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Interferon induces morphologic reversion with elimination of extrachromosomal viral genomes in bovine papillomavirus-transformed mouse cells

(transformation reversion/papovavirus/interferon cure/bovine papillomavirus plasmid)

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ABSTRACT **The effect of mouse L-cell interferon on bovine papillomavirus type 1 (BPV-1) transformation of murine cells was examined. Mouse interferon reduced the level of BPV-1-induced transformation of mouse C127 cells by 95%. Long-term treatment of established BPV-1-transformed mouse cell clones with mouse L-cell interferon led to a decrease in the average number of the plasmid viral genomes present in these cells to 1/3 to 1/8. Although revertant lines could not be isolated from these lines in the absence of treatment with interferon, flat revertants were easily selected from two independent clonal transformed lines carried for 60 generations in the continued presence of 200 units of interferon per ml. These flat revertants had the biological characteristics of nontransformed C127 cells and could be retransformed by BPV-1. Southern blot hybridization failed to detect BPV-1 DNA in any of eight independent revertant lines examined under conditions that could detect 0.2 copies per cell. We conclude that interferon treatment has resulted in a selective reduction of the amount of extrachromosomal BPV-1 DNA in transformed cells and has cured some treated cells completely of their viral DNA.**

The bovine papillomavirus type 1 (BPV-1) causes fibropapillomas in cattle and, unlike most other papillomaviruses, induces fibroblastic tumors in a variety of foreign host species, including hamsters (1) and rabbits (2). This virus and its cloned DNA are also capable of inducing tumorigenic transformation of murine fibroblasts *in vitro* (3–5). BPV-1-transformed cells exhibit an array of properties related to cellular transformation such as growth to high saturation densities, anchorage-independent growth in suspension culture, and tumor formation in athymic or syngeneic mice (3). BPV-1 transformants contain multiple copies of nonintegrated supercoiled circular BPV-1 DNA molecules in their nuclei (4, 6), in contrast to polyoma or simian virus 40 (SV40)-transformed rodent cells. Cells transformed by SV40 usually contain only one or a few copies of viral DNA per cell, these copies often represent only part of the viral genome, the viral DNA may be rearranged compared to virion DNA, and the viral DNA is usually present in an integrated state (7–11).

Interferon treatment reduces the number of infected cells and viral yield in acute productive infection with many transforming viruses, such as SV40 (12), *Herpesvirus saimiri* (13, 14). *Herpesvirus ateles* (13), Epstein–Barr virus (14), and the Kirsten murine sarcoma virus (15, 16). Whereas interferon treatment can reduce the numbers of SV40 transformants in acutely-infected cultures, the treatment of established SV40 transformants by interferon has no effect on their neoplastic phenotype or on the maintenance of the viral genome (17–19). In view of the unique plasmid nature of the viral genome in papillomavirus-transformed cells, we have studied the effect of mouse L-cell interferon treatment on mouse C127 cells acutely infected with BPV-1 and on two established BPV-1-transformed C127 cell lines, ID13 and ID14 (3, 4). Interferon treatment was found to reduce the efficiency of BPV-1-induced transformation, to lower the average number of BPV DNA copies in cells transformed by BPV-1, and to induce morphological reversion in some transformed cells with complete elimination of the plasmid BPV-1 genomes, thus leading to a "cure" of the BPV-1 infection and the virus-encoded neoplastic phenotype.

MATERIALS AND METHODS

Cells and Viruses. The flat murine C127 cells (20) were used in acute infection. Established C127-derived BPV-1 transformants, clonal lines ID13 and ID14, have been described (3, 4). The BPV-1 strain 307 was isolated and purified from a bovine fibropapilloma and stored in suspension at −70°C (21).

Interferon. Mouse interferon produced in L cells after stimulation with Newcastle disease virus was purified on an antibody affinity column in the laboratory of the late K. Paucker (22). The preparation used in these studies had a specific activity of 9×10^7 units/mg of protein.

