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Induction of Type-C RNA Virus by Cycloheximide: Increased Expression of Virus-Specific RNA

(viral RNA transcription/protein synthesis inhibition/tumor virus/cancer)

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ABSTRACT **Mouse cells contain the genetic information for multiple endogenous type-C RNA viruses. The mechanisms by which the cell controls expression of these naturally integrated viruses are not yet known. Recently, chemicals that inhibit protein synthesis have been shown to induce a specific type-C virus at high frequency from BALB/c mouse embryo cells. In the present studies, virus activation in response to a representative translational inhibitor, cycloheximide, is demonstrated to be transient, with virus release primarily occurring within the first 12–24 hr following drug exposure. Analysis of virus-specific RNA in cells by molecular hybridization revealed an absolute increase in viral RNA concentration in cycloheximide-treated cells. This was blocked by simultaneous exposure of the cells to actinomycin D. Further, inhibition of RNA synthesis during but not subsequent to cycloheximide exposure prevented virus activation. These findings show that virus induction by cycloheximide requires *de novo* RNA synthesis during but not after drug exposure and suggest that the required RNA species may be that of the virus itself. The present results are consistent with the hypothesis that translational inhibitors prevent synthesis of a labile protein whose normal action is to inhibit viral RNA transcription or to cause degradation of viral RNA.**

Mouse cells contain the genetic information for multiple (1–6) biologically distinguishable (4, 7) type-C RNA viruses. One of these endogenous viruses, induced from virus-negative cells in culture, has recently been shown to induce lymphatic leukemia *in vivo* (8). Thus, study of the mechanisms involved in cellular regulation of naturally integrated type-C viral genes may be of importance in the development of methods to prevent expression of their malignant potential. In a recent report, several chemicals that inhibit different steps in protein synthesis in eukaryotes have been demonstrated to induce type-C virus at high frequency from virus-negative mouse cells (9). In the present studies, the mechanism of action of one of these chemicals, cycloheximide, has been investigated. We report that cycloheximide causes an increase in the cellular concentration of type-C viral RNA and that this effect is dependent upon *de novo* RNA synthesis. Thus, cycloheximide affects the pathways involved in type-C virus release directly or indirectly at the level of viral RNA.

METHODS

Cultures were grown in Dulbecco's modification of Eagle's medium supplemented with 10% calf serum (Colorado Serum Co., Denver, Colo.). Cells included the continuous mouse lines, BALB/3T3 and BALB/3T12-3 (10), and the normal rat kidney (NRK) line (11). A clonal BALB/3T3 line, K-BALB, nonproductively transformed by the Kirsten strain of murine sarcoma virus (KiMSV), has also been reported (12). Viruses included BALB:virus-2 (7), a xenotropic endogenous mouse type-C virus, and the Kirsten strain of mouse leukemia virus (KiMuLV) (12).

Virus Assays. Virus induction from BALB/c mouse cells was analyzed by a biologic assay utilizing cells nonproductively transformed by murine sarcoma virus. Chemical activation of these cells results in rescue of the sarcoma virus genome in the envelope of the cell's endogenous helper viruses (7, 13, 14). While assays for helper leukemia viruses invariably require multiple cycles of replication, transformation by murine sarcoma virus requires only a single cycle of infection (15). Thus, this technique provides one of the most sensitive and quantitative biologic techniques available for studying induction of type-C viruses. (9, 13, 14, 16). Activated sarcoma virus was measured in tissue culture fluids as previously described (16). An infectious center assay to quantitate the fraction of sarcoma virus-activated cells has also been reported (9, 16).

Cell Colony-Forming Efficiency. Cells were detached from the petri dish with 0.1% trypsin in phosphate-buffered saline and transferred at 10-fold dilutions to new petri dishes. Cell counts were performed 24 hr later by hemocytometer. At 12 days, cultures were fixed with formalin and stained with 1% hematoxylin. Colonies consisting of more than 20 cells were scored with the aid of a dissecting microscope. The colony-forming efficiency was determined as the number of colonies measured at 12 days per total cells present 24 hr following transfer.

Chemicals. Cycloheximide and actinomycin D were generously provided by the Drug Development Branch, National Cancer Institute. Mitomycin C and cordycepin were purchased from Sigma Chemicals, St. Louis, Mo.

