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Increased nuclease activity in cells treated with pppA2′p5′A2′p5′A

(protein synthesis/interferon action/reticulocyte lysates/calcium phosphate coprecipitation)

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ABSTRACT **A series of 2′-5′-linked oligo(adenylic acid) triphosphate (2′-5′A) inhibitors of protein synthesis were described recently. These inhibitors are synthesized from ATP by an enzyme activated in interferon-treated cell extracts or rabbit reticulocyte lysates by double-stranded RNA. We show here that 2′-5′A is a potent inhibitor of protein synthesis in intact cells of different origin (human, monkey, hamster, and mouse). At a concentration of 10 nM (in AMP equivalents), protein synthesis is inhibited by 50–85%. There is also a secondary effect on the total RNA synthesis which becomes evident several hours after inhibition of protein synthesis. All of these effects, however, are transient and, after a recovery period, both RNA and protein synthesis resume rates comparable to the appropriate controls. A nuclease activity is detected in cells after treatment with 2′-5′A. The total polyadenylylated RNA is much reduced in comparison to that from untreated cells, and electrophoretic analysis in polyacrylamide slab gels provides evidence for its degradation. Similarly, there is an apparent degradation of ribosomal RNA. Consistent with these results, extracts from cells that had been treated with 2′-5′A manifest an enhanced nuclease activity *in vitro* on incubation with exogenous RNA. Here, we propose that, as in cell-free systems, the mechanism of action of 2′-5′A in intact cells involves activation of a nuclease. This activation is transient, but the nuclease remains sensitive to further activation by the inhibitor.**

Protein synthesis in cell-free systems from interferon-treated cells shows an enhanced sensitivity to inhibition by double-stranded RNA (dsRNA) (1–3). A protein kinase(s), 2′-5′A synthetase [responsible for the formation of a series of 2′-5′-linked oligo(adenylic acid) triphosphate (2′-5′A) inhibitors of protein synthesis in which the trimer pppA2′p5′A2′p5′A is predominant], and a nuclease may all be involved in this inhibition (4–12). The kinase is thought to phosphorylate the smallest subunit of initiation factor eIF2, as appears to be the case in rabbit reticulocyte lysates, on inhibition of protein synthesis by a number of agents, including dsRNA (12–16). The 2′-5′A synthetase could be retained on a column of dsRNA bound to a solid support and conveniently used in this form to synthesize large quantities of the inhibitor (7, 16). An identical series of such oligonucleotide inhibitors is also formed on incubation of reticulocyte lysates with dsRNA and ATP (ref. 17; E. M. Martin, N. G. M. Birdsall, R. E. Brown, and I. M. Kerr, personal communication). These oligonucleotide inhibitors are effective at subnanomolar concentrations in cell-free systems prepared from control cells, and their effect seems to be mediated, at least in part, by a nuclease that degrades mRNA (18–20).

Recently, Williams and Kerr (21) reported that 2′-5′A inhibits protein synthesis in BHK21 cells made permeable by incubation in hypertonic medium (22). Here, we confirm and extend this observation by using a calcium phosphate coprecipitation technique (23) to introduce 2′-5′A into a variety of different cell types. This latter technique appears to be less harmful, since no gross apparent effects were observed on the morphology or the metabolism of the cell after calcium phosphate treatment, and thus allows *in vivo* analysis of the development, duration, and mechanism of action of the inhibitory process.

MATERIALS AND METHODS

Materials. All radiochemicals were supplied by the Radiochemical Centre (Amersham, England). Vesicular stomatitis virus (VSV) [^{3}H]mRNA was prepared and purified by affinity chromatography on oligo(dT)-cellulose (24, 25). Rabbit reticulocyte lysates and L cell-free systems were as described (26, 27). Turnip yellow mosaic virus RNA (generous gift of J. Bové) was translated in the L cell-free system under conditions similar to those reported for encephalomyocarditis virus RNA (27, 28). 2′-5′A was synthesized from ATP by the use of an enzyme fraction from rabbit reticulocyte lysates bound to poly(I)-poly(C)-Sepharose (17). Its partial purification and assay by means of inhibition of ^{14}C-labeled amino acid incorporation in rabbit reticulocyte lysate and L cell-free systems were as described (7). The concentration of 2′-5′A in AMP equivalents was calculated from absorbance on the basis of A_{260} of 15.6×10^3/M·cm for AMP. In addition, the dilution of 2′-5′A required to produce a 50% inhibition of cell-free protein synthesis corresponds to an AMP concentration of about 0.5 nM, as has been described (8, 17). ^{3}H-Labeled 2′-5′A was prepared and purified according to Hovanessian *et al.* (7). Hamster fibroblast BHK21 and hamster sarcoma virus-transformed BHK21 cells (29), VERO cells (a continuous line of African Green Monkey kidney cells that have lost the capacity to produce interferon but remain fully susceptible to its antiviral action) (30), human embryonic fibroblasts (MRC5), and mouse L-929 cells were from frozen stocks.