Infection with BPV-1. BPV-1 was used to infect C127 cells as follows. Cells were plated at 5.0×10^5 cells per 60-mm culture dish 24 hr prior to infection. The virus was adsorbed in a 0.3-ml inoculum for 2 hr at 37°C, the inoculum was removed, and the cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum in the presence or absence of 200 international units of mouse L-cell interferon per ml. The medium was changed every 3rd day, and fresh interferon was added. The plates were fixed and stained at 14–21 days to assay the number of transformed foci. The same protocol was used to assess the susceptibility of revertants to retransformation by BPV-1.

Analysis of Intracellular BPV-1 DNA. Total cellular DNA was extracted from ID13 and ID14 cell lines at each passage and assayed for the number of BPV-1 genomes by Southern blot hybridization (4). The DNA was cleaved with *Bam*HI, electrophoresed through a 1% agarose gel, and transferred to a nitrocellulose filter as described (4). Cloned BPV-1 DNA (23) was nick-translated to high specific activity (24) and hybridized to DNA immobilized on the filter (4, 25). After washing, the filters

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Abbreviations: BPV-1, bovine papillomavirus type 1; SV40, simian virus 40.

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were exposed to Kodak x-ray film at −70°C. The quantitative analysis was performed on densitometer tracings of the resulting autoradiographs. The quantitation was achieved by using a reconstruction analysis in which known amounts of cloned BPV-1 DNA were added to uninfected C127 cell DNA.

RESULTS

Interferon Inhibits BPV-1 Transformation of Mouse C127 Cells. To determine the influence of interferon on the efficiency of BPV-1-induced transformation in acute infection, mouse C127 cells were infected with BPV-1 at serial 1:10 dilutions and subsequently maintained in the presence of 200 units of mouse L-cell interferon per ml. This concentration of interferon protected C127 cells and two BPV-1-transformed C127 cell lines, ID13 and ID14, from encephalomyocarditis virus-induced cytopathic effect. The interferon treatment had a slight inhibitory effect on the proliferative capacity of the C127 cells and of the BPV-1-transformed cell lines. The doubling time of the interferon-treated transformed ID13 cells was 20.6 hr vs. 19.0 hr in the controls, and the doubling time for the uninfected C127 cells carried in the presence of interferon was 32.4 hr as opposed to 28.8 hr for the untreated controls. The saturation density of C127 cells was $5.0–7.0 \times 10^4$ cells per cm^2 in the presence or in the absence of interferon. ID13 cells with or without interferon reached saturation at $1.4–1.7 \times 10^5$ cells per cm^2. The saturation densities of uninfected C127 cells and their BPV-1 transformants were unaffected, therefore, by interferon treatment.

Preliminary experiments showed that short-term (24 hr) treatment with interferon did not influence the efficiency of BPV-1 transformation after viral infection. To determine the influence of continuous interferon treatment on the efficiency of transformation in acute infection, mouse C127 cells were infected with serial dilutions of BPV-1 and maintained in the presence of 200 units of mouse L-cell interferon per ml. Infected cultures carried in the presence of interferon showed a reduction by 95% in the number of BPV-1-induced foci (Table 1). Because the number of foci was similar when assayed at 3 wk or at 2 wk (Table 1, experiment 2), the lower level of transformation in the interferon-treated cells is due to the inhibition of transformation rather than merely to a delay in the appearance of the transformed foci.

Interferon Lowers the Plasmid Copy Number of BPV-1 Genomes in Established Transformed Mouse Cells. One hypothesis to explain the above results with acute infection would be that interferon had selectively inhibited BPV-1 DNA replication. Therefore, we examined the effect of interferon on the quantity of BPV-1 DNA in established transformants. Two BPV-1-transformed mouse cell clones were used in these experiments: ID14, containing ≈40–50 BPV-1 copies per cell (4), and ID13, with 150–175 BPV-1 DNA molecules per cell. These C127-derived transformants were carried in the presence of interferon for 10 cell passages or about 60 cell divisions. DNA was extracted from the cells at each passage (approximately six divisions), treated with *Bam*HI endonuclease, and analyzed by Southern blotting. A reconstruction experiment, in which different amounts of linearized BPV-1 DNA from *Bam*HI-cleaved pBPV-1 (8–2) was added (23) to uninfected C127 cell DNA, was used to determine the copy number of the BPV-1 genomes in the cells. The BPV-1 copy number remained unchanged for the first five passages for both lines in the interferon-treated cells and in the untreated controls. Whereas the relative abundance of BPV-1 genomes remained constant in the control BPV-1 transformants through all 10 passages, it was reduced significantly in interferon-treated ID13 and ID14 cells between passages 6 and 10 (Fig. 1). A quantitative analysis of the Southern blot shown in Fig. 1 revealed an approximate decrease to 1/3 to 1/8 in the number of BPV-1 genome equivalents in the transformed lines with continual treatment with interferon (Fig. 2).

