

# Kinetics of decay in the expression of interferon-dependent mRNAs responsible for resistance to virus

(interferon action/antiviral kinetics/5,6-dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole)

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**ABSTRACT** We used 5,6-dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole (DRB), a selective and reversible inhibitor of mRNA production, to investigate the regulation of the pathway leading to resistance to viruses in cells treated with interferon (IF). DRB allows initiation of transcription but promotes premature termination of the nucleotide chains, so that it abolishes interferon-dependent protection against viruses. When the DRB is removed, synthesis of complete mRNAs can resume. Mouse L-929 cells were exposed to 100  $\mu$ M DRB before and during a 1-hr pulse of IF followed by treatment with antibody to IF to prevent cell-to-cell spread of IF after that time. At different intervals thereafter the cells were washed and the DRB was replaced by medium; after further incubation, the cells were infected with vesicular stomatitis virus. Resistance to virus was inversely proportional to the duration of the block imposed by DRB. When the DRB was removed soon after the IF pulse, substantial protection from virus ensued, but none developed when removal of the DRB was deferred for 5-6 hr. Cells exposed to DRB for 5 hr, then pulsed with IF for 1 hr, still mounted a strong antiviral response. The data show that the ability of cells to resist viral infection decays within 5-6 hr after treatment with IF. Whether the decay is due to shutoff of transcription of mRNAs, or to their destruction or degradation, or whether regulation takes place at one or more subsequent steps in the antiviral pathway, remains to be determined.

The fact that the protection against viruses conferred upon cells by exposure to homologous interferon (IF) depends upon new mRNA synthesis was inferred from experiments in which actinomycin D was found to abolish the antiviral response when it was administered at the same time as the interferon (1, 2). As the time between treatment with IF and the addition of actinomycin was extended, so that longer periods of mRNA synthesis were permitted, the antiviral response reappeared and increased, until after several hours of delay in administering actinomycin, the drug no longer blocked establishment of the antiviral state (3, 4). It was deduced that the amount of protection from viral infection is proportional to the amount of IF-dependent mRNAs made and that synthesis of a protective quantity of such mRNAs is achieved by the time that actinomycin treatment no longer prevents resistance to viruses. Experiments with enucleated cells supported these findings (5, 6), as have recent studies of enzymes that inhibit mRNA translation (7-9). They include oligoisoadenylate synthetase and eukaryotic initiation factor 2 (eIF-2) protein kinase. These enzymes, and also a 2',5'-phosphodiesterase, have recently been isolated from IF-treated cells (10-13), as has an associated mRNA (12). The enzymes display IF dose dependence similar to that shown for development of resistance to viruses (13), and the induction of the isolated IF-dependent proteins is blocked by exposure of the cells to actinomycin early after IF treatment (12-14).

The experiments reported here had a different aim from

those just mentioned. The goal of our experiments was to estimate how long the cell remains committed to the expression of the IF-dependent transcriptional responses that result in virus resistance, once transcription is completely switched on through the action of transmembrane or other IF-induced signal molecules. Such experiments are now practicable with the aid of 5,6-dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole (DRB), a selective and reversible inhibitor of heterogeneous nuclear RNA and mRNA syntheses (15-17).

Inhibition of mRNA synthesis imposed by DRB is rapid and the inhibition is promptly reversed after removal of the DRB by medium change (18). Recent studies indicate that DRB permits uninterrupted initiation of transcription but promotes premature termination of nucleotide chains (19, 20). It has only a minor effect on protein synthesis when cells are treated for several hours (15, 16, 18).

We postulated that cells treated with IF would be capable of initiating transcription of IF-dependent mRNAs during a period of DRB exposure but that the premature termination of the nucleotide chains would render them incapable of coding for the respective antiviral proteins. We presumed that if the interval between treatment with IF and removal of the DRB were not excessive, complete mRNA chains would begin to appear soon after the DRB block was lifted and that this would be manifested as the acquisition of resistance to viral infection. Within limits, a subsequent decay or shutoff in the synthesis or expression of IF-dependent mRNA could also be observed if the interval between IF treatment and DRB removal were sufficiently prolonged. Data from experiments designed to test these assumptions indicate that cellular commitment to the expression of IF-dependent mRNAs for protection against viruses does decay within a relatively few hours after the administration of a 1-hr pulse of interferon terminated by antibody to interferon, perhaps due to negative regulation of one or more steps in the antiviral pathway.