Measurement of RNA and Protein Synthesis in Intact Cells. Cells were grown in monolayer culture in Falcon flasks and tissue culture dishes in basal medium (Eagle's) with Earle's salts containing 10% newborn calf serum (growth medium) at 37°C in a humidified, 5% CO_2 atmosphere. For the experiments described in Figs. 1 and 2, cells plated at $1.0–1.5 \times 10^5$ per 16-mm tissue culture dish (Linbro, Hamden, CT) were incubated for 1–3 days before the experiment. To measure RNA or protein synthesis, we labeled cells in growth medium (0.5 ml) with [^{3}H]uridine (1 μCi/dish, 40 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) or in growth medium (0.5 ml) lacking methionine but containing [^{35}S]methionine (2 μCi/dish, 750–900 Ci/mmol) for 60 min or as otherwise indicated. At the end of the assay period, cells were washed with phosphate-buffered saline, and 1 ml of 5% trichloroacetic acid was added for 30 min at 4°C. The cells were washed twice with 5% trichloroacetic acid, digested in 1.0 ml of 0.1 M NaOH for 60 min at 40°C, and neutralized with 0.1 ml of 1.0 M HCl. The radioactivity of the entire sample was measured after dilution into 10 ml of liquid scintillant prepared according to Bray (31).

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Abbreviations: 2′-5′A, oligo(adenylic acid) triphosphate (pppA2′p5′A2′p5′A); VSV, vesicular stomatitis virus; dsRNA, double-stranded RNA.

