Induction of mouse type-C virus by translational inhibitors: Evidence for transcriptional derepression of a specific class of endogenous virus
(gene regulation/cancer/molecular hybridization/inhibition of protein synthesis)

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ABSTRACT Several biologically distinguishable type-C RNA viruses are genetically transmitted in mouse cells. In the present report, chemicals that inhibit several different steps in protein synthesis are shown to cause marked increases in the cellular concentration of viral RNA and the subsequent induction of virus. Analysis of the effect of translational inhibitors on mouse embryo cells of different genotypes indicates that activation of viral RNA is specific for one endogenous virus class and is a dominant genetic characteristic. Two lines of evidence favor the hypothesis that the induction of viral RNA involves transcriptional derepression rather than an alteration in its post-transcriptional processing. First, nuclear and cytoplasmic fractions of induced cells are shown to demonstrate similar increases in their concentrations of virus-specific RNA. Second, the decay of induced viral RNA following inhibition of further RNA synthesis by actinomycin D is not prevented by continued exposure to the inducer. These findings weigh against the possibility that translational inhibitors act to stabilize viral RNA post-transcriptionally. The results are consistent with a model in which the expression of one class of endogenous virus is regulated by a labile repressor protein acting at a transcriptional level.

Type-C RNA viruses are genetically transmitted within mouse cellular DNA (1-4). Several biologically distinguishable viruses have been demonstrated (5-8), and there is increasing evidence that the expression of each virus is specifically regulated (7, 9). One approach to the determination of the cellular controls affecting endogenous viruses has come from the discovery that inhibitors of protein synthesis temporarily disrupt the normally dominant repression of endogenous virus release (10). It has previously been shown that during the period of translational inhibition there is a marked increase in cellular concentration of type-C viral RNA. Following release from protein synthesis inhibition, virus is transiently activated from a high frequency of cells. Both effects are dependent upon de novo cellular RNA synthesis during the time of drug exposure (11). These findings have suggested that the mechanism of virus activation by translational inhibitors may involve increased transcription of viral RNA or an alteration of its post-transcriptional processing. In the present report, evidence is presented that virus induction by protein synthesis inhibitors occurs at a transcriptional level and that this alteration is specific for one endogenous virus class.

METHODS

Cells and Viruses. The derivations of cells used in these studies have been previously described (7, 8, 12). Embryo cell cultures were also established from a wild mouse colony maintained by J. Parker, Microbiological Assoc. Viruses included BALB: virus-2 (7), an inducible xenotropic endogenous virus of BALB/c mouse cells, and xenotropic viruses of NIH Swiss and NZB (8, 13) strains. The latter two viruses are immunologically and biochemically indistinguishable, but can be readily differentiated from BALB: virus-2 (14, 15).

Virus Induction. An infectious center assay, which measures the frequency of virus activation of K-BALB, a line of nonproducer cells transformed by Kirsten murine sarcoma virus, has been reported (10).

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

Synthesis of Virus-Specific DNA Probes. 3H-Labeled DNA complementary to BALB: virus-2 RNA was synthesized in the presence of 20 μg/ml of actinomycin D using detergent-disrupted virus (16-18). In some cases, 75 μg/ml of calf thymus DNA, which had been previously digested by DNase for 6 hr, was added to the reaction (19). Following incubation at 37° for 6 hr, cDNA was extracted as previously reported (16), separated from nucleotides on a Sephadex G-200 column, lyophilized, and stored at -20°. The specific activity of the cDNA probe was 2 × 107 cpm/μg. The complexity of BALB: virus-2 cDNA was estimated by hybridization to its homologous viral 32P]RNA (17). At an equal molar ratio, around 70% of the 70S 32P]RNA was made resistant to 10 μg/ml of pancreatic RNase.

DNA-RNA Hybridization. Hybridization of approximately 1000 cpm of viral 32P]cDNA with 20-500 μg of cellular RNA or 1-100 ng of viral RNA was for 2 days at 68°, in a 0.05 ml reaction volume containing 0.6 M NaCl, 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA. Hybridization was assayed with S1 nuclease (18, 20). By comparing the Ct (moles of RNA nucleotide per liter times incubation time in seconds) values for percent hybridization at half-saturation of the different preparations to that of 70S viral RNA (2.0 × 10-2 mol/sec/liter), it was possible to calculate the percent virus-specific RNA in each sample.