RNA·DNA Hybridization. Single-stranded reverse transcriptase [^{3}H]DNA product was prepared from sucrose gradient-purified BALB:virus-2 by methods previously described (17). The viral DNA, uniformly labeled with all four deoxynucleoside triphosphates, had a specific activity of 3×10^3 cpm/ng. It was shown to contain sequences representative of at least 70% of its viral RNA by hybridization of limiting ^{32}P-labeled 70S viral RNA with excess ^{3}H-viral DNA (18). Cellular and viral RNAs were prepared by the hot phenol method. Hybridization of 0.3 ng ^{3}H-viral DNA product with 20–500 μg of cellular RNA or 1–100 ng of viral RNA was for 7

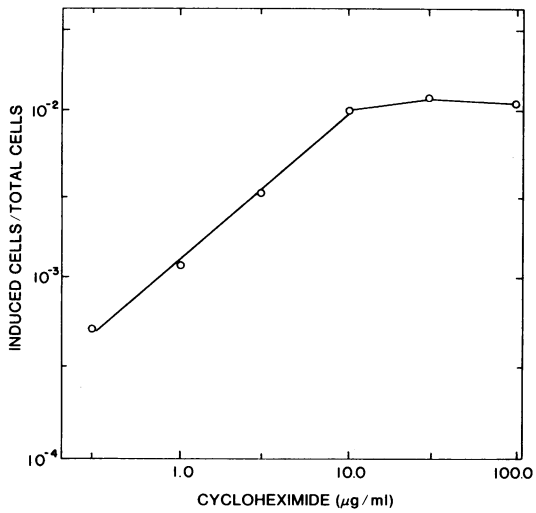

FIG. 1. Dose response for cycloheximide induction of type-C virus. Around 5×10^5 K-BALB cells were exposed to various concentrations of cycloheximide for 18 hr. Following drug removal, the cells were washed twice and exposed to mitomycin C (25 μg/ml) for 1 hr. After another cycle of washes, the cells were detached from the surface of the petri dish with 0.1% trypsin in phosphate-buffered saline and transferred to new petri dishes containing 10^5 NRK cells that had been plated 24 hr earlier in medium containing 2 μg/ml of Polybrene (Abbott Laboratories, Chicago). Sarcoma virus focus formation was assayed 7–9 days later as previously described (16). The fraction of virus-activated cells was determined from the number of murine sarcoma virus infectious centers divided by the total cells as measured by cell count at 24 hr following transfer. The results represent the mean values of three separate experiments.

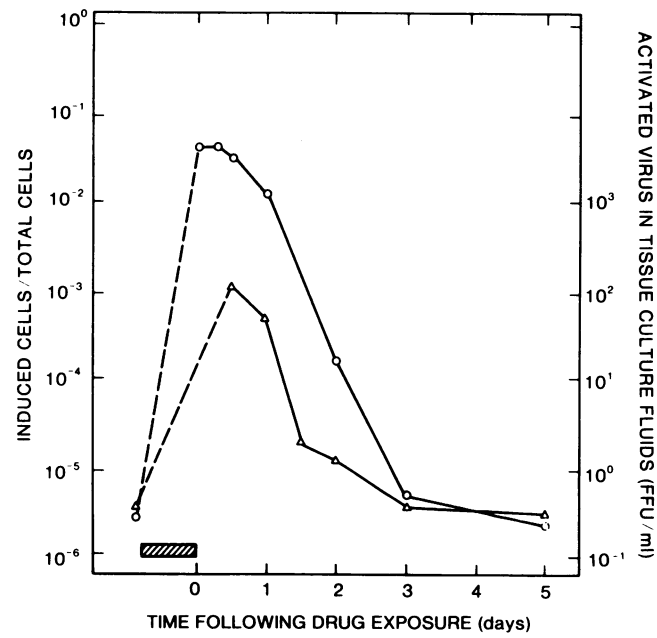

FIG. 2. Kinetics of type-C virus induction in response to cycloheximide. Around 5×10^5 K-BALB cells were exposed to cycloheximide (10 μg/ml) (shaded bar) for 12 hr, washed twice, and transferred for infectious center assay to NRK cells as described in the legend to Fig. 1 at the times indicated (O——O). Sarcoma virus release was quantitated by assay of 12-hr harvests of tissue culture fluids for focus-forming virus on NRK cells (Δ——Δ). The number of cells in the culture was measured by hemocytometer at the time of each virus collection. The results are expressed as focus-forming units (FFU)/ml of sarcoma virus released per 10^6 K-BALB cells.

days at 43°, in a 0.05 ml reaction volume containing 38% formamide, 1 mM EDTA, 15 mM Tris·HCl, pH 7.5, and 150 mM NaCl. Hybridization was assayed with nuclease S-1 (19, 20).

