JOURNAL OF VIROLOGY, Jan. 1988, p. 313–324
0022-538X/88/010313-12$02.00/0

Vol. 62, No. 1

Progression of the Phenotype of Transformed Cells after Growth Stimulation of Cells by a Human Papillomavirus Type 16 Gene Function

TETSUO NODA, HAJIME YAJIMA, AND YOSHIAKI ITO*

Institute for Virus Research, Kyoto University, Sakyo-Ku, Kyoto 606, Japan

Received 30 June 1987/Accepted 18 September 1987

Alteration of the growth properties of the established murine fibroblast cell lines NIH 3T3 and 3Y1 was studied in monolayer cultures and in cells suspended in semisolid medium after introduction of a cloned human papillomavirus type 16 (HPV16) DNA. HPV16 DNA stimulated both cell lines to grow beyond their saturation densities in monolayer cultures without any apparent morphological changes or tendency to pile up. These cells were also stimulated to grow in soft agar. Since essentially all the cells that received the viral gene were stimulated to grow, the growth-stimulatory activity of HPV16 appeared to be due to the direct effect of a viral gene function. The NIH 3T3 cells showed an additional change in growth properties upon prolonged incubation of dense monolayers of cells containing the HPV16 DNA; morphologically recognizable dense foci appeared at a frequency of about 10^{-3}. These cells, when cloned from the foci, grew more rapidly in soft agar than the parental cells and were morphologically transformed. In other words, there were two sequential steps in cell transformation induced by HPV16. Practically all the viral DNAs were present in the cells as large rearranged multimers and were integrated into host chromosomal DNA. There was no obvious difference in the state of viral DNA in the cells of the original clone or the three subclones derived from it as dense foci. There was no difference in the amount or the number of viral RNA species expressed in the cells at these two stages. The secondary changes in the growth properties of NIH 3T3 cells appear to be due to some cellular alterations.

In 1933, Shope (52) reported that an infectious agent induced papillomatosis in rabbits. Since the discovery of this agent, Shope papillomavirus of cottontail rabbits, various types of papillomavirus have been found from many different animal species. Most of these papillomaviruses primarily induce benign papillomas or benign fibropapillomas (26, 41). Malignant conversion of these benign tumors has been observed frequently in the hyperplastic region induced by several types of papillomavirus including the type 4 bovine papillomavirus and cottontail rabbit papillomavirus (19, 23). This sequential process seems to be a typical feature of carcinogenesis induced by papillomaviruses. Extensive analysis of this process has suggested the involvement of some factors other than papillomavirus in malignant transformation (5, 19, 22, 39, 45).

Recently, human papillomaviruses (HPVs) have been strongly implicated as etiological agents of human cervical carcinomas by clinical, epidemiological, and virological studies combined with molecular analysis (4, 15). Of the more than 40 types of HPV isolated from human tissues, HPV type 16 (HPV16), -18, -31, -33, and -35 have been found in preneoplastic lesions of the uterine cervix or in genital tumors, including cervical carcinoma (2, 3, 9, 12, 28, 29). Molecular hybridization studies have shown that a high proportion of biopsies obtained from cervical carcinomas contain the genomes of these types of HPV (3, 12, 15, 36, 40). Previous clinical observations suggest that the carcinogenic process of cervical cancer also involves several steps (25). To elucidate the role of HPV in this process, it is essential to study the biological function of the HPV genes.

The in vitro cell transformation system has been successfully used in studies of tumor viruses. Until recently, neoplastic transformation by HPV of cells in tissue culture did not occur readily. Interestingly, rodent fibroblasts could be easily transformed in vitro but only by a certain group of papillomaviruses, those which induce fibropapillomas in vivo. Therefore, in vitro cell transformation by the bovine papillomavirus, which causes fibropapillomas in cattle, has been studied extensively as a model of neoplastic transformation by papillomaviruses (27, 30, 46, 47). Two transforming genes, E5 and E6, have been identified. They cooperate in inducing the full transformation phenotype in mouse fibroblasts (48, 62).

More recently, however, HPV16 has been shown to transform established mouse (NIH 3T3) and rat (3Y1) fibroblast cell lines (21, 63) as well as primary human fibroblasts and keratinocytes (42). Furthermore, cellular DNA containing the HPV16 genome and isolated from an adenocarcinoma of the uterine cervix had transforming activity, and the DNA preparation that had this transforming activity, even in the second-cycle transfection experiment, contained the HPV16 sequence (58). Therefore, the carcinogenic potential of HPV can be examined directly in the quantitative in vitro cell transformation system.

We report here the results of our analysis of transformation of NIH 3T3 and 3Y1 cells by HPV16. Our findings suggest that the growth of cells in monolayers and in suspension in semisolid medium is stimulated directly by a viral gene function and that, in addition, some cellular events take place at a certain frequency to further transform the cells.

MATERIALS AND METHODS

Cell lines and culture conditions. NIH 3T3 cells were obtained from the American Type Culture Collection (Rockville, Md.) and maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% calf serum (Colorado Serum Company, Denver, Colo.). 3Y1 cells (24) were

* Corresponding author.

a gift of Genki Kimura and were maintained in DMEM supplemented with 10% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.). To assay cell growth in soft agar (33), we suspended NIH 3T3 cells and cell lines derived from NIH 3T3 cells in DMEM containing 0.33% Bacto-Agar (Difco Laboratories, Detroit, Mich.), 10% tryptose phosphate broth (Difco), and 10% calf serum. The 3Y1 cells and the cell lines derived from them were suspended in DMEM containing 0.28% Seakkem agarose (FMC Corp., Marine Colloids Div., Rockland, Maine) and 10% fetal calf serum.

Doubling time and saturation density. A total of 2×10^4 cells of each cell line derived from the 3Y1 cells were plated in 50-mm dishes and incubated for 1 month. The culture medium was changed every 3 days. Cells were trypsinized and counted every 2 days during week 1 or every 4 days during the last 3 weeks. The doubling time of each cell line was calculated from the number of cells on days 2 and 4, when their growth was exponential. The cell number at 4 weeks after plating was regarded as the cell number at saturation.

Colony-forming activity and diameter of colonies. A total of 10^4 cells of each 3Y1-derived cell line were suspended in 1.5 ml of DMEM containing 0.28% soft agarose and 10% fetal calf serum and incubated for 4 weeks. Colonies consisting of more than 10 cells were counted, and the mean diameter of the colonies was calculated from the diameters of 200 randomly selected colonies.

[^{3}H]thymidine uptake and autoradiography. A total of 5×10^5 NIH 3T3 cells were plated in a 50-mm dish and incubated in DMEM supplemented with 10% calf serum. On day 4, the medium was changed to DMEM with 5% calf serum. [^{3}H]thymidine (0.2 μCi/ml; New England Nuclear Corp., Boston, Mass.) was added to the medium on day 6, and the cells were incubated for 20 h. Then the cells were washed with phosphate-buffered saline three times and fixed by the addition of −20°C methanol. For emulsion autoradiography, the cells were rehydrated in phosphate-buffered saline and coated with nuclear track emulsion (NTB2; Eastman Kodak Co., Rochester, N.Y.). After 24 h at 4°C, the emulsion was developed with half-strength developer (D-19; Kodak). The labeling index of the cells was obtained by counting the labeled nuclei of 2,000 cells.

Transfection, *neo* selection, and cell cloning. NIH 3T3 and 3Y1 cells were transfected with plasmids by the calcium phosphate precipitation method (16, 60). Form I of plasmid DNAs was used for transfection. To select cells expressing the bacterial neomycin resistance gene (8), we incubated transfected cultures in culture medium containing 400 μg of G418 (GIBCO) per ml. Cells were cloned by limiting dilution.

Plasmids. The monomer and the head-to-tail dimer of the HPV16 genome linearized at its unique *Bam*HI site were obtained from plasmid p622-1 (a gift from Peter Howley) and cloned into a *Bam*HI site of pSV2-neo (55). These constructs were designated pSVHPV16-M and pSVHPV16-D, respectively. In these plasmids, the early region of HPV16 is transcribed in the same direction as the neomycin gene driven by the simian virus (SV40) promoter.

Southern blot analysis. Cellular DNA was analyzed by the method of Southern (54), using the standard protocol (34). Briefly, high-molecular-weight cellular DNA was extracted by dissolving EDTA-dispersed cells with 0.5 M sodium perchloride–0.3% sodium dodecyl sulfate, followed by two cycles of extraction with an equal-volume mixture of phenol and chloroform. The aqueous phase was dialyzed extensively against 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA and was then used for the restriction enzyme analysis. Samples (10 μg) of the DNA were digested completely with various restriction enzymes, separated by 0.6% agarose gel electrophoresis, and blotted to nitrocellulose filters. For Southern blot analysis with two-dimensional agarose gels, 5 μg of cellular DNA restricted with endonuclease *Xba*I was electrophoresed through a 0.5% agarose gel and then through a 1.0% agarose gel. Baked filters were hybridized with nick-translated DNA fragments (43) at 42°C in 50% formamide–5× Denhardt solution–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–10% dextran sulfate–50 mM Tris hydrochloride (pH 7.4)–0.1% sodium dodecyl sulfate–100 μg of sheared calf thymus DNA per ml. Hybridized filters were washed in 0.2× SSC–0.1% sodium dodecyl sulfate at 68°C and processed for autoradiography. To distinguish episomal DNA from other cellular DNA fragments (digested with restriction endonucleases), 5 μg of total cellular DNA digested with restriction enzymes was treated with 12 U of ATP-dependent DNase (Toyobo, Osaka, Japan) for 3 h in 66.7 mM glycine (pH 9.4)–30 mM $MgCl_2$–8.3 mM 2-mercaptoethanol–0.5 mM ATP and subjected to Southern blot analysis.