## MATERIALS AND METHODS

**Cell Cultures and Viruses.** Mouse L-929 cells obtained from the American Type Culture Collection were grown in Eagle's basal medium (BME) plus 10% fetal calf serum (FCS). Coverslip monolayers used in experiments contained approximately  $8 \times 10^4$  cells in BME/2% FCS. They were incubated at 37°C in 35-mm plates. Like cultures in Microtiter wells, coverslip monolayers require only small reagent and inoculum volumes (0.1 ml); but unlike Microtiter cultures, the cells remain attached after repeated fluid additions and washings. Mouse C-243 cells were obtained from R. H. Bassin, National Institutes of Health.

Abbreviations: DRB, 5,6-dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole; IF, interferon; MIF, mouse interferon; anti-MIF, antibody to mouse interferon; eIF-2, eukaryotic initiation factor 2; BME, Eagle's basal medium; FCS, fetal calf serum; VSV, vesicular stomatitis virus; NDV, Newcastle disease virus; PFU, plaque-forming units.

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They were cultivated in BME plus 10% heat-inactivated FCS. Vesicular stomatitis virus (VSV), Indiana serotype, was plaque purified three times in L-929 cells before a stock virus pool of a large-plaque variant was grown in secondary chicken embryo fibroblasts. The stock virus titer was  $4 \times 10^8$  plaque-forming units (PFU)/ml. Newcastle disease virus (NDV) was propagated in the allantoic cavity of 10- to 11-day-old embryonated chicken eggs and yielded 2000 hemagglutinating units per ml of allantoic fluid. NDV used for IF induction was pelleted by centrifugation for 2 hr at  $66,000 \times g$  at  $4^\circ\text{C}$ , and the virus was resuspended in half the original volume with cold phosphate-buffered saline. Samples of all viruses were kept at  $-70^\circ\text{C}$ .

**Interferon and Antibody to Interferon.** Mouse interferon (MIF) was induced in C243-3 cells with NDV by the method of Tovey *et al.* (21). Cells were exposed to NDV for 1 hr at  $37^\circ\text{C}$  at a multiplicity of 10 PFU/ml. Residual virus was removed by rinsing and cells were re-fed with BME/2% FCS. After further incubation at  $37^\circ\text{C}$  for 24 hr, the culture fluid was harvested, clarified by low-speed centrifugation, and treated with 1 M HCl to bring the pH to 2.0. After further treatment for 5 days at  $4^\circ\text{C}$  the clarified supernatant containing the MIF was readjusted to pH 7.5 with 1 M NaOH, then centrifuged at  $100,000 \times g$  for 1 hr at  $4^\circ\text{C}$  and filtered through a Millipore 0.22- $\mu\text{m}$ -pore-diameter membrane. Samples of the processed MIF were stored at  $-70^\circ\text{C}$ . Infectivity tests on monolayers of chicken fibroblasts or in embryonated eggs showed that no infectious NDV was present in the MIF preparations. The preparations satisfied the criteria of Lockart (22) for virus-induced interferons. The antiviral activity of the crude MIFs, when assayed by the procedure of Dahl and Degre (23), closely approximated that of National Institute of Allergy and Infectious Diseases reference MIF (no. G002-904-511).

Partially purified fibroblast MIF and antibody to mouse fibroblast IF (anti-MIF) were gifts from Kurt Paucker, Medical College of Pennsylvania. The MIF had been partially purified by antibody affinity chromatography and titered  $6.8 \times 10^5$  units/ml. The anti-MIF inhibited  $3.8 \times 10^3$  reference units of fibroblast MIF per ml.