RESULTS

Induction of Type-C Virus as a Function of Cycloheximide Concentration. A type-C virus, designated BALB:virus-2, that is endogenous to BALB/c mouse cells, is noninfectious for mouse cells but transmits to cells of several other species (7). This virus has been shown to be very efficiently induced by treatment of the cells with inhibitors of protein synthesis. A BALB/c clonal line, K-BALB, was exposed to different concentrations of a representative protein synthesis inhibitor, cycloheximide. As shown in Fig. 1, the fraction of virus-induced cells capable of registering as infectious centers on NRK assays cells increased in approximately linear fashion with exposure to cycloheximide at concentrations above 0.3 μg/ml. A plateau in the frequency of virus-activated cells (1.2×10^{-2}) was reached at a dose of 10 μg/ml. At no drug concentration tested were virus-positive cells observed when treated cells were plated for infectious center assay on either NIH/3T3 or BALB/3T3 mouse cells (data not shown). Since BALB:virus-1, another BALB/c endogenous virus, grows preferentially in NIH Swiss mouse cells (2, 7), these findings confirm that BALB:virus-2 but not BALB:virus-1 was activated by cycloheximide.

Time Course of Virus Synthesis Following Exposure to Cycloheximide. The time course of virus activation from BALB/c cells in response to cycloheximide was next investigated. As shown in Fig. 2, the fraction of cells registering as virus-induced was maximal when the cells were transferred for infectious center assay within the first 12 hr after drug treatment. Cells assayed at later times showed a rapid decline in virus activation; by 72 hr, the fraction remaining virus-induced was no longer distinguishable from the spontaneous activation frequency of around 3×10^{-5} observed with untreated cultures (16). Similar results were obtained by assay of tissue culture fluids; virus release was maximal within 12 hr of drug removal (100 focus-forming units/ml) and was no longer detectable 72 hr later (Fig. 2).

The return to the virus-restricted state following cycloheximide induction was much more rapid than has previously been shown with virus activation by another class of inducers, halogenated pyrimidines. With these latter drugs, type-C virus release from BALB/c cells begins within 24–48 hr and peaks at around 72 hr; however, the cells remain virus-activated at high frequency for several more days (12, 16).

Studies were performed to test the reinducibility of cultures after cycloheximide induction. As shown in Table 1, when cells were plated for infectious center assay immediately after a single exposure to cycloheximide, 1.6% registered as virus-induced. Of cells treated identically but transferred for infectious center assay 72 hr later, only 0.006% remained virus-positive. When re-exposed to cycloheximide at 72 hr and assayed immediately thereafter, the cells showed a striking return to a high level of virus-induced cells. Around 1.0% registered as virus-positive under these conditions (Table 1).

TABLE 1. *Virus induction following single or repeated exposure to cycloheximide*

Cell treatment schedule*		
Exposure to cycloheximide for hours:	Transfer for infectious center assay at hour:	Virus-induced cells (%)
0–12	12	1.6
0–12	84	0.006
0–12 and 72–84	84	1.0

* Cultures of exponential phase K-BALB cells containing around 5×10^5 cells were exposed to 10 μg/ml of cycloheximide according to the above treatment schedule and transferred for infectious center assay at the designated times. The infectious center assay was performed according to the methods described in the legend to Fig. 1. The results represent the mean values of three separate experiments.

While these results do not exclude the possibility that selective drug toxicity to initially induced cells accounted for the transient release of virus, they argue that transient activation was not due to generalized toxicity affecting the whole cell population.