Preparation of Cellular RNA. Cellular RNA was extracted by the hot phenol method (21). Nuclear and cytoplasmic fractions were isolated by the method of Busch (22). This technique led to less than 2% contamination of nuclear with cytoplasmic RNA and less than 0.5% contamination of cytoplasmic with nuclear RNA. Nuclear RNA preparations were chromatographed on Sephadex G-200 prior to the lyophilization step to remove DNA oligonucleotides. RNA was determined by A250 and orcinol methods (23).

RESULTS

Effects of Inhibitors of Different Steps in Protein Synthesis on Virus-Specific RNA Expression. Previous studies have demonstrated that cycloheximide, an inhibitor of poly-

Abbreviations: Ct, product of RNA concentration (moles of nucleotide/liter) and time (seconds); Tm, melting temperature.
peptide chain elongation, causes an increase in the cellular concentration of endogenous virus-specific RNA (11). In the first series of studies, inhibitors of various steps in protein synthesis were tested for their effects on viral RNA expression and concomitantly for the efficiency at which each caused type-C virus activation from BALB/c embryo cells. Fig. 1 shows the kinetics of the response of cell-associated viral RNA to pactamycin, an inhibitor of polypeptide chain initiation. The level of viral RNA in untreated cells remained constant over the 16-hr time course of the study. In contrast, the cellular concentration of virus-specific RNA in pactamycin-exposed cultures was detectably increased by 4 hr and achieved a level 12.5-fold above that observed in the untreated cells by 16 hr. Under these conditions, there was no more than a 10% change in total RNA per cell (data not shown). Thus, the measured increase in virus-specific RNA reflected an absolute increase in the content of viral RNA in pactamycin-treated cells. As shown in Fig. 1, [3H]leucine incorporation by cultures exposed to pactamycin under these conditions was rapidly inhibited to less than 5% that of untreated cells within 1 hr. Cellular RNA synthesis was also inhibited, but not nearly as severely.

Table 1 shows the results of cell exposure to pactamycin, cycloheximide, or puromycin. This last drug causes premature termination of the growing polypeptide chain, thus acting by a different mechanism than either of the other translational inhibitors. With each inhibitor, markedly increased levels of viral RNA were demonstrated at drug concentrations that caused virus activation at high frequency. Induction, as measured both by viral RNA increase and frequency of virus-activated cells, was greater at optimal concentrations of pactamycin or puromycin than with cycloheximide. This may be due to somewhat more severe cell toxicity associated with cycloheximide exposure. Invariably, however, a marked induction response was only observed under conditions where protein synthesis was rapidly and severely inhibited (Table 1). The above results were all consistent with the hypothesis that virus activation was initiated by inhibition of translation, shortly thereafter associated with a rise in the intracellular concentration of type-C virus-specific RNA.

Evidence That Induction of Viral RNA Is Specific for BALB-Virus-2. Inbred mouse strains such as NIH Swiss and NZB lack chemically inducible type-C virus of the class represented by BALB-virus-2 (14). However, these strains are known to contain information for a different xenotropic endogenous virus (8, 13, 14). This latter virus is spontaneously released at readily detectable levels by NZB embryo cells in tissue culture, whereas its release appears to be completely re-

Table 1. Induction of virus-specific RNA by inhibitors of different steps in protein synthesis

<table>
<thead>
<tr>
<th>Drug treatment</th>
<th>Concentration (μg/ml)</th>
<th>Relative increase in virus-specific RNA* (drug-treated/untreated)</th>
<th>Induction frequency† (virus-induced cells/total cells)</th>
<th>[3H]Leucine incorporation‡ (percent of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>1.0</td>
<td>1.0×10^-1</td>
<td>100</td>
</tr>
<tr>
<td>Pactamycin</td>
<td>0.1</td>
<td>6.3</td>
<td>1.5×10^-1</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>9.5</td>
<td>2.4×10^-1</td>
<td>&lt;3</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>12.8</td>
<td>4.1×10^-1</td>
<td>&lt;3</td>
</tr>
<tr>
<td>Puromycin</td>
<td>10</td>
<td>1.4</td>
<td>5.0×10^-1</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>15.5</td>
<td>6.2×10^-1</td>
<td>&lt;3</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>0.1</td>
<td>1.9</td>
<td>2.0×10^-4</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>5.2</td>
<td>5.0×10^-3</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>6.3</td>
<td>1.5×10^-2</td>
<td>&lt;3</td>
</tr>
</tbody>
</table>

