# Ribosomal RNA cleavage, nuclease activation and 2-5A ($ppp(A2'p)_nA$) in interferon-treated cells

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## ABSTRACT

Ribosomal RNA (rRNA) in intact ribosomes is cleaved into discrete products on incubation of reticulocyte lysates or L-cell extracts with $ppp(A2'p)_3A$. Cleavage of rRNA may, therefore, provide a useful assay for 2-5A ($ppp(A2'p)_nA$; n = 2 to 4) or for the presence of a 2-5A-dependent nuclease. The results with reticulocyte lysates differed from those obtained in the L-cell-free system in that (a) a different RNA cleavage pattern was produced (with added L-cell ribosomes) and (b) cleavage was fully activated by the analogue $ppp(A2'p)_3A3'pCp$. As might be expected from the relatively high levels of 2-5A present in interferon-treated, encephalomyocarditis virus (EMC)-infected L-cells, rRNA extracted from these cells was also cleaved. The cleavage pattern observed overlapped with that obtained on incubation of an L-cell-free system with 2-5A. Thus, not only is 2-5A present, but the 2-5A-dependent nuclease also appears to be active, in interferon-treated, EMC-infected L-cells.

## INTRODUCTION

Interferon treatment of cells induces the synthesis of several proteins among which is an enzyme, 2-5A synthetase, which, in the presence of double-stranded RNA (dsRNA) and ATP, synthesises 2-5A ($ppp(A2'p)_nA$ where n = 2 to 4) (1). The 2-5A in turn activates an RNase which can inhibit protein synthesis by RNA degradation (2-6). The levels of 2-5A synthetase vary with both growth and hormone status in a variety of tissues and cell types (7). The 2-5A system may, therefore, have wide significance.

The 2-5A-dependent endonuclease can degrade endogenous messenger RNA (mRNA) or RNA added to cell-free systems (2-6). Its effect on ribosomal RNA (rRNA) in intact ribosomes, however, has not been directly investigated, although Hovanessian et al. observed that rRNA isolated from cells into which 2-5A had been deliberately introduced was partially degraded (8). Cleavage of rRNA has also been reported to occur in interferon-treated, SV40 virus-infected cells late after infection (9). We have recently presented evidence for the natural occurence of 2-5A in interferon-treated, encephalomyocarditis virus (EMC)-infected mouse L-cells (10,11). It is not known, however, whether this endogenous 2-5A activates the 2-5A dependent endonuclease and/or performs some alternative function.

Here we show that on addition of ppp(A2'p)$_3$A to rabbit reticulocyte lysates or L-cell extracts rRNA present in intact ribosomes is cleaved to yield characteristic patterns of discrete products. In accord with this and the natural occurrence of 2-5A in interferon-treated, EMC-infected L-cells rRNA from such cells shows a similar cleavage pattern.

## MATERIALS AND METHODS

Cell culture. Mouse L-cells grown in spinner culture in Eagle's minimal essential medium supplemented with glutamine, antibiotics and 10% newborn calf or foetal serum, were either treated with interferon (1 to 5 x $10^7$ reference units/mg protein, 400 reference, 20 effective, units/ml), EMC infected or both interferon- treated and EMC infected according to published procedures (11). Where indicated, L-cells were labelled by incubation as above with $^{14}$C-sodium formate (2μCi/ml, 50-60 Ci/mole, Radiochemical Centre, Amersham, U.K.).

Preparation of cell extracts. Reticulocyte lysates were prepared as described previously (12,13). Extracts were prepared from L-cells washed three times in buffered saline, resuspended in 1.5 volumes of 20mM KCl, 5mM Tris HCl pH7.6, 1.25mM magnesium acetate and 1.25% glycerol, swollen for 5 min at 0°C and homogenised with 20 strokes of a glass Dounce homogeniser. The homogenates were centrifuged for 15min at 16,000xg. The supernatant solutions were stored in aliquots at -70°C.

Isolation of ribosomes. Ribosomes were obtained by centrifuging L-cell extracts prepared as above through a cushion of 1M sucrose, 5mM Tris HCl pH7.6, 1mM dithiothreitol (DTT) and 0.1mM EDTA for 5hr at 150,000xg at 4°C. The ribosomes were resuspended (7 mg/ml) in 0.25M sucrose, 5mM Tris HCl, pH7.6, 1mM DTT and 0.1mM EDTA.