Table 1. Inhibition of BPV-1 transformation of mouse cells by interferon (IFN)

	Number of transformed foci*					
	Exp. 1†		Exp. 2†		Exp. 2‡	
Dilution of virus stock	No IFN	With IFN	No IFN	With IFN	No IFN	With IFN
10^2	TMTC	TMTC	TMTC	53	TMTC	80
10^3	TMTC	19	TMTC	7	140	8
10^4	34	1	15	1	17	1
10^5	1	0	1	0	0	0
Control	0	0	0	0	0	0

Infection of C127 cells with BPV-1 was carried out as described. IFN-treated cultures were maintained in the presence of 200 units of mouse L-cell IFN per ml. Medium was changed every 3 days, and freshly thawed IFN was added at the same time.
* TMTC, too many to count, >150 foci per plate.
† Focus determinations were made on day 14.
‡ Focus determinations were made on day 21.

Interferon Induces Morphologic Reversion in BPV-1-Transformed Cells. Because in BPV-1 transformants treated with interferon there was a decrease to 1/3 to 1/8 in the average copy number of viral genomes, an intriguing possibility was that some cells in the interferon-treated population might have been "cured" of the resident viral genome. If the presence and continuous expression of the BPV-1 genome were required for the maintenance of the transformed state, such cells would be expected to revert to the nontransformed phenotype.

The interferon-treated ID13 and ID14 cells at passage 10 were subcloned in the absence of interferon to screen for cell colonies with revertant morphology. Untreated ID13 cells were subcloned at low density in parallel as a control. No revertant colonies were detected in the untreated ID13 cells, indicating that the frequency of revertance in these cells was below 1×10^{-5}. In the interferon-treated ID13 and ID14 cultures, however, between 1% and 10% of the cells reverted to the flat morphology typical of uninfected C127 cells. Several independent flat colonies were subcultured. The morphological characteristics of two representative revertant cell lines (ID13-if-R) in comparison to that of uninfected C127 cells and ID13 cells are shown in Fig. 3.

DNA was extracted from three transformed subclones of the interferon-treated ID13 culture and from eight flat ID13-if revertants, treated with *Bam*HI endonuclease, and analyzed by Southern transfer (Fig. 4). Each of the ID13-if cell lines (ID13-if-T) examined that expressed the transformed phenotype harbored unintegrated BPV-1 DNA. The ID13-if-T2 and ID13-if-T3 lines contained ≈50 and ≈100 BPV-1 genome equivalents per cell, respectively (Fig. 4). However, none of the eight revertants (ID13-if-R1 through -R8), contained detectable BPV-1 sequences (<0.2 copies per cell) either in plasmid form or in an integrated state covalently linked to host-associated DNA sequences. The analysis of the DNA of six of these revertant lines is shown in Fig. 4. In accordance with the flat morphology of the ID13-if-R revertant lines, these flat cells lost the capacity to grow to high saturation densities and to form colonies in soft agar medium (Table 2). Moreover, the two revertant lines examined (ID13-if-R1 and ID13-if-R7), were approximately as susceptible to BPV-1 retransformation as were uninfected parental cells (Table 2).