* Exponentially growing BALB/3T12-3 cells were treated with the appropriate drug for 16 hr. RNA extraction and hybridization to BALB-virus-2 cDNA were performed as described in Methods. The percent viral RNA in untreated cells (0.95×10^-2) remained constant over the time course of the experiment. The results represent mean values of at least two separate experiments.
† Exponentially growing K-BALB cultures containing around 5×10^6 cells in 50 mm petri dishes were exposed to the appropriate drug for 16 hr, then washed twice, and treated with mitomycin C (25 μg/ml) for 1 hr. Following another series of washes, the cells were transferred to new petri dishes containing 10^6 NRK cells plated 24 hr earlier in medium containing 2 μg/ml of Polypebre (Abbott Laboratories, Chicago). Focus formation by induced sarcoma virus was assayed 7-9 days later as previously described (10). The fraction of virus-activated cells was determined from the number of sarcoma virus infectious centers divided by the total cells determined by hemocytometer 24 hr following cell transfer. The results represent mean values of three separate experiments.
‡ At 1 hr following drug treatment BALB/3T12-3 cultures, cells were pulse-labeled for 30 min with [3H]leucine in continued drug presence. Incorporation relative to that of untreated cells was determined as described in the legend to Fig. 1, and the results represent the mean values of two separate experiments.
Table 2. Analysis of cycloheximide-induced viral RNA in mouse cells of different genotypes

<table>
<thead>
<tr>
<th>Strain of embryo cell</th>
<th>Percent viral RNA (\times 10^3)*</th>
<th>Percent maximum hybridization†</th>
<th>(t_m) of hybrids (°C)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>Untreated 1.0</td>
<td>Treated 4.1</td>
<td>Untreated 60</td>
</tr>
<tr>
<td>NZB</td>
<td>Untreated 10.5</td>
<td>Treated 10.8</td>
<td>Untreated 55</td>
</tr>
<tr>
<td>NIH Swiss</td>
<td>Untreated 2.1</td>
<td>Treated 2.1</td>
<td>Untreated 53</td>
</tr>
<tr>
<td>Wild mouse</td>
<td>Untreated 3.3</td>
<td>Treated 3.3</td>
<td>Untreated 49</td>
</tr>
<tr>
<td>(NIH Swiss × BALB/c)F&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Untreated 1.4</td>
<td>Treated 3.8</td>
<td>Untreated 58</td>
</tr>
<tr>
<td>(Wild mouse × BALB/c)F&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Untreated 2.6</td>
<td>Treated 6.8</td>
<td>Untreated 58</td>
</tr>
</tbody>
</table>

* Exponentially growing mouse embryo cells were exposed to cycloheximide (10 μg/ml) for 16 hr, after which time the percent viral RNA was determined as described in Methods. For NZB, NIH Swiss, and wild mouse embryo cultures, the level of virus-specific RNA was determined by comparing \(C_{4t}\) of their cellular RNAs to that of NZB type-C viral 70S RNA on the BALB:virus-2 cDNA probe.
† The maximum hybridization represents the percent of BALB:virus-2 cDNA hybridized to each cellular RNA to a final \(C_{4t}\) of 10<sup>4</sup>. The maximum hybridizations achieved with 70S viral RNAs of BALB:virus-2, NZB, and NIH Swiss virus were 95%, 55%, and 55%, respectively.
‡ The melting temperatures \(t_m\) (values) of hybrids formed between each of the cellular RNAs and BALB:virus-2 cDNA at a \(C_{4t}\) of 10<sup>4</sup> were determined as previously described (16). The \(t_m\) values of hybrids formed between this probe and 70S viral RNAs of BALB:virus-2, NZB, and NIH Swiss type-C viruses were 86°, 79°, and 79°, respectively.

stricted by NIH Swiss embryo cells (9). As shown in Table 2, the level of viral RNA in untreated NZB embryo cells was approximately 5-fold higher than that present under steady-state conditions in NIH Swiss embryo cells. These findings were consistent with the known higher levels of xenotropic virus release by NZB cells.

It was of interest to investigate the effect of protein synthesis inhibitors on viral RNA of the xenotropic virus endogenously to NIH Swiss and NZB strains. Whereas cycloheximide-induced BALB/c cultures showed a 4.1-fold increase in virus-specific RNA, neither NIH Swiss nor NZB embryo cells demonstrated detectable alteration in their viral RNA concentration (Table 2). Wild mouse embryo cell cultures are not virus-inducible by inhibitors of protein synthesis (unpublished observations). As shown in Table 2, these cells also demonstrated no increase in their content of viral RNA following treatment with cycloheximide. Exposure of either (NIH Swiss × BALB/c) F<sub>1</sub> or (wild mouse × BALB/c) F<sub>1</sub> hybrid embryo cells to cycloheximide resulted in an increase in the concentration of virus-specific RNA, similar to that observed with BALB/c cells. Thus, inducibility of viral RNA was a dominant genetic characteristic in crosses between the virus-inducible BALB/c and either of two nonvirus inducible strains.

