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Polyoma Virus Replication in BHK-21 Cells: Semi-Permissiveness Is Due to Cellular Heterogeneity[1]

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Normal and transformed BHK-21 cells are heterogeneous in their capacity to support replication of polyoma virus. *In situ* hybridization has been used to demonstrate rare cells in which large numbers of polyoma genomes accumulate following infection by wild-type virus or induction of polyoma *A* gene mutants. Different transformed cell lines vary in the frequency of occurrence of these rare cells. Such cells may be an important determinant of the frequency of transformation of BHK-21 cells by polyoma virus.

Cell transformation by polyoma virus is studied almost exclusively with Syrian hamster or rat cells. Cell lines of both species are semipermissive for replication of virus or viral DNA. (Borgaux 1964; Fogel and Sachs, 1969; Folk, 1973; Prasad *et al.*, 1976; Near *et al.*, 1977). Virus replication kills BHK-21 hamster cells. As a result, transformed cells containing only defective genomes eventually predominate (Watkins and Dulbecco, 1967; Summers and Vogt, 1970; Folk, 1973; Hutchinson *et al.*, 1979). However, BHK-21 cells transformed by conditionally defective *A* gene mutants of polyoma virus do not undergo such a selection if they are kept at high temperature. They retain intact viral genomes which will begin replicating when the temperature is lowered to a level where the *A* gene product is functional (Folk, 1973; Folk and Bancuk, 1976). With transformed rat F2408 cells, a similar phenomenon occurs (Zouzias *et al.*, 1977). In these cells, after a shift to low temperature, integrated viral genomes are excised and replicate vegetatively in rare cells (Basilico *et al.*, 1979; Gattoni *et al.*, 1980). Replication of virus in isolated foci of infected BHK-21 cells was suggested, but never conclusively shown by early experiments in which accumulation of virion antigen was followed by immunofluorescence (Fraser *et al.*, 1966). In this report we confirm that a normal BHK-21 cell line contains rare cells which serve as foci for vegetative DNA replication, and we demonstrate that the same pattern is observed upon induction of viral DNA replication in *A* gene-transformed cells. Furthermore, we show that various transformed cell lines differ with respect to the frequency that such foci occur.

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MATERIALS AND METHODS

Cells and viruses. The sources of the cell lines and viruses, and the media and procedures used for culturing cells have been described previously (Folk, 1973; Folk and Bancuk, 1976; Bendig *et al.*, 1979).

Infection or induction of cells. Secondary whole mouse embryo cells (WME) were seeded in 60-mm plates with 1-cm^2 coverslips, and after incubation overnight they were infected with large plaque polyoma virus at multiplicities of 4 and 40 PFU/cell. After 2 days at 37° coverslips were processed as described below.

Untransformed or transformed BHK-21 cell lines were added at low densities (1×10^5 cells) to 60-mm plates containing 1-cm^2 coverslips. After incubation overnight at 37° (untransformed cells) or 39.5° (transformed cells) the plates were shifted to 33° (induction) or infected with polyoma

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virus at a multiplicity of 40 PFU/cell. Those that were infected were either kept at 39.5° (preventing replication of ts-a virus) or shifted to 33°. At the stated time, coverslips were removed and processed for *in situ* hybridization as described below. Transformed cells maintained at 39.5°, and uninfected BHK-21 cells maintained at 37° exhibited no labeled nuclei (less than one per 150,000 cells).

In Experiment 1 of Table 1, samples of induced cells were also seeded in soft agar over monolayers of WME cells to measure infectious centers at 33° as described previously (Folk 1973).

