High-Frequency C-Type Virus Induction by Inhibitors of Protein Synthesis

Abstract. *When inhibitors of protein synthesis are added to BALB/c mouse cells in culture, induction of naturally integrated C-type RNA virus occurs in a high percentage of cells. The action of protein synthesis inhibitors differs from that of halogenated pyrimidines, another class of virus inducers, in their effects on biologically distinguishable viruses. The use of such inhibitors to study integrated virus expression provides a means for studying gene regulation in mammalian cells.*

RNA C-type viruses are present in an unexpressed form in all mouse cells (*1, 2*). Recent genetic (*3*) and biochemical (*4*) evidence indicates that the structural genes for multiple viruses are naturally integrated within the cell genome. The study of cellular regulation of these endogenous viruses provides the opportunity to investigate how cellular controls affect their oncogenic potential and to gain a more basic understanding of the processes involved in mammalian gene regulation.

Mouse cells normally restrict the complete expression of their integrated viral genes, since spontaneous virus activation occurs at only a very low frequency in cells of different strains (*1, 2, 5*). Halogenated pyrimidines increase virus induction (*2, 6*), although the mechanism of action is not yet known. Studies of gene regulation in other mammalian systems have indicated that during differentiation (*7*) or after exposure to hormones (*8*) enzyme induction may be affected by exposure of cells to inhibitors of macromolecular synthesis. These inhibitors have also been reported to induce certain enzymes directly (*9*), possibly by interfering with a labile intracellular inhibitor that acts during or after transcription (*7–9*). If C-type virus activation were restricted by a labile control molecule or molecules, then inhibition of its production might result in virus induction. Tests of chemicals that interfere with different aspects of macromolecular synthesis have led to the discovery that inhibitors of protein synthesis are very efficient inducers of C-type virus.

Cells were grown in Dulbecco's modification of Eagle's medium, supplemented with calf serum (10 percent) (Colorado Serum Co.). The derivation of clonal lines of contact-inhibited BALB/3T3 and NIH/3T3 mouse cells (*10*) and normal rat kidney (NRK) cells (*11*) have been reported. Chemicals including actinomycin D, cytosine arabinoside, cycloheximide, puromycin, anisomycin, sparsomycin, and mitomycin C were provided by the Drug Development Branch, National Cancer Institute.

A sensitive biologic assay for C-type virus induction has been described. When cells transformed by murine sarcoma virus (MSV) in the absence of coinfection with helper leukemia virus (*12*) are induced, they release sarcoma virus in the envelope of the activated endogenous C-type viruses of those cells (*6, 13*). The biologic assay for sarcoma virus transformation requires only a single cycle of infection (*12*), while assays for helper leukemia virus generally require many cycles of virus replication. Thus, the sarcoma virus provides an indirect but sensitive indicator for induction of inefficiently replicating endogenous viruses. A clonal line of Kirsten (K) MSV-transformed nonproducer BALB/c cells [K-BALB (*12*)] was used. Induced sarcoma virus was assayed in tissue culture fluids (*13*) or by infectious center assay of virus-activated cells (*5*).

The results of treatment of K-BALB cells with inhibitors of macromolecular synthesis are shown in Table 1. Each inhibitor of protein synthesis induced virus from a high percentage of cells. Puromycin at optimum concentration (10 μg/ml) activated virus from 15 percent of the cells, an increase of more than 10^5-fold above the spontaneous frequency of virus activation (*5*). Cycloheximide, anisomycin, and sparsomycin induced virus with almost comparable efficiency. Activation was observed over a 1000-fold dose range with cycloheximide and over a 100-fold range with the other protein synthesis inhibitors. Incorporation of radioactive amino acids into protein in parallel cultures indicated that virus activation occurred only when protein synthesis was impaired by more than 90 percent (*14*). At these concentrations, there was associated, but delayed, 30 to 60 percent inhibition of both DNA and RNA synthesis. In contrast, other chemicals that inhibited RNA or DNA synthesis over a range of 30 to 90 percent but did not inhibit protein synthesis had no detectable virus-inducing activity (Table 1).

