JOURNAL OF VIROLOGY, Sept. 1980 p. 865-875
0022-538X/80/09-0865/11$02.00/0

Vol. 35, No. 3

Growth Dynamics of a Latent Primate Papovavirus

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The stumptailed macaque papovavirus strain HD was discovered in a persistently infected cell line of primate origin designated Vero 76 (K. Bosslet and G. Sauer, J. Virol. **25**:596-607, 1978; W. Waldeck and G. Sauer, Nature [London] **269**:171-173, 1977). In clonal derivatives of Vero 76 cells a minor and variable proportion of cells is engaged in the productive synthesis of the HD virus strain. A combination of immunofluorescence using simian virus 40 polyoma subgroup-specific antiserum and in situ hybridization with HD complementary RNA revealed that only those cells which harbor discernible amounts of HD DNA also contain the subgroup-specific antigen. Treatment with arabinofuranosylcytosine caused irreversible disappearance of the antigen, whereas actinomycin D, in contrast, reversibly inhibited both HD DNA replication and synthesis of the subgroup-specific antigen. The proportion of HD DNA and subgroup-specific antigen-synthesizing cells in Vero 76 clonal lines could be either decreased or increased by the mode of passaging of the cell cultures. When cell cultures were split every 3 to 7 days at a 1:4 ratio, the amount of HD DNA sequences as revealed by DNA-DNA reassociation and by the Southern blotting technique fell below the level of detection after only a few passages. Furthermore, expression of the viral subgroup-specific antigen was no longer discernible. However, viral DNA persists in such latently infected cells, because a change in the splitting protocol to a 2-week passaging rhythm led to reinitiation of both viral DNA replication and expression of the subgroup-specific antigen. The HD DNA is perpetuated in a restricted state in latently infected cells in an episomal, unintegrated form as shown by Southern blot analysis. This finding complies with the fact that HD DNA-free subclones could be derived from persistently infected clonal Vero 76 cells. Such subclones have lost the viral genomes, probably owing to segregation during cell division.

The HD virus strain of stumptailed macaque virus (7) was discovered in a Vero cell line of primate (*Cercopithecus aethiops*) origin (18). HD virus is propagated in this cell line, which was designated Vero 76, without an apparent cytopathic effect. Because cellular growth is not impaired by synthesis of HD virus, it was possible to establish clonal lines of virus-producing Vero 76 cells (1).

Besides Vero 76 cells and clonal derivatives, there are as yet no permissive cells available which would permit lytic growth of HD virus as in the case, for example, of the closely related simian virus 40 (SV40)-CV-1 cell system. The initially successful attempts to grow HD virus at least to a limited extent in RITA cells of *C. aethiops* origin (1) failed recently, probably because of the current higher passage level of the cells. Moreover, attempts to establish a productive infection in originally HD virus-free Vero cells by inoculation of either HD virus or HD DNA were unsuccessful, although Vero cells, upon infection with HD virus, may acquire the ability to grow both in soft agar and under low serum concentration. Such cells fail, however, to produce HD virions (unpublished data). Thus, unlike other papovavirus-host cell systems, one cannot readily distinguish between permissive and nonpermissive cells.

The virus-producing Vero 76 cells display another unusual feature which will be dealt with in this paper. Although the virus occasionally apparently disappears from the Vero 76 cells and also from clonal derivates thereof the reappearance of HD virus synthesis was observed after continued passage of the cultures. We operationally define virus-producing cells as cells exhibiting positive fluorescence with the SV40 polyoma subgroup-specific antigen (13) since there is no infectivity assay for HD virus available.

Depending on the passage conditions, cultures harboring the viral genomes in a latent state may reinitiate abundant synthesis of viral com-

ponents and virions. Therefore, the HD-Vero 76 virus-host cell system represents an excellent model for the analysis of the conditions accounting for viral latency. As will be shown, the abundance of viral genomes within the cell cultures directly depends on the growth kinetics of the cells.

MATERIALS AND METHODS

Cells and virus. Vero cells, CV-1 cells, Vero 76 cells, and Vero 76 clones A and D (1) were grown in Eagle minimal essential medium supplemented with 10% calf serum. Unless otherwise stated, the cultures were passaged at 2-week intervals. For infectivity assays the virus was harvested as described in detail (18). Since plaque tests are not available, infectivity was determined by immunofluorescence assays.

Immunofluorescence. A cross-reactive serum which contains antibodies against the capsid protein(s) of all members of the SV40 polyoma subgroup (13) was used for indirect immunofluorescence assays. For determination of the proportion of positively fluorescing nuclei (4), cover slip cultures were fixed 2 days after seeding, and not less than 10^3 cells were examined in each sample.

In situ hybridization. Two-day-old cover slip cultures were used for in situ hybridization unless otherwise stated in the figure legend. Either the method described by Jones (8) or the method of Stuart and Porter (16) was employed. As radioactive probes, ^{3}H-labeled complementary RNA (19) or nick-translated ^{32}P-labeled HD DNA (11) were used. Autoradiography was performed with Kodak AR10 stripping film.

DNA-DNA reassociation kinetics. DNA reassociation was performed as described (14). Superhelical HD DNA was isolated for nick translation from Vero 76 cells (1). Cellular DNA was isolated according to the method of Gross-Bellard et al. (6).

Blotting. Transfer of DNA sequences to nitrocellulose filters and hybridization with nick-translated ^{32}P-HD DNA were performed as described (15).

Inhibitors. Arabinofuranosylcytosine (ara-C) was used at a final concentration of 20 μg/ml. Actinomycin D (Boehringer) was used at a final concentration of 0.5 μg/ml, and the cells were exposed to the drug in the dark.

