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Genetic Analysis of *in Vitro* Progression of Human Papillomavirus-transfected Human Cervical Cells[1]

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ABSTRACT

The authors have established an *in vitro* model system which demonstrates the progression of the transformed phenotypes of human cervical epithelial cells transfected with human papillomavirus (HPV) type 16 and 18 DNAs. Both viral DNAs exhibit immortalizing potential; however, only HPV 18-immortalized cell lines progress to exhibit anchorage-independent growth and, in a limited number of cases, tumorigenesis. In this paper, the authors have examined the genetic basis for this *in vitro* progression step by step, including immortalization, anchorage-independent growth, and tumorigenicity of the HPV-transfected human cervical epithelial cells by cell fusion. The results suggest that (*a*) all three transformed phenotypes, *i.e.*, immortalization, anchorage-independent growth, and tumorigenesis, in this *in vitro* cervical carcinogenesis model are a result of recessive changes in genes or processes involved; (*b*) inactivation of p53 and retinoblastoma protein is not sufficient for immortalization of human cervical epithelial cells; (*c*) HPV expression *per se* does not account for immortalization of human cervical epithelial cells; (*d*) immortalization of human cervical epithelial cells initiated by HPV can occur through different processes, although one of them is the most preferred; and (*e*) probably only one group of recessive genes appears to be involved in the loss of anchorage-dependent growth for HPV-immortalized human cervical cells.

INTRODUCTION

HPV[4] DNAs, in particular HPV 16 and 18, have been found at high frequency (80%) in human cervical cancers (1). We and others have demonstrated that HPV 16 and 18 can immortalize normal human cervical epithelial cells *in vitro* (2, 3). Since cellular senescence is a basic characteristic of normal human cells in culture (4, 5), the ability of HPV 16 and 18 to immortalize human cervical epithelial cells argues for their initiating role in human cervical carcinogenesis.

Recently, it has been shown that DNA tumor viruses, including SV40, adenoviruses, and HPV 16 and 18, share some common mechanism associated with their transforming activity (6, 7). Two tumor suppressor gene products, *i.e.*, p53 and pRB, have been found associated with oncogene products of these DNA tumor viruses (8, 9). The SV40 T antigen, adenovirus E1a proteins, and the E7 proteins of HPV 16 and 18 bind pRB, while SV40 T antigen, adenovirus E1b proteins, and the E6 proteins of HPV 16 and 18 bind p53. It has been suggested that these viruses execute their immortalizing and transforming functions through binding and inactivation of p53 and pRB by these viral oncogene products (10).

Carcinogenesis is a multistep process (11) which may include functional inactivation of p53 or pRB or both. In our *in vitro* model of cervical carcinogenesis, there are several distinct stages, including at least immortalization, anchorage-independent growth, and tumorigenicity. *In vitro* evidence shows that HPV 16/18 can immortalize normal human epithelial cells (2, 3); however, only in rare cases, the HPV 18-immortalized cells become tumorigenic at higher passages (12, 13). This indicates that additional cellular changes are necessary to render the HPV-immortalized cells tumorigenic.

In this paper, we have investigated the genetic characteristics of each specific step, *i.e.*, immortalization, anchorage-independent growth, and tumorigenesis, in an *in vitro* model of cervical carcinogenesis by cell fusion techniques and observed that each of these steps is under the control of recessive regulatory processes.

MATERIALS AND METHODS

Cell Lines. The HPV 16 and 18 immortalized human cervical keratinocyte cell lines used here have been characterized and described in detail (12), and their properties are summarized in Table 1. C33A was purchased from the American Type Culture Collection. Normal cervical epithelial cells and fibroblasts were initiated and maintained as described (2, 12).

Transfection and Cell Fusion. Each cell line was transfected with pSV2neo or pSV2hyg by lipofection (Lipofectin, BRL) as recommended by the manufacturer. G418 (250 to 300 μg/ml; Gibco)-resistant colonies and hygromycin (100 μg/ml; Boehringer)-resistant colonies were isolated and expanded as the parental cells. In order to isolate a "universal hybridizer," G418-resistant Z213A cells (identified as Z213Aneo) were exposed to 100 μM 6-thioguanine (Sigma), and neo$^+$HPRT$^-$ clones were isolated and tested in G418 (300 μg/ml) and HAT medium (Gibco).