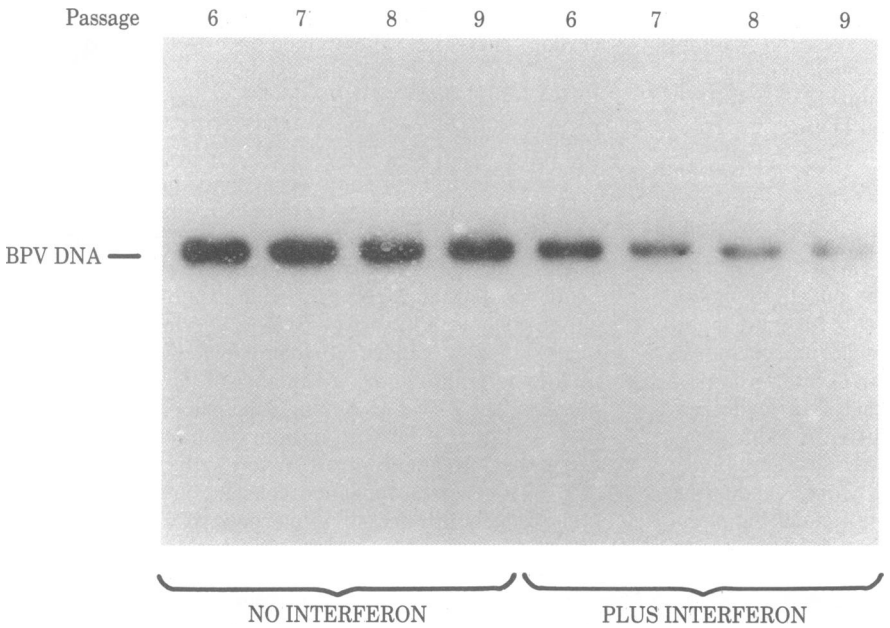

FIG. 1. Southern transfer analysis of BPV-1 DNA in the transformed cell clone ID14 carried with or without interferon treatment. The cells were maintained in the presence or absence of 200 units of mouse L-cell interferon per ml for the indicated number of passages and split at a ratio of 1:100. Total cellular DNA was extracted from the cultures at each passage and assayed for the quantity of BPV-1 DNA by Southern blot hybridization. The DNA was cleaved with *Bam*HI (which cleaves BPV-1 DNA once), and the resulting fragment was separated on a 1% agarose gel, denatured *in situ*, transferred, immobilized on nitrocellulose, and hybridized with ^{32}P-labeled nick-translated BPV-1 DNA as described (4).

DISCUSSION

Treatment with interferon is known to inhibit acute infection with many transforming viruses (12–19). Thus, it is not surprising that interferon treatment of C127 cells acutely infected with BPV-1 leads to reduced numbers of transformed cell foci; however, the interferon-mediated elimination of viral genomes and morphologic reversion has not been observed previously in established viral transformants. These data establish that the continuous presence (and presumably expression) of the viral genome is required for the maintenance of fibroblast transformation.

The capacity of interferon to "cure" the cells of BPV-1 DNA may be a function of the plasmid state of BPV genomes in the nuclei of the transformed cells. Most other DNA tumor viruses require integration of the viral genome into the host DNA (6–9, 24, 25). Possible exceptions include *Herpesvirus saimiri*, *Herpesvirus ateles*, and the Epstein–Barr virus; these herpesviruses also may be present in multiple, unintegrated copies in the transformed cell. It is of interest to note that interferon treatment of cells infected with *Herpesvirus ateles* or *Herpesvirus saimiri* not only suppressed the cytopathic effect induced by these viruses but also eliminated latent virus infection when continued over 14 or more passages (13). It is possible that in this case, the effect of interferon is mediated by a mechanism similar to that in murine BPV-1 transformants.

The precise mode of interferon action in acute BPV-1 infection and in established BPV transformants is unclear. Interferon treatment did not lead to any gross alterations in the restriction patterns of the plasmid BPV-1 DNA, either in the total cell population of ID13 or ID14 cells, which were maintained in the presence of interferon over 10 passages, or in the individual transformed subclones derived from the treated cells. In the Southern blot analysis with the single-cut enzyme *Bam*HI (Figs. 1 and 4) or with multiple-cut enzymes (not shown), we did not