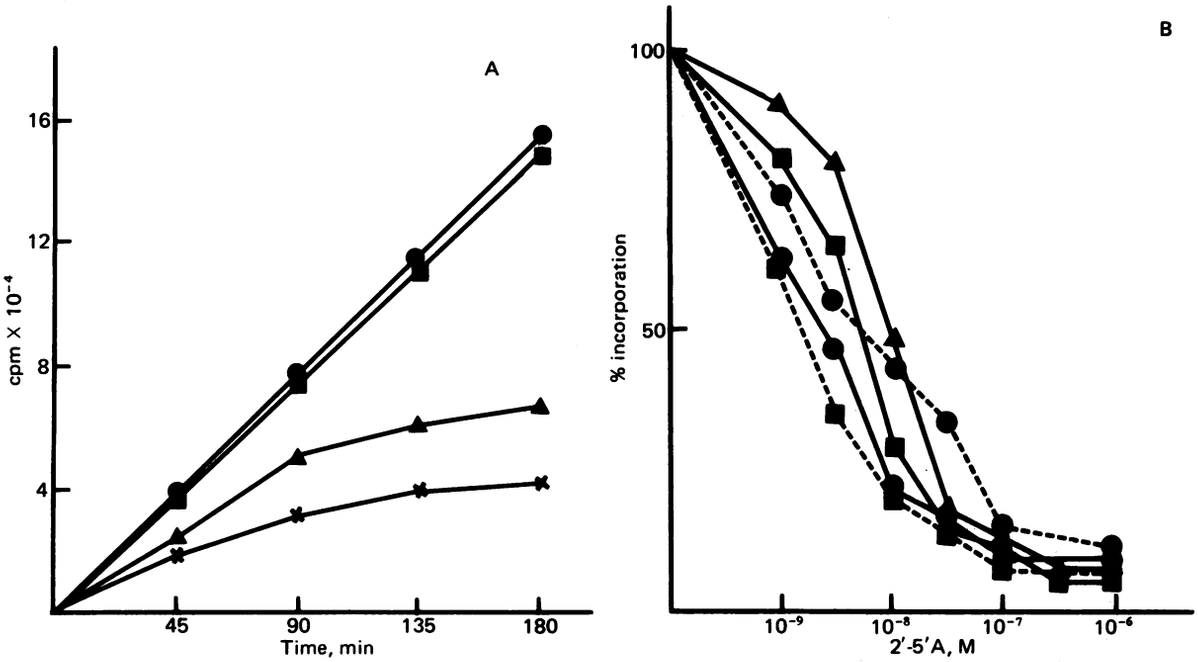

FIG. 1. *In vivo* inhibition of protein synthesis by 2'-5'A. (*A*) Kinetics of protein synthesis in cells treated with 2'-5'A. MRC5 cells were incubated in 0.5 ml of medium lacking methionine (●) or in Hepes-buffered saline (pH 7.05) containing $CaCl_2$ (125 mM) without (■) or with 2'-5'A at 10^{-8} M (▲) and 5×10^{-8} M (x) and [^{35}S]methionine (4 μCi/ml, 900 Ci/mmol). Incubations were at room temperature for the initial 45 min before addition of medium (0.5 ml) to all of the samples and further incubation at 37°C. At the times indicated, cell monolayers were washed with phosphate-buffered saline and processed. The ordinate represents the total [^{35}S]methionine cpm per dish (the mean value of four similar samples). (*B*) Specific inhibitory activity of 2'-5'A on protein synthesis in intact cells. Monolayers of MRC5 (■—■), BHK21 (●- -●), sarcoma virus-transformed BHK21 (●—●), VERO (■- -■), and mouse L929 (▲- -▲) cells were treated with different concentrations of 2'-5'A at 20°C for 45 min before incubation at 37°C for 60 min. Incorporation of [^{35}S]methionine into the cells in the presence of different concentrations of 2'-5'A is expressed as a percentage of the control value (no 2'-5'A). The total [^{35}S]methionine cpm incorporated into the different cells in the absence of 2'-5'A was not less than 50,000 cpm.

Treatment of Cells with 2'-5'A. Growth medium was aspirated and the cell monolayers were incubated in Hepes-buffered saline (pH 7.05) (8.0 g of NaCl, 0.37 g of KCl, 0.125 g of $Na_2HPO_4 \cdot 2H_2O$, 1.0 g of glucose, and 5.0 g of Hepes per liter) (23) containing different concentrations of 2'-5'A as indicated in individual experiments and $CaCl_2$ at a final concentration of 125 mM. After an initial incubation at room temperature for 45 min, the treatment solution was diluted with an equal vol-