In cell fusion experiments, 10^6 cells of each parental cell line were plated in 60-mm dishes. For fusion between cell lines, one parental line was G418 resistant, and the other was hygromycin resistant; for fusion between Z213A and normal epithelial cells or fibroblasts, Z213A neo$^+$HPRT$^-$ was used. After overnight incubation, the Petri dishes were washed once with HBSS (Gibco), treated with 48% (w/v) PEG 1000 (Baker) in Opti-MEM (Gibco) for 50 s, and washed 3 times with HBSS. The cells were then fed with fresh medium. The following day (for Z213A × normal epithelial cells 2 days after fusion), the cells were replated at 5×10^4 cells per 100-mm plate for selection. As control, the dishes containing either parental cells were also subjected to PEG treatment and replated for selection. In some cases, the dishes containing a mixture of both parental cells were directly replated for selection without PEG treatment. For cell fusions between cell lines, the hybrid cells were selected with G418 (300 μg/ml) plus hygromycin (100 μg/ml). For Z213A × normal epithelial cells or fibroblasts, the hybrid cells were selected with G418 (300 μg/ml) plus HAT. In both controls, there were no viable cells at the end of the selection period. The clones of hybrid cells were isolated and subcultivated with selection medium until five passages after isolation. Only hybrid clones having undergone more than 16 PD were included in the results (15). In each fusion experiment, 7 to 10 hybrid colonies were subcultivated until senescence as defined by the inability of reaching 1 PD in 4 wk (16) or immortalization as defined by reaching 100 PDs (15). Thus, senescence or loss of immortalization was determined on a minimum of 7 to 10 independent clones for each cell pair, derived from 3 to 4 different fusion experiments. To confirm the occurrence of cell fusions, the DNA content of hybrid cells and both parental cells was measured by DNA flow cytometry (Becton Dickinson; Lysis II version 1.0) of propidium iodide-stained cells (17).

RT-PCR. Since the numbers of senescing cells were not sufficient for Northern analysis, RT-PCR was performed to examine expression of the HPV genomes. Total RNA of senescing hybrid cells, immortal hybrid cells, and parental cells was extracted by the acid guanidinium thiocyanate-phenol chloroform method (18). In the case of senescing clones, parallel plates were maintained for an additional month to exclude the possible existence of segregant proliferating subpopulations. Total RNA (100 ng) from each sample was subjected to RT-PCR as described (19). To avoid possible contamination of genomic DNA in the total RNA preparation, the PCR primers were specifically

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[4] The abbreviations used are: HPV, human papillomavirus; SV, simian virus; pRB, retinoblastoma protein; HAT, 10^{-4} M hypoxanthine:4×10^{-7} M aminopterin:1.6×10^{-5} M thymidine; HBSS, Hanks' balanced salt solution; PEG, polyethylene glycol; PD, population doublings; RT-PCR, reverse-transcription polymerase chain reaction; PCR, polymerase chain reaction.

Table 1 *Properties of HPV cell lines*

Designation	Patient source	Cell line	HPV type	Passage	CFE[a] (%)	Tumorigenicity[b]
A	1	Z132	16	>50	<0.1	−
B	2	Z172	16	>50	<0.1	−
C	3	Z202	16	>50	<0.1	−
D	3	Z208	16	>50	0.16	ND
E	1	Z133	18	>50	3.91	−
F	2	Z183A	18	>50	4.22	+
G	2	Z183B	18	>50	7.58	+
H	3	Z213A	18	>50	4.10	−
I	4	Z243	18	>50	2.78	−

[a] CFE, colony-forming efficiency in soft agar; ND, not done.
[b] Data from Ref. 12.

designed to amplify HPV 18E6* (spliced form of E6) (20). The upstream Primer 1 was GAGGA TCCAA CACGG CGACC (nucleotides 117-136) (21). The downstream Primer 2 was CGCAG GCACC TCTGT AAGTT (nucleotides 223-233, 416-424) (20). The pair of primers can amplify E6* (predicted size, 126 base pairs) but not the full-length E6; these sequences were selected in order to exclude the possibility of contaminating HPV 18 genomic DNA in the reaction. PCR was performed for 30 cycles as follows: 30 s at 95°C; 30 s at 60°C; and 1 min at 72°C. The PCR products were then separated in 1.8% agarose gel and blotted on Nytran (Schleicher & Schuell) which was then hybridized with the ^{32}P-radiolabeled HPV 18 E6 probe (22).