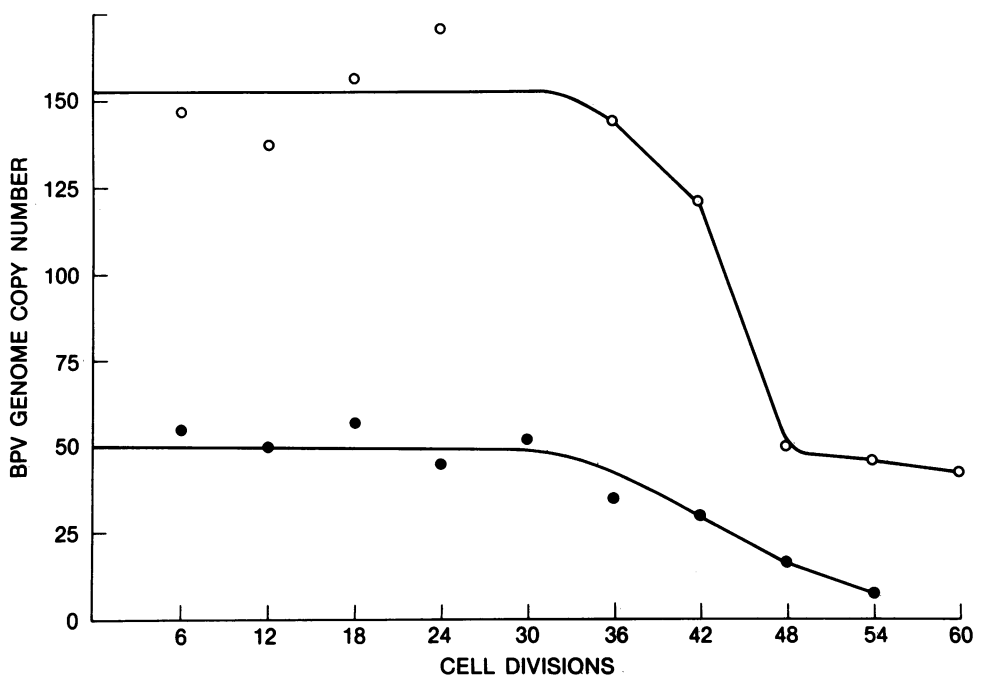

FIG. 2. Quantitation of BPV-1 DNA copy numbers in the transformed clonal lines ID13 (○) and ID14 (●) from densitometer tracings of Southern blot autoradiograms similar to that in Fig. 1. The copy number was interpolated from the tracings of bands from samples containing known amounts of cloned BPV-1 DNA included in the same gel.