In situ hybridization and autoradiography. Coverslips containing cells were removed from culture dishes, rinsed with phosphate-buffered saline (PBSa), and processed essentially as described by Langelier *et al.* (1975). Cells were fixed in acetic acid–ethanol (1:3) for 15 min at room temperature, then rinsed in PBSa, and dehydrated through 70% ethanol and two 95% ethanol rinses. They were air dried. To denature DNAs, the coverslips were placed in boiling 0.1 × SSC (SSC: 0.15 *M* NaCl, 0.015 *M* sodium citrate, pH 7.0) for 5 min, and then immersed in ice-cold 1 × SSC. Then they were dehydrated and air dried as described above. For *in situ* hybridization, the coverslips were inverted on microscope slides containing 25 μl of polyoma [^{3}H] cRNA (8.3×10^7 dpm/μg; 0.5 μg/ml in 5× SSC with 100 μg/ml *Escherichia coli* tRNA. The cRNA was synthesized by *E. coli* RNA polymerase transcription of polyoma form 1 DNA essentially as described by Kamen *et al.* (1974). The edges of the coverslips were sealed with Permount, and the slides were incubated in a moist chamber at 65° for 18 hr. The coverslips were pried off with a razor blade, washed several times with 2× SSC, and then treated with boiled RNase A (20 μg/ml) in 2× SSC for 60 min at 37°. They were then repeatedly washed with 2× SSC at room temperature, dehydrated by three ethanol washes at 0°, and air dried. They were dipped in Kodak NTB-3 emulsion diluted 1:2 with water, and exposed for 8–10 days. After development of the emulsion, they were stained with Giemsa and examined at 100–400× power. In all cases, at least 10,000 cells were examined, and often greater than 200,000 were examined. Only cells with nuclear grain densities substantially greater than that of the surrounding area were scored as positive.

RESULTS

After infection of BHK-21 cells with polyoma virus, the production of progeny virus cannot be detected over the background of input virus, and it is difficult to detect significant incorporation of label into progeny DNA (Borgaux, 1964; Basilico *et al.*, 1970; Folk, 1973). Immunofluorescent tracing of virion capsid antigen was used in early experiments to demonstrate that rare cells accumulate large amounts of capsid protein, and it was suggested that these might be foci for vegetative replication (Fraser and Gharpure, 1963; Fraser *et al.*, 1966). The use of inducible *A* gene virus-transformed cell lines to study viral DNA replication circumvents the problem of detecting the formation of new virus, for before virus synthesis is induced there is no background of free virus (Folk, 1973). However, when such induced cells are examined for capsid antigen synthesis by immunofluorescent straining, they are uniformly negative (Folk, 1973). Thus it is unclear if the synthesis of viral DNA in an induced population of cells occurs uniformly throughout all the cells, or if foci of cells support vegetative growth of the virus.

To directly look for cells synthesizing viral DNA we have employed *in situ* hybridization as an assay (Gall and Pardue, 1971; Langelier *et al.*, 1975). This technique permits the detection of cells containing large quantities of viral DNA, but under normal circumstances it is not sufficiently sensitive to detect the few viral genomes present in transformed cells prior to a temperature shift. When BHK-21 cells transformed by *A* gene mutants are shifted to 33°, autonomous viral DNA replication is initiated and 30–100 genomes per cell accumulate over a period of 72 hr (Folk, 1978; Folk and Bancuk, 1976). Such a measure is an average of the amount of DNA replication throughout the population. If only rare cells support DNA replication, they might

TABLE 1

PERCENTAGE NUCLEI LABELED AT 33°

	Experiment 1[a]		Experiment 2[b]		Experiment 3[c]	
			−Polyoma	Large plaque + polyoma	−Polyoma	Large plaque + polyoma
WME	0					
WME + 4 PFU/cell	16					
WME + 40 PFU/cell	19					
BHK-21	ND[d]		<0.0006	0.003	<0.007	0.008
2a2 cl d No. 7	0.006	5[e]	ND	ND	ND	0.006
2a2 cl d No. 11	0.005	1	0.011	0.016	0.003	0.028
25 17e No. 3	0.005	1	0.015	0.08	0.013	0.05
25 17e No. 6	0.07	1	0.016	0.017	0.03	0.1
tsa-6	<0.01	0.3	0.013	10	ND	5
tsa-7	0.016	2	ND	ND	0.013	0.16

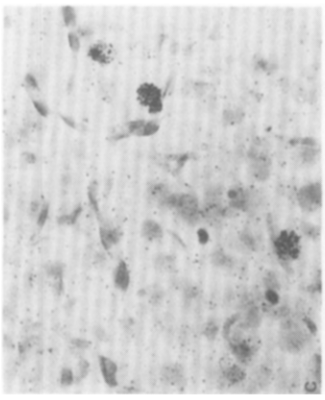
[a] Incubation for 70 hr. (Only the WME cells were infected).
[b] Incubation for 75 hr.
[c] Incubation for 73 hr.
[d] Not done.
[e] Percentage infectious centers.