Cellular RNAs of induced BALB/c, (NIH Swiss × BALB/c)F<sub>1</sub>, and (wild mouse × BALB/c)F<sub>1</sub> embryo cells showed between 75 and 80% homology to the BALB:virus-2 cDNA probe (Table 2). These values were significantly higher than those achieved with any of the other cellular RNAs tested and were also greater than the maximum hybridization of 50–55% achieved with 70S RNAs of NZB or NIH Swiss xenotropic viruses. The \(t_m\) values of hybrids formed between BALB:virus-2 cDNA and cellular RNAs of induced BALB/c parental or F<sub>1</sub> hybrid cells involving this strain were around 85°, 4° to 5° higher than observed with any of the other cellular RNAs tested. The \(t_m\) of the hybrid formed between BALB:virus-2 cDNA and its homologous 70S viral RNA was also 85°. The above results indicate that viral RNA induced by translational inhibitors from BALB/c parental cells or from hybrids of this inducible strain and either of two noninducible strains was primarily that of BALB:virus-2.

The lower \(t_m\) and extent of hybridization observed with control BALB/c cellular RNA suggests that the viral RNA detected here represents that of another virus. BALB/c embryo cells are known to express type-C viral antigens in the absence of overt virus release. These antigens have been identified as belonging to an endogenous virus that is immunologically distinguishable from BALB:virus-2 (9, 14, 15). Thus, if the viral RNA present in untreated BALB/c cells, in large part, represents this other virus, the observed increase in viral RNA during induction by translational inhibitors may well reflect an increase in BALB:virus-2 RNA of much larger magnitude.

**Virus-Specific RNA in Nucleus and Cytoplasm of Induced Cells.** The increased concentration of virus-specific RNA in cells exposed to protein synthesis inhibitors might be due to its increased transcription or altered processing. If the former, viral RNA might be expected to be proportionally increased in both the nucleus and cytoplasm of drug-treated cells. However, a block to viral RNA processing might lead to a relative build-up of viral RNA in either subcellular compartment, depending upon the site at which this block occurred. To examine this question, BALB/c cells were exposed to cycloheximide (10 μg/ml) for 16 hr and then fractionated into their nuclear and cytoplasmic components. Each was then assayed for its content of type-C virus-specific RNA by molecular hybridization.

As shown in Fig. 2, nuclear and cytoplasmic components of drug-treated cells showed similar 3-fold increases in virus-specific RNA. In addition, the relative increase in viral RNA synthesis was greater in nuclear than in cytoplasmic fractions. This result indicates that the viral RNA is more highly concentrated in the nucleus than in the cytoplasm.
specific RNA concentration as compared to untreated cells. In each case, the increase in viral RNA was absolute since the total RNA of each subcellular component was found not to change by more than 10% during the period of drug exposure (data not shown). These results were consistent with the possibility that protein synthesis inhibitors increased virus-specific RNA at a transcriptional level.

Effect of Inhibition of RNA Transcription on Decay of Virus-Specific RNA in Induced Cells. The fate of induced viral RNA was next analyzed under conditions in which further RNA transcription was blocked. Under these conditions, the induced viral RNA should decay as a result of cellular degradative processes. If translational inhibitors primarily act to increase the rate of virus-specific RNA synthesis, the levels of viral RNA would decrease in the absence of further RNA synthesis with or without continued exposure to the inducer. However, if these drugs act to stabilize nascent viral RNA, the level of induced viral RNA would tend to be maintained under conditions of continued treatment with the inducer in the absence of any further RNA synthesis.

Cells were first exposed to cycloheximide for 8 hr. This resulted in a rise of approximately 2-fold in the cellular concentration of viral RNA (Fig. 3). Preinduction of cells insured that the viral RNA species whose half-life would be measured was that of the induced virus. Following preinduction with cycloheximide, cultures were washed, and fresh medium containing cycloheximide with or without actinomycin D was immediately added. Other preinduced cultures received medium either alone or in combination with actinomycin D. The percent viral RNA in cells under each set of conditions was then measured at specific intervals over the next 16 hr.

As shown in Fig. 3, cultures maintained in the presence of cycloheximide following the initial 8-hr exposure exhibited a further 1.5-fold increase in virus-specific RNA, demonstrating persistence of the induced state over the time course of the experiment. In contrast, cultures released from protein synthesis inhibition at 8 hr exhibited a decrease in their concentration of viral RNA over the next 16 hr, indicating that the induced state was maintained only in the presence of cycloheximide. The decay of induced viral RNA in cells exposed to actinomycin D was more rapid, as would be expected under conditions where further RNA synthesis was inhibited. Its half-life was approximately 8 hr (Fig. 3). Most importantly, exposure of actinomycin-D-treated cells to cycloheximide did not prevent viral RNA decay.