As a test of nonspecific cell toxicity, which might impair the ability of in-

Table 1. C-type virus induction by inhibitors of protein synthesis. Growing cultures containing 5×10^5 K-BALB cells were exposed to different inhibitors for 18 hours at 37°C. After drug removal, the cells were washed twice and exposed to mitomycin C (25 μg/ml) for 60 minutes. One hour later, the cells were transferred to petri dishes containing 10^5 NRK cells that had been plated 24 hours earlier in medium containing polybrene (2 μg/ml). Sarcoma virus focus formation was assayed 7 to 9 days later (*5, 13*). Mitomycin inhibited cell division of K-BALB cells, making it easier to detect MSV-induced infectious centers. Similar frequencies of induction were observed with each drug in the absence of mitomycin exposure. The percentage of virus-activated cells was determined from the number of MSV infectious centers divided by the total cells as measured by cell count at 24 hours after transfer. The maximum induction frequency for each drug was determined from at least five separate experiments and is expressed as the percentage of virus-activated cells.

Inhibitor of synthesis	Amount (μg/ml)		Virus induction (%)
	Range	Optimum	
None			< 0.001
Protein			
Puromycin	0.1–100	10	15
Cycloheximide	0.1–100	10	10
Anisomycin	1–100	100	6
Sparsomycin	1–100	100	4
RNA			
Actinomycin D	0.1–10		< 0.001
DNA			
Cytosine arabinoside	0.1–10		< 0.001

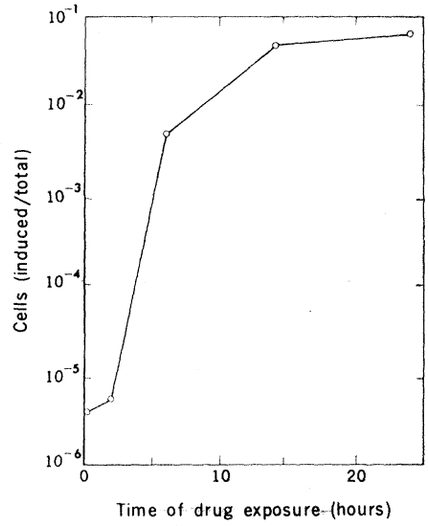

Fig. 1. Effect of duration of cycloheximide exposure on C-type virus induction. K-BALB cells (5×10^5) were exposed for varying periods to cycloheximide (10 μg/ml) and then transferred for infectious center assay on NRK cells as described in the legend to Table 1. The results are the average of two experiments.

duced cells to register as virus-positive, the colony-forming efficiencies of cells exposed to cycloheximide, actinomycin D, or cytosine arabinoside were compared. At concentrations of cycloheximide that induced 5 to 10 percent of the cells, there was a 50 to 90 percent decrease in colony-forming efficiency. Actinomycin or cytosine arabinoside concentrations that inhibited RNA or DNA synthesis comparably to cycloheximide were no more detrimental to cell viability. The above findings indicate that protein synthesis inhibitors activate virus by a mechanism involving inhibition of protein synthesis.

The duration of cycloheximide exposure necessary for virus activation is shown in Fig. 1. There was more than a 10^3-fold increase above the spontaneous activation level within 5 hours of drug treatment. While there was some further increase with exposure for up to 24 hours, the results demonstrate that a relatively short period of protein synthesis inhibition was sufficient to cause a striking induction response.

In the above studies, the frequency of virus-activated cells was determined by infectious center assay. Virus was also detected in tissue culture fluids of induced cells. Between 0.3×10^2 to 3×10^2 focus-forming units per milliliter were released in the first 24 hours after cycloheximide treatment. Activated virus subsequently declined to levels below detection over the next 3 days. That the protein synthesis inhibitors acted at a step (or steps) in the induction process instead of enhancing virus replication, as was observed with certain steroid hormones (*15*), was indicated by the fact that cycloheximide caused impairment of virus production by K-BALB cells that had either been previously or newly infected with Rauscher mouse leukemia virus.

BALB/c mouse cells contain at least two independently segregating C-type viruses (*3*). These viruses can be distinguished by host range and serologic characteristics. For example, BALB: virus-1 is infectious for both NIH Swiss and NRK cells, while BALB: virus-2 transmits only to NRK (*3*). The effect of protein synthesis inhibitors on activation of the two viruses was compared with that of halogenated pyrimidines, bromodeoxyuridine (BrdU), and iododeoxyuridine (IdU). As shown in Table 2, K-BALB cultures exposed to BrdU or IdU registered as virus-activated with similar efficiencies on NRK and NIH/3T3 cells. In contrast, puromycin- or cycloheximide-activated cells registered as virus-induced at high frequency only when plated as infectious centers on NRK cells. There was no detectable focus induction on NIH/3T3 cells. These findings indicate that the protein synthesis inhibitors, unlike halogenated pyrimidines, specifically activated only BALB: virus-2.