Soft Agar Assay and Tumorigenicity Assay. The parental cells and hybrid cells were assayed for anchorage-independent growth (2). Tumorigenicity was determined as previously described (12). The nude mice were followed for 12 wk or until they died.

Northern Analysis. Total cellular RNA was prepared, and Northern analysis was performed as described (22).

RESULTS

Analysis of the Immortalization Stage. The cellular senescence exhibited by normal human cervical epithelial cells *in vitro* can be overcome by transfection with HPV 16 and HPV 18 DNAs but not HPV6 DNA (2, 3). Furthermore, this difference correlates with the ability of the HPV 16 and HPV 18, but not HPV6 E6 and E7 proteins, to bind and presumably inactivate the tumor suppressor gene products p53 and pRB, respectively. We were interested to determine (*a*) whether HPV 16 and 18 expression *per se* can account for immortalization of normal cervical epithelial cells, *i.e.*, whether the immortalization process by HPV is dominant; and (*b*) whether inactivation of both p53 and pRB is sufficient for immortalization of normal cervical epithelial cells.

To answer these questions, we hybridized one HPV 18-immortalized human cervical cell line (Z213A) with normal human cervical epithelial cells or fibroblasts (Table 2). If the immortalization of human epithelial cells by HPV is a dominant process, the hybrid (Z213A × primary epithelial cells or fibroblasts) cells should always be immortal. However, only one of 19 clones became immortal, suggesting recessivity of this trait. Furthermore, similar results were obtained in hybridization experiments of Z213A and Z183A with C33A cells, a HPV-negative human cervical line that has mutations in the p53 and pRB genes (10). Before accepting this conclusion, alternative possibilities should be excluded.

First, senescence may occur because of inhibition of HPV mRNA expression in the hybrid cells. To test for this possibility, we examined senescing hybrid cells for HPV mRNA using the RT-PCR method because of the low number of available cells. When the total RNA of senescing cells was first reverse transcribed and then amplified by PCR, all the senescing hybrid clones tested (Fig. 1, *Lanes b* to *e* and *g*) like their parental cells (*Lanes f* and *i*), and one immortalized hybrid clone (*Lane h*) expressed HPV 18 E6* mRNA. Thus, senescence of the hybrid cells is not due to loss of HPV 18 mRNA expression, although a relative decrease of the HPV 18 mRNA in the senescing hybrid cells cannot be excluded.

Second, the E6 and E7 expression in the HPV-immortalized cells may be sufficient to overcome "one dose" of p53 and pRB, but not the "double dose" of hybrid cells or alternatively, immortalization involves additional cellular processes directly inactivated by HPV gene products or by other random or specific mechanisms. If inactivation of p53 and pRB and/or other cellular processes by HPV gene products is sufficient for immortalization, then hybrids between HPV-immortalized cell lines would be immortal.

To test for this possibility, we fused nine HPV-immortalized cervical cell lines (four HPV 16 and five HPV 18 cell lines; Table 1) with each other. As shown in Table 3, in some combinations including the autologous fusions, all of the hybrid clones were immortal, while in other combinations, most of the hybrid clones senesced (about 1/5 to ¼ of hybrid clones escaped from senescence in these combinations, probably due to chromosomal segregation during cell division).

Based on our results, several points may be suggested. First, the observed senescence in HPV-immortalized × normal hybrids cannot be attributed solely to the inability of the E6 and E7 gene products to overcome a "double dose" of p53 and pRB. Second, inactivation of p53 and pRB and/or other cellular processes by HPV gene products is not sufficient for immortalization.

The nine HPV lines tested fall in at least four complementation groups (Table 4), suggesting that there are at least four groups of cellular processes involved in immortalization initiated by HPV 16 and 18. Among them, Group II is the process most frequently used for HPV immortalization. The cellular process involved in the immortalization of individual HPV cell lines was not related to the HPV types (Table 4), to the type of plasmid constructs, to the patients from whom

Table 2 *Fusions between HPV18 cell lines and normal cells*

Cell hybrid	Immortal clones/total clones	Range of PD at cessation of division
Z213A[a] × C × K[b]	1/9	<30
Z213A[a] × C × Fb[c]	0/10	<30

[a] HPV 18-immortalized cell lines.
[b] Normal cervical epithelial cells.
[c] Normal cervical fibroblasts.