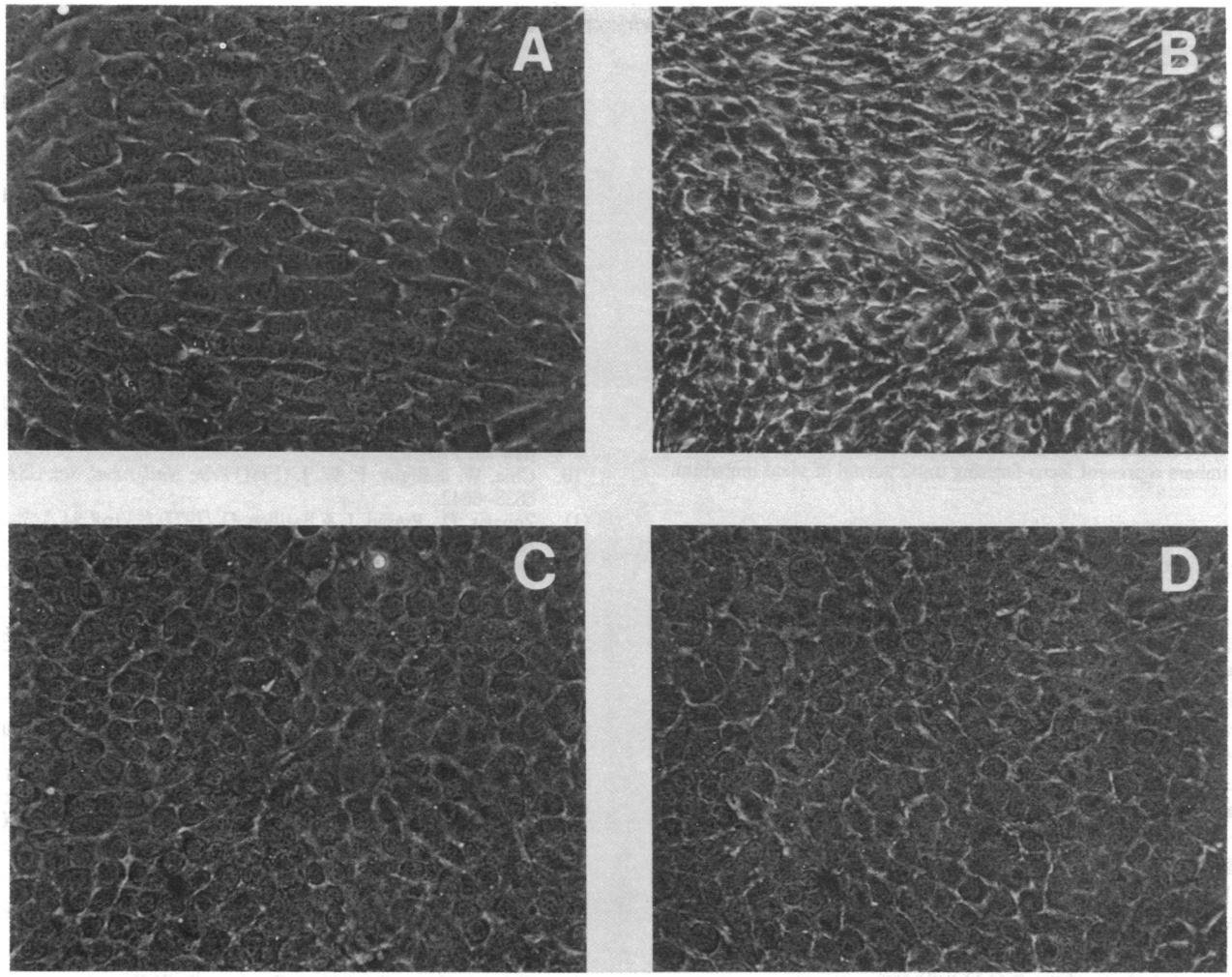

FIG. 3. Morphology of flat, uninfected C127 cells (*A*), the established transformed cell clone ID13 (*B*), and two representative revertant subclones derived from the interferon-treated ID13 cell population at passage 10, ID13-if-R1 (*C*) and ID13-if-R7 (*D*). (Phase contrast = ×100.)

detect joint fragments suggestive of integration or gross deletions or rearrangements of the viral DNA in these interferon-treated cells. The revertant cell lines which were "cured" of persistent BPV-1 genomes were as susceptible to BPV-1 retransformation as uninfected C127 cells. This observation indicates that interferon treatment neither permanently altered the cell's ability to support BPV-1 DNA replication nor selected for BPV-1-resistant cell variants.

From the indirect evidence discussed above, the target of interferon effect may be a viral (or possibly cellular) function

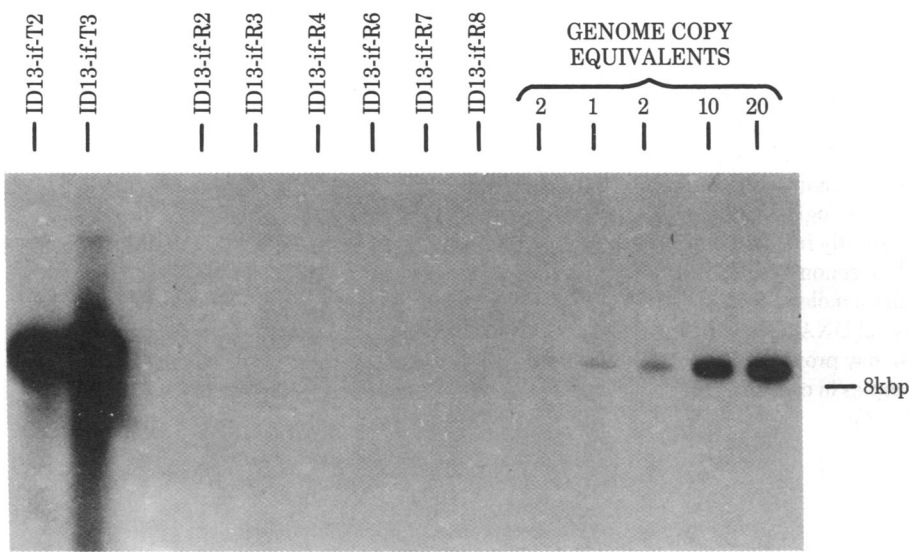

FIG. 4. Analysis of BPV-1 DNA in transformed (left two lanes) and revertant subclones derived from the interferon-treated ID13 cells at passage 10. Total cellular DNA was isolated from the respective cell lines, digested with *Bam*HI, and analyzed by Southern blotting. Reconstruction lanes with uninfected C127 cell DNA and known amounts of cloned BPV-1 DNA are in the five right lanes. One copy per cell equals 5 pg of cloned BPV-1 DNA per 5 μg of total cellular DNA. kbp, Kilobase pairs.