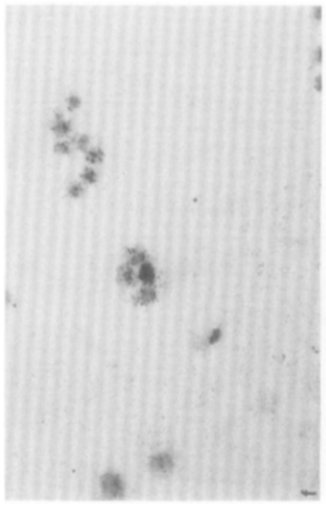
contain the bulk of the viral genomes and be detected by *in situ* hybridation.

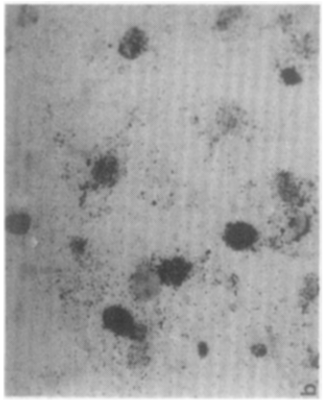
The confidence with which cells containing polyoma genomes can be detected was established by measuring the fraction of polyoma-infected WME (permissive whole mouse embryo) cells which exhibited significant hybridization with polyoma [^{3}H]-cRNA. After infection with 40 PFU/cell, when most susceptible cells are supporting vegetative replication, approximately 20% of the cells are scored as positive by *in situ* hybridization (Table 1; Figs. 1a, b). A rough estimate of the number of polyoma genomes in these cells can be obtained from the yield (1–5 μg) of polyoma DNA obtained by Hirt extraction (Hirt, 1967) of such an infected population divided by the number of cells (7×10^6). The average amount of viral DNA for each positive cell is likely to be near 10^5 viral genomes.

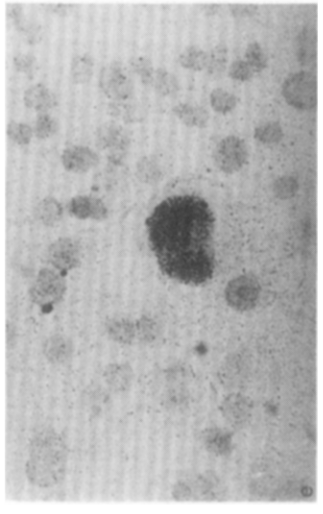
Normal BHK-21 cells as well as six cloned polyoma *A* gene mutant transformed cell lines were studied. Two subclones of each of two independently transformed lines previously described (2a2cl-d and 25-17E; Folk and Bancuk, 1976) and two other independently ts-a-transformed lines (tsa-6 and tsa-7) have been maintained in continuous culture at 39.5° for several years, and are all inducible for virus replication at 33°. Between 0.3 and 5% of the cells of each cell line form infectious centers when suspended in soft agar for 20 days at 33° over a monolayer of whole mouse embryo cells (Table 1; Folk, 1973). This figure reflects the capacity of such cells to produce one or more infectious virus particles over 2 to 3 weeks, but does not reveal the extent of DNA replication that occurs soon after a temperature shift.

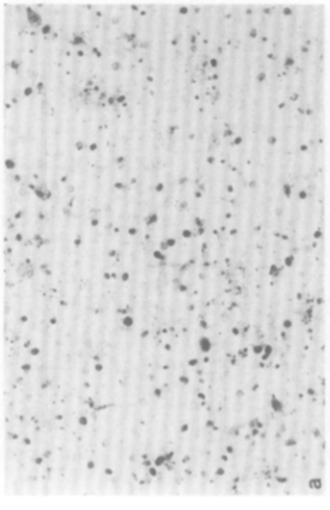
The *in situ* hybridization assay with polyoma [^{3}H]cRNA permits early and direct detection of cells supporting vegetative replication of viral DNA molecules. Upon infection of BHK-21 cells with polyoma virus, polyoma sequences are detected in rare cells (Table 1). Similarly, at 3 days after a temperature shift, 0.003 to 0.07% of the transformed cells contain sufficient viral DNA to be scored as positive (Table 1, Figs. 1c, d, e). These cells may contain amounts of hybridizable polyoma DNA comparable to that present in infected whole mouse embryo cells (compare Fig. 1b with Figs. 1c, d, e). In that case, much (if not most) of the 20–100 viral genomes per cell that is detected by infectivity measurements and