RESULTS

HD virus DNA replication and expression of late viral gene functions. Less than 0.1% of Vero 76 cells were shown to react with monospecific antiserum against stumptailed macaque virus, and the same fraction carried the SV40 polyoma subgroup-specific antigen (7, 13), which is a component of the viral capsid. This observation suggests that only a minute minority of the cell population is engaged in synthesis of HD viral components. We have noticed, however, that the proportion of cells harboring the subgroup-specific antigen(s) was subject to considerable fluctuations during passage of Vero 76 cells or clonal derivatives (at split ratios of 1:4).To determine the reasons for these changes, we first attempted to investigate whether the presence of HD DNA in individual cells is always correlated with the presence of the subgroup-specific antigen.

In Fig. 1 it is shown that both synthetic ^{3}H-HD complementary RNA and nick-translated ^{32}P-HD DNA are suitable probes to reveal homologous HD DNA sequences in individual nuclei of Vero 76 Cl A cells after in situ hybridization. Uninfected CV-1 cells and Vero 72 cells (the ancestor line from which Vero 76 cells were derived) (Fig. 1 E and F) did not react with the radioactive probes. A combination of immunofluorescence and autoradiography after in situ hybridization (Fig. 2) revealed that the subgroup-specific antigen was always expressed when the nuclei contained discernible amounts of HD DNA. On the other hand, nuclei displaying the subgroup-specific antigen, yet lacking detectable amounts of HD DNA, were never observed.

The limit of detection of HD genome equivalents per cell by in situ hybridization is not known. We failed to reveal, for example, one integrated SV40 genome equivalent per cell in transformed cell lines. Hence, nuclei containing one (or a few) HD genome equivalent(s) whose size almost equals the size of the SV40 genome would also remain undetected in the autoradiographs, a situation which, as will be elaborated below does occur. Such nuclei are lacking the subgroup-specific antigen, although they harbor HD genomes at a low concentration (see Fig. 10).

Synthesis of the subgroup-specific antigen depends on the physiological state of the cells. Other than during the course of a one-step growth cycle, the synthesis of viral components in a carrier culture probably occurs heterochronously. One might conceive that the physiological state of the cell, i.e., either active growth or the resting state, might exert an influence on the replication of HD virus. To test for this possibility, Vero 76 Cl A cells were seeded sparsely on cover slips, and at various periods of time after seeding, cultures were fixed simultaneously for in situ hybridization with ^{32}P-HD DNA. Positively fluorescing nuclei accumulated consistently up to 2 days after seeding. After that time a plateau value comprising between 5 and 7% of the cells was reached (Fig. 3A). The relative amount HD DNA sequences present within the cultures at various periods of time after seeding was quantitatively determined by in situ hybridization. ^{32}P-HD DNA was hybridized to cover slip cultures of Vero 76 Cl A cells in triplicate, and the specifically bound radioactivity was measured in a liquid scintillation counter as Cerenkov counts. The individually

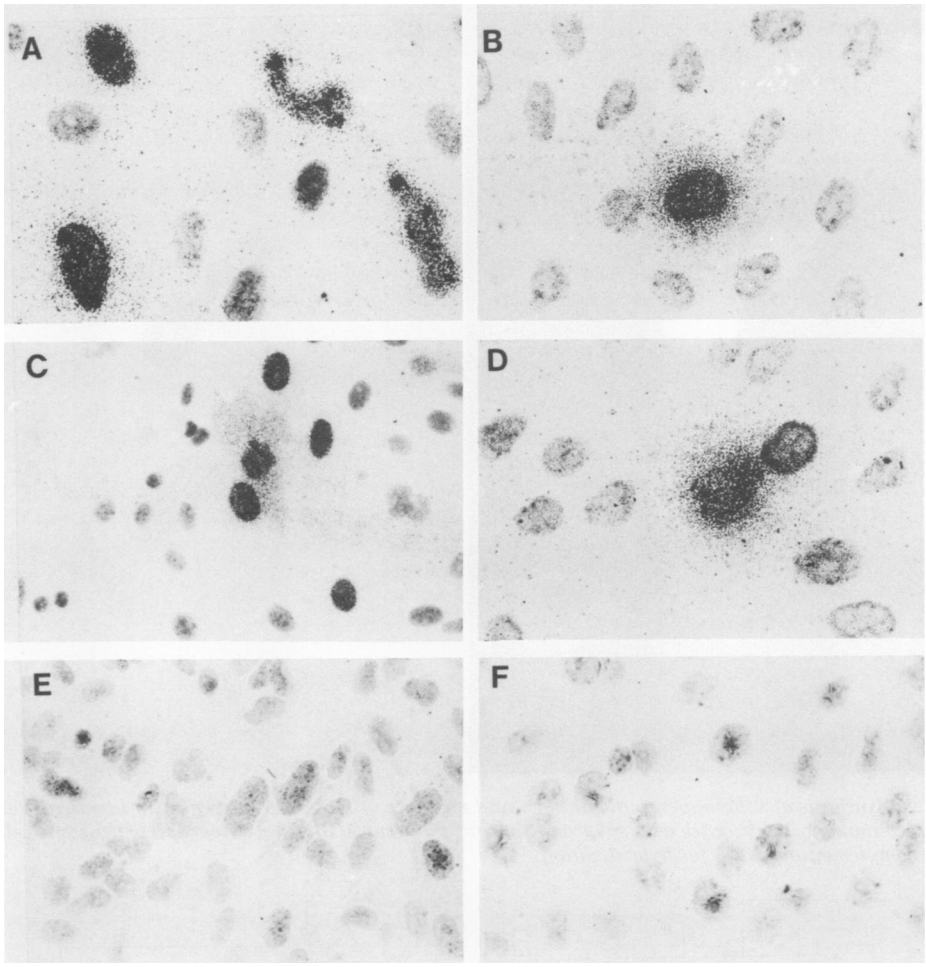

FIG. 1. *Detection of HD DNA in Vero 76 Cl A cells by in situ hybridization. (A) to (D) Vero 76 Cl A cells; (E) CV-1 cells; (F) Vero 72 cells. In (A), (C), and (E), 10^5 cpm of ^{3}H-HD complementary RNA was used for hybridization; in (B), (D), and (F), 2×10^4 cpm of ^{32}P-HD DNA was used for hybridization.*

