig. 3 and its legend. Cells were seeded and cultured in the agarose medium for 3 weeks before photomicrographs were taken. The identity of each clone is denoted on the top.

clones 16, 17, 19, 20, and 21, which contained various levels of pulse-labeled E7 (Fig. 3), were examined for colony formation in soft agar. As shown in Fig. 4, the clones with lower E7 levels tended to form smaller colonies in an agarose medium. The efficiency of soft agar colony formation in clone 19 was one-third to one-half of that of clone 16, 17, 20, or 21. Thus, the anchorage-independent growth of 3Y1HP-1T clones, or the malignancy acquired by the cell during repeated passages, appeared to be affected by the level of the HPV oncoproteins.

DISCUSSION

In summary we have shown that the cervical cancer cell lines (C4-1, HeLa, CaSki, and SiHa), the HPV 16-transformed HEK cells, and some of the HPV 16-transformed rat cells are dependent on expression of the HPV 16/18 oncoproteins to grow efficiently *in vitro*, and that the malignancy acquired after repeated passages of rat 3Y1HP-1 cells appears to be affected by the level of the E7 oncoprotein. The results support the hypothesis that the oncoproteins can be factors determining the malignancy of tumor cells. These results are consistent with those reported earlier; that the HPV 16 E7 gene can induce cell DNA synthesis of rat 3Y1 cells,[25] that E7 expression is required for maintenance of the HPV 16 and EJ-*ras* mediated transformation phenotype,[26] and that the growth of C4-1 and HeLa cells is modulated by the HPV 18 oncoproteins.[6,7]

The significance of the difference in susceptibility between the human and rat cell lines and between the rat cell lines remains to be investigated. The difference may be partly ascribable to the fact that rodent cells are spontaneously transformed *in vitro* much more frequently than human cells. This may complicate viral transformation of rodent cells. Perhaps the immortalizing function of the HPV oncoproteins is not the major target of the antisense assay, because both unimmortalized HPV 16-transformed HEK cells and some of the immortalized rat 3Y1 transformants were susceptible to the antisense plasmids. Probably, the antisense plasmids mostly affect the functions of the HPV oncoproteins to stimulate cell growth dependent on or independent of anchorage.

The inhibitory effects of antisense plasmids on cell growth were specific to the HPV type and, although untestable in the assay described above, probably resulted from the decrease in expression of HPV oncoproteins caused by antisense RNA. The growth inhibition of C4-1 cells has been shown to be accompanied by a reduction of the E7 protein level caused by antisense RNA expressed from the introduced expression plasmid.[6] Unlike intracellular introduction of the plasmids expressing antisense RNA,[6] direct application of synthetic nuclease-resistant oligonucleotides to the CaSki cultures has been reported to have both specific anti-HPV 16 and other non-specific effects on cell growth and synthesis of viral proteins.[27] Perhaps the extracellular application of antisense oligonucleotides has yet-unidentified additional effects different from those of the intracellularly expressed antisense RNA.

The findings in this study strongly suggest that the HPV 16/18 oncoproteins, which are believed to be involved in the initial stage of carcinogenesis by immortalizing the target cells,[3,4] also play an important role in maintenance of the transformed or cancerous phenotype by stimulating cell growth. However, it is unclear at present whether the HPV 16/18 oncoproteins are involved in the process of increasing malignancy of the immortalized cells. Because the previous[6-8] and present data were obtained with cell lines established from cervical carcinoma (selected and cultured *in vitro*), a question arises as to whether the growth or maintenance of cancer cells *in vivo* (without selection by cell cultivation) is generally affected by expression of the HPV oncoproteins. Detailed analyses of the HPV transcripts in the *in vivo* cancer cells may partly answer this question.

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