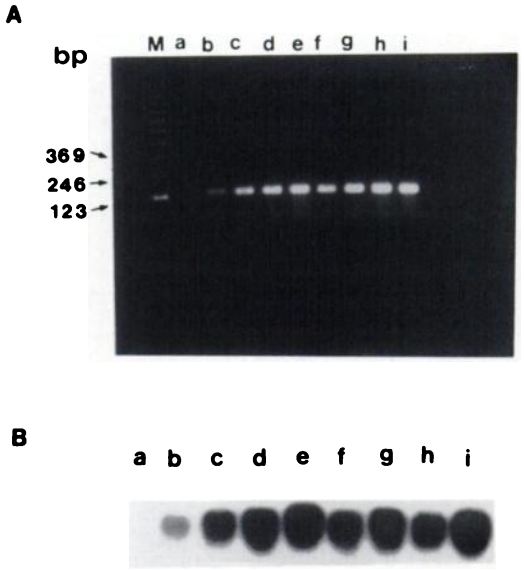

Fig. 1. Expression of HPV 18 mRNA in senescing hybrid cells. Total RNA (100 ng) from senescing hybrid clones (*Lanes b* to *e* and *g*), an immortalized hybrid clone (*Lane h*), and parental cell lines (*Lanes f* and *i*) was reverse transcribed (except *Lane a*) and then amplified by PCR using primers specific for HPV 18 E6* (see "Materials and Methods"). The PCR products were separated on 1.8% agarose gel (*A*) and then blotted to Nytran, which was hybridized with a ^{32}P-labeled HPV 18 E6 probe (*B*). *Lane a*, Z213A neo (without RT); *Lane b*, Z213A × CxK, clone 4; *Lane c*, Z213A × CxK, clone 6; *Lane d*, Z213A × C33A, clone 8; *Lane e*, Z213A × C33A, clone 36; *Lane f*, Z213A neo; *Lane g*, Z183A × C33A, clone 17; *Lane h*, Z183A × C33A, clone 2; and *Lane i*, Z183A neo. *bp*, base pair. *Lane M*, molecular weight marker (123 bp).

Table 3 *Fusions between HPV cell lines*

		A Z132	B Z172	C Z202	D Z208	E Z133	F Z183A	G Z183B	H Z213A	I Z243	HeLa
A	Z132	+[a]	–	–	–	–	–	–			
B	Z172	–		–	+	+	+	–			
C	Z202	–	–		–			–			
D	Z208	–	+	–							
E	Z133	–	+			+	+	–	+	+	
F	Z183A	–	+			+		–	+	+	+
G	Z183B	–	–	–		–	–		–		–
H	Z213A					+	+	–			
I	Z243					+	+				
	Hela						+	–			

[a] +, immortalization of all hybrid clones; –, senescence in most of the hybrid clones.

Table 4 *Assignment of HPV cell lines to different complementation groups for immortalization*

I	II	III	IV
Z132 (HPV 16)	Z172 (HPV 16)	Z202 (HPV 16)	Z183B (HPV 18)
	Z208 (HPV 16)		
	Z133 (HPV 18)		
	Z183A (HPV 18)		
	Z213A (HPV 18)		
	Z243 (HPV 18)		
	HeLa (HPV 18)		

the cells were derived (Tables 1 and 4), or to the G418 selections (data not shown). When HeLa cells were fused with Z183A or Z183B cells, respectively, the HeLa × Z183A hybrid clones, but not the HeLa × Z183B hybrids, were consistently immortal (Table 3). This indicates that the cellular process in HeLa is the preferred pathway for HPV immortalization (Table 4) of human cervical epithelial cells.

Analysis of Anchorage-independent Growth Stage. In the *in vitro* model of carcinogenesis, anchorage-independent growth represents a transformation step beyond immortalization. In our nine HPV 16 and 18 immortalized cervical cell lines, the five HPV 18 cell lines always generate colonies in soft agar at a similar frequency, while all four HPV 16 cell lines do not (Table 1). This is consistent with clinical findings indicating that HPV 18-containing carcinomas are more aggressive than HPV 16-containing tumors (23). To determine whether the anchorage-independent growth of HPV 18 cell lines is due to HPV expression *per se* or to cellular events, *i.e.*, activation of oncogenes or inactivation of suppressor genes occurring in later stage, we examined the phenotype of individual hybrids. To avoid the chromosomal segregation occurring during continuous passages, we examined all the cell hybrids at the same early passage (passage 2). The results were unequivocal (Table 5; Fig. 2). HPV 16 × HPV 18 hybrids were unable to grow in soft agar, while all HPV 18 × HPV 18 hybrids, like the parental HPV 18 cells, were able to form colonies at a similar frequency. When two HPV 16 × HPV 18 hybrid clones (BE1 and BE7) were examined for anchorage-independent growth at a later passage (passage 14), the ability to grow in soft agar increased about 10-fold compared to the same clones at passage 2 (Table 5). Total RNAs from parental cell lines and early and late passage hybrid clones were hybridized with E6/E7 probes of HPV 16 and 18, and the mRNA expression levels and patterns of E6/E7 of HPV 16 and 18 were not different among these cell lines (Fig. 2).