MIF was warmed to  $37^\circ\text{C}$  before use.

**DRB.** This chemical was obtained from Calbiochem. It was dissolved in dimethyl sulfoxide, then diluted 1:100 in BME to give a solution containing 1000  $\mu\text{g}/\text{ml}$  and filtered through an 0.22- $\mu\text{m}$  Millipore membrane. It was diluted in serum or amino-acid-free BME/2% FCS before use at 100  $\mu\text{M}$  final concentration in MIF, anti-MIF, or BME/2% FCS.

**Incorporation of [ $^3\text{H}$ ]Uridine into RNA.** We used the method devised by Tamm *et al.* (18) to determine the rapidity and extent of the inhibition of RNA synthesis of L-929 cells exposed to 100  $\mu\text{M}$  DRB and the release of the inhibition after a medium change. After exposure to [ $^3\text{H}$ ]uridine (ICN) at a concentration of 2.5  $\mu\text{Ci}/\text{ml}$  (1 Ci =  $3.7 \times 10^{10}$  becquerels), cells were washed four times with ice-cold phosphate-buffered saline and the drained monolayers were frozen at  $-70^\circ\text{C}$  and scraped into 1 ml of water. Each determination was done in triplicate.

## RESULTS

**Action of DRB on L-929 Cells.** We needed to confirm that, as Tamm *et al.* (18) reported, inhibition of L cell RNA synthesis by DRB promptly reverses after removal of the DRB, and that protein synthesis is not materially suppressed during the treatment periods to be employed in experiments. Tamm *et al.* (18) found that 90  $\mu\text{M}$  DRB inhibits RNA synthesis in L-929 cells almost maximally within 15 min after the beginning of treatment. On the basis of their dose-response curves, we employed 100  $\mu\text{M}$  DRB to verify that the L-929 cells we used

would respond similarly. This dose of DRB inhibited RNA synthesis 55% after 1 hr of treatment and 69% after 5 hr of treatment and, within 15 min after the DRB was removed, incorporation of uridine into acid-precipitable form was restored to pretreatment rates (Table 1). Our findings with 1-hr and 5-hr periods of treatment with 100  $\mu\text{M}$  DRB were consonant with published observations (15, 16, 18) of both the extent and reversibility of inhibition of RNA synthesis by DRB and verified the facts that DRB in doses that maximally depress RNA synthesis has only a minor effect on protein synthesis for a period of several hours and that L cells rapidly recover their ability to make protein after removal of DRB.

### IF-Induced Resistance to Virus After Removal of DRB.

Preliminary experiments showed that, for our purposes, total VSV output was a consistent and reliable index of IF-induced antiviral activity and was technically more convenient than other available assays. Although replication of a number of viruses is restricted by halobenzimidazole ribonucleosides such as DRB (15), we found that VSV exhibited the same single-cycle kinetics of growth in the presence of 100  $\mu\text{M}$  DRB as in its absence (Fig. 1). This result assured us that treatment of cells with DRB followed by its removal would not skew experiments that relied upon measurements of VSV output to quantify antiviral protection.

It was necessary to determine a precise time zero in order to establish accurate intervals between the cessation of IF treatment and withdrawal of the DRB block by medium change. MIF was administered mixed with DRB. After the MIF was removed at the end of 1 hr, the cells were treated with anti-MIF in DRB. This prevented repeated cycles of elution and re-binding of IF; the results of direct cell-to-cell transfer experiments (unpublished data) have recently fortified prior evidence (24, 25) that recycling takes place and that anti-MIF prevents it.

Two separate experiments with 2048 units of crude MIF showed that, as the interval between the termination of IF treatment and the removal of the DRB increased, protection from virus decreased (Fig. 2). The cells became completely susceptible to viral infection after 4-5 hr. The disappearance of the IF-mediated inhibition of viral replication was not due to inability of the cells to respond to IF after 5 hr of exposure to DRB; cells exposed first to DRB for 5 hr, then treated for an additional hour with MIF in DRB, followed by anti-MIF in DRB for 15 min, still mounted a strong antiviral response.