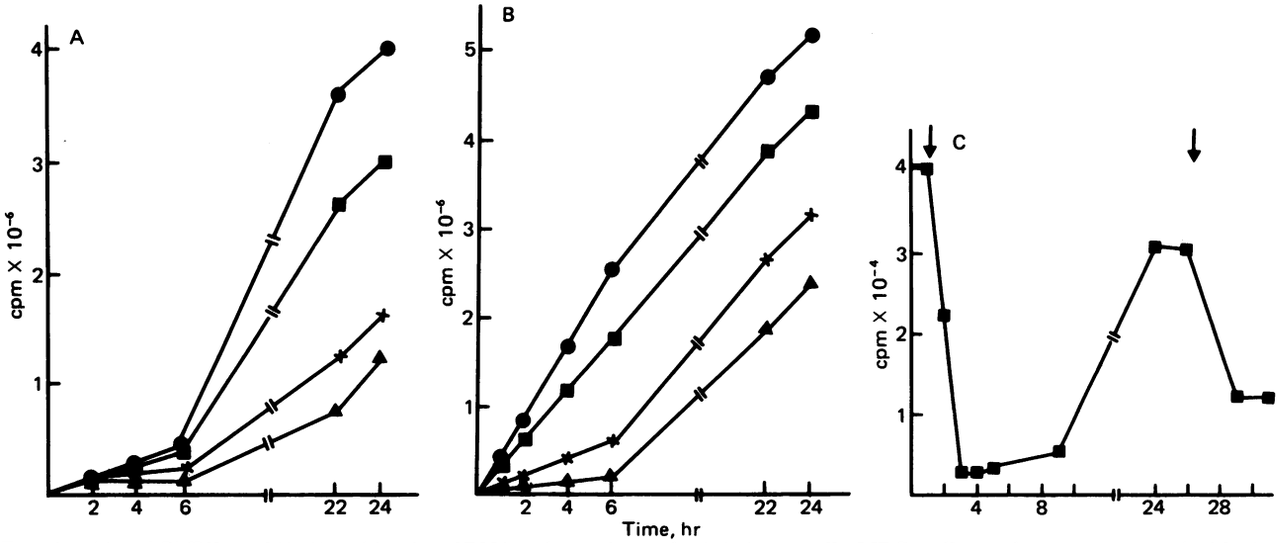

FIG. 2. (*A* and *B*) Effect of 2'-5'A on kinetics of RNA and protein synthesis in intact cells. MRC5 cell monolayers were treated with 2'-5'A, and RNA (*A*) or protein (*B*) synthesis was measured after removal of the inhibitor (0 time). Cells preincubated with medium (●) or with Hepes-buffered saline (pH 7.05) containing $CaCl_2$ in the absence (■) or presence of 2'-5'A at a final concentration of 10^{-8} M (x) and 5×10^{-8} M (▲) were washed with medium and incubated in growth medium containing [^{3}H]uridine (2 μCi/dish) in *A* or [^{35}S]methionine (1.5 μg/ml, 5 μCi/dish) in *B*. At the times indicated, cells were washed with phosphate-buffered saline and processed. The slow rate of RNA synthesis for the first 6 hr may have been due to the incubation of cells in serum-free medium during treatment with 2'-5'A. There was no apparent difference in the number of cells at 24 hr between the control and 2'-5'A-treated cultures. The rate and extent of incorporation of [^{3}H]uridine or [^{35}S]methionine into control cells not treated with Hepes-buffered saline and $CaCl_2$ were not affected by the presence of 2'-5'A during the incubation period (data not shown). (*C*) *In vivo* inhibition of protein synthesis by consecutive treatments with 2'-5'A. The rate of protein synthesis was measured in MRC5 cell monolayers before, during, and after treatment with 2'-5'A (5×10^{-8} M). Arrows indicate the start of each treatment. Each point (■) represents the end of a labeling period (60 min) in methionine-free medium with 10% serum and [^{35}S]methionine (2 μCi/dish). During the 2'-5'A treatment, [^{35}S]methionine was added directly in the treatment solution.

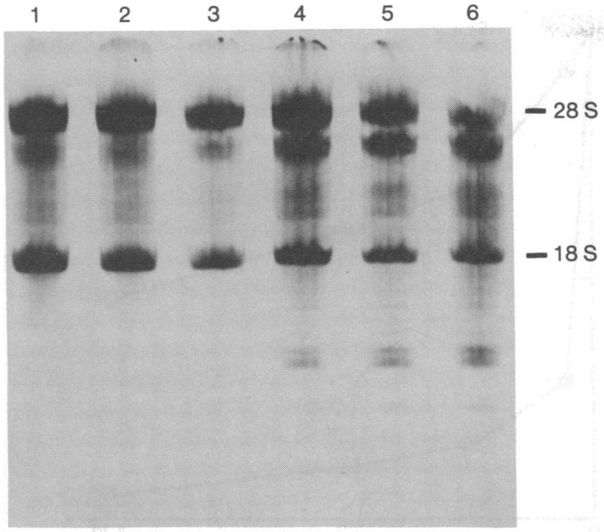

FIG. 3. Electrophoretic profiles of [^{3}H]uridine-labeled RNA from MRC5 cells. Confluent cells (7.5×10^6) in 150-mm Falcon dishes were labeled with 15 ml of medium containing [^{3}H]uridine (25 μCi/ml) for 20 hr at 37°C. The cultures were then washed and incubated with medium (channel 1) or with Hepes-buffered saline (pH 7.05) containing $CaCl_2$ in the absence (channel 2) or presence of 2′-5′A at a final concentration of 10^{-9} M (channel 3), 10^{-8} M (channel 4), 5×10^{-8} M (channel 5), and 10^{-7} M (channel 6) for 45 min at room temperature followed by a further incubation at 37°C for 90 min. The cell monolayers were then washed with phosphate-buffered saline and scraped with a rubber policeman and RNA was extracted as described (33). ^{3}H cpm were 72.1, 94.7, 47.8, 51.4, 44.5, and 39.6×10^5 in 10-μl aliquots of each sample (1 ml), channels 1–6, respectively. Aliquots containing 10^6 cpm were analyzed in 2.6% acrylamide slab gels containing 6 M urea and 0.1% sodium dodecyl sulfate (34, 35). Fluorography was as described (36).

ume of serum-free medium and the cells were incubated at 37°C for 90 min. This liquid medium was then aspirated and the cell monolayers were incubated in growth medium for assay of RNA and protein synthesis as described above. Cell density appeared to have some effect on sensitivity of cells to treatment with 2′-5′A, and subconfluent monolayers gave more reproducible results.

RESULTS

Inhibition of Protein Synthesis by 2′-5′A in Intact Cells. 2′-5′A is a potent inhibitor of protein synthesis in intact cells when its uptake is facilitated by incubation in Hepes-buffered saline and $CaCl_2$ (see *Materials and Methods*). Upon addition of $CaCl_2$ (70–125 mM) to solutions of 2′-5′A (^{3}H-labeled) in the buffered saline a precipitate is formed consisting of 2′-5′A and calcium phosphate which sediments onto the cells, becomes absorbed to the cell membrane, and then can be taken up by the cell. (^{3}H radioactivity is associated with acid-insoluble material when the cells are treated with ^{3}H-labeled 2′-5′A as described for the unlabeled material in *Materials and Methods*.) Inhibition of protein synthesis measured by the incorporation of [^{35}S]methionine into acid-insoluble radioactivity was detectable as soon as 20 min after addition of $CaCl_2$, with maximum inhibitory effects (70–95%) at 90 min (Fig. 1A). In the absence of $CaCl_2$, however, different concentrations of 2′-5′A had no apparent effect on *in vivo* protein synthesis (data not shown). Fig. 1*B* shows the specific inhibitory activity of 2′-5′A in intact cells of different origin: mouse, human, hamster, and monkey. At a concentration of 10 nM, 2′-5′A results in the inhibition of protein synthesis by 50–80%. This is reasonable in comparison with the concentrations required to inhibit protein synthesis in cell-free systems and in intact cells (7, 8, 17, 21).