determined data were in good agreement with each other (Fig. 3B) and reproducibly permitted an accurate assessment of the relative amounts of viral DNA sequences present in the cultures. Although cover slip cultures of uninfected CV-1 cells bound only 150 cpm (at either 1 or 5 days after seeding), we found, on the average, 4,700 cpm bound to Vero 76 Cl A cell cultures between days 3 and 5. After that time the relative amount of HD DNA sequences in the cultures decreased rapidly such that at day 7 after seeding less than 10^3 cpm were measured. This decline may be explained by the release of virions into the medium, since we have noticed that increasing amounts of DNase-resistant HD-DNA sequences were shed into the medium particularly from day 5 after seeding on. They were isolated by centrifugation and revealed by filter hybridization using a ^{32}P-HD DNA probe (data not shown here). During the same period of time the relative proportion of nuclei harboring HD DNA sequences, as determined by evaluation of autoradiographs, did not change (data not shown here) and remained constant at a level between 5 and 7%.

Attempts to synchronize the infectious cycle. Several inhibitory drugs were employed in an attempt to force the heterochronously occurring infectious cycles that take place within the carrier cultures into synchrony. Ara-C, which inhibits DNA synthesis, is known to permit expression of early viral functions such as synthesis of SV40 T-antigen while completely blocking late transcription and, therefore, also virus maturation (2, 10). When ara-C was added to Vero 76 Cl D cultures 3 h after the cells were

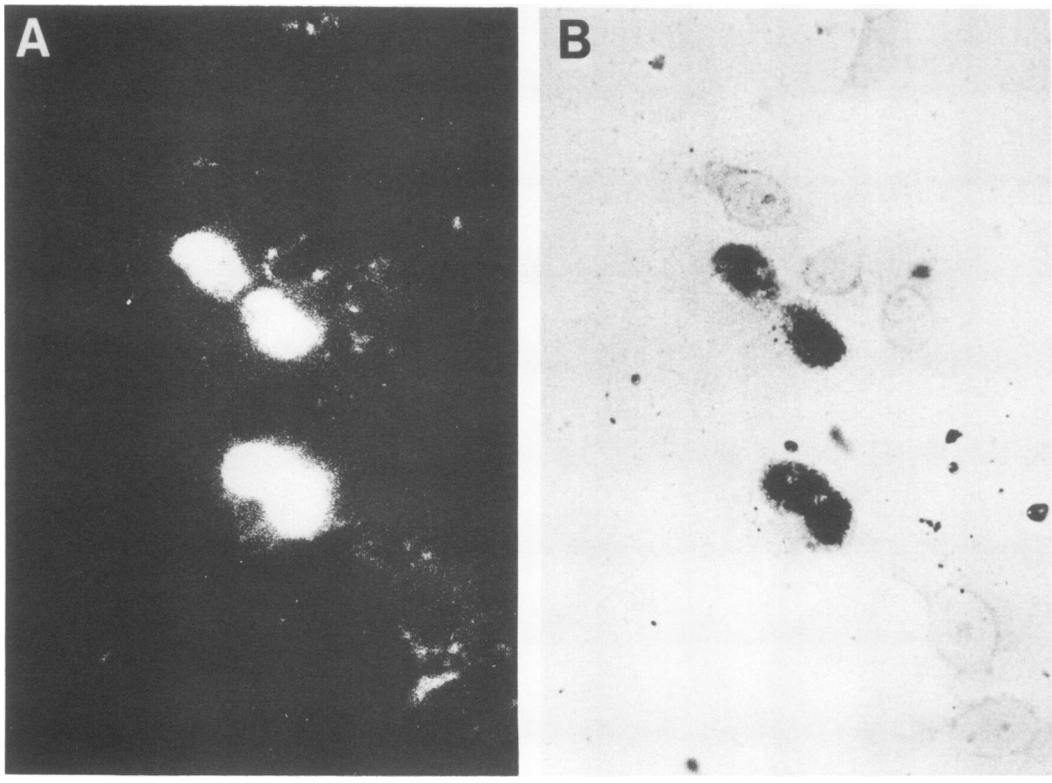

FIG. 2. *Synthesis of subgroup-specific antigen and presence of HD-DNA sequences in identical Vero 76 Cl A cells. (A) Immunofluorescence with subgroup-specific antiserum. (B) Autoradiograph of the same cells using ^{3}H-HD complementary RNA for hybridization.*

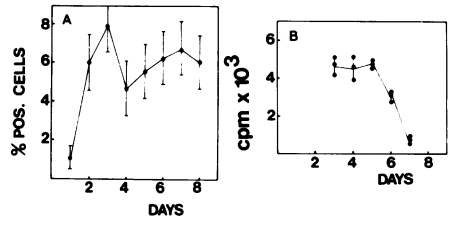

FIG. 3. *The influence of trypsinization on the expression of viral functions. (A) Relative proportion of Vero 76 Cl A cells with subgroup-specific antigen in the cultures at various periods of time after seeding. (B) In situ hybridization with ^{32}P-HD DNA. At various periods of time, cover slip cultures in triplicate were fixed and hybridized with 3.4 × 10^{4} cpm of ^{32}P-HD DNA. After removal of nonspecifically bound DNA, the radioactivity on the cover slips was determined in a liquid scintillation counter separately for each cover slip culture. Similarly treated uninfected CV-1 cover slip cultures bound 150 cpm.*