Northern blot analysis. Northern (RNA) blot analysis was performed essentially as described previously (38). Briefly, total cellular RNA was isolated from cells by lysing them in GTC solution (5.7 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% *N*-lauroylsarcosine, 0.1 M 2-mercaptoethanol), followed by centrifugation through 5.7 M cesium chloride (7). The pelleted RNA fraction was extracted with phenol and precipitated with ethanol. Heat- and formamide-denatured total RNA (20 μg) was electrophoresed through 1.0% agarose gels containing 2.2 M formaldehyde in a morpholinepropanesulfonic acid (MOPS)-acetate buffer for 12 h at 25 V. The RNA was blotted onto nitrocellulose filters and baked in a vacuum oven at 80°C for 2 h. The nick-translated DNA fragments were hybridized in the same solution as the one used in Southern blotting together with 100 μg of yeast tRNA. Filters were washed in 0.2× SSC–0.1% sodium dodecyl sulfate at 68°C and processed for autoradiography.

RESULTS

Growth properties of NIH 3T3 cells transfected with pSV2-neo, pSVHPV16-M, or pSVHPV16-D. Recombinant plasmids containing either a monomer (pSVHPV16-M) or a head-to-tail dimer (pSVHPV16-D) of the HPV16 genome, in addition to the bacterial neomycin resistance gene, were constructed (Fig. 1). The plasmid containing the head-to-tail dimer was expected to contain at least one intact copy of all the HPV16 genes without interruption by the *Bam*HI site (51).

Monolayers of NIH 3T3 cells were transfected with pSVHPV16-M, pSVHPV16-D, or a backbone vector, pSV2-neo, and the G418-resistant cell populations were selected. Since both the neomycin resistance gene and the HPV16 genome were present in the same constructs, the majority of the cells expressing the neomycin resistance gene, and thereby resistant to G418, were expected to contain the HPV16 genes. We later confirmed that all the randomly selected cell clones isolated from the population of G418-resistant cells indeed contained the HPV DNA.

At confluency, cells transfected with pSV2-neo (P-NIH 3T3) were indistinguishable from parental NIH 3T3 cells (Fig. 2, A1). Cells transfected with either pSVHPV16-M (M-NIH 3T3) (Fig. 2, B1) or pSVHPV16-D (D-NIH 3T3) (Fig. 2, C1), however, formed denser monolayers, although we found no apparent morphological changes in individual

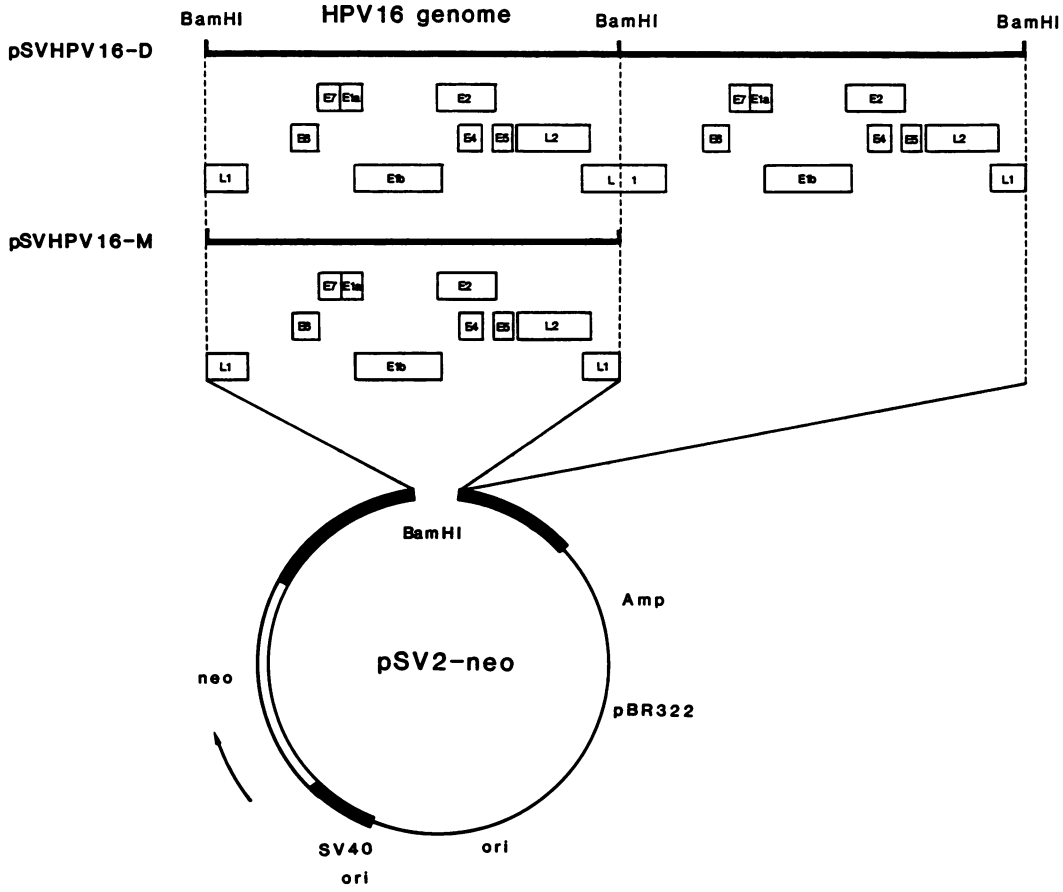

FIG. 1. Structures of recombinant plasmids pSVHPV16-M and pSVHPV16-D. The HPV16 DNA linearized at the unique *Bam*HI site was inserted into the *Bam*HI site of pSV2-neo (55) as a monomer (pSVHPV16-M) or as a head-to-tail dimer (pSVHPV16-D). Open boxes indicate putative open reading frames. ori, Origin.

cells. To test whether the denser cell sheet formed by cells containing the HPV16 genome indicated that these cells did not stop growing at confluency, we labeled these three sets of cells with [^{3}H]thymidine after the cells became confluent, and autoradiograms were made. The cells containing only pSV2-neo did not significantly incorporate the radioactive thymidine (Fig. 2, A2), whereas those containing the HPV16 DNA incorporated it well (Fig. 2, B2 and C2). Since most of the viral DNA in these cells was integrated into host cell DNA, the grains seen in Fig. 2 were considered to represent the chromosomal DNA synthesis, rather than the extrachromosomal replication of viral DNA (see below).

When these cells were suspended in soft agar, the cells containing the HPV16 DNA formed slow-growing colonies (Fig. 2, B3 and C3), while the cells containing pSV2-neo did not divide (Fig. 2, A3).

When the monolayers of the cells containing the HPV16 DNA were cultured for 25 days, morphologically recognizable dense foci appeared (Fig. 2, B4 and C4), whereas the control culture remained as a flat monolayer (Fig. 2, A4). These foci appear to correspond to the foci described by Yasumoto et al. (63).

Quantitative data of the results shown in Fig. 2 are summarized in Table 1. Saturation densities of the cells containing either the monomer or the dimer of the HPV16 DNA were less than twofold higher than those of cells containing the control vector and were lower than expected from the data shown in Fig. 2, B2 and C2. This was probably due to the tendency of the cells containing the HPV16 DNA to come off the plates readily and to be lost during the manipulation. As to the efficiency of colony formation, only about 10% of the suspended cells formed colonies. We observed that NIH 3T3 cells usually form colonies at this frequency regardless of the agents used for cell transformation, and therefore, the efficiency that we observed probably means that the majority of the cells containing the HPV DNA would form colonies in soft agar.

The frequency with which dense foci appeared in rather dense monolayers of NIH 3T3 cells containing the HPV16 DNA was about 10^{-3}. Cells obtained from these foci looked

TABLE 1. Growth properties of NIH 3T3 cells transfected with pSV2-neo, pSVHPV16-M, or pSVHPV16-D

Cell line	Cell no. at saturation (10^6 cells/60-mm dish)	% Labeled nuclei[a]	Efficiency of colony formation (%)	No. of foci/60-mm dish[b]
P-NIH 3T3	2.57	0.8	0.1	0.1
M-NIH 3T3	4.39	48.4	11.2	12.3
D-NIH 3T3	4.56	58.0	8.2	18.6

[a] Percentage of cells with radioactive nuclei of 1,000 cells examined.
[b] Average of 10 dishes.

morphologically transformed; they tended to pile up when they became confluent and to show crisscross formation.

To examine whether the cells forming the dense foci shown in Fig. 2, B2, and C2, acquired this property secondarily during the prolonged cultivation of growth-stimulated cells or at the time of transfection, we performed the following clonal analysis.

Fifteen random cell clones were isolated from the culture of D-NIH 3T3 cells soon after the population of G418-resistant cells was obtained without prolonged cultivation as confluent monolayers. Seven cell clones were also isolated from similar cultures but received only the backbone vector. Since less than 100 dense foci were expected to appear after prolonged incubation in the plate originally seeded at 2×10^4 cells, the chance that any of the 15 clones isolated would form dense foci was very small. As expected, the cells from all 14 clones formed flat monolayers at an earlier stage. (Clone 4 was lost since the cells came off the plate before analysis.) In most cases, the cell densities of these monolayers were higher than that of parental NIH 3T3 cells. Of the 15 randomly selected clones, 12 formed small colonies in soft agar at about 10% plating efficiency (data not shown). Therefore, virtually all the clones which received the HPV16 DNA appeared to be able to form small colonies in soft agar. Upon prolonged incubation, dense foci ranging from less than 10 to about 200 per plate appeared in most plates (Fig. 3, B1 through B15, except for B4). All the eight clones tested contained the viral DNA (data not shown). Therefore, the majority of the clones were expected to contain the viral DNA. It is not known whether clone 15, which did not produce dense foci, contained the viral DNA. Only one dense focus was formed (Fig. 3, A5) in one of the seven clones that received the control construct (Fig. 3, A1 through A7). These results suggest that any of the cell clones that received the HPV16 DNA had the potential to form small colonies in soft agar and to give rise to cells which form dense foci.