Table 2. Growth properties of revertants and their susceptibility to retransformation with BPV-1

Cells	Saturation density $\times 10^{-4}$*	Agar colony formation†	Retransformation with BPV-1 $\times 10^{-5}$‡
C127	5.4	$<10^{-5}$	5.2
ID13-if-R1	6.6	$<10^{-5}$	2.5
ID13-if-R7	6.9	$<10^{-5}$	2.7
ID13	14.0	9.6×10^{-2}	NA

NA, not applicable.

* Cell density at saturation was obtained by plating 5×10^5 cells per 60-mm dish and changing the culture medium daily. Values given represent the number of cells per cm^2 and were obtained at day 8–10 for the transformed ID13 cells and on day 9–11 for the flat C127 cells and the revertant clones.

† Cell growth in semisolid agar suspension medium was determined as described (4, 5). Value given is relative colony-forming efficiency.

‡ Flat C127 cells and revertants were plated and infected as described. Numbers represent focus-forming units per ml of viral inoculum.

responsible for the plasmid BPV-1 genome replication. This target function could be impaired both in acute infection, resulting in a low efficiency of viral transformation, and in established BPV-1 transformants, leading to a gradual reduction of BPV-1 copy number. Although such a mechanism would explain both of these phenomena, the effect on acute infection and that on BPV-1 copy numbers in established BPV transformants may alternatively be mediated by two different modes of interferon action. Previous experiments have demonstrated that the numbers of BPV-1 genomes vary between individual subclones of established BPV-transformed mouse cell lines, probably reflecting an only approximately equal distribution of BPV-1 plasmids at mitosis (4). It is conceivable that interferon treatment could impair this process and lead to unequal distribution of BPV-1 molecules to daughter cells. Analysis of multiple individual subclones from interferon-treated transformants would be necessary to confirm this hypothesis.

Papillomaviruses are responsible for a wide variety of human pathologic conditions, which include juvenile laryngeal papillomatosis, condylomata accuminata, flat warts of the uterine cervix, and plantar, flat, and common warts (26). The human papillomavirus type 5 (HPV-5) is responsible for a macular lesion recognized in patients with epidermodysplasia verruciformis, which frequently progresses into squamous cell carcinoma (27). Unintegrated human papillomavirus type 5 DNA has been found both in these benign macular lesions and in carcinomas arising in association with these lesions (27, 28). The effect of interferon on certain papillomavirus-induced human diseases including juvenile laryngeal papillomatosis and epidermodysplasia verruciformis is being evaluated in ongoing clinical trials (29, 30). In the absence of an *in vitro* tissue culture system that would enable us to study the productive papillomavirus–host cell interaction, the bovine papillomavirus-transformed mouse system provides a model for examining the biological and biochemical effect of interferon on cells persistently infected with a papillomavirus. The elimination of BPV-1 genomes from established BPV-1 transformants also provides a molecular model to investigate the mechanism of episomal viral DNA persistence and replication. Interferon treatment also may prove useful for modulating the number of plasmid DNA copies in cells carrying BPV-based eukaryotic cloning vectors (31–33).