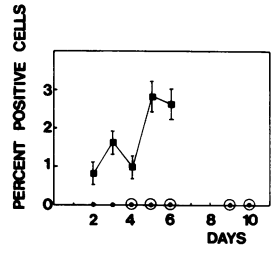

FIG. 4. *The influence of ara-C on the synthesis of the subgroup-specific antigen. Three hours after seeding of Vero 76 Cl D cells, 20 μg of ara-C per ml was added to the cultures, and at various intervals of time the proportion of fluorescent nuclei was determined* (●). *In some cultures the ara-C was removed by washing after an incubation period of 3 days* (○). (■) *Values from untreated Vero 76 Cl D cells.*

seeded, the synthesis of subgroup-specific antigen was completely blocked within 2 days (Fig. 4). The inhibitory effect could not be reversed by removal of the ara-C–containing medium after a 3-day exposure of the cells to the drug (Fig. 4). This result shows that in the case of HD virus, as with other papovaviruses, DNA replication is required for the expression of late viral functions such as synthesis of capsid constituents.

In the case of DNA viruses, actinomycin D precludes in a similar fashion the synthesis of

viral progeny, although it inhibits neither early viral RNA synthesis (3) nor the expression of early functions (10, 12). Actinomycin D was administered to Vero 76 Cl A cover slip cultures at 3 h after seeding, and the cells were exposed for various periods of time to the drug before fixation for immunofluorescence and in situ hybridization. The drug was removed from part of the cultures after 3 days.

In agreement with the data shown in Fig. 3A, approximately 6% of the untreated Vero 76 Cl A cells contained the subgroup-specific antigen (Fig. 5A). On the other hand, exposure to the drug for only 1 day led to complete disappearance of positively fluorescing cells from the cultures (Fig. 5A). Interestingly, not only was the inhibitory effect reversible, but, in fact, temporary exposure of the cells to actinomycin D for a period of 3 days, followed by removal of the drug, lead to an enhancement of the number of subgroup-specific antigen-synthesizing cells in the culture (Fig. 5A). Two days after the removal of the block, the proportion of positively fluorescing nuclei exceeded by several percent the relative number that is present in untreated controls, and at 3 days after the removal of the drug the number of cells with positively fluorescing nuclei was four times higher than in control cultures. After this peak value had been reached a steady decrease was noticed, although when the curve leveled off after another 2 days, the actinomycin-treated cultures still contained two times more cells with subgroup-specific antigen than untreated Vero 76 Cl A cultures.

The relative amount of HD DNA sequences present in actinomycin-treated and untreated Vero 76 Cl A cells was determined after in situ hybridization with ^{32}P-HD DNA according to the procedure described in Fig. 3B. It may be seen from Fig. 5B that actinomycin reversibly inhibited the synthesis of HD DNA. One day after release of the block, concomitantly with the increase in the number of subgroup-specific antigen-containing cells, a rapid increase in the amount of HD DNA sequences was noticed (Fig. 5A). A peak was reached 3 days after the removal of the drug. At this time twice as much labeled probe was bound to actinomycin pulse-treated cultures than to untreated controls. Then the HD-specific hybridization declined to the control level.

To further assess that DNA replication is reversibly blocked by actinomycin D, petri dish cultures of Vero 76 Cl A cells were treated as

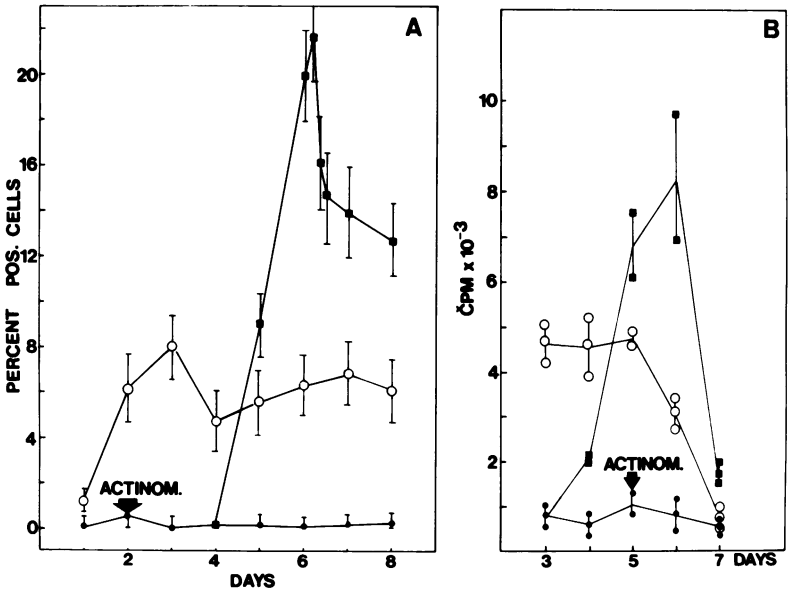

FIG. 5. *The influence of actinomycin D on the synthesis of the subgroup-specific antigen and on the presence of HD DNA in Vero 76 Cl A cells. Vero 76 Cl A cells were treated with 0.5 μg of actinomycin D per ml for a period of 3 days (●). In some cultures the drug was removed by washing after this period of time (■). (○) Untreated Vero 76 Cl A cells. These data are separately shown in Fig. 3. (A) Percentage of cells with subgroup-specific antigen. (B) In situ hybridization with ^{32}P-DNA. At various periods of time, cover slip cultures in triplicate were fixed and hybridized with 3.4 × 10^4 cpm of ^{32}P-HD DNA. After removal of nonspecifically bound DNA, the radioactivity on the cover slips was determined in a liquid scintillation counter separately for each cover slip culture.*

described above with the drug. [^{3}H]thymidine was added to the medium of actinomycin-treated and untreated cultures for a period of six days, and, to actinomycin pulse-treated cultures (which had been exposed to actinomycin for a period of 3 days), the isotope was added 3 days after the removal of the drug. The DNA was isolated and subjected to dye-buoyant density centrifugation to test whether the label was incorporated into superhelical HD DNA. As may be seen in Fig. 6A, there was no DNA replication in the presence of actinomycin D. In contrast, synthesis of superhelical DNA was resumed after removal of the drug (Fig. 6B). For comparison, DNA isolated from untreated Vero 76 Cl A cells is shown in Fig. 6C.