Growth properties of cells isolated from dense foci. As mentioned above, cells of all 14 clones isolated from D-NIH 3T3 formed a flat monolayer at earlier stages. As two examples, the cells of clone 2 (cl-2) and cl-14 were suspended in soft agar. As expected, they formed colonies like those shown in Fig. 2, C3 (Fig. 4, 1A and 2A, respectively). The cells isolated from dense foci, however, generally grew much more rapidly in soft agar and formed larger colonies. Three subclones each isolated from three independent dense foci from the cultures of cl-2 and cl-14 were suspended in soft agar. Figure 4, 1B, 1C, and 1D, and Fig. 4, 2B, 2C, and 2D, respectively, show the colonies formed from cl-2 and cl-14. Although the size of the colonies varied slightly, the cells isolated from the dense foci generally formed larger colonies than those formed by the parental cells. Morpho-

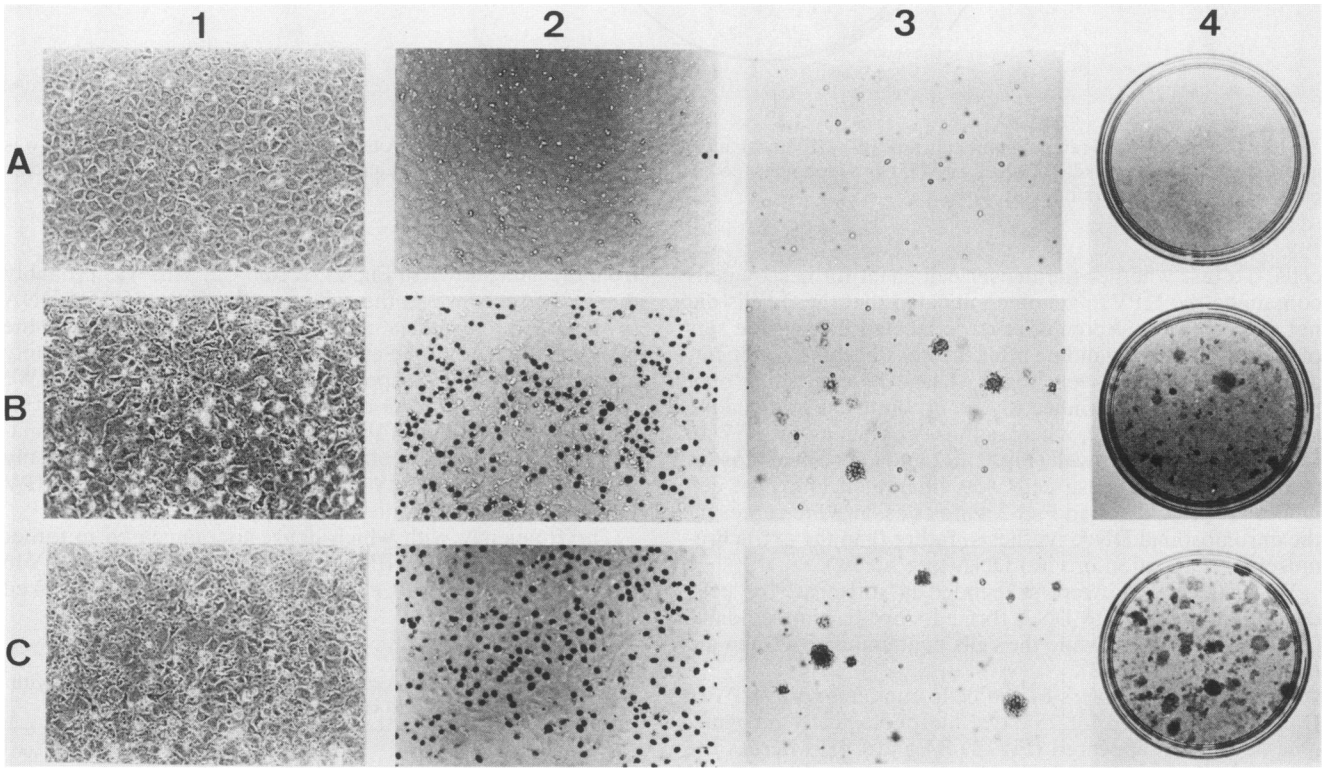

FIG. 2. Effect of HPV16 DNA on morphology and growth properties of NIH 3T3 cells. Cells (3×10^4) were plated in 50-mm dishes and incubated for 1 week in DMEM containing 10% calf serum. The medium was changed on day 4. Cells were photographed with a phase-contrast microscope (×200) (row 1). Similarly prepared cells were labeled with 1 μCi of [^{3}H]thymidine per plate during the last 20 h of 1 week of cultivation. They were fixed with cold methanol and processed for autoradiography (×100) (row 2). Cells (10^4) were suspended in 0.33% soft agar containing 10% calf serum, and colonies appearing after 3 weeks of incubation were photographed under an inverted microscope (×100) (row 3). The same number of cells as in panel A were incubated for 25 days in a 50-mm dish and stained with Giemsa solution after fixation with an equal volume mixture of methanol and acetone (row 4). (A) P-NIH 3T3 cells; (B) M-NIH 3T3 cells; (C) D-NIH 3T3 cells.

logically, the cells isolated from dense foci were all transformed.

Growth properties of 3Y1 cells after introduction of HPV16 DNA. Since there were no differences in biological effects between pSVHPV16-M and pSVHPV16-D, pSVHPV16-M was used in the studies with 3Y1 cells. The population of G418-resistant cells transfected with the monomer containing the plasmid (M-3Y1mix) formed a denser monolayer (Fig. 5, 1C) than did the parental 3Y1 cells (Fig. 5, 1A) or 3Y1 cells transfected with pSV2-neo (P-3Y1mix) (Fig. 5, 1B). Figure 5, 1D, 1E, and 1F, shows the three independent clones isolated randomly from the G418-resistant cell populations M-3Y1 cl-1, cl-2, and cl-3, respectively. cl-1 and cl-3 formed dense cell sheets, while cl-2 looked less dense. Three additional clones examined, cl-4, cl-5, and cl-6, formed dense cell sheets similar to those formed by cl-1 and cl-3 (data not shown). These cell clones were also tested for their growth in soft agar. The clones M-3Y1 cl-1 and cl-3 as well as M-3Y1mix grew slowly in suspension in soft agar (Fig. 5, 2D, 2F, and 2C, respectively), while the parental cells as well as P-3Y1mix did not divide under the conditions used. Three independent clones containing the backbone vector, P-3Y1 cl-1, cl-2, and cl-3, were indistinguishable from the P-3Y1mix (data not shown). M-3Y1 cl-2 formed very small colonies (Fig. 5, 2E). There was, therefore, very good correlation between the density of cells in monolayers and the size of the colonies formed by such clones.

Formation of dense cell sheets by the cells transfected with the HPV16 DNA was more quantitatively examined by counting the number of cells per plate at 4-day intervals after plating. The growth kinetics of P-3Y1mix and P-3Y1 cl-1, cl-2, and cl-3 were indistinguishable from those of the parental 3Y1 cells (Fig. 6A). M-3Y1mix, as well as five clones isolated from it, grew to severalfold-higher densities at confluency (Fig. 6B). cl-2, as expected from the results shown in Fig. 5, 1E and 2E, did not grow significantly better than the parental 3Y1 cells. We later confirmed that cl-2 contained the HPV16 DNA (data not shown). Why this clone has a phenotype markedly different from that of the other clones is not known.

Quantitative data of the growth characteristics of these cells are shown in Table 2. There was little difference in doubling time among various clones transfected with the HPV16 DNA or the control vector. The cell clones transfected with the HPV16 DNA, however, reached two- to ninefold-higher cell densities at confluency, except for cl-2. This is in contrast to the results with NIH 3T3 shown in Table 1, for which the saturation densities of the cells transfected with the HPV16 DNA were less than twofold higher than those of the parental cells. The NIH 3T3 cells containing the