These results suggest that the anchorage-independent growth of HPV 18 cells is not dominant and that this phenotype is due to genetic changes (probably loss of suppressor genes) occurring at late passage. Interestingly, since all of HPV 18 × HPV 18 hybrids which have been tested can grow in soft agar (Table 5), it is probable that only one major group of recessive genes is involved in the regulation of the anchorage-independent growth phenotype in HPV 18-transformed cells.

Analysis of Tumorigenesis Stage. As described, two HPV 18-immortalized cell lines, Z183A and Z183B, have progressed to tumorigenesis (Table 1), and we decided to investigate the nature of this step from anchorage-independent growth to tumorigenesis. We selected to fuse Z183A TCL-1 with Z213A, since they belong to the same complementation group for immortalization and anchorage-independent growth (Tables 4 and 5), in order to avoid additional factors masking the "anchorage-independent growth to tumorigenesis" step. The cell line Z183A TCL-1, which was derived from a tumor induced by Z183A, is more tumorigenic than parental Z183A. The cell line Z213A, another HPV 18 cell line, is immortal and anchorage independent, but nontumorigenic. The parental Z183A TCL-1 cells, as expected, induced tumors in nude mice (Table 6). However, the hybrids Z213A × Z183A TCL-1 clone 1, clone 5, and clone 9 could not produce tumors in nude mice, although they still exhibited the phenotype of anchorage-independent growth (Table 6), and there was no decrease in the mRNA level (data not shown). We can then also suggest that the stage from anchorage-independent growth to tumorigenesis of this *in vitro* model of cervical carcinogenesis is recessive.

DISCUSSION

In this paper we have demonstrated by cell fusion experiments that, in an *in vitro* model of cervical carcinogenesis by HPV, all three transformed phenotypes, *i.e.*, immortalization, anchorage-independent growth, and tumorigenesis, are recessive.

The results of three sets of cell fusion experiments (Tables 2 and 3) clearly suggest that immortalization of human epithelial cells by HPV is a recessive process and also indicate that HPV *per se* cannot account for immortalization. HPV expression may extend the life span of normal epithelial cells but may not immortalize them unless other cellular processes have been activated or inactivated. This notion is

Table 5 *Anchorage-independent growth of hybrids*

Hybrid clone	HPV types	Passage	CFE[a] (%)
BF[b] 5	16 × 18	2	0.09
BF 6	16 × 18	2	0.11
AE 13[c]	16 × 18	2	0.14
AG 4[d]	16 × 18	2	0.24
BE 1	16 × 18	2	0.13
BE 1	16 × 18	14	1.83
BE 7	16 × 18	2	0.19
BE 7	16 × 18	14	2.23
EE 2	18 × 18	2	4.30
EE 5	18 × 18	2	4.73
HE 1	18 × 18	2	3.44
HE 2	18 × 18	2	3.40
IE 1	18 × 18	2	6.77
IE 2	18 × 18	2	4.01
FH 2	18 × 18	2	4.17
FH 3	18 × 18	2	4.50
FI 1	18 × 18	2	3.06
FI 2	18 × 18	2	3.56

[a] CFE, colony-forming efficiency in soft agar.
[b] See Table 1 for designation of cell lines.
[c] AE 13 eventually senesced.
[d] AG 4 is an immortal segregant.