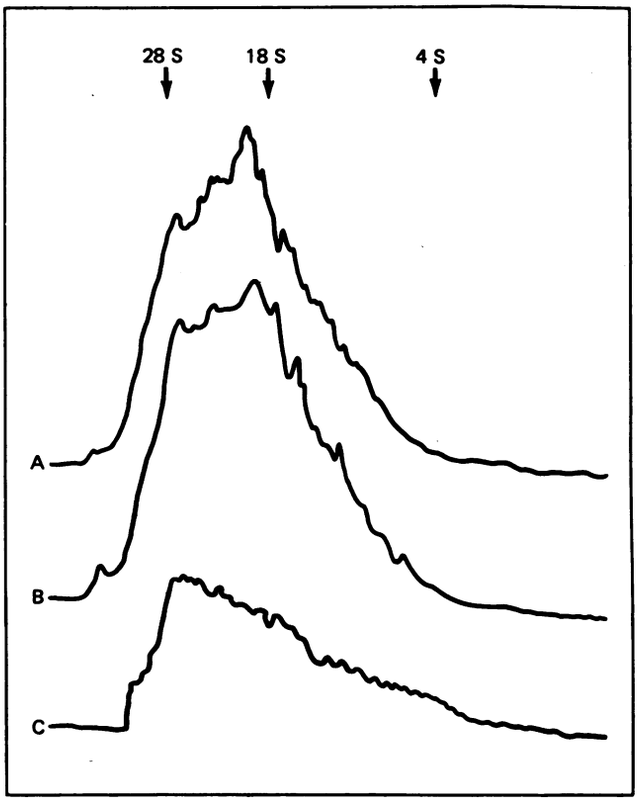

FIG. 4. Electrophoretic profiles of [^{3}H]uridine-labeled polyadenylylated RNA from MRC5 cells. Total ^{3}H-labeled RNA from cells incubated in medium (curve A) or in Hepes-buffered saline containing $CaCl_2$ in the absence (curve B) or presence (curve C) of 2′-5′A at a final concentration of 5×10^{-8} M was prepared as described in the legend of Fig. 3. Polyadenylylated RNA was then prepared from each sample by poly(U)-Sepharose chromatography (Pharmacia). ^{3}H cpm were 64.6, 82.7, and 42.5×10^6 in 10-μl aliquots of each sample (1 ml), curves A–C, respectively, before poly(U)-Sepharose chromatography and 5.1, 4.9, and 1.7×10^6 after chromatography. A scan of the electrophoretic pattern of ^{3}H-labeled polyadenylylated RNA from the fluorography is shown. 28 S and 18 S represent the position of ^{3}H-labeled MRC5 cell marker ribosomal RNA analyzed in parallel.

Kinetics of Inhibition of RNA and Protein Synthesis in Intact Cells after Treatment with 2′-5′A. *In vivo* RNA and protein synthesis after preincubation in Hepes-buffered saline with $CaCl_2$ was not less than 80% of the synthesis in cells that had been preincubated in normal medium (Fig. 2 *A* and *B*). In the presence of 2′-5′A, however, both processes were inhibited at different times. Inhibition of protein synthesis was detectable during the treatment with 2′-5′A (Fig. 1A) at a time when there was no apparent effect on RNA synthesis (data not shown). Inhibition of RNA synthesis was not detectable until 2 hr after removal of 2′-5′A treatment medium (Fig. 2*A*), when protein synthesis was still inhibited (Fig. 2*B*). These results suggest that the observed inhibition of protein synthesis by 2′-5′A is not a consequence of inhibition of RNA synthesis (which was most strongly affected at 4–6 hr after removal of the inhibitor). Such inhibition is probably a secondary consequence of the shutdown of protein synthesis resulting from activation of a nuclease discussed below. Both of these inhibitory effects on RNA and protein synthesis were transient. After a period of recovery, *in vivo* RNA and protein synthesis resumed rates comparable to those of the appropriate controls (Fig. 2 *A* and *B*; incorporation at 22–24 hr). The cells, however, remained sensitive to further treatment by the inhibitor (Fig. 2*C*).