The superhelical DNA in Fig. 6B was identified as HD DNA by virtue of the cleavage pattern after digestion with endo R·*Eco*RI and comparison with the pattern of authentic HD DNA (data not shown here).

Thus, as shown by these data, HD DNA synthesis in the Vero 76 Cl A carrier cell system can be reversibly blocked by using actinomycin D.

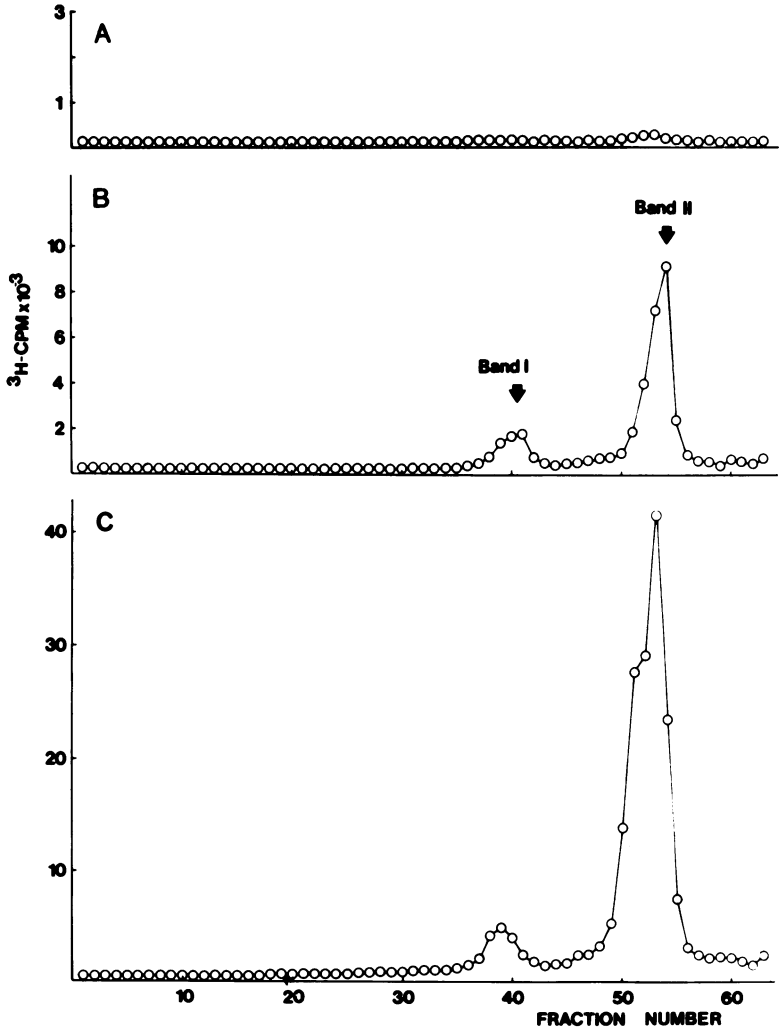

FIG. 6. *Influence of actinomycin D on the synthesis of superhelical HD DNA as shown by dye-buoyant density equilibrium gradients. Vero 76 Cl A cells in Roux flasks were labeled immediately after seeding with 6 μCi of [^{3}H]thymidine per ml for a period of 6 days. The HD-DNA was then selectively extracted and analyzed in cesium chloride-ethidium bromide gradients. (A) DNA selectively extracted from a culture which had been exposed to 0.5 μg of actinomycin per ml throughout the period of 6 days. (B) DNA selectively extracted from actinomycin-inhibited cultures 3 days after removal of the block. (C) DNA selectively extracted from untreated Vero 76 Cl A cells.*

This observation permits the analysis of transcriptional processes occurring either in the presence or absence of HD DNA replication. It is possible now to examine whether the transcription of the HD genome occurs sequentially, as in the case of other papovaviruses, where "early" regions are being transcribed before the onset of viral DNA replication.

The rate of cell passaging determines the yield of virus. Vero 76 Cl A cultures when split at a 1:4 ratio at 3-day intervals rapidly lost subgroup-specific antigen-producing cells (Fig. 7A). After three passages the cultures already contained less than 1% of positively fluorescing cells. Similarly, when the cultures were being passaged at weekly intervals, the proportion of subgroup-specific antigen-containing cells decreased rapidly, and after four passages no positively fluorescing cells were discernible. This is a general feature which was observed in the case of Vero 76 cells and of different HD-producer clones (Fig. 7B and C). The apparent lack of subgroup-specific antigen was maintained over a period of at least 2 months provided the cultures were split at the same pace (Fig. 7C). Despite maintenance of the 1:4 splitting protocol every 3 days over prolonged periods of time, the cell density of the cultures remained unaltered at approximately 1.2×10^7 cells per Roux flask before trypsinization because the cells undergo, on the average, two divisions within 3 days.