Virus-Specific RNA in Cycloheximide-Induced Cells. Cellular control of naturally-integrated type-C viral information might be exerted at the level of viral RNA transcription as in the case of the λ repressor (21) or at a post-transcriptional level (see refs. 22 and 23). As a step in elucidating the processes involved, single-stranded DNA product of BALB:virus-2 was used to examine by molecular hybridization the effect of cycloheximide on the cellular concentration of virus-specific RNA. RNAs from untreated BALB/3T12-3 cells and from parallel cultures exposed to 10 μg/ml of cycloheximide for 8 hr were hybridized to BALB:virus-2 [^{3}H] DNA. As shown in Fig. 3, RNA from untreated cells showed up to 60% homology to the viral DNA probe; the level of virus-specific RNA in the cells was not sufficient to achieve either complete or plateau hybridization with the highest amount of cell RNA tested (500 μg). The relative concentration of BALB:virus-2-homologous RNA in cycloheximide-treated cells was definitely increased (Fig. 3). Here essentially complete hybridization of the viral DNA probe was achieved with 200 μg of cell RNA. In other studies, reciprocal hybridization of viral RNA's of BALB:virus-2 and another BALB/c endogenous virus, BALB:virus-1, with their respective DNA products, has revealed that the genetic information of the two viruses is around 70% homologous (unpublished observations). The fact that RNA from cycloheximide-induced cells was able to almost completely hybridize the BALB:virus-2 DNA probe provides biochemical evidence that the increased level of virus-specific RNA in induced cells was due at least in part to a rise in concentration of BALB:virus-2 RNA. Whether this reflects a quantitative increase in virus-specific sequences normally present in untreated cells or a qualitative increase as well is not resolved.

The kinetics of the increase in virus endogenous viral RNA in cycloheximide-treated cells are shown in Fig. 4. The results were standardized to the concentration of viral RNA in untreated cells at zero time. Since the total RNA per treated cell did not change by more than 20% during exposure to cycloheximide under these conditions, any increase in virus-specific RNA above this level must be absolute rather than relative. A representative plot according to the method of Wetmur and Davidson (28) of hybridization data for induced cell RNA at 12 hr compared to that of noninduced cells is shown in the insert to Fig. 4. The relative viral RNA levels can be determined from comparison of the slopes of the two curves. The approximately 3-fold increase in slope observed reflects a 3-fold increase in virus-specific RNA in the induced cells at this time.

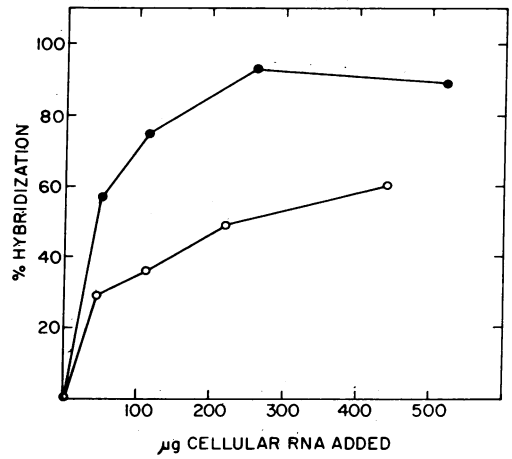

FIG. 3. Type-C virus-specific RNA in cycloheximide-treated cells. Exponential phase BALB/3T12-3 cells were grown at 37° in Bellco roller bottles and exposed either to cycloheximide (10 μg/ml) for 8 hr or left untreated. The cultures were then harvested by scraping, centrifuged at 2000 × *g* to remove medium, and frozen at −70° prior to extraction of RNA. Hybridization to BALB:virus-2 [^{3}H]DNA product was performed as described in the *Methods*. Background hybridization achieved with saturating levels of RNA from Fisher rat embryo cells, the cells in which BALB:virus-2 had been propagated, was 10% and was subtracted from the total [^{3}H]DNA input counts. Under the assay conditions, 100 ng of BALB:virus-2 RNA from purified virus hybridized over 95% of BALB:virus-2 [^{3}H]DNA. BALB/3T12-3 control, O; cycloheximide-treated, ●.

The kinetics of viral RNA expression in cells, as determined by this method, revealed a significant increase within 3 hr of drug exposure. At 16 hr, the peak in viral RNA was observed at a level 3-fold above that in untreated cultures. The decay in cell-associated viral RNA after drug removal was rapid, such that by 36 hr it had decreased by at least a factor of 2 from its peak concentration. By 72 hr, the viral RNA level was indistinguishable from that of untreated cells (data not shown). These kinetics closely resembled the kinetics of virus release as determined by biologic assay (Fig. 2).