Activation of a Nuclease in Cells Treated with 2′-5′A. Studies on the mechanism of action of 2′-5′A in cell-free systems

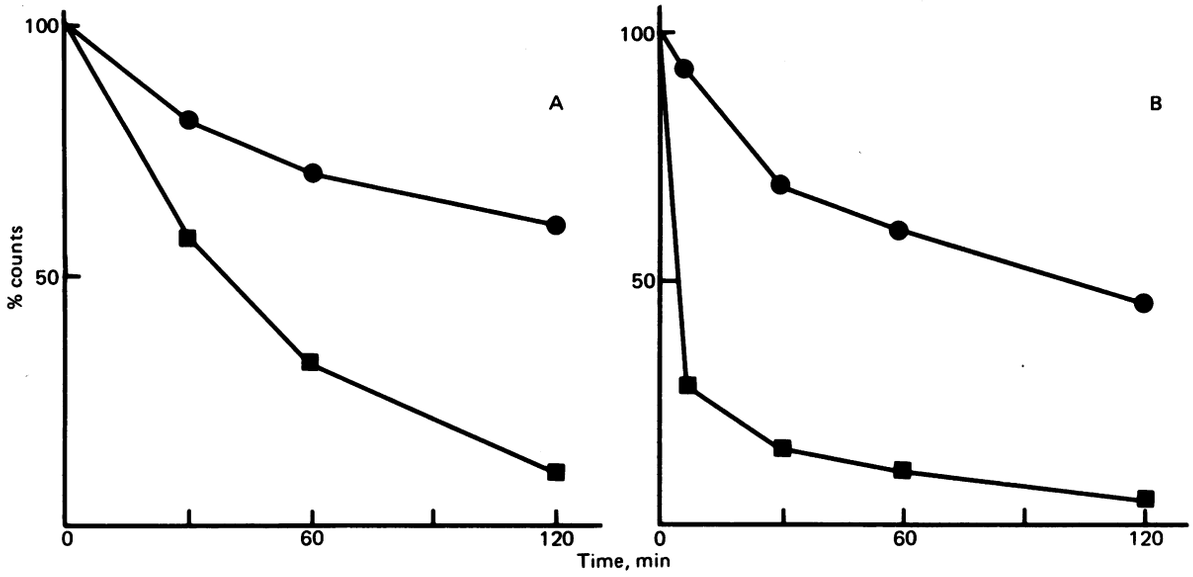

FIG. 5. Involvement of a nuclease in the mechanism of action of 2′-5′A. (*A*) Breakdown of VSV [^{3}H]mRNA on incubation in a control cell-free system from mouse L-929 cells (32) in the absence (●) or presence (■) of 2′-5′A at a final concentration of 5×10^{-8} M. (*B*) Breakdown of VSV [^{3}H]mRNA on incubation in extracts prepared from MRC5 cells (2×10^7) treated in Hepes-buffered saline in the absence (●) or presence (■) of 2′-5′A as above. After 60 min, the cells were washed with Hepes-buffered saline (pH 7.5), scraped, and lysed with an equal volume of buffer [10 mM Hepes, pH 7.5/10 mM KCl/2 mM $Mg(OAc)_2$/7 mM 2-mercaptoethanol]. The concentration of KCl was then brought to 90 mM. The mixture was centrifuged at 10,000 × *g* for 10 min and the supernatant was incubated with VSV [^{3}H]mRNA without any further additions. In *A* and *B*, the VSV [^{3}H]mRNA (2×10^5 cpm/μg) was added to 16 μg/ml and incubated in the cell extract at 30°C. The ordinates give the residual trichloroacetic acid-insoluble radioactivity per 5-μl aliquot as a percentage of the zero time value (6200 cpm).

have indicated that it activates a nuclease which then degrades mRNA. This activatable nuclease is present in cell extracts from control cells (20, 32). It was of interest, therefore, to analyze the RNA extracted from cells after treatment with the inhibitor. For this purpose, cells were incubated in medium containing [^{3}H]uridine for 20 hr before removal of the residual label and treatment with 2′-5′A. The total and polyadenylylated RNA from control cells or cells preincubated in Hepes-buffered saline (pH 7.05) containing $CaCl_2$ in the absence and presence of 2′-5′A were analyzed by electrophoresis in polyacrylamide slab gels (Figs. 3 and 4).

It is apparent from these figures that $CaCl_2$ (125 mM) in the absence of 2′-5′A did not cause any modification in the electrophoretic profiles of both total and polyadenylylated RNA (Fig. 3, channels 1 and 2; Fig. 4, curves A and B). After treatment with the inhibitor, however, both total and polyadenylylated RNA, measured by ^{3}H radioactivity, were reduced by 50% and 60–70%, respectively. In accord with this, electrophoretic profiles of total RNA indicated an apparent degradation of 28S and 18S ribosomal RNA into several small molecular weight components (Fig. 3, channels 3–6). Moreover, the residual polyadenylylated RNA-enriched fraction gave rise to a wider spectrum of products on electrophoresis with a tendency to a higher amount of small molecular weight components (Fig. 4, curve C). Contrary to these results, tRNA did not seem to be degraded in cells treated with 2′-5′A (data not shown). Some minor degradation or modification of tRNA species, however, cannot be excluded.