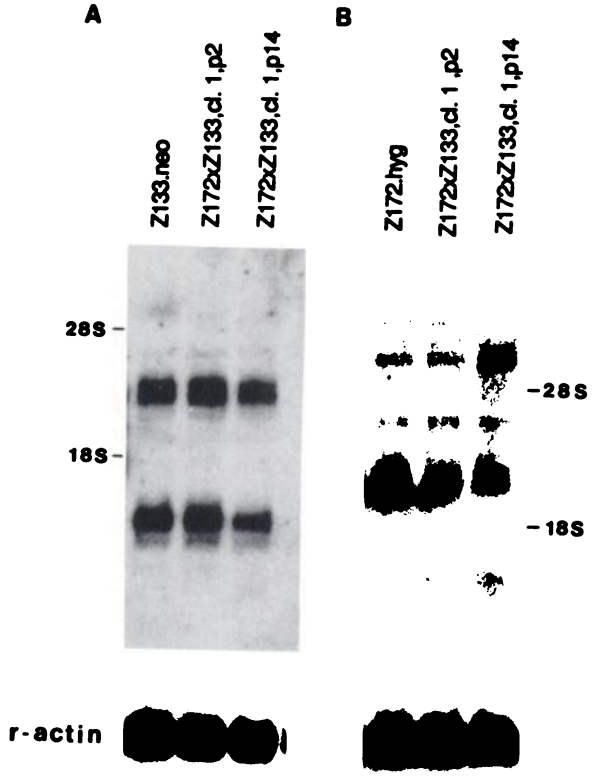

Fig. 2. Expression of HPV 16 and 18 mRNA in parental and hybrid cells at different passages. Total RNA (15 μg) was size fractionated on 1.2% agarose gel and transferred to nitrocellulose filters. The filters were hybridized with ^{32}P-labeled HPV 18 E6/E7 probe (*A*) or HPV 16 E6/E7 probe (*B*). After the probes were stripped, the same filters were hybridized with ^{32}P-labeled γ-actin (human) probe.

Table 6 *Tumorigenicity of hybrid cells*

Cell line or hybrid	No. of tumors/No. of injections	CFE[a] (%)
Z183A TCL-1	4/5	3.45
Z213A × Z183A TCL-1 clone 1	0/5	3.77
Z213A × Z183A TCL-1 clone 5	0/5	5.62
Z213A × Z183A TCL-1 clone 9	0/5	3.19

[a] CFE, colony-forming efficiency in soft agar.

supported by the experimental evidence that immortalization of human epithelial cells by HPV is a very rare event compared to transfection efficiency (24). The fact that the senescing hybrids continue to express HPV 18 mRNA indicates that senescence is not necessarily due to selective inhibition of HPV transcription by putative negative factors present in the normal epithelial cells as it has been suggested (25).

It is also apparent that functional inactivation of p53 and pRB, postulated as an effector mechanism of HPV activity, is not sufficient to confer immortalization, but may contribute to the process. This is supported by the finding that HPV 16 E6 + E7 or adenovirus E1a + E1b can replace a temperature-sensitive SV40 T antigen in the immortalization of human fibroblasts at the nonpermissive temperature. (26) Furthermore, although HPV E6 can antagonize the antiproliferative effect of wild-type p53 (22), overexpression of exogeneous wild-type p53 inhibits the growth of HPV-immortalized cells,[5] suggesting that HPV E6 may overcome endogenous wild-type p53 but may not be sufficient to counteract overexpressed wild-type p53. This may partially account for the early senescence of Z213A × CxK and Z213A × CxFb hybrids.

From the cross-hybridization experiments between HPV 16 and HPV 18 lines, it appears that there are 4 different complementation groups involved in immortalization, one of which, Group II, shared with HeLa, is predominant. Assignation to any of these groups is independent of the HPV type used in transformation. Since most of the SV40-transfected lines in the work of Pereira-Smith and Smith *et al.* (15) have been assigned to a group different from the one for HeLa, it indicates that although both SV40 and HPV inactivate p53 and pRB, their mechanisms of induction of immortalization must operate through different pathways. This hypothesis is reinforced by the fact that a phenomenon of "crisis" almost always occurs in SV40-transformed human cells (27, 28), while very rarely, if ever, with HPV-transformed human epithelial cells (2, 3, 29).