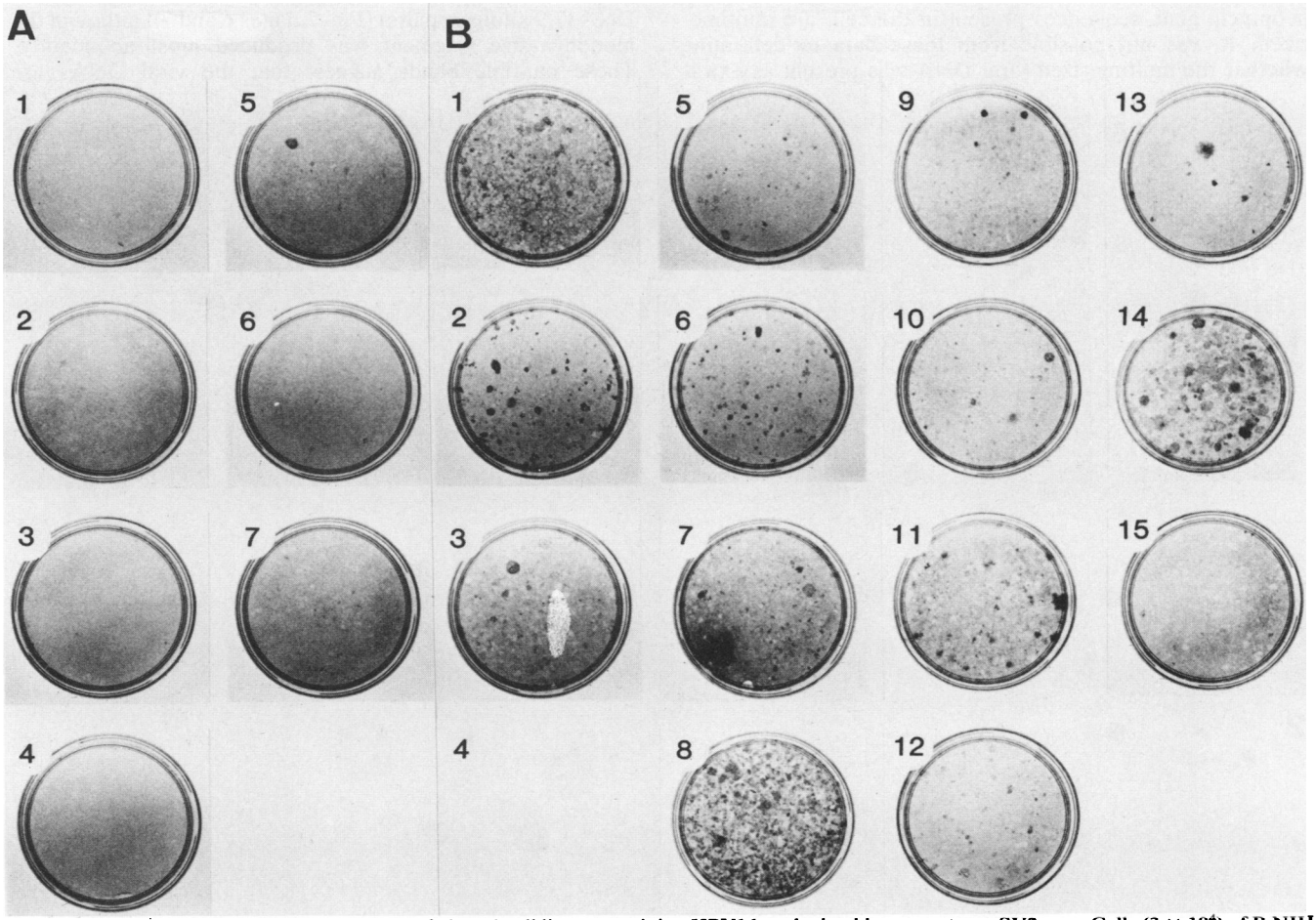

FIG. 3. Focus formation in monolayers of cloned cell lines containing HPV16 or the backbone vector, pSV2-neo. Cells (3×10^4) of P-NIH 3T3 cl-1 to cl-7 (A1 to A7) or D-NIH 3T3 cl-1 to cl-15 (B1 to B15) were incubated in a 50-mm dish for 25 days. The medium was changed every 3 days. Cells were fixed with ice-cold methanol and stained with Giemsa solution.

HPV DNA may be loosely attached to the plastic surface and tend to be lost during medium change or other manipulations, while 3Y1 cells do not have this tendency.

Tables 1 and 2 show that there were marked differences in the efficiency of colony formation between NIH 3T3 and 3Y1 cells. The finding that nearly 90% of the cells of at least one clonal population suspended in soft agar could divide and grow strongly suggests that virtually all cells that received the HPV16 DNA are stimulated to grow in soft agar. This stimulation, therefore, must be due to the direct effect of an HPV16 gene function.

Southern blot analysis. To determine whether there is any difference in the state of viral DNA between the cells that form flat and dense monolayers and grow moderately in soft agar and those that form foci in monolayers and large colonies in soft agar, we performed Southern blot analysis on the DNA extracted from a set of NIH 3T3 cells. High-molecular-weight DNA was extracted from two representative cell clones, D-NIH 3T3 cl-6 and D-NIH 3T3 cl-14. The restriction endonucleases *Xba*I and *Xho*I do not have recognition sites in the pSVHPV16-D DNA. Each of these endonucleases generated a cluster of bands of over 23 kilobases in both DNA preparations. These bands hybridized both with viral DNA (Fig. 7, lanes 2 through 5) and with pSV2-neo DNA (Fig. 7, lanes 8 through 11). Since the original construct containing the dimer of the HPV DNA was 21.5 kilobase pairs, the results shown in Fig. 7 mean that virtually all the viral DNA, together with plasmid and neomycin gene sequences present in the cell, are multimerized. It was not possible from these data to determine whether the multimerized viral DNA was present as extrachromosomal plasmids or integrated into the host chromosome. Digestion with *Bam*HI endonuclease, which cleaves the inserted viral DNA, reproducibly generated multiple bands, especially in the DNA from D-NIH 3T3 cl-14, at above and below the size of the monomer of the HPV16 DNA (7.9 kilobase pairs) (Fig. 7, lanes 6 and 7), although the monomer-size fragment was produced most abundantly. These multiple bands suggest that the viral DNAs are

TABLE 2. Growth properties of 3Y1 cells transfected with pSV2-neo or pSVHPV16-M

Cell clone	Doubling time (h)	Cell no. at saturation (10^6 cells/ 60-mm dish)	Efficiency of colony formation (%)	Avg diam of colonies (μm)
3Y1	16.9	1.50	0.0	
P-3Y1mix	16.1	1.38	1.3	54.4[a]
cl-1	16.3	1.51	1.6	56.8[a]
cl-2	17.7	1.38	0.0	
cl-3	16.9	1.23	0.0	
M-3Y1mix	16.9	5.33	62.7	83.3
cl-1	16.3	5.80	78.6	63.9
cl-2	17.7	1.85	6.0	54.6[a]
cl-3	14.9	8.66	87.2	86.7
cl-4	18.4	3.85	77.3	72.9
cl-5	14.6	3.05	44.6	63.6
cl-6	18.9	7.33	73.5	83.3

[a] Average of 100 colonies (others are the average of 200 colonies).

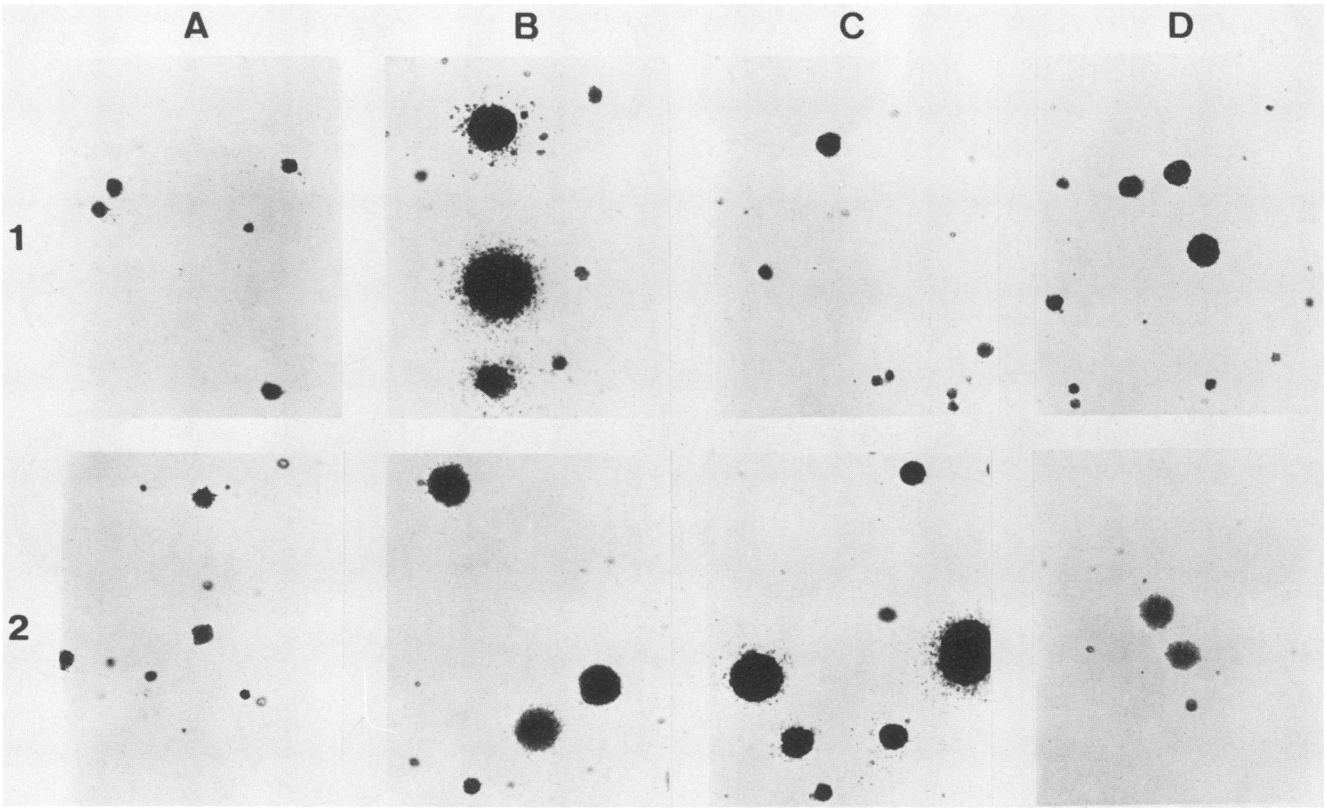

FIG. 4. Colony formation in soft agar of cloned NIH 3T3 cell lines containing HPV16 or the backbone vector, pSV2-neo. Cells (10^4) of D-NIH 3T3 cl-2 (1A), D-NIH 3T3 cl-2f1 to -f3 (1B to 1D), D-NIH 3T3 cl-14 (2A), and D-NIH 3T3 cl-14f1 to -f3 (2B to 2D) were suspended in 0.33% soft agar and incubated for 21 days. Colonies were photomicrographed (×100).