DISCUSSION

The discoveries that certain drugs (3, 4, 10) efficiently induce endogenous type-C virus from mouse cells have made it possible to investigate the cellular mechanisms that normally regulate the expression of these naturally integrated viral genes. The present report demonstrates that drugs that inhibit several different steps in translation were each capable of inducing marked elevations of virus-specific RNA, supporting the contention that this effect is mediated by translational inhibition. The rapid rise in viral RNA following exposure to the inducer suggests that this may be a proximal event in the induction process.

Previous studies have shown that inbred mouse strains contain genetic information of several distinguishable endogenous viruses, only one of which is efficiently induced by protein synthesis inhibitors (9, 10). NIH Swiss and NZB strains lack this inducible virus but contain information and express antigens of another virus class. In the present studies, the steady state levels of RNA of this latter virus in NIH Swiss and NZB cells were shown to be unaffected by translational inhibitors under conditions where induction of viral RNA was readily demonstrated with BALB/c and with F1 hybrid embryo cells of BALB/c crossed to either of two different noninducible strains. Analysis of the induced viral RNA by the extent of hybridization achieved with the BALB:virus-2 cDNA probe and the $f_m$ of the hybrids formed indicated that the induced virus-specific RNA represented that of BALB:virus-2. Thus, the present findings support the hypothesis that translational inhibitors specifically induce viral RNA of this class of endogenous virus and, further, that its induction is a dominant genetic characteristic.

Two lines of evidence indicate that the increase in viral RNA associated with protein synthesis inhibition reflects transcriptional derepression rather than an alteration in viral RNA processing. First, the levels of virus-specific RNA were proportionally increased in the nucleus and cytoplasm of the drug-treated cell. These data are consistent with a mechanism involving increased viral RNA transcription, since an alteration in viral RNA processing might be expected to lead to a relative accumulation of viral RNA in one or the other of these subcellular compartments (24). More direct evidence for a transcriptional effect was derived from studies of the rates of decay of cycloheximide-induced viral RNA under conditions where further cellular RNA synthesis was blocked by actinomycin D. Findings that the decay of induced viral RNA in actinomycin-D-treated cells was not prevented by exposure to the inducer weighed heavily against the possibility that translational inhibitors act to stabilize viral RNA. Instead, the present results strongly suggest that the increased levels of viral RNA resulting from translational inhibition are mediated at the level of viral RNA transcription.
The half-life of induced viral RNA, measured in the presence of actinomycin D, was around 8 hr. This is in good agreement with the reported functional half-life of Rauscher mouse leukemia viral RNA in chronically infected mouse cells treated with actinomycin D (25). By a variety of techniques, the half-lives of several other messenger RNAs in mouse cells were found to range from 8 to 12 hr (26–28). Actinomycin D has been reported to decrease the half-lives of some cellular messenger RNAs (29). In the present studies, the half-life of viral RNA determined in the presence of a high concentration (50 μg/ml) of the adenine analogue cordycepin was very similar to that observed with actinomycin D. Since this drug inhibits cellular RNA synthesis by a mechanism that differs from that of actinomycin D, it is unlikely that actinomycin D selectively increases the degradation of induced viral RNA.

The above experimental findings are most consistent with a model in which the expression of one class of endogenous mouse type-C virus is regulated by a labile repressor protein acting at the transcriptional level. According to this model, protein synthesis inhibition would lead to decay of this protein, resulting in derepression of viral RNA synthesis. The actual release of virus would be expected to occur only following release from translational inhibition and would be likely to persist only until the control protein returned to its pretreatment level. This hypothesis is consistent with previous studies indicating that induction in response to inhibitors of protein synthesis requires cellular RNA synthesis during drug exposure and is rapidly reversed following drug removal (11).

Disruption of normal cellular metabolism by a mechanism involving translational inhibition has been reported to affect gene regulation in other systems as well. For example, amino acid deprivation can induce colicins in bacteria (30) and cause release of the DNA tumor virus simian virus 40 from nonpermissively transformed hamster cells (31). The availability of specific inducers as well as probes for the transcriptional and translational products of endogenous type-C viral genes makes it possible to study how genetic factors affect the biologic expression of these viruses. Such studies may also be useful in developing a better understanding of general mechanisms involved in eukaryotic gene regulation.

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