It can also be seen in Fig. 4 that simultaneous treatment of cells with cycloheximide and an inhibitor of RNA synthesis, actinomycin D, effectively prevented the increase in virus-specific RNA observed with cycloheximide alone. These findings indicate that cycloheximide treatment is associated with an increase in the cellular concentration of BALB:virus-2 RNA, and that this effect can be blocked by inhibition of cellular RNA synthesis.

Time of RNA Synthesis Required for Cycloheximide Induction. Studies were next performed to determine the time period during which RNA synthesis was necessary in order for cycloheximide to induce. In these experiments, cells were exposed to cycloheximide in the presence or absence of actinomycin D

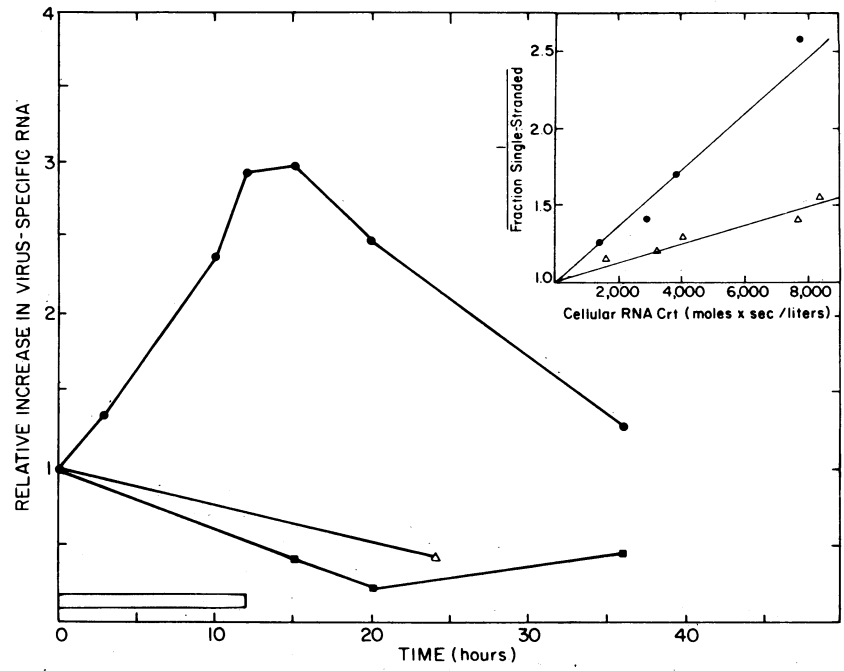

FIG. 4. Effect of cycloheximide on the cellular concentration of type-C viral RNA. Exponential-phase BALB/3T12-3 cells growing in Bellco roller bottles were exposed to various drugs (stippled bar). At 12 hr, cultures were washed twice, and fresh medium was added. At designated times during and after drug treatment cells were harvested as described in the legend to Fig. 3. Hybridization of cell RNA to BALB:virus-2 [^{3}H]DNA product was performed as described in the *Methods*. Virus-specific RNA in cell relative to that at time zero in untreated BALB/3T12-3 cells, △; exposed to cycloheximide (10 μg/ml), ●; exposed to cycloheximide (10 μg/ml) plus actinomycin D (1 μg/ml), ■. The insert shows a Wetmur–Davidson plot (28) of hybridization data with cellular RNAs from zero hour control (△) and 12 hr-cycloheximide-treated cells (●). The concentration of virus-specific RNA at the other points relative to that in untreated cells (zero time) was determined in an analogous way.

or cordycepin. The latter drug inhibits RNA synthesis by a mechanism different from that of actinomycin D (24). At the concentrations of actinomycin D and cordycepin utilized, RNA synthesis was inhibited by more than 90% within 2 hr of cell exposure to either drug. As shown in Table 2, under conditions where cycloheximide treatment resulted in virus activation from 1.2% of cells, treatment with either RNA synthesis inhibitor alone resulted in no detectable virus release. Simultaneous exposure of cells to cycloheximide in the presence of either RNA synthesis inhibitor effectively blocked virus induction; less than 0.01–0.05% cells registered as virus-positive under these conditions (Table 2). In striking contrast, when cells were exposed first to cycloheximide and then subsequently treated with either actinomycin D or cordycepin, virus induction was not detectably inhibited. In each case, around 1.0% of the cells registered as virus-activated, a frequency very similar to that of cells treated with cycloheximide alone (1.2%). These results indicate that virus induction by cycloheximide requires continued cellular RNA synthesis during but not subsequent to cycloheximide exposure.