As has been reported (14, 20, 22), incubation of cell-free systems with 2′-5′A resulted in an enhanced level of nuclease activity (Fig. 5*A*). This was assayed by a reduction in acid-insoluble radioactivity from VSV [^{3}H]mRNA (32). Degradation of this mRNA was at a much higher rate when extracts were prepared from MRC5 cells that had been treated with 2′-5′A (Fig. 5*B*). This was probably due to the fact that the nuclease was already in its activated form since there was no residual 2′-5′A activity in those extracts (measured by its heat-stable inhibitory activity in cell-free systems). In accord with these results, Williams *et al.* (32) have recently shown that 2′-5′A is rapidly broken down in cell-free systems and in this process a heat-labile inhibitory component is formed which is a ribonuclease. An enhanced degradation of exogenously added cellular ^{3}H-labeled polyadenylylated and ribosomal RNA was also observed in extracts from 2′-5′A-treated cells (data not shown).

DISCUSSION

The effect of 2′-5′A in intact cells is dependent on its penetration into the cell. This may occur after treatment of cells in hypertonic medium (21) or by adsorption of 2′-5′A onto the cell membrane (*Materials and Methods*). Here, this latter method was used to investigate the mechanism of action of 2′-5′A in intact cells.

2′-5′A is a potent inhibitor of protein synthesis in intact cells at a concentration of less than 10 nM (in AMP equivalents) (Fig. 1). This is in accord with the activity of 2′-5′A in cell-free systems (8, 17, 37). In the latter, the kinetics of inhibition by 2′-5′A showed a lag of 10–15 min before onset of inhibition (7). In view of this and the effectiveness of 2′-5′A at subnanomolar concentrations, it is suggested that 2′-5′A is not directly inhibitory but that it, in turn, regulates the formation of an inhibitor(s) or subsequent inhibitory events. Interestingly, consistent with earlier reports of an ATP- and dsRNA-dependent nuclease activity in extracts from interferon-treated cells (9, 10), several workers have presented evidence for a model for the mechanism of action of 2′-5′A in cell-free systems (18–20). According to this model, 2′-5′A activates a nuclease which then degrades mRNA. Such a nuclease is present in control and interferon-treated cell extracts at similar levels (32) and does not appear to be specific, since it degrades both viral and host mRNAs (18, 20).

Here, evidence is provided to suggest that such activation of a nuclease may also be involved in the mechanism of action of 2′-5′A in intact cells (Figs. 3 and 4). The degradation of both

polyadenylylated and ribosomal RNAs are observed after treatment with 2′-5′A. This result is difficult to explain on any basis other than an enhanced level of nuclease activity, since it represents degradation of previously labeled RNA. Accordingly, extracts from 2′-5′A-treated cells manifested an enhanced nuclease activity *in vitro* on viral and cellular RNAs (Fig. 5). Once again, therefore, there was no specificity in the action of the nuclease. An interesting aspect of the activated nuclease is that its effects are transient (Fig. 2). This is of great advantage because the cell survives after a period when mRNA species are degraded and thus provides a suitable mechanism for the elimination of undesirable messages. In accord with this, preliminary studies indicated that viral RNA synthesis can be reduced to background levels after treatment with 2′-5′A (unpublished results). It is possible, therefore, that the antiviral action of interferon may at least in part be mediated by the action of 2′-5′A-activated nuclease—i.e., treatment of cells with interferon enhances levels of 2′-5′A synthetase which, in the presence of viral replicative intermediate (dsRNA) and ATP, forms 2′-5′A. The latter in turn activates a nuclease responsible for destroying cytoplasmic mRNA, cellular and viral alike. By this process, viral replication could be eliminated or limited to a certain extent while the cell may or may not survive. Whatever the situation, however, little virus is produced.

The results described here emphasize the potential value of the calcium phosphate coprecipitation technique for penetration of 2′-5′A into cells which provides the basis for a more detailed characterization of the *in vivo* activated nuclease, its mode of action, and its shutoff.