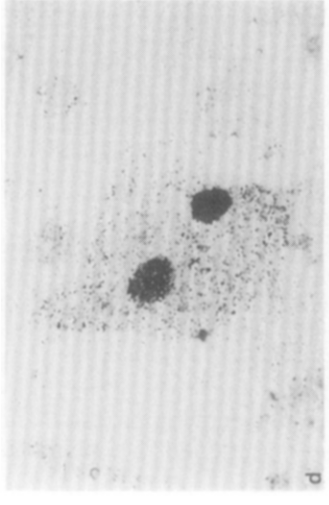












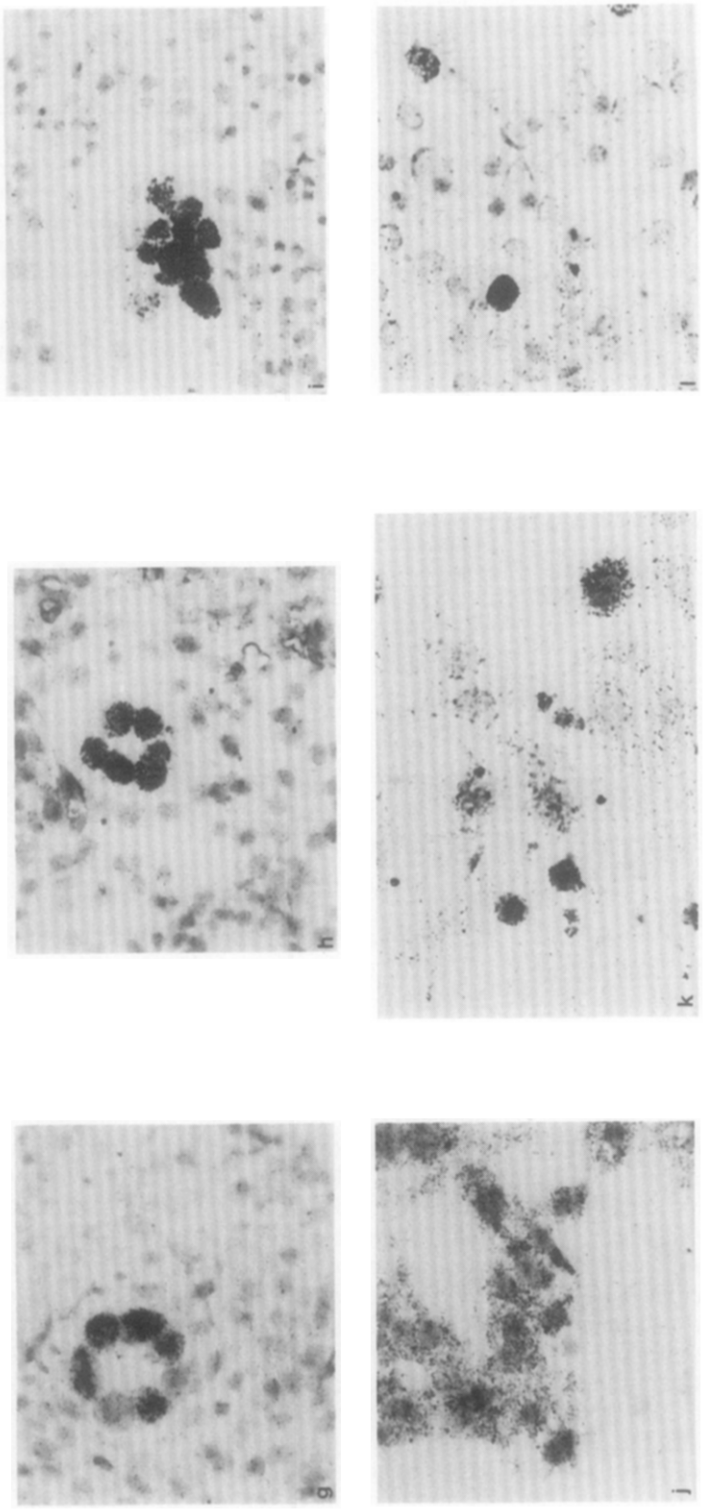


FIG. 1. Cells containing polyoma genomes detected by *in situ* hybridization. (a) Whole mouse embryo cells, 70 hr after infection with large plaque polyoma virus (100×). (b) Whole mouse embryo cells, 70 hr after infection with large plaque polyoma virus (400×). (c) 2a2 cl d No. 7, 70 hr after induction at 33° (400×). A rare field with three labeled cells. (d) 25 17e No. 6, 70 hr after induction at 33° (400×). Two nearby cells with different grain densities. (e) tsa-7, 70 hr after induction at 33° (400×). A labeled multinucleated cell. (f) 2a2 cl d No. 7, 24 hr after infection (and induction) at 33° with large plaque polyoma virus (400×). (g) 2a2 cl No. 7, 120 hr after infection (and induction) at 33° with large plaque polyoma virus (400×). (h) 25 17E No. 3, 96 hr after infection (and induction) at 33° with large plaque polyoma virus (400×). (i) 2a2 cl d No. 11, 120 hr after infection (and induction) at 33° with large plaque polyoma virus (400×). (j) tsa-6, 24 hr after infection (and induction) at 33° with large plaque polyoma virus (400×). Extensive cytoplasmic labeling at early times after infection prevents accurate definition of labeled nuclei. We do not understand the basis of this cytoplasmic labeling. (k) tsa-6 73 hr after infection (and induction) at 33° with large plaque polyoma virus (400×). Cytoplasmic labeling has diminished. (l) tsa-6 96 hr after infection (and induction) at 33° with large plaque polyoma virus (400×). Cytoplasmic labeling has disappeared. Extensive labeling is apparent, as several labeled cells in a field is common.

TABLE 2

PERCENTAGE NUCLEI LABELED AT 33° AND 39.5°

	Percentage nuclei labeled at 33°					
	+Wild-type polyoma virus				+ts-a virus	
	24 hr	75 hr	96 hr	120 hr[a]	72 hr	96 hr