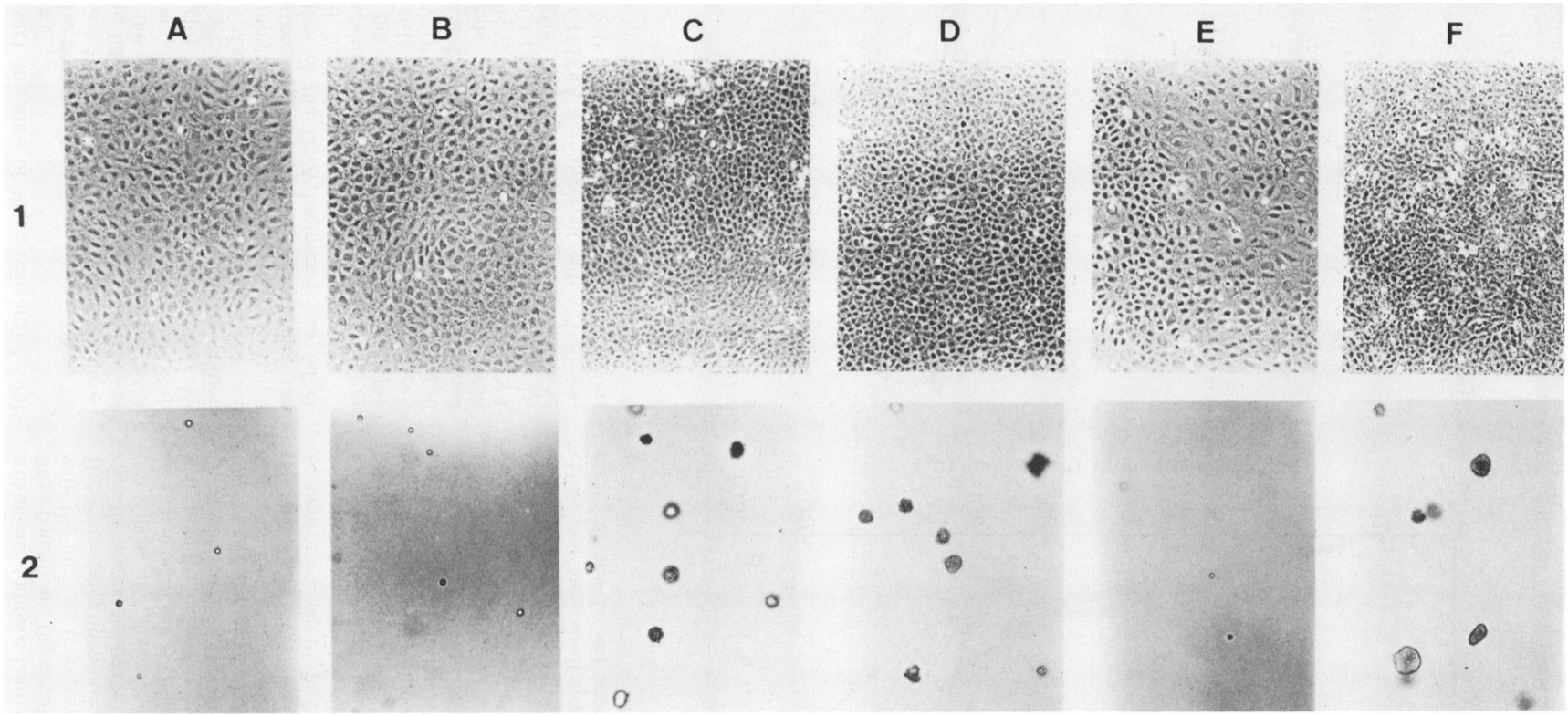

FIG. 5. Growth of 3Y1 cells transfected with pSV2-neo or pSVHPV16-M. Cells (2×10^4) of each clone were plated in 50-mm dishes and incubated in DMEM containing 10% fetal calf serum. The culture medium was changed every 3 days. Cells were photographed under an inverted phase-contrast microscope after a 3-week incubation (row 1). Cells (10^4) of each clone were suspended in 0.28% soft agarose containing DMEM supplemented with 10% fetal calf serum. After 4 weeks of incubation, cells were photomicrographed (row 2). (A) 3Y1 cells; (B) P-3Y1mix cells; (C) M-3Y1mix cells; (D to F) M-3Y1 cl-1 to cl-3, respectively.

rearranged. The exact structure of these rearranged viral DNAs is not known.

Integration of viral DNA into host chromosomal DNA. To test whether the viral DNA was integrated into host chromosomal DNA, the DNA isolated from D-NIH 3T3 cl-14 was digested with the noncutting restriction enzyme *Xba*I and analyzed by two-dimensional gel electrophoresis with two concentrations of agarose (59) (data not shown). Although the results were consistent with the interpretation that the viral DNA was integrated into cellular DNA, we could not say unambiguously that the viral DNA was not circular and linearized by the *Xba*I digestion, since the distance of migration of the DNA fragments was rather short, owing to their large sizes.

To test further whether the viral DNA was integrated or not, we digested the DNA with an exonuclease, ATP-dependent DNase (61). The *Xba*I-digested NIH 3T3 DNA mixed with the pSVHPV16-D DNA was electrophoresed in agarose gel. Lane 1 in Fig. 8A shows the total DNA stained with ethidium bromide. After blotting and hybridization with ^{32}P-labeled viral DNA, forms I and II of the plasmid DNA containing HPV16 DNA were visualized (Fig. 8B, lane 1). Similarly mixed NIH 3T3 DNA digested with *Xba*I and plasmid pSVHPV16-D DNA were digested with ATP-dependent DNase. The bulk of the DNA was digested by this treatment (compare lane 3 with lane 2 in Fig. 8A). Under these conditions, the circular plasmid DNA was not digested with the enzyme (compare lane 3 with lane 2 in Fig. 8B). However, when the plasmid DNA was opened at a unique *Hin*dIII site before mixing with the *Xba*I-digested NIH 3T3 cell DNA, the plasmid DNA was also digested by the treatment with the ATP-dependent DNase (compare lane 5 with lane 4 in Fig. 8A and B). The high-molecular-weight DNA isolated from D-NIH 3T3 cl-14 was digested with three types of restriction enzyme that had no recognition sites within the plasmid pSVHPV16-D DNA, and it was then digested with the ATP-dependent DNase. The virus-specific sequences present in the high-molecular-weight DNA preparation were completely digested by this treatment (Fig. 8B, lanes 7 through 12). The results demonstrate that most of the HPV16 DNA in D-NIH 3T3 cl-14 was integrated into host chromosomal DNA. High-molecular-weight DNA was also isolated from several other clones of cells and examined. In all cases tested, viral DNA isolated from the cells transfected with the dimer containing plasmid-generated DNA species above 23 kilobases upon digestion with several noncutting enzymes (data not shown).

Comparison of viral DNA and its expression between the clones that form small colonies and the subclones that form large colonies in soft agar. High-molecular-weight DNAs

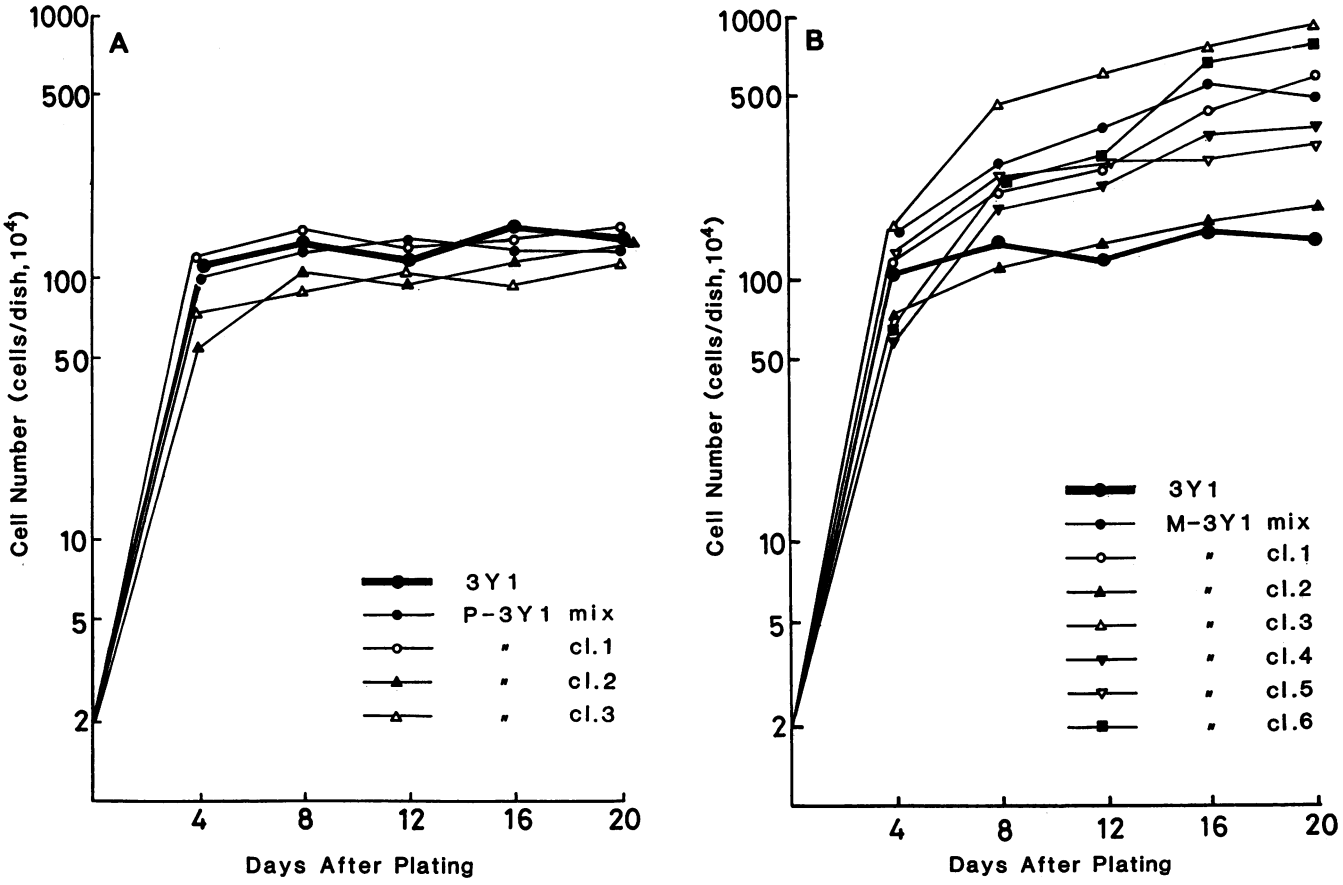

FIG. 6. Growth curves of 3Y1 cells transfected with pSV2-neo (P-3Y1) or pSVHPV16-M (M-3Y1). Cells (2×10^4) of each clone were plated in 50-mm dishes and incubated in DMEM containing 10% fetal calf serum. Culture medium was changed every 3 days. Cells were trypsinized and counted on days 4, 8, 12, 16, and 20 by dye exclusion. (A) P-3Y1; (B) M-3Y1.