Table 2. Comparison of induction of two BALB/c endogenous viruses by inhibitors of protein synthesis and halogenated pyrimidines. Growing cultures (5×10^5 K-BALB cells) were exposed to the appropriate inducer for 18 hours at 37°C. The cells were then treated with mitomycin C (25 μg/ml) for 1 hour and transferred for infectious center assay on either NRK or NIH/3T3 cells as described in the legend to Table 1. The results are the average of two experiments with each inducer.

Inducer	Amount (μg/ml)	Induction frequency (% virus-activated cells)	
		NRK	NIH/3T3
Cycloheximide	10	8.3	<0.001
Puromycin	10	12	<0.001
IdU	30	3.8	2.4
BrdU	30	1.6	0.8

Our studies demonstrate that inhibitors of protein synthesis are very efficient inducers of C-type virus. Several lines of evidence favor the hypothesis that their action directly or indirectly results from interference with this aspect of macromolecular synthesis. First, each of several chemicals that inhibit different steps in polypeptide formation (*16*) were active as virus inducers. Second, induction occurred only in association with a severe impairment of amino acid incorporation into cellular protein. Finally, induction was not observed in our studies with inhibitors of either RNA or DNA synthesis nor with a large number of other chemicals that include mutagens, inhibitors of DNA repair, and chemical carcinogens (unpublished observations).

It is possible that the drugs specifically interfere with a labile protein whose function is to prevent virus release. Such a protein might act either during or after transcription. Since protein synthesis is also required for virus assembly, the production of this inhibitory protein would have to be more sensitive or have a faster turnover or reaccumulate more slowly after release from inhibition by the chemical. The imbalance would result in virus activation. It is also possible that induction results from some indirect effect of protein synthesis inhibition on cellular metabolism (*17*). The evidence that the drugs induce one but not another BALB/c endogenous virus and that other classes of chemicals that produce a multiplicity of effects on cellular metabolism lack activity as virus inducers leads us to favor the hypothesis that the chemicals interfere with a specific protein inhibitor of virus induction.

In comparison to the detailed knowledge of the interactions of phage with its bacterial cell host, understanding of the relation of naturally integrated C-type viruses to normal cells is not nearly as advanced. However, the genetic approaches that made the regulation of phage amenable to study are now developing in this eukaryotic system. Conditional lethal mouse C-type virus mutants (*18*) and cells which contain definable genetic factors related to endogenous viruses and their regulation (*3, 19*) have been obtained. Our data shows that two classes of highly efficient chemical inducers are able to differentially affect the control of biologically distinguishable integrated viruses. With the availability of sensitive and specific biologic and biochemical assays for detection of these viruses and their gene products, the study of cellular control of endogenous C-type viruses provides an opportunity for determining the molecular mechanisms involved in gene regulation in eukaryotic cells.

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14. About 5×10^5 K-BALB cells were incubated at 37°C with inhibitor in the presence of [^{3}H]leucine (10 μc/ml) (Schwarz/Mann). At sequential time points, cultures were washed twice, and incubated a further 30 minutes with regular medium containing a 100-fold excess of unlabeled leucine. The medium was then removed, and cells were dissolved in 1.0 ml of sodium dodecyl sulfate lysing buffer. Radioactivity in the acid-precipitable fraction was measured in a toluene-based liquid scintillation system. Results are the percent inhibition of incorporation in the absence of drug treatment. RNA and DNA synthesis were measured in the same way except with [^{3}H]uridine and [^{3}H]thymidine (10 μc/ml), respectively.
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Beta Cell Protection to Alloxan Necrosis by Anomers of D-Glucose

Abstract. *Various concentrations of either the α or β anomers of* D-*glucose were injected into fasted rats prior to a diabetogenic dose of alloxan. Plasma concentrations of glucose were measured 24 hours later. There was a significantly greater protection of the pancreatic beta cells by the α anomer of* D-*glucose as compared to the β anomer, which was evidenced by concentrations of glucose in the plasma, and morphology of beta cells.*