Most interestingly, however, if the splitting protocol was changed such that the cultures were divided every 2 weeks at a 1:4 ratio, the proportion of subgroup-specific antigen-synthesizing cells (Fig. 7D) increased. When this protocol was applied, cultures that were originally almost virus free could be converted within three passages to active virus producers. The proportion of subgroup-specific antigen-synthesizing cells increased steadily up to 15%, declined slightly, and increased again to reach almost 30% after approximately 3 months of passaging at 2-week intervals. Thereafter, again, a decrease could be noticed. Apparently the virus production occurs in waves. A period of very active synthesis of virus probably affects the viability of the cells such that, on the average, fewer producer cells survive than during a period of reduced virus synthesis. This may account for the regularly observed fluctuation in the proportion of cells with positively fluorescing nuclei.

To determine the HD-DNA content in actively dividing and in resting cells, DNA-DNA reassociation kinetics were performed (Fig. 8). Cultures that had been passaged at 2-week intervals contained 2×10^4 genome equivalents per cell. When the mode of passaging was changed to a 3-day rhythm, the viral DNA content was reduced quickly to 1% of its original value within four passages (2×10^2 genome equivalents per cell at passage 17), and after another one to two passages, the viral DNA content was reduced well below the level of detection. These observations were substantiated by filter hybridization experiments according to the method of Southern (15) (see photograph in Fig. 8). It is also shown in Fig. 8 that after changing the protocol of passaging of the cultures to a 2-week rhythm, after only two passages at the slower pace there was clearly an accumulation of HD-DNA sequences within the

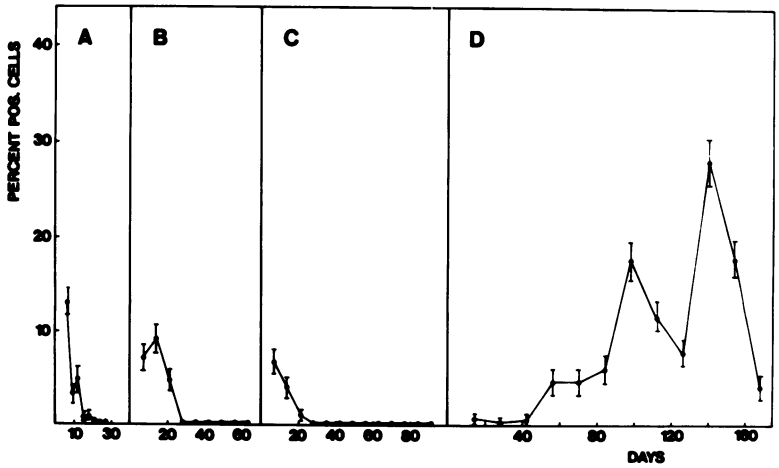

FIG. 7. *Relationship between the mode of passaging of Vero 76 cells and the synthesis of the subgroup-specific antigen. The cultures were split at a 1:4 ratio: (A) Vero 76 Cl A every 3 days; (B) Vero 76 Cl D every 3 days; (C) Vero 76 Cl A every 7 days; and (D) Vero 76 Cl A every 14 days.*

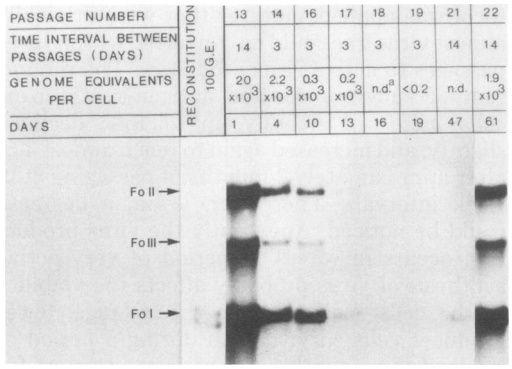

FIG. 8. *Relationship between the mode of passaging of Vero 76 Cl A cells and the synthesis of HD DNA in the cell cultures. DNA samples (1 μg) from each individual passage were electrophoresed in 1.4% agarose gels after digestion with endo R·BamHI and transferred to a nitrocellulose filter after denaturation, and the HD-DNA sequences were revealed in the autoradiograph after hybridization with ^{32}P-HD DNA. The reconstitution experiment in the first lane shows equal amounts of both the smaller and larger HD DNA classes corresponding to 100 genome equivalents (G.E.) per cell. The DNA samples extracted during various passages in the following lanes display a preponderance of the larger HD DNA size class. The number of genome equivalents per cell was determined as described in Table 1 by DNA-DNA reassociation kinetic experiments. nd., Not determined.*

cultures, recognized both in the Southern blots and in DNA-DNA C_0t reassociation kinetics (1.9 $\times 10^3$ genome equivalents per cell in passage 22). These results corroborate the data shown in Fig. 7, which were obtained by evaluation of the proportion of cells with the subgroup-specific antigen.

Furthermore, the data in Fig. 8 permit a conclusion on the physical state of the HD DNA in the clonal Vero 76 cells. All DNA samples were digested with the restriction endonuclease *Bam*HI which does not cleave the HD DNA (unpublished data). The viral DNA exists exclusively in a free state as circular FOI, as FOII, and as linear FOIII DNA. Overexposure of the autoradiograph (not shown here) failed to reveal integrated viral DNA sequences which would migrate differently from those mentioned above. This conclusion will be substantiated in more detail below in Fig. 10.