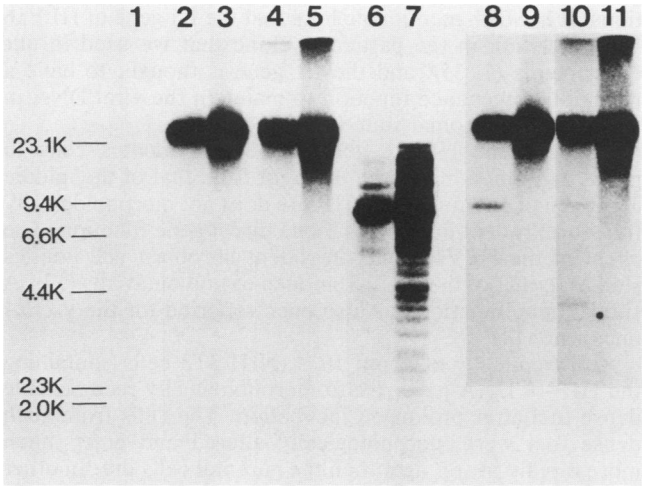

FIG. 7. Southern blot analysis of the DNA extracted from NIH 3T3 cells transfected with pSVHPV16-D. DNA (10 μg) extracted from NIH 3T3 cells (lane 1), D-NIH 3T3 cl-6 (lanes 2, 4, 6, 8, and 10), and D-NIH 3T3 cl-14 (lanes 3, 5, 7, 9, and 11) was digested with *Xba*I (lanes 1, 2, 3, 8, and 9), *Xho*I (lanes 4, 5, 10, and 11), or *Bam*HI (lanes 6 and 7) and electrophoresed through 0.6% agarose gels. The DNA blotted to a nitrocellulose membrane was hybridized with ^{32}P-labeled HPV16 DNA (lanes 1 through 7) or pSV2-neo DNA (lanes 8 through 11). The positions of size markers (lambda bacteriophage DNA restricted with *Hin*dIII) are indicated (K, kilobase pairs).

were isolated from D-NIH 3T3 cl-14 and from its three subclones isolated from foci, D-NIH 3T3 cl-14f-1, -f-2, and -f-3. These DNAs were digested with *Xba*I and *Bam*HI and analyzed by Southern blotting with the HPV16 DNA as a

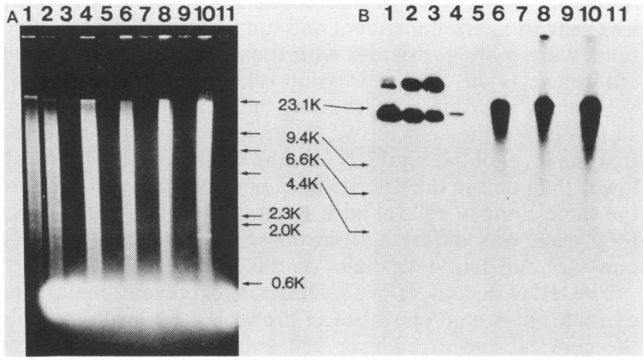

FIG. 8. (A) ATP-dependent DNase digestion of the DNA isolated from NIH 3T3 cells transfected with pSVHPV16-D. Plasmid pSVHPV16-D (200 pg) mixed with 5 μg of *Xba*I-digested NIH 3T3 DNA (lanes 2 and 3), 40 pg of pSVHPV16-D linearized with *Hin*dIII and mixed with 5 μg of *Xba*I-digested NIH 3T3 DNA (lanes 4 and 5), and 5 μg of cellular DNA of D-NIH 3T3 cl-14 digested with *Xba*I (lanes 6 and 7), *Sac*I (lanes 8 and 9), or *Eco*RV (lanes 10 and 11) were digested with ATP-dependent DNase (lanes 3, 5, 7, 9, and 11) or treated in a same manner without the enzyme (lanes 2, 4, 6, 8, and 10). The DNA was extracted with phenol, precipitated with ethanol after the addition of 5 μg of yeast tRNA, and electrophoresed through 0.6% agarose. pSVHPV16-D (200 pg) mixed with 5 μg of *Xba*I-digested NIH 3T3 DNA without any treatment was also electrophoresed as a control (lane 1). (B) The DNA was blotted to a nitrocellulose filter and hybridized with ^{32}P-labeled HPV16 DNA. K, Kilobase pairs.

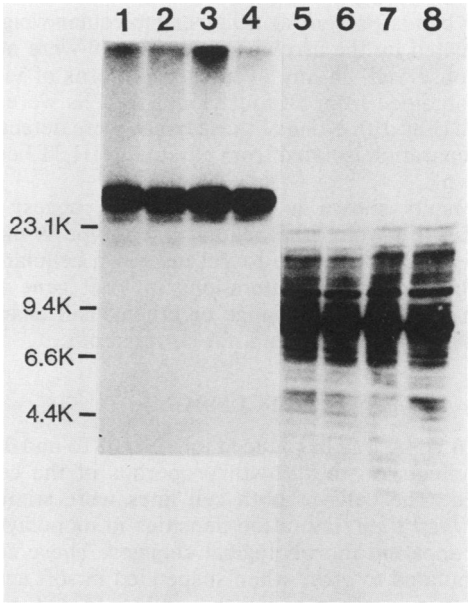

FIG. 9. Southern blot analysis of the DNA extracted from NIH 3T3 cells transformed by HPV16 and three subclones obtained from foci. DNA (10 μg) extracted from D-NIH 3T3 cl-14 (lanes 1 and 5) and D-NIH 3T3 cl-14f-1 (lanes 2 and 6), -f-2 (lanes 3 and 7), and -f-3 (lanes 4 and 8) was digested with *Xba*I (lanes 1 to 4) or *Bam*HI (lanes 5 to 8) and electrophoresed through 0.6% agarose gels. The DNA blotted to nitrocellulose membranes was hybridized with ^{32}P-labeled HPV16 DNA. Positions of size markers (lambda phage DNA restricted with *Hin*dIII) are indicated (K, kilobase pairs).

radioactive probe. The hybridization signals of the HPV16-positive DNAs of all the cell clones tested were indistinguishable (Fig. 9.) There was also no difference in the patterns generated by digestions with *Sac*I and *Eco*RV (data not shown).

Total RNA was isolated from the four cell clones whose DNA was analyzed above and examined by Northern blot analysis for virus-specific RNA. Three species of virus-specific RNAs, 7.5, 4,8, and 1.9 kilobases, were reproducibly observed in all cases (indicated by three arrows in Fig.

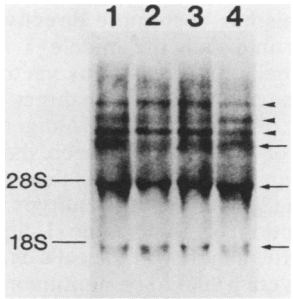

FIG. 10. Northern blot analysis of the RNA obtained from NIH 3T3 cells transformed by HPV16 and their subclones obtained from foci. Total cellular RNA (20 μg) extracted from D-NIH 3T3 cl-14 (lane 1) and D-NIH 3T3 cl-14f-1 (lane 2), -f-2 (lane 3), and -f-3 (lane 4) was electrophoresed through 1.0% agarose gels containing 2.2 M formaldehyde. RNA blotted to nitrocellulose membranes was hybridized with ^{32}P-labeled HPV16 DNA. The positions of 28S and 18S RNAs are indicated at left side. Arrows and arrowheads are defined in the text.

10). The bands present at a higher-molecular-weight range and indicated by the arrowheads in Fig. 10 were not reproducibly observed. In any event, the patterns of viral RNA species obtained from all four clones of cells were indistinguishable (Fig. 10). None of these bands were detected in the RNA preparation isolated from parental NIH 3T3 cells (data not shown).

The results shown in Fig. 9 and 10 suggest that the mechanism by which the cellular phenotype is induced to change is likely to be due to yet unknown cellular changes rather than to genetic alterations in viral gene function, although a single base change or other small changes in a critical viral sequence could not be ruled out.

DISCUSSION

HPV16 DNA was introduced into NIH 3T3 and 3Y1 cells, and its effects on the growth properties of the cells were examined. The cells of both cell lines were stimulated to grow beyond their saturation densities in monolayers without any apparent morphological changes. These cells were also stimulated to grow when suspended in soft agar. Since there was no difference in the ability to induce growth stimulation of the cells between the monomeric viral DNA linearized at its *Bam*HI site and the head-to-tail dimer of the viral DNA, the gene that causes this effect is not disrupted by cleavage at the *Bam*HI site. When NIH 3T3 cells were stimulated to grow by HPV16, morphologically altered transformed cells appeared. This sequential event occurred at a frequency of about 10^{-3}.