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1. Kerr, I. M., Brown, R. E. & Ball, L. A. (1974) *Nature (London)* **250,** 57–59.
2. Content, J., Lebleu, B., Nudel, U., Zilberstein, A., Berissi, H. & Revel, M. (1975) *Eur. J. Biochem.* **54,** 1–10.
3. Kerr, I. M., Brown, R. E., Clemens, M. J. & Gilbert, C. S. (1976) *Eur. J. Biochem.* **69,** 551–561.
4. Roberts, W. K., Hovanessian, A. G., Brown, R. E., Clemens, M. J. & Kerr, I. M. (1976) *Nature (London)* **264,** 477–480.
5. Lebleu, B., Sen, G. C., Shaila, S., Cabrer, B. & Lengyel, P. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 3107–3111.
6. Zilberstein, A., Federman, P., Shulman, L. & Revel, M. (1976) *FEBS Lett.* **68,** 119–124.
7. Hovanessian, A. G., Brown, R. E. & Kerr, I. M. (1977) *Nature (London)* **268,** 537–540.
8. Kerr, I. M., Brown, R. E. & Hovanessian, A. G. (1977) *Nature (London)* **268,** 540–542.
9. Ratner, L., Sen, G. C., Brown, G. E., Lebleu, B., Kawakita, M., Cabrer, B., Slattery, E. & Lengyel, P. (1977) *Eur. J. Biochem.* **79,** 565–577.
10. Lewis, J. A., Falcoff, E. & Falcoff, R. (1978) *Eur. J. Biochem.* **86,** 497–509.
11. Eppstein, D. A. & Samuel, C. E. (1978) *Virology* **89,** 240–251.
12. Kerr, I. M. & Brown, R. E. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 256–260.
13. Farrel, P. J., Balkow, K., Hunt, T., Jackson, R. J. & Trachsel, H. (1977) *Cell* **11,** 187–200.
14. Farrel, P. J., Sen, G. C., Dubois, M. F., Ratner, L., Slattery, E. & Lengyel, P. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 5893–5897.
15. Zilberstein, A., Kimchi, A., Schmidt, A. & Revel, M. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 4734–4738.
16. Hovanessian, A. G. & Kerr, I. M. (1979) *Eur. J. Biochem.* **93,** 515–526.
17. Hovanessian, A. G. & Kerr, I. M. (1978) *Eur. J. Biochem.* **84,** 149–159.
18. Clemens, M. J. & Williams, B. R. G. (1978) *Cell* **13,** 565–572.
19. Baglioni, C., Minks, M. A. & Maroney, P. A. (1978) *Nature (London)* **279,** 684–687.
20. Schmidt, A., Zilberstein, A., Shulman, L., Federman, P., Berissi, H. & Revel, M. (1978) *FEBS Lett.* **95,** 257–264.
21. Williams, B. R. G. & Kerr, I. M. (1978) *Nature (London)* **276,** 88–89.
22. Castellot, J., Jr., Miller, M. R. & Pardee, A. B. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 351–355.
23. Graham, F. L. & Van Der Eb, A. J. (1973) *Virology* **52,** 456–467.
24. Grubman, M. J., Moyer, S. A., Banerjee, A. K. & Ehrenfeld, E. (1975) *Biochem. Biophys. Res. Commun.* **62,** 531–538.
25. Aviv, H. & Leder, P. (1972) *Proc. Natl. Acad. Sci. USA* **68,** 1408–1412.
26. Clemens, M. J., Henshaw, E. C., Rahaminoff, H. & London, I. M. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 2946–2950.
27. Kerr, I. M., Olshevsky, U., Lodish, H. F. & Baltimore, D. (1976) *J. Virol.* **18,** 627–635.
28. Bénicourt, C., Père, J. P. & Haenni, A. L. (1978) *FEBS Lett.* **86,** 268–272.
29. Lage-Davila, A. & Montagnier, L. (1977) *Biochem. Biophys. Res. Commun.* **79,** 577–584.
30. Desmyter, J., Melnick, J. L. & Rawls, W. R. (1968) *J. Virol.* **2,** 955–961.
31. Bray, G. A. (1960) *Anal. Biochem.* **1,** 279–285.
32. Williams, B. R. G., Kerr, I. M., Gilbert, C. S., White, C. N. & Ball, L. A. (1978) *Eur. J. Biochem.* **92,** 455–462.
33. Scherrer, K. (1968) in *Fundamental Techniques in Virology,* eds. Habel, K. & Salzman, N. P. (Academic, New York), pp. 413–422.
34. Floyd, R. W., Stone, M. P. & Joklik, W. K. (1974) *Anal. Biochem.* **59,** 599–608.
35. Peacock, A. C. & Dingman, C. W. (1968) *Biochemistry* **7,** 668–674.
36. Bonner, W. & Laskey, R. (1974) *Eur. J. Biochem.* **46,** 83–88.
37. Ball, A. L. & White, C. N. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 1167–1171.