DISCUSSION

The HD strain of the stumptailed macaque primate papovavirus can assume the properties of a latent virus within permissive cells. Whereas abundant viral DNA replication and virus production take place in contact-inhibited and slowly passaged Vero 76 cells, the expression of "late" viral genes, such as synthesis of the subgroup-specific antigen and viral DNA replication, can either cease or be reduced below the level of detection in cells maintained in actively dividing cultures over prolonged periods of time. Although such originally virus-producing cells apparently seem to have lost the viral genomes because they appear to be phenotypically normal, they revert to virus producers when passaged for prolonged periods of time at a slower pace. As we have shown, viral DNA synthesis and expression of late viral gene functions are resumed in such cell cultures. By a combination of in situ hybridization and immunofluorescence we have also correlated the presence of discernible amounts of viral DNA and the synthesis of the subgroup-specific antigen within individual cells. Such cells accumulate within resting cultures, and they vanish completely from cultures which are kept in an actively dividing state. Nevertheless, HD viral DNA molecules must persist at least within a few cells over a period of several months because, as shown in this work, production of viral components can be reinitiated. An increasing number of cells with expressed viral genes are recruited within such cultures, provided the mode of passaging is altered and the cells are maintained over prolonged periods of time in a contact-inhibited state.

The question arises, therefore, in which physical state the HD DNA is being perpetuated in cells that apparently lack viral genomes. Experiments using reversible inhibitors of viral DNA synthesis such as actinomycin D may facilitate an answer to this question, which has a bearing on the general problem of viral latency. We have shown in accordance with previous data (3) that actinomycin D reversibly inhibits the expression of papovavirus genes and the replication of the viral DNA, but we have also, at the same time, activated and revealed by removal of the drug the presence of a large number of initially inapparent viral genomes within originally phenotypically normal cells (i.e., within cells that did not synthesize the subgroup-specific antigen).

We conclude, therefore, that HD-DNA can persist within permissive Vero 76 cells in different states: either as an actively replicating molecule which expresses late viral functions, such as virion production, or as a DNA molecule whose replication and expression are repressed. Regarding the relationship between viral DNA replication and the physiological state of the host cell, the following considerations have to be taken into account: the viral DNA content in actively dividing cell cultures is no longer discernible by the Southern blot technique, which

would reveal one viral genome within every tenth cell. Since the cell number remained constant within cultures that were split at a 1:4 ratio every 3 days, it follows that on the average the cells underwent two divisions every 3 days. Clearly, one can deduce that within such cultures viral DNA molecules must replicate at a slower rate than cellular DNA because, if not so, this would be detected by the analytical methods available. It appears from our data that replication of cellular DNA governs the replication of HD DNA rather than vice versa, as is the case with most other cytocidal viruses which eventually kill the host cell by employing its metabolic pathways for their own replication and expression. Attempts to detect replicating HD DNA molecules in synchronized Vero 76 Cl A producer cells which had been harvested as mitotic figures have failed owing to the small amounts of viral DNA sequences that were present. On the other hand, Vero 76 Cl A producer cells, when split at a 1:4 ratio, where shown to reach confluency at 5 days after seeding. From then on they discontinued further growth and remained stationary (unpublished data). One might assume, therefore, the existence of a cellular replication factor whose binding affinity to the replicating DNA of the host cell is greater than to the viral DNA. Therefore, in resting cell cultures which have ceased their DNA replication, such a hypothetical factor might be available in quantities sufficiently large to enable viral molecules to replicate at a faster rate than the host DNA.

The questions remain to be answered (i) why it is that not all cells of the virus-producing cultures are productively infected and (ii) why it was never possible to establish a productive infection in originally HD virus-free Vero cells either by using lysates from virus-producing Vero 76 cells (18) or by transfection with viral DNA (unpublished data). So far we have only established transformed clonal lines of Vero cells after infection with HD virus (18), which have never expressed late viral gene functions after more than 3 years of in vitro passaging although they harbor varying amounts of free HD-DNA sequences (unpublished data). It appears, therefore, that we are dealing here with a proportion of "competent" cells whose permissiveness can be altered by extraneous influences, such as the mode of passaging of the cell cultures. As demonstrated here, one can never observe either an apparent productive or a latent infection of all cells within the HD virus-producing culture. Within the cultures some cells are always present which prove to be refractory against infection with the HD genome. This is substantiated by cloning experiments which were carried out with HD-producing cloned Vero 76 cells during a period of active cell growth (Table 1). From seven subclones that were established from a Vero 76 Cl A line, there were three which proved to be devoid of HD DNA. Although these clonal lines had been maintained at a slow split ratio (1:4 every 2 weeks for five passages), we could not demonstrate any HD virus-specific DNA. Segregation of viral genomes can occur, therefore, in the absence of the synthesis of infectious virion production. The HD DNA-containing clones (Table 1), however, did not display any subgroup-specific antigen when reacted with antisera. Thus, there are cells in the cultures which harbor viral DNA in a restricted state such that late viral genes are not expressed.

To analyze carefully the physical state of the HD DNA in the subclones enlisted in Table 1, we first determined the level of sensitivity at which HD genomes can be detected by the Southern technique. The presence of one (linear FOIII)HD genome was detected in every 10th and possibly even in every 20th cell (Fig. 9). By applying the same sensitivity of detection, the total DNA from the Vero 76 Cl A from which the subclones were derived and the DNA from

TABLE 1. *Distribution of HD DNA in subclonal lines derived from a Vero 76 Cl A culture*

Source of DNA[a] (designation of subclones)	DNA concn (mg/ml of reassociation mixture)	$\Delta s \times 10^{-3}$[b]	Genome equivalents per cell[c]
Cl1	2	0.05	0.1
Cl2	2	0.13	0.3
Cl3	2	1.75	4.1
	0.2	0.23	5.5
Cl4	2	ND[d]	ND
Cl5	2	0.47	1.1
	0.2	0.15	3.6
Cl6	2	ND	ND
Cl7	2	ND	ND

[a] Clonal lines designated Cl1 to Cl7 were established from Vero 76 Cl A colonies derived originally from soft agar (1) by plating the 76 Cl A cells and nonselectively isolating the resulting colonies. Once the clones were grown to the size of a small Falcon flask, they were passaged five times according to the protocol described in Fig. 7D to permit accumulation of HD DNA in the cultures. Then DNA was isolated and assayed in DNA-DNA reassociation kinetic experiments for quantitative determination of the content of HD DNA sequences.