The transforming function of the HPV16 genes is worth comparing with that of other papovaviruses, polyomavirus and SV40, which have been extensively characterized. Stoker (56) showed that infection of baby hamster kidney cells with polyomavirus at a high multiplicity of infection transiently stimulated virtually every cell to grow in soft agar, while only a small proportion of such cells became permanently transformed and formed large colonies. This phenomenon, called abortive transformation, is interpreted to mean that the polyomavirus has an acute transforming gene (middle T antigen gene [18] or middle and small T antigen genes [38]), and therefore, the cell that is infected with the polyomavirus genome is stimulated to grow. However, since the integration of viral DNA into host chromosomal DNA is inefficient, only those cells in which successful integration occurred could be stimulated to grow permanently. This has been more directly demonstrated in the experiments in which the middle T antigen gene was introduced into cells by a retrovirus vector (11, 20), which confirmed that the middle T antigen directly transforms cells (20). In contrast, the SV40 large T antigen, the main transforming protein of the virus, has been shown to be far less efficient as a transforming protein than the polyomavirus middle T antigen (20, 44). What phenotype is induced in cells by the direct action of SV40 large T antigen is not well understood, but the majority of the cells expressing the SV40 large T antigen were found to be nontumorigenic (20). These results suggest the involvement of cellular changes for full transformation. The HPV16 cells isolated after transfection and coselection with the neomycin resistance gene had a colony formation rate of nearly 90%. This suggests that one of the HPV16 genes stimulates the cells to grow directly in monolayers as well as in soft agar. Since the properties of the HPV16-containing cells are stable, the integration of the HPV16 DNA into chromosomal DNA appeared to have occurred rather efficiently under our experimental conditions. It is worth mentioning here that the E1 gene of HPV16 has a deletion in the particular clone that we used in our experiments (1, 35), and the E1 gene is thought to have a plasmid maintenance function to maintain the viral DNA in an extrachromosomal state (31, 32).

Although the HPV16 gene directly stimulates cells to grow, this function is quite different from that of the middle T antigen of polyomavirus; HPV16 does not morphologically transform rodent fibroblasts by its direct gene function. The effect of the HPV16 gene on rodent fibroblast cell lines is similar to that of the small T antigen of polyomavirus (37). A similar gene function has also been reported for the v-*erbA* oncogene (14).

At a frequency of about 10^{-3}, NIH 3T3 cells containing the HPV16 DNA gave rise to morphologically recognizable dense foci after prolonged incubation. The cells from such dense foci were morphologically altered and grew much more rapidly in soft agar than the parental cells did. In other words, two distinct sequential events occur in the process of transformation of NIH 3T3 cells by the HPV16 DNA. The frequency of conversion from the first stage to the second seems to be quite high owing to a genetic alteration of a cellular gene. Continued growth stimulation by an HPV16 gene is presumably the condition that induces the second event. We observed that cells at the second stage rarely appeared from cells that were at the first stage and growing exponentially, while the cells remaining in a confluent monolayer gave rise to dense foci, which represented the second stage.

Similarly, two distinct phenotypes without apparent correlation with the state of viral DNA have been observed in rat fibroblast cells transformed by bovine papillomavirus type 1 (17). More recently, Yasumoto and his collaborators (64) reported two distinct stages in the transformation of NIH 3T3 cells by the HPV16 DNA. At the first stage, the transfected cells acquired the ability to grow in the presence of reduced serum growth factor(s) although the cells were nontumorigenic, while at the second stage, the cells became less growth factor dependent and tumorigenic. Our observations were quite consistent with theirs. The only important difference is in the expression of the viral sequences. Whereas we did not observe any differences in the expression between the cells at two different stages, they reported that the level of the viral RNA in the second stage was much lower than that in the first stage. Since they did not compare the same clone of cells at both stages and since the construct they used was different from ours, we could not directly compare our data with theirs on this point.

The HPV16 and HPV18 DNAs are present mainly as extrachromosomal episomes in preneoplastic lesions, while the cervical cancer cells or cell lines obtained from them contain mainly integrated copies of the viral DNA (10, 13). The change in the state of the viral DNA from episomal to integrated has been suggested to correspond to premalignant-to-malignant stages (13). In the present study, even the cells at the earliest time after transfection contained mainly integrated copies. Furthermore, the growth properties of the cells containing the HPV16 DNA changed stepwise without any detectable change in the viral DNA. Therefore, the integration of viral DNA per se does not necessarily determine a certain phenotype of the cells. These results also suggest that the changes responsible for the conversion of the cells from the first to the second stage are unlikely to be due to the changes in the viral genes but rather to be due to the alterations in cellular functions. It could not be ruled out that changes in viral sequences undetectable by

the method used here were responsible for the phenotypic change. If this were the case however, the altered viral sequence, which would have strong transforming activity, should easily be isolated. Since the first stage that we observed is stimulation of cell growth, it seems reasonable to assume that the first stage corresponds to the preneoplastic stages of cervical cancer in which epithelial cells are stimulated to grow without any typical malignant phenotype. If this assumption is correct, the second stage that we observed would correspond to the malignant cell stage itself or one or more steps before the malignant status.

There have been conflicting reports on the mechanism of oncogenic transformation by the bovine papillomavirus and the HPVs. Jarrett and his collaborators (6) reported that bovine papillomavirus type 4 was found in benign papillomas but that no viral sequence was detected in the malignant cells, which were considered to be converted from benign papillomas. In human cervical carcinomas, a very high proportion of clinical material obtained from different laboratories contained the HPV sequence, the most prevalent being types 16 and 18 (3, 12). Most of the cell lines originating from cervical cancer have also been found to contain the same types of HPV sequence (40, 57, 65). In some cancer cells as well as cell lines, the viral sequences were found to be expressed as RNAs (50, 53). Furthermore, a certain region of the viral genome is commonly present, and there is some regularity in the pattern of viral RNA in these cell lines. These results suggest that the function of a certain viral gene is required to maintain at least a part of the phenotype of the cancer cells (49). The region of the HPV16 or the HPV18 DNA frequently found in cervical cancer cells encodes the E6 and E7 genes (49, 50). It is of great interest to see whether either one of these two genes has the growth-stimulating activity that we observed in the present study.

ACKNOWLEDGMENTS

We thank Peter Howley, National Cancer Institute, Bethesda, Md., for the gift of the HPV16 DNA clone, which was originally isolated by Harald zur Hausen, German Cancer Center, Heidelberg, Federal Republic of Germany. We also thank Genki Kimura, Kyushu University, for his gift of 3Y1 cells and Hideo Yamagishi, Kyoto University, for valuable advice on ATP-dependent DNase.

LITERATURE CITED

1. **Baker, C. C., W. C. Phelps, V. Lindgren, M. J. Braun, M. A. Gonda, and P. M. Howley.** 1987. Structural and transcriptional analysis of human papillomavirus type 16 sequences in cervical carcinoma cell lines. J. Virol. **61**:962–971.
2. **Beaudenon, S., D. Kremsdorf, O. Croissant, S. Jablonska, S. Wain-Hobson, and G. Orth.** 1986. A novel type of human papillomavirus associated with genital neoplasias. Nature (London) **321**:675–681.
3. **Boshart, M., L. Gissmann, H. Ikenberg, A. Kleinheinz, W. Scheurlen, and H. zur Hausen.** 1984. A new type of papillomavirus DNA and its presence in genital cancer biopsies and in cell lines derived from cervical cancer. EMBO J. **3**:1151–1157.
4. **Broker, T. R., and M. Botchan.** 1986. Papillomaviruses: retrospectives and prospectives. Cancer Cells **4**:17–36.
5. **Campo, M. S., and W. F. H. Jarrett.** 1986. Papilloma virus infection in cattle: viral and chemical cofactors in naturally occurring and experimentally induced tumors. CIBA Found. Symp. **120**:117–135.
6. **Campo, M. S., M. H. Moar, M. L. Sartirana, I. M. Kennedy, and W. F. H. Jarrett.** 1985. The presence of bovine papillomavirus type 4 DNA is not required for the progression to, or the maintenance of, the malignant state in cancers of the alimentary canal in cattle. EMBO J. **4**:1819–1825.
7. **Chirgwin, J. M., A. E. Pryzybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5499.
8. **Colbere-Grapin, F., F. Horodniceanu, P. Kourilsky, and A. C. Grapin.** 1981. A new dominant hybrid selective marker for higher eukaryotic cells. J. Mol. Biol. **150**:1–14.
9. **Crum, C. P., H. Ikenberg, R. M. Richart, and L. Gissmann.** 1984. Human papillomavirus type 16 and early cervical neoplasia. N. Engl. J. Med. **310**:880–883.
10. **Crum, C. P., M. Mitao, R. U. Levine, and S. Silverstein.** 1985. Cervical papillomaviruses segregate within morphologically distinct precancerous lesions. J. Virol. **54**:675–681.
11. **Donoughue, D. J., C. Anderson, T. Hunter, and P. L. Kaplan.** 1984. Transmission of the polyoma virus middle T gene as the oncogene of a murine retrovirus. Nature (London) **308**:748–750.
12. **Dürst, M., L. Gissmann, H. Ikenberg, and H. zur Hausen.** 1983. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. Proc. Natl. Acad. Sci. USA **80**:3812–3815.
13. **Dürst, M., E. Schwarz, and L. Gissmann.** 1986. Integration and persistence of human papillomavirus DNA in genital tumors, p. 273–280. *In* R. Peto and H. zur Hausen (ed.), Banbury report 21: viral etiology of cervical cancer. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Gandrillon, O., P. Jurdic, M. Benchaibi, J.-H. Xiao, J. Ghysdael, and J. Samarut.** 1987. Expression of the v-erb A oncogene in chicken embryo fibroblasts stimulates their proliferation in vitro and enhances tumor growth in vivo. Cell **49**:687–697.
15. **Gissmann, L., and A. Schneider.** 1986. Human papillomavirus DNA in preneoplastic and neoplastic genital lesions, p. 217–224. *In* R. Peto and H. zur Hausen (ed.), Banbury report 21: viral etiology of cervical cancer. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
17. **Grisoni, M., G. Meneguzzi, O. DeLapeyriere, B. Binétruy, M. Rassoulzadegan, and F. Cuzin.** 1984. The transformed phenotype in culture and tumorigenicity of Fisher rat fibroblast cells (FR3T3) transformed with bovine papilloma virus type 1. Virology **135**:406–416.
18. **Ito, Y.** 1980. Organization and expression of the genome of polyoma virus, p. 447–480. *In* G. Klein (ed.), Viral oncology. Raven Press, New York.
19. **Jarrett, W. F. H., P. E. McNeil, W. T. R. Grimshaw, I. E. Selman, and W. I. M. McIntyre.** 1978. High incidence area of cattle cancer with a possible interaction between an environmental carcinogen and a papilloma virus. Nature (London) **274**:215–217.
20. **Jat, P. S., C. L. Cepko, R. C. Mulligan, and P. A. Sharp.** 1986. Recombinant retroviruses encoding simian virus 40 large T antigen and polyomavirus large and middle T antigens. Mol. Cell. Biol. **6**:1204–1217.
21. **Kanda, T., S. Watanabe, and K. Yoshiike.** 1987. Human papillomavirus type 16 transformation of rat 3Y1 cells. Gann **78**:103–108.
22. **Kidd, J. G., and P. Rous.** 1937. Effect of the papillomavirus (Shope) upon tar warts of rabbits. Proc. Soc. Exp. Biol. Med **37**:518–520.
23. **Kidd, J. G., and P. Rous.** 1940. Cancers deriving from the virus papillomas of wild rabbits under natural conditions. J. Exp. Med. **71**:469–494.
24. **Kimura, G., A. Itagaki, and J. Summers.** 1975. Rat cell line 3Y1 and its virogenic polyoma- and SV40-transformed derivatives. Int. J. Cancer **15**:694–706.
25. **Koss, L. G.** 1986. Chairman's concluding remarks: sequence of events in carcinogenesis of the uterine cervix, p. 179–186. *In* R. Peto and H. zur Hausen (ed.), Banbury report 21: viral etiology of cervical cancer. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Lancaster, W. D., and C. Olson.** 1982. Animal papillomaviruses. Microbiol. Rev. **46**:191–207.