Recently, Shay *et al.* (29) and Radna *et al.* (30) have proposed a two-phase immortalization model (29) to explain SV40 immortalization of human fibroblasts. In this model SV40 can overcome the M_1 phase (by inactivation, for example, of p53 and pRB) but cannot overcome the M_2 phase which occurs as random successive events. Our data support this model as applicable to HPV immortalization of human epithelial cells, in the sense that HPV can also overcome the M_1 but not the M_2 phase. The M_2-phase genes are also recessive, and they are not inactivated directly by HPV or SV40, since not all of HPV or SV40 cell lines are assigned to the same complementation group (Refs. 15 and 31; Table 4). Furthermore, the processes of the M_2 phase must be distinctive for HPV or SV40, since the prevalent complementation groups are different for each of the two virus-transformed cells. Recently, the cell senescence-related gene(s) of HeLa have been demonstrated on chromosome 4 (32), while alterations of chromosome 6 have been found in many SV40-immortalized human fibroblast cell lines (33). Work is in progress to test if a normal human chromosome 4 can drive our HPV cell lines into senescence.

HPV 18 cell lines, but not HPV 16 cell lines, can grow in soft agar. HPV 16 × HPV 18 hybrids cannot grow in soft agar at an early passage, but are able to do so at a late passage probably because of chromosomal segregation. Since we have not detected any gross differences of HPV 16 and 18 expression among the parental cells and the early passage and late passage cell hybrids, it seems that the anchorage-independent growth phenotype is recessive. The fact that HPV 18-transfected cells at very early passage do not exhibit anchorage-independent growth also supports the recessive nature of this property.[6] It then appears that HPV 18 has a stronger capacity than HPV 16 to engender cellular genetic changes which may result in inactivation of certain suppressor gene(s). This is consistent with the fact that clinically, HPV 18 is always associated with more advanced cervical intraepithelial neoplasia and cervical cancers than HPV 16 (23). *In vitro* studies also indicate the HPV 18 is more effective in immortalization and transformation than HPV 16 (2, 24). Since all the HPV 18 × HPV 18 hybrids tested retain the property of anchorage-independent growth without much variation in their colony-forming efficiency, the recessive process(es) controlling this transformed phenotype in cervical epithelial cells is singular or very limited in number. The recessive characteristic of anchorage-independent growth has also been demonstrated in hamster cells transformed by either oncogenes (34) or chemical carcinogens (35).

Deletion of human chromosome 11p has been associated with induction of anchorage-independent growth by HPV 16 in human fibroblasts (36), and introduction of one human chromosome 11 abrogates the tumorigenicity of HeLa and SiHa cells (37, 38). Putative tumor suppressor gene(s) may exist on this chromosome. Actually, one cell line, Z183B, in which one chromosome 11 has been deleted, exhibits tumorigenicity and a higher capacity to grow in soft agar (Table 1).

[5] T-M. Chen *et al.*, manuscript in preparation.

[6] G. Pecoraro *et al.*, unpublished data.

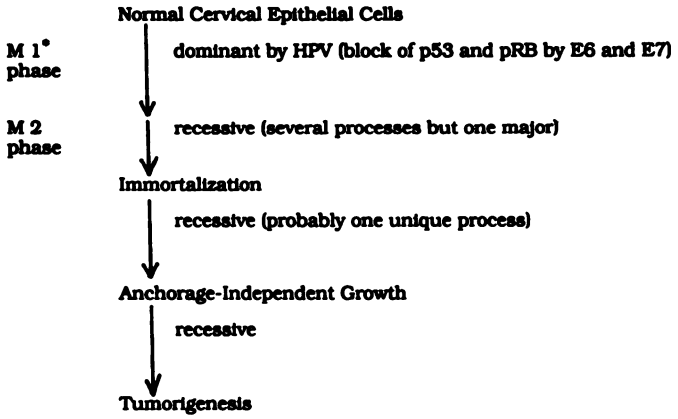

Fig. 3. *In vitro* model of cervical carcinogenesis by HPV. *, see Ref. 29.

Finally, we also defined the recessive characteristics of the stage specifically between anchorage independence and tumorigenesis. Since only one tumor cell line could be examined, we do not know how many possible groups of recessive genes are involved in this stage.

In this paper, we have demonstrated the characteristics of each specific step in an *in vitro* model of cervical carcinogenesis (Fig. 3). According to this model, tumor suppressor genes, either directly or indirectly affected by HPV, are involved in each stage of *in vitro* progression. However, our results do not preclude the possibility that dominant genes may contribute to *in vitro* progression. In fact, introduction of the activated H-*ras* oncogene can drive HPV-immortalized human cervical epithelial cells into a tumorigenic phenotype (39). We have also identified activation of some cellular oncogenes which probably contribute to the tumorigenesis stage of the HPV 18 tumorigenic cell lines (40).

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