[b] Δs, Increase of the slope of the regression curve after the addition of the test DNA.

[c] As determined from reconstruction experiments where 2 ng of HD DNA per ml, corresponding to 1.14 genome equivalents per cell, revealed a Δs of 0.37×10^{-3} (min^{-1}).

[d] ND, Not detectable.

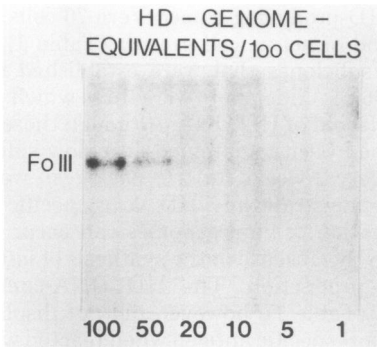

FIG. 9. *Sensitivity of detection of HD DNA sequences by the Southern technique. Vero cell DNA (5 μg per track) was mixed with 4.4, 2.2, 0.88, 0.44, 0.22, and 0.044 pg of HD DNA, corresponding to 100, 50, 20, 10, 5, and 1 HD genome equivalents per 100 cells, respectively. After digestion with the restriction endonuclease TaqI, which cleaves HD DNA only once, the DNA mixture was electrophoresed into a 1.4% agarose gel (16 h, 60 V), transferred after denaturation to a nitrocellulose filter by the Southern procedure, and hybridized with nick-translated ^{32}P-HD DNA. The time of exposure of the X-ray film was 2 weeks.*

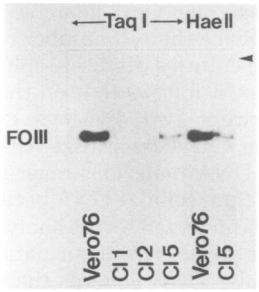

FIG. 10. *Detection of free HD DNA in clonal and subclonal lines of Vero 76 cells. Total DNA was isolated from Vero 76 Cl A cells which had undergone a rapid splitting protocol (four times, at a 3-day cycle). The total DNA from the subclonal derivatives designated Cl 1, 2, and 5 had been isolated from slowly passaged cultures as described in the legend to Table 1. The DNA was digested either with TaqI or with HaeII, which both cleave HD DNA only once. A 5-μg portion of each sample was electrophoresed into a 1.4% agarose gel at 60 V for 16 h and further treated as described in the legend to Fig. 9. The arrow indicates the top of the gel.*

various subclones were examined. Two different single-cut restriction endonucleases (*Taq*I and *Hae*II) were employed for digestion of the DNA (Fig. 10). HD DNA sequences were detected only in the Vero 76 Cl A cells and in subclone 5 (and subclone 3, data not shown), whereas subclones 1 and 2 did not contain detectable HD DNA sequences. This result is in agreement with the data in Table 1. As shown in Fig. 10, the HD DNA was present exclusively as full-length linear FOIII. No HD DNA sequences were found to migrate along with high-molecular-weight DNA. Thus, the cleavage products obtained either with the no-cut endonuclease *Bam*HI (Fig. 8) or with the two different single-cut endonucleases *Taq*I and *Hae*II show that HD DNA is present in the persistently infected lines only in free circular form. When enriched from the Hirt supernatant fraction, the free HD genomes present in subclone 3 could be analyzed in greater detail. The double digest with *Hin*dIII plus *Eco*RI shown in Fig. 11 revealed the presence of all five authentic fragments that are represented in the wild-type HD DNA.

The general validity of this system for the study of viral latency is obvious. We have elaborated parameters which influence the expression and the control of viral genomes in primate cells. Very similar observations were reported recently in the case of the human BK papovavirus (17), cytomegalovirus (9), and Epstein-Barr virus producer lines (5). In all systems,

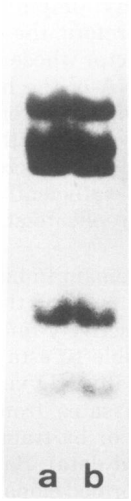

FIG. 11. *Comparison of the restriction enzyme cleavage patterns of wild-type HD DNA from Vero 76 Cl A with HD DNA from subclone 3. Total DNA from Vero 76 Cl A cells and DNA selectively isolated from the sodium chloride-sodium dodecyl sulfate supernatant from subclone 3 cells (to enrich the free circular HD DNA) were subjected to a double digestion with HindIII plus EcoRI and analyzed as described in the legend to Fig. 9. The time of exposure was 3 days.*

virus production is repressed in the majority of cells, and the maintenance of the viral genomes within the persistently infected cultures is not dependent on virus-producing cells. Spontaneous activation of virus production in a minority of the cell population has been observed in

all cases reported. We have shown that this phenomenon is correlated with the physiological state of the persistently infected cultures in which the viral genomes persist in the form of free circular and unintegrated molecules (Fig. 8). The persistence as an episomal structure can be correlated with a restricted gene expression where late viral genes are apparently repressed by host cell factors. Furthermore, being present as a free molecule at low copy number, the HD genome can get lost owing to segregation from persistently infected cells. We are currently studying the regulation of HD DNA transcription under various physiological conditions to understand the factors which influence the expression of this episomal papovavirus genome.

ACKNOWLEDGMENTS

We thank Beate Klein for technical help, Walter Keller for critical reading of the manuscript, Harvey Ozer for kindly providing the subgroup-specific antiserum, and Eberhard Amtmann for help with the nick translation.

This work was supported by the Stiftung Volkswagenwerk.

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