Alloxan (mesoxalylurea) produces diabetes in various animal models by necrosis of the beta cell in the islets of Langerhans (*1*). However, D-glucose injected into animals prior to the diabetogenic agent has been shown to diminish or to prevent this effect of alloxan (*2, 3*). Crystalline D-glucose when placed in solution reaches an equilibrium in which 64 percent is in the β and 36 percent in the α configuration (*4*). When the near pure glucose anomers, at various concentrations, are immediately dissolved and injected into rats before significant mutarotation, a difference in the degree of protection between the α and β anomers to alloxan-induced necrosis is observed, the former being more active.

Male rats (Charles River strain) weighing between 190 to 200 g were given free access to Purina Chow and then fasted for 24 hours. A No. 21 butterfly (3-inch tubing Minicath Prn, Deseret Pharmaceutical Co., No. 5084) was placed in a tail vein after dilation induced by hot water. Alpha and beta glucose (Sigma Chemical G-5250, beta lot 052e-0810, analyzed as 99.2 percent β and 0.8 percent α; and Sigma Chemical G-5000 alpha lot 091C-1690 analyzed as 97.6 percent α and 2.4 percent β) were each rapidly dissolved in normal saline by vigorous shaking just before use in each animal. The glucose was administered in a volume of 0.5 ml at doses of 250, 500, or 750 mg per kilogram of body weight. An additional 0.2 ml of saline was injected to flush the tubing and then alloxan (Eastman, 40 mg/kg), freshly dissolved in normal saline in a volume of 0.5 ml, was injected. A final 0.2 ml of saline was then injected. The animals were randomly selected for the experiment, and alloxan-injected animals that were not given glucose were included as controls at the beginning and end of each series. The animals were then allowed free access to their food. Twenty-four hours later a blood sample was obtained by cutting a small section of the tail, and approximately 0.5 ml of blood was captured in a heparinized pipette, and centrifuged; the plasma was analyzed with the use of the Beckman glucose oxidase analyzer. Blood samples, taken 48 or 72 hours later, did not differ from those taken 24 hours after alloxan. Statistical analysis was performed by means of the unpaired *t*-test (Table 1).

Table 1. Either a glucose anomer or saline was injected intravenously 4 to 6 seconds before administration of alloxan (40 mg per kilogram of body weight) to fasted rats, and glucose in the plasma was determined 24 hours later. Similarly treated animals not receiving alloxan showed a mean glucose concentration of 164 ± 2 mg per 100 ml of plasma ($N = 21$). The plasma glucose is given in milligrams per 100 ml of plasma.

Anomer (mg/kg)	Plasma glucose (mean ± S.E.M.)		N	P
	α Anomer	β Anomer		
0	533 ± 21	533 ± 21	38	
250	266 ± 31	409 ± 31	12	< .01
500	240 ± 15	341 ± 20	44	< .001
750	163 ± 4	174 ± 9	23	< .3

Although the exact mechanism in which glucose protects against the diabetogenic effect of alloxan is unknown (*5, 6*), L-glucose, the nonmetabolizable optical isomer of D-glucose has been shown not to protect against alloxan, excluding a purely chemical reaction, while 3-*O*-methyl-D-glucose and 2-deoxy-D-glucose have produced protection as evidenced by permeability in vitro (*6*). The abolition of protection by prior treatment with mannoheptulose has placed credence on the theory that the protective site is moderately stereospecific, probably at the beta cell membrane and not through a common protective metabolic intermediate (*3*). Although the mutarotation of α- or β-glucose into an equilibrium state is rapid in a physiologic setting (≃ 7 minutes) (*7*), the beta cells in the islets of Langerhans are able to distinguish between the injected anomers, as evidenced by the different degree of protection. To further corroborate this observation, the animals protected with α-glucose were morphologically compared with those with β-glucose and those with alloxan alone. Beta cell necrosis was noted in those receiving only alloxan, and, in contrast, "protected islets" demonstrated slight to moderate degranulation of beta cells and only minimal or absent evidence of injury, both proportionate to the protection against diabetes as evidenced by concentrations of glucose in the plasma (*8*).

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