There was not only a more pronounced suppression of virus

Table 1. Reversibility of the inhibitory effect of 100  $\mu\text{M}$  DRB on [ $^3\text{H}$ ]uridine incorporation in L-929 monolayers

Medium		[ $^3\text{H}$ ]Uridine incorporation			
Before pulse	During 15-min pulse	Protein, cpm	Protein, $\mu\text{g}$	cpm/ $\mu\text{g}$ protein	% of control
BME, 1 hr	BME (control)	2661	178	14.9	100
DRB, 1 hr	DRB	1809	270	6.7	45
DRB, 1 hr	BME	7639	222	34.4	230
DRB, 5 hr	DRB	975	210	4.6	31
DRB, 5 hr	BME	3362	220	15.2	102

Each part of the experiment was done in triplicate; the data are averages. Incomplete monolayers of L-929 cells in 35-mm plates were exposed to 2 ml of either serum-free BME (control) or of 100  $\mu\text{M}$  DRB in serum-free BME. They were incubated at  $37^\circ\text{C}$  and the medium was removed from the plates either 1 hr or 5 hr thereafter. The medium was then replaced with either control (after washing four times) or DRB-containing serum-free BME and [ $^3\text{H}$ ]uridine (specific activity 27 Ci/mmol) was added to a final concentration of 2.5  $\mu\text{Ci}/\text{ml}$ . Acid-precipitable cpm/ $\mu\text{g}$  of protein were then determined in duplicate by the method described by Tamm *et al.* (18).

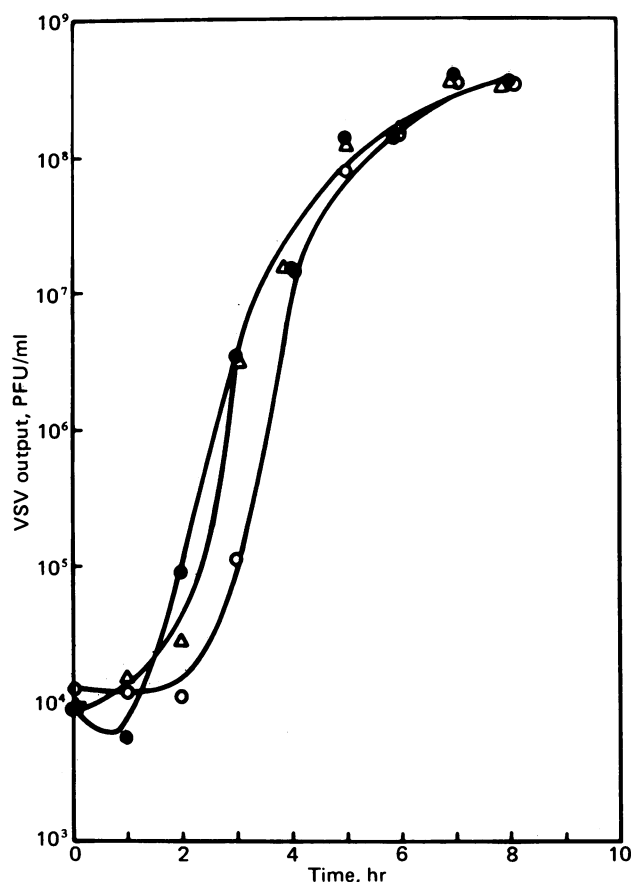


FIG. 1. Kinetics of replication of VSV in L-929 cells at 37°C in DRB and after removal of DRB. DRB was 100  $\mu$ M in BME/2% FCS. Cells in 35-mm plates were treated with DRB for 1 hr, then washed three times with BME/2% FCS (●); were untreated (○); or were treated with DRB for the duration of the experiment (△). They were exposed to  $5 \times 10^6$  PFU of VSV in 0.1 ml for 1 hr, washed three times with BME/2% FCS, and incubated in the same medium. At intervals plates were frozen and thawed and VSV output was assayed by plaque titration.

replication but also a slower decay of virus resistance revealed by DRB in cells treated with high concentrations of purified IF compared with cells exposed to 2048 units/ml of IF (Fig. 3). The decay curve for 23,000 units/ml of MIF was approximately the same as that for 230,000 units/ml, suggesting that 23,000 units/ml of MIF induced a maximal mRNA response. Although the crude 2048-units/ml MIF used in this experiment was a different sample from the samples employed in the prior experiments (Fig. 2), decay curves for the three different 2048-units/ml samples closely resembled one another.