BHK-21	<0.003	<0.0005	0.002	0.003	<0.0004	0.004
2a2 cl d No. 7	0.02	0.006	0.01	0.008	0.002	0.01
2a2 cl d No. 11	0.006	0.002	0.031	0.012	0.0008	0.01
25 17 E No. 3	0.003	0.043	0.16	0.10	0.004	0.038
25 17 E No. 6	<0.001	<0.001	0.005	0.01	0.0005	0.005
Tsa-6	0.09	1.2	2.9	0.02	0.02	0.2
Tsa-7	<0.001	0.006	0.04	0.1	0.0003	0.006
	Percentage nuclei labeled at 39.5°					
	+Wild-type polyoma virus				+ts-a virus	
	12 hr	24 hr	48 hr	75 hr[a]	48 hr	72 hr
BHK-21	<0.006	<0.002	0.003	0.004	<0.0008	<0.0003
2a2 cl d No. 7	<0.004	<0.002	<0.0003	0.0003	0.004	0.0004
2a2 cl d No. 11	<0.004	<0.001	<0.0002	<0.0001	0.0003	<0.0002
25 17 E No. 3	<0.003	0.001	0.07	0.03	<0.0004	<0.0002
25 17 E No. 6	<0.004	<0.02	0.0006	0.0005	<0.0003	<0.0003
Tsa-6	0.005	0.025	0.35	0.09	<0.0008	<0.0003
Tsa-7	<0.005	<0.002	0.0005	0.0002	<0.0004	<0.0003

[a] Cells were overgrown and detaching from the plate by this time.

by DNA reassociation kinetics as an average throughout the transformed cell population may be contained in a small number of induced, positive cells. It is apparent that vegetative replication of polyoma DNA in these cell lines occurs predominantly in rare cells.

To gain some insight into whether this heterogeneity of the induction of viral DNA replication in the transformed cell lines is due to the availability of free viral DNA templates, or to a variable ability of BHK-21 cells to support polyoma DNA replication, each of the transformed cell lines was superinfected with wild-type polyoma virus and examined for viral DNA replication by *in situ* hybridization (Table 1). After infection, in five transformed BHK-21 cell lines, the fraction of cells with labeled nuclei was the same or somewhat greater than that observed after induction alone at 33°. One cell line (tsa-6) exhibited many more positive cells after infection than after induction.