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1. Friedman, J. C., Levy, J. P., Lasneret, J., Thomas, M., Boiron, M. & Bernard, J. (1963) *Comptes Rendus Acad. Sci. Paris* **257**, 2328–2331.
2. Breitburd, F., Favre, M., Zoorob, R., Fortin, D. & Orth, G. (1981) *Int. J. Cancer* **27**, 693–702.
3. Dvoretzky, I., Shober, R. & Lowy, D. R. (1980) *Virology* **103**, 369–375.
4. Law, M.-F., Lowy, D. R., Dvoretzky, I. & Howley, P. M. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 2727–2731.
5. Lowy, D. R., Dvoretzky, I., Shober, R., Law, M.-F., Engel, L. & Howley, P. M. (1980) *Nature (London)* **287**, 72–74.
6. Lancaster, W. D. (1981) *Virology* **108**, 251–255.
7. Ketner, G. & Kelly, T. J., Jr. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 1102–1106.
8. Botchan, M., Topp, W. & Sambrook, J. (1976) *Cell* **9**, 269–287.
9. Botchan, M., Stringer, J., Mitchison, T. & Sambrook, J. (1980) *Cell* **20**, 143–152.
10. Chia, W. & Rigby, P. W. J. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 6638–6642.
11. Zouzias, D., Prasad, I. & Basilico, C. (1977) *J. Virol.* **24**, 142–150.
12. Revel, M. (1979) *Interferon* **1**, 101–163.
13. Daniel, M. D., Tamulevich, R., Bekesi, J. G., King, N. W., Falk, L. A., Silva, D. & Holland, J. F. (1981) *Int. J. Cancer* **27**, 113–121.
14. Lvovsky, E., Levine, P. H., Fuccillo, D., Ablashi, D. V., Bengall, Z. H., Armstrong, G. R. & Levy, H. B. (1981) *J. Natl. Cancer Inst.* **66**, 1013–1019.
15. Morris, A. G. & Clegg, C. (1978) *Virology* **88**, 400–402.
16. Avery, R. J., Norton, J. D., Jones, J. S., Burke, D. C. & Morris, A. G. (1980) *Nature (London)* **288**, 93–95.
17. Todaro, G. J. & Baron, S. (1965) *Proc. Natl. Acad. Sci. USA* **54**, 752–756.
18. Oxman, M. N. & Black, P. A. (1966) *Proc. Natl. Acad. Sci. USA* **55**, 1133–1140.
19. Oxman, M. N., Baron, S., Black, P. H., Takemoto, K. K., Habel, K. & Rowe, W. P. (1967) *Virology* **32**, 122–127.
20. Lowy, D. R., Rands, E. & Scolnick, E. M. (1978) *J. Virol.* **26**, 291–298.
21. Lancaster, W. D. & Olson, C. (1978) *Virology* **89**, 372–379.
22. Ogburn, C. A., Berg, K. & Paucker, K. (1973) *J. Immunol.* **111**, 1206–1218.
23. Howley, P. M., Law, M.-F., Heilman, C., Engel, L., Alonso, M. D., Lancaster, W. D., Israel, M. A. & Lowy, D. R. (1980) in *Viruses in Naturally Occurring Cancers*, eds. Essex, M., Todaro, G. & zur Hausen, H. (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), Vol. 7, 233–247.
24. Sharp, P. A., Pettersson, U. & Sambrook, J. (1974) *J. Mol. Biol.* **86**, 709–716.
25. Frenkel, N., Locker, H., Batterson, W., Hayward, G. & Roizman, B. (1976) *J. Virol.* **20**, 527–531.
26. Howley, P. M. (1982) *Arch. Pathol. Lab. Med.* **106**, 429–432.
27. Orth, G., Favre, M., Breitburd, F., Croissant, O., Jablonska, S., Obalek, S., Jarzabek-Chorzelska, M. & Rzesa, G. (1980) in *Viruses in Naturally Occurring Cancers*, eds. Essex, M., Todaro, G. & zur Hausen, H. (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), Vol. 7, 259–282.
28. Ostrow, R. S., Bender, M., Niimura, M., Seki, T., Kawashima, M., Pass, F. & Faras, A. J. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 1634–1638.
29. Haglund, S., Lundquist, P.-G., Cantell, K. & Strander, H. (1981) *Arch. Otolaryngol.* **107**, 327–332.
30. Blanchet-Bardon, C., Puissant, A., Lutzner, M., Orth, G., Nutini, M. T. & Guesry, P. (1981) *Lancet* **i**, 274.
31. Sarver, N., Gruss, P., Law, M.-F., Khoury, G. & Howley, P. M. (1981) *Mol. Cell. Biol.* **1**, 486–496.
32. DiMaio, D., Treisman, R. & Maniatis, T. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 4030–4034.
33. Sarver, N., Byrne, J. C. & Howley, P. M. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 7147–7151.