### DISCUSSION

Kinetic experiments such as those reported here mandate the establishment of a time zero. To accomplish this we took advantage of the facts that IF prewarmed to 37°C binds to cells rapidly (26) and that its spontaneous elution (24, 27) and re-binding (25) to other cells can be interrupted by exposure to anti-IF (28). Termination of a 1-hr IF pulse with anti-MIF prevented consecutive cycles of elution and re-binding of MIF, which might have continued seriatim for the duration of the experiment and resulted in stimulation of some cells with MIF after removal of the DRB. This would have generated uninterpretable data.

Indirect evidence that IF-mediated mRNA (1, 2) and protein (29) syntheses are required for the development of virus resis-

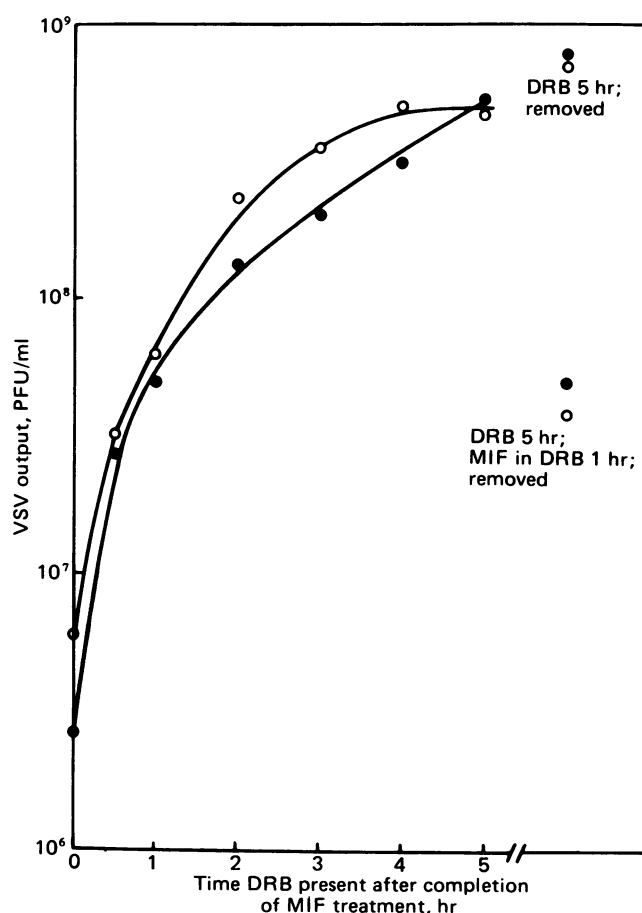


FIG. 2. Decay in resistance to virus with increasing duration of exposure to DRB after completion of an MIF pulse delivered to DRB-inhibited L-929 cells. Two separate experiments are shown (● and ○). They were done in triplicate. MIF and anti-MIF antibody always contained 100  $\mu$ M DRB made up in BME/2% FCS. Coverslip monolayers containing approximately  $8 \times 10^4$  cells were successively treated as follows: 100  $\mu$ M DRB for 1 hr; MIF for 1 hr, removed by five washes of 100  $\mu$ M DRB; anti-MIF antibody for 15 min; and continued exposure to 100  $\mu$ M DRB until the DRB was removed at different intervals and replaced (after wash) with BME/2% FCS. Cells were then incubated at 37°C for 18–20 hr to allow development of antiviral activity, inoculated with  $10^3$  PFU of VSV, and incubated again for 24 hr. Supernatant fluids from triplicate coverslips were assayed for VSV output. The output of coverslips treated with DRB for 5 hr but not exposed to an MIF pulse is labeled "DRB 5 hr; removed." It closely approximated the output of coverslips treated with neither DRB nor MIF (not shown). The output of coverslips treated only with an MIF pulse (not shown) approximated the plotted output of those coverslips treated with both DRB and MIF from which DRB was removed at time zero, the end of the MIF pulse. Also plotted is the output of coverslips exposed to DRB for 5 hr before being treated with MIF for 1 hr and anti-MIF antibody for 15 min. The DRB was then removed by washing, as the label indicates, and further incubation and assay for VSV output were carried out as described above.