27. **Law, M.-F., D. R. Lowy, I. Dvoretzky, and P. M. Howley.** 1981. Mouse cells transformed by bovine papillomavirus contain only extrachromosomal viral DNA sequences. Proc. Natl. Acad. Sci. USA **78**:2727–2731.
28. **Lorincz, A. T., W. D. Lancaster, R. J. Kurman, A. B. Jenson, and G. F. Temple.** 1986. Characterization of human papillomaviruses in cervical neoplasias and their dectection in routine clinical screening, p. 225–238. *In* R. Peto and H. zur Hausen (ed.), Banbury report 21: viral etiology of cervical cancer. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Lorincz, A. T., W. D. Lancaster, and G. F. Temple.** 1986. Cloning and characterization of the DNA of a new human papillomavirus from a woman with dysplasia of the uterine cervix. J. Virol. **58**:225–229.
30. **Lowy, D. R., I. Dvoretsky, R. Shober, M.-F. Law, L. Engel, and P. M. Howley.** 1980. *In vitro* transformation by a defined subgenomic fragment of bovine papillomavirus DNA. Nature (London) **287**:72–74.
31. **Lusky, M., and M. R. Botchan.** 1984. Characterization of the bovine papilloma virus plasmid maintenance sequences. Cell **36**:391–401.
32. **Lusky, M., and M. R. Botchan.** 1985. Genetic analysis of bovine papillomavirus type 1 *trans*-acting replication factors. J. Virol. **53**:955–965.
33. **MacPherson, I., and L. Montagnier.** 1964. Agar suspension culture for the selective assay of cells transformed by polyoma virus. Virology **23**:523–529.
34. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
35. **Matsukura, T., T. Kanda, A. Furuno, H. Yoshikawa, T. Kawana, and K. Yoshiike.** 1986. Cloning of monomeric human papillomavirus type 16 DNA integrated within cell DNA from a cervical carcinoma. J. Virol. **58**:979–982.
36. **McCance, D. J., M. J. Campion, P. K. Clarkson, P. M. Chesters, D. Jenkins, and A. Singer.** 1985. Prevalence of human papillomavirus type 16 DNA sequences in cervical intraepithelial neoplasia and invasive carcinoma of the cervix. Br. J. Obstet. Gynaecol. **92**:1101–1105.
37. **Noda, T., M. Satake, T. Robbins, and Y. Ito.** 1986. Isolation and characterization of NIH 3T3 cells expressing polyomavirus small T antigen. J. Virol. **60**:105–113.
38. **Noda, T., M. Satake, Y. Yamaguchi, and Y. Ito.** 1987. Cooperation of middle and small T antigens of polyomavirus in transformation of established fibroblast and epitheliallike cell lines. J. Virol. **61**:2253–2263.
39. **Orth, G.** 1986. Epidermodysplasia verruciformis: a model for understanding the oncogenicity of human papillomaviruses. CIBA Found. Symp. **120**:157–174.
40. **Pater, M. M., and A. Pater.** 1985. Human papillomavirus types 16 and 18 sequences in carcinoma cell lines of the cervix. Virology **145**:313–318.
41. **Pfister, H.** 1984. Biology and biochemistry of papillomaviruses. Rev. Physiol. Biochem. Pharmacol. **99**:111–182.
42. **Prisi, L., S. Yasumoto, M. Feller, J. Doniger, and J. A. DiPaolo.** 1987. Transformation of human fibroblasts and keratinocytes with human papillomavirus type 16 DNA. J. Virol. **61**: 1061–1066.
43. **Rigby, P. D., M. D. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
44. **Risser, R., and R. Pollak.** 1974. A nonselective analysis of SV40 transformation of mouse 3T3 cells. Virology **53**:477–489.
45. **Rous, P., and J. G. Kidd.** 1936. The carcinogenic effect of a virus upon tarred skin. Science **83**:468–469.
46. **Sarver, N., M. S. Rabson, Y.-C. Yang, J. C. Byrne, and P. M. Howley.** 1984. Localization and analysis of bovine papillomavirus type 1 transforming functions. J. Virol. **52**:377–388.
47. **Schiller, J. T., W. C. Vass, and D. L. Lowy.** 1984. Identification of a second transforming region in bovine papillomavirus DNA. Proc. Natl. Acad. Sci. USA **81**:7880–7884.
48. **Schiller, J. T., W. C. Vass, K. H. Vousden, and D. R. Lowy.** 1986. E5 open reading frame of bovine papillomavirus type 1 encodes a transforming gene. J. Virol. **57**:1–6.
49. **Schneider-Gadicke, A., and E. Schwarz.** 1986. Different human cervical carcinoma cell lines show similar transcription patterns of human papillomavirus type 18 early genes. EMBO J. **5**: 2285–2292.
50. **Schwarz, E., U. K. Freese, L. Gissmann, W. Mayer, B. Roggenbuck, A. Stremlau, and H. zur Hausen.** 1985. Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. Nature (London) **314**:111–114.
51. **Seedorf, K., G. Krammer, M. Dürst, S. Shuhai, and W. Rowekamp.** 1985. Human papillomavirus type 16 DNA sequence. Virology **145**:181–185.
52. **Shope, R. E.** 1933. Infectious papillomatosis of rabbits. J. Exp. Med. **58**:607–624.
53. **Smotkin, D., and F. O. Wettstein.** 1986. Transcription of human papillomavirus type 16 early genes in a cervical cancer and a cancer-derived cell line and identification of the E7 protein. Proc. Natl. Acad. Sci. USA **83**:4680–4684.
54. **Southern, E.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**: 503–517.
55. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1**:327–341.
56. **Stoker, M.** 1968. Abortive transformation by polyoma virus. Nature (London) **218**:234–238.
57. **Tsunokawa, Y., N. Takebe, S. Nozawa, T. Kasamatsu, L. Gissmann, H. zur Hausen, M. Terada, and T. Sugimura.** 1986. Presence of human papillomavirus type 16 and type 18 DNA sequences and their expression in cervical cancers and cell lines from Japanese patients. Int. J. Cancer **37**:499–503.
58. **Tsunokawa, Y., N. Takebe, T. Kasamatsu, M. Terada, and T. Sugimura.** 1986. Transforming activity of human papillomavirus type 16 DNA sequences in a cervical cancer. Proc. Natl. Acad. Sci. USA **83**:2200–2203.
59. **Wettstein, F. O., and J. G. Stevens.** 1982. Variable sized free episomes of Shope papillomavirus DNA are present in all non-virus-producing neoplasms and integrated episomes are detected in some. Proc. Natl. Acad. Sci. USA **79**:790–794.
60. **Wigler, M., A. Pellicer, S. Silverstein, and R. Axel.** 1978. Biochemical transfer of single-copy eukaryotic genes using total cellular DNA as donor. Cell **14**:725–731.
61. **Yamagishi, H., T. Tsuda, S. Fujimoto, M. Toda, K. Kato, Y. Maekawa, M. Ueno, and M. Arai.** 1983. Purification of small polydisperse circular DNA of eukaryotic cells by use of ATP-dependent deoxyribonuclease. Gene **26**:317–321.
62. **Yang, Y.-C., H. Okayama, and P. M. Howley.** 1985. Bovine papillomavirus contains multiple transforming genes. Proc. Natl. Acad. Sci. USA **82**:1030–1034.
63. **Yasumoto, S., A. L. Burkhardt, J. Doniger, and J. A. DiPaolo.** 1986. Human papillomavirus type 16 DNA-induced malignant transformation of NIH 3T3 cells. J. Virol. **57**:572–577.
64. **Yasumoto, S., J. Doniger, and J. A. DiPaolo.** 1987. Differential early viral gene expression in two stages of human papillomavirus type 16 DNA-induced malignant transformation. Mol. Cell. Biol. **7**:2165-2172.
65. **Yee, C., I. Krishnan-Hewlett, C. C. Baker, R. Schlegel, and P. M. Howley.** 1985. Presence and expression of human papillomavirus sequences in human cervical carcinoma cell lines. Am. J. Pathol. **119**:361–366.