In an effort to exclude the possibility that cell toxicity associated with exposure to these chemicals might have influenced the above results, the colony-forming efficiencies of cells were compared under each set of treatment conditions. At the concentration utilized, actinomycin D caused little or no additional impairment to cell growth (Table 2). While there was obvious cell toxicity with simultaneous exposure to cycloheximide and cordycepin, the colony-forming efficiencies were no worse than those of cells sequentially treated with the same drugs. These findings tend to exclude the possibility that selective cell toxicity during simultaneous drug exposure was responsible for the block to virus release.

DISCUSSION

The discovery that inhibitors of protein synthesis efficiently induce a specific class of endogenous mouse type-C viruses has provided a potentially useful tool for analysis of cellular processes that normally restrict integrated type-C viral expression (9). In the present report, a representative protein synthesis inhibitor, cycloheximide, has been shown to cause an increase in the level of virus-specific RNA in treated cells. The findings that virus-specific RNA rises only around 3-fold in the total cell population while the number of induced cells increases by 10^3- to 10^4-fold may reflect the need for only a relatively small increase in viral RNA above a threshold level in order for a high frequency of cells to register as virus-induced. Alternatively, if *only* cells that register as induced exhibit an increase in virus-specific RNA, the level of viral RNA in those cells may increase by a much greater factor.

Simultaneous but not subsequent exposure of cells to actinomycin D was shown to prevent both the increase in cell-associated viral RNA and virus release in response to cycloheximide. This demonstrates a requirement for *de novo* RNA synthesis during but not after cycloheximide exposure in order for virus activation and suggests that the required RNA species may be that of the virus itself. These results are consistent with a mechanism whereby cycloheximide causes decay of a labile control protein that either inhibits viral RNA transcription or acts at a post-transcriptional level to degrade viral RNA. While an increase in viral RNA could also result from feedback from a primary target of the inducer at a very late step in virus assembly, the early rise in virus-specific RNA makes this less likely. The rapid return of cells to the non-virus expressed state would presumably be due to re-

TABLE 2. *Effect of inhibitors of RNA synthesis on virus induction by cycloheximide*

Treatment schedule time (hr)		Virus-induced cells (% of total)	Colony-forming efficiency (% of untreated cells)
0–10	10–20		
Cycloheximide	—	1.2	30
Actinomycin D	—	<0.01	33
Cordycepin	—	<0.05	1.7
Cycloheximide + actinomycin D	—	<0.01	25
Cycloheximide + cordycepin	—	<0.05	1.8
Cycloheximide	Actinomycin D	1.3	22
Cycloheximide	Cordycepin	0.8	2.3

Cultures containing around 5×10^5 exponential phase K-BALB cells were exposed to 10 μg/ml of cycloheximide in the presence or absence of actinomycin D (1 μg/ml) or cordycepin (50 μg/ml) from 0 to 10 hr and washed three times with regular medium. The cells were then treated with actinomycin or cordycepin, or were untreated from the 10th to 20th hour. At 20 hr, cell cultures were washed twice and transferred for infectious center assay, as described in the legend to Fig. 1, or for determination of colony-forming efficiency, as described in the *Methods*. The colony-forming efficiency of untreated cells was 15%. The results represent the mean of three separate experiments.

accumulation of the control protein following return of protein synthesis within the cells.

The class of endogenous virus induced from BALB/c cells by cycloheximide is of biologic interest in that it appears to completely lack the ability to exogenously infect cells in which it normally resides (7, 25). There is evidence that similar viruses exist in cells of other mouse strains, including those with both high and low incidence of naturally-occurring leukemia (7, 25–27). These viruses have been termed "xenotropic" on the basis of the nonpermissiveness of mouse cells and the permissiveness of cells of other species for their growth (26). Whether cycloheximide and other protein synthesis inhibitors will specifically induce such viruses from cells of other mouse strains and other species remains to be determined.

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