tance has recently been validated by the detection or isolation of mRNAs (12) and proteins (11–14) from IF-treated cells. Several proteins have been identified. Two are double-stranded-RNA-dependent enzymes. The first, oligo(adenylate synthetase, catalyzes the synthesis of  $\text{pppA}(2'\text{p}5'\text{A})_n$ , in which  $n$  is 2 to 5 (10). The trimer  $\text{pppA}2'\text{p}5'\text{A}2'\text{p}5'\text{A}$  is predominant. These oligo(adenylate products activate a ribonuclease, already present in untreated cells, that degrades mRNA (30–34). The second enzyme is a specific protein kinase that phosphorylates both a 67,000  $M_r$  protein and the small subunit of eIF-2, inactivating the latter and thus preventing mRNA translation

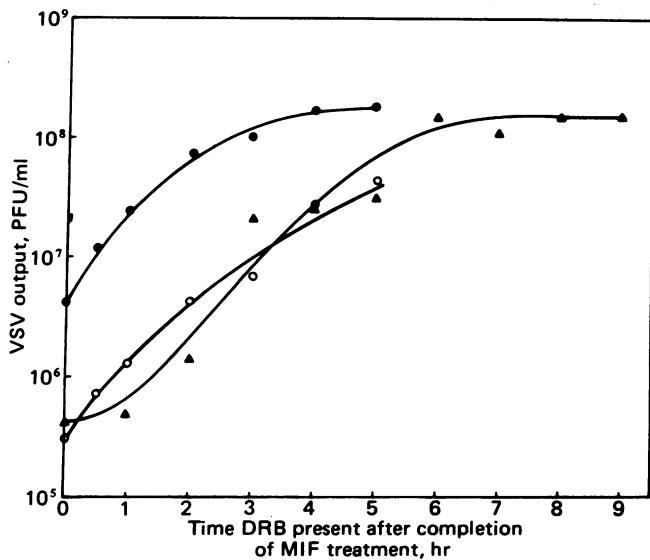


FIG. 3. Comparison of cells exposed to low and high doses of MIF with respect to the decay of IF-mediated virus resistance during treatment with DRB for various periods after completion of MIF treatment. ●, Crude MIF at 2048 units/ml; ○, purified MIF at 23,000 units/ml; ▲, purified MIF at 230,000 units/ml. Procedures and controls were those described for Fig. 2. VSV outputs of controls, which closely resembled those presented in Fig. 2, are not plotted. The sample of crude MIF containing 2048 units/ml was different from the samples used in the experiments shown in Fig. 2.

(7–9, 13, 31). Both enzymes exhibit IF dose dependence paralleling that for the appearance of resistance to viruses (13).