To confirm that vegetative polyoma DNA replication, and not simply accumulation of viral DNA from the adsorbed virus is being measured in these cells, we repeated the experiment described above with cells infected by wild-type or ts-a virus and placed the cells at 33° or 39.5° (at the higher temperature autonomous ts-a viral DNA replication is restricted; Di Mayorca *et al.*, 1969; Eckhart, 1969; Fried, 1970). As seen in Table 2, silver grains are detected in the nuclei of cells infected by wild-type virus at both temperatures, but only in the ts-a-infected cells at 33°, confirming that vegetative DNA replication is being measured. In other experiments we established that there is no apparent difference in the absorption of ts-a and wild-type virus to these cells.

Thus, in five of the transformed cell lines, rare cells are capable of supporting large amounts of viral DNA replication when templates are provided by induction or by superinfection. However, the majority of the cells do not support sufficient vegetative viral DNA replication to be detected by this assay, regardless of how viral templates are presented. One cell line (tsa-6) contains a substantially larger number of cells capable of supporting vegetative DNA replication upon infection by virus than is observed upon induction by a temperature shift to 33°. This indicates that many of these cells have the capacity to replicate viral DNA, but without superinfection, viral templates are limiting. Such a limitation may be imposed by the state of the viral DNA in these cells. Although it is not rigorously proven, it is likely that at the nonpermissive temperature, the majority of the viral sequences in these lines are covalently joined to cell DNA (Folk and Bancuk, 1976; V. Rothwell, J. Truden, and W. R. Folk, unpublished observations).

DISCUSSION

The BHK-21 cell line has been widely used to study viral and chemical transformation. Because of the relatively high efficiency with which it can be transformed and the obvious advantages of studying cellular responses to viral infections with homogeneous cell populations, it replaced hamster embryo cell cultures as a model system for studying polyoma virus transformation. Our results demonstrate that even these cloned cell lines are not homogeneous, and that rare cells differ in their response to polyoma virus.

It is conceivable that this heterogeneity determines the frequency of transformation by polyoma virus. At multiplicities of approximately 50 PFU/cell the frequency of transformation is between 0.01 and 0.1% (Stoker and Abel, 1962; Folk, unpublished observation). This is similar to the observed frequency of cells supporting large amounts of virus replication. If an early event that is required for transformation by polyoma virus is amplification of the number of DNA copies by replication, only those cells that permit this may be transformed. This would explain the requirement in transformation for functional *A* gene product soon after infection by virus (Fried, 1965; DiMayorca et al., 1969; Eckhart, 1969). As has recently been shown, transfection of hamster cells with polyoma DNA bypasses the need for functional *A* gene product (Moore *et al.*, 1980). Presumably this occurs because the cells receive a much greater dose of DNA when it is presented in this fashion, and it need not be amplified by replication.

Once transformation is established, functional *A* gene product acts to select against cell survival, as continuous viral DNA replication may kill permissive cells. At present we do not know why BHK-21 cell lines segregate susceptible cells or why the tsa-6 cell line appears to be more permissive than most others. In previous studies of the clonal distribution of BHK-21 cells supporting virion antigen synthesis, Fraser *et al.* (1966) postulated that mutants arose spontaneously which have increased sensitivity to vegetative growth of the virus. Our results support this proposal, although the change need not be heritable. In several instances, cells supporting viral DNA replication were adjacent to one another, as if they were clonally derived (Fig. 1, panels d–i).

As of yet, the underlying basis for the difference in permissivity to polyoma virus of different cells is unknown. Some recent observations on the ability of polyoma virus to replicate in embryonal carcinoma cells indicate that rearrangements of the viral genome can bypass cellular blocks to replication (Vasseur *et al.*, 1980, Katinka *et al.*, 1980). However, the viruses that replicate in BHK-21 cells do not have large genomic alterations (Anderson and Folk, 1977). Furthermore, we have searched among the induced viruses for mutants which are adapted to grow on hamster cells, but have not found any. The capacity of the virus to grow in BHK-21 cells seems to be due to cellular variability, and not to viral mutation.

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