The action of these double-stranded-RNA-dependent enzymes might be regulated by two other enzymes also found in IF-treated cells (13). One is a phosphoprotein phosphatase that dephosphorylates both the 67,000  $M_r$  protein and eIF-2. It might act to restore translation of viral mRNA and hence regulate the antiviral activity of IF. The second enzyme, a phosphodiesterase, cleaves the 2'-phosphodiester bonds of  $\text{ppp}(2'\text{-p5'A})_n$  and also degrades tRNA. After IF treatment, this enzyme appeared more rapidly than the others, reaching a maximum in 8 hr under the conditions employed, and it has been suggested (13) that it might regulate the amount of protein synthesis not only by lysing  $\text{ppp}(2'\text{-p5'A})_n$  but also perhaps by damaging tRNA. When actinomycin was added 3 hr after the beginning of IF treatment there was an augmented yield of both oligoisoadenylate synthetase and of eIF-2 protein kinase, implying that these IF-mediated protein products accumulated (13). Enhancement of virus resistance by actinomycin administration during IF treatment had previously been noted (4). These findings are those expected if there is negative control of IF-dependent mRNA expression achieved by means of actinomycin-sensitive transcription of an mRNA for a regulatory protein.

Our kinetic experiments measured only the total of virus resistance attained when the synthesis of competent mRNAs for antiviral effector or regulatory proteins was prevented by administering DRB for various periods of time after IF treatment was completed. The amount of virus resistance must represent the outcome of an interplay between the activation of distinct antiviral pathways, two of which are now recognized (31), and their regulation by several possible mechanisms. Our studies became feasible because of recent progress in elucidating the mechanism of action of DRB, a specific and reversible inhibitor of mRNA synthesis (15, 16). We corroborated the fact that, in L cells, DRB inhibits RNA synthesis without significantly affecting protein synthesis during the 5- to 6-hr periods

employed for our experiments and that both the imposition and removal of the DRB block are relatively rapid. Insight into the mechanism of DRB action has come from studies of adenovirus and HeLa cell transcription. Although adenovirus mRNA synthesis continues during exposure to DRB, the nucleotide chains are prematurely terminated or broken (19, 35, 36), and DRB increases the frequency of early termination of heterogeneous nuclear RNA precursor molecules in uninfected HeLa cells (20). We inferred that IF-dependent mRNAs might respond likewise to DRB, that the resulting incomplete mRNA nucleotide chains might be incapable of coding for antiviral proteins, and that after removal of DRB, any IF-mediated mRNAs made could be faithfully translated if the mRNAs remained intact. The ability of cells to resist infection with a challenge virus would therefore reflect the amount of competent IF-dependent mRNAs available after the termination of the IF pulse. In concert with transcriptional or posttranscriptional regulation of mRNA expression, DRB-mediated effects would be revealed as changes in the amount of protection against viruses, the kinetics of which we could determine. We found that if there is a DRB-imposed delay of 5–6 hr in permitting complete IF-dependent mRNA chains to be synthesized, the antiviral state is not established, although control experiments showed that cells exposed to DRB for that length of time, then treated with IF, were responsive to IF and capable of resisting infection with the challenge virus.

To the best of our knowledge, DRB has not previously been used to investigate the antiviral pathway induced by IF. It has, however, been employed in interesting studies of IF production. Properly timed treatment of cultures with inhibitors of RNA or protein synthesis after administration of IF inducers leads to "superinduction," i.e., a marked increase of IF yields (37). This is due to a posttranscriptional mechanism which, among other possible effects, appears to inactivate or degrade the mRNA for IF protein synthesis 6–8 hr after the cells are treated with IF inducers (38). Superinduced cells continue to secrete IF for as long as 4 days. Removal of DRB at any time during the first 24 hr triggers quick cessation of IF synthesis (39); hence the cells must remain committed for 24 hr to the synthesis of a mRNA that regulates IF production.

Our data indicate that there is also a mechanism that regulates the ultimate manifestation of virus resistance of IF-dependent mRNAs for antiviral proteins. The step at which regulation takes place has not been identified. Whether the mechanism is shutoff of mRNA transcription degradation or destruction of the mRNAs, restriction of enzyme action, lysis of intermediates such as oligoisoadenylate (perhaps by 2'-phosphodiesterase) or phosphorylated eIF-2 (perhaps by phosphoprotein phosphatase), or whether (as is likely) different mechanisms operate for the different antiviral pathways that apparently exist is unknown. It appears, however, that after an IF pulse the cell is committed for only a few hours to the full expression of the mRNAs that code for antiviral effector proteins.

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