Activation of the 2-5A-dependent endonuclease in cell extracts. Incubations (50μl) were at 30°C under reticulocyte protein synthesis conditions except for the omission of creatine phosphokinase and contained reticulocyte lysate (with 20μM haemin) or L-cell extract (20μl), 1mM ATP, 0.2mM GTP, 10mM creatine phosphate, 2mM glucose, 75mM KCl, 2mM magnesium acetate 10mM Tris HCl buffer pH7.6 a mixture of 19 amino acids (excluding cysteine) and, where indicated, tetramer 2-5A (ppp(A2'p)$_3$A) prepared as described (14).

RNA extraction. Cell extracts (prepared as above) were diluted 10-20 -fold with 100mM Tris HCl pH8, 100mM NaCl, 1mM EDTA and extracted with an equal volume of water saturated phenol : chloroform (1:1). The aqueous phase was re-extracted twice with fresh phenol : chloroform, made 100mM in sodium acetate pH5.5 and the RNA precipitated with 2.5 volumes of ethanol at -20°C.

Electrophoretic analysis of RNA on agarose gels. Glyoxal denatured RNA samples were electrophoresed on 1.8% agarose gels in 10mM sodium phosphate buffer pH7.0 (15) or in 25mM sodium citrate pH 3.5 in the presence of 7M urea (16). The gels were stained with 1µg/ml of ethidium bromide and the RNA bands in the wet gels visualised under UV light (302nm). Fluorography of the dried gels was as previously described (17).

## RESULTS

2-5A induced ribosomal RNA cleavages in the reticulocyte lysate. The 2-5A-dependent endonuclease can, in the presence of 2-5A, degrade purified viral, messenger, and rRNAs (2,3,6,11). To see whether this nuclease can cleave rRNA in the intact ribosome, total RNA from a reticulocyte lysate which had been incubated either in the presence or absence of tetramer 2-5A ($ppp(A2'p)_3A$)[3] was extracted, denatured and analysed by electrophoresis on agarose gels. RNA from control lysates showed prominent 28S and 18S rRNA species (Fig.1A), whereas RNA extracted from 2-5A-treated lysates showed a new band between 28S and 18S rRNAs and some enhancement of a band below the 18S RNA (▷, Fig.1A). These latter, however, are barely discernible from the figure. Accordingly, purified $^{14}C$-labelled L-cell ribosomes were added to a reticulocyte lysate and incubated in the presence or absence of tetramer 2-5A. The labelled rRNA from the incubation with 2-5A was partially cleaved to discrete products confirming that rRNA in intact ribosomes is susceptible to the 2-5A-dependent endonuclease (Fig.1B).

2-5A-induced ribosomal RNA cleavage in L-cell extracts. The generality of this phenomenon was investigated using a cell-free system derived from mouse L-cells. RNA isolated from L-cell extracts which had been incubated at 30°C with and without tetramer 2-5A was analysed on agarose gels as above. That from control extracts showed little if any breakdown - the major species were 28S and 18S rRNAs and tRNA. That from incubations with 2-5A showed discrete cleavage products between the 28S and 18S RNAs and 3 prominent bands below the 18S species. The cleavages appeared on incubation of the extracts for 30min with 100nM tetramer 2-5A (Fig.2A). Incubation with increasing concentrations of tetramer 2-5A for 120min at 30°C showed that $\geq$10nM was sufficient to induce a similar cleavage pattern (Fig.2B).

Confirmation that the products were derived from rRNA present in intact ribosomes was once again obtained by adding purified $^{14}C$-labelled L-cell ribosomes to the incubations. Labelled RNA species (detected by fluorography Fig.2C) appeared at identical positions to the cleavage products observed by ethidium bromide staining (Fig.2B).

Comparison of the 2-5A-dependent nuclease activity in L-cell extracts and reticulocyte lysates. The 2-5A-dependent endonucleases in reticulocyte lysates and L-cell extracts were compared by their activities on added $^{14}C$-labelled L-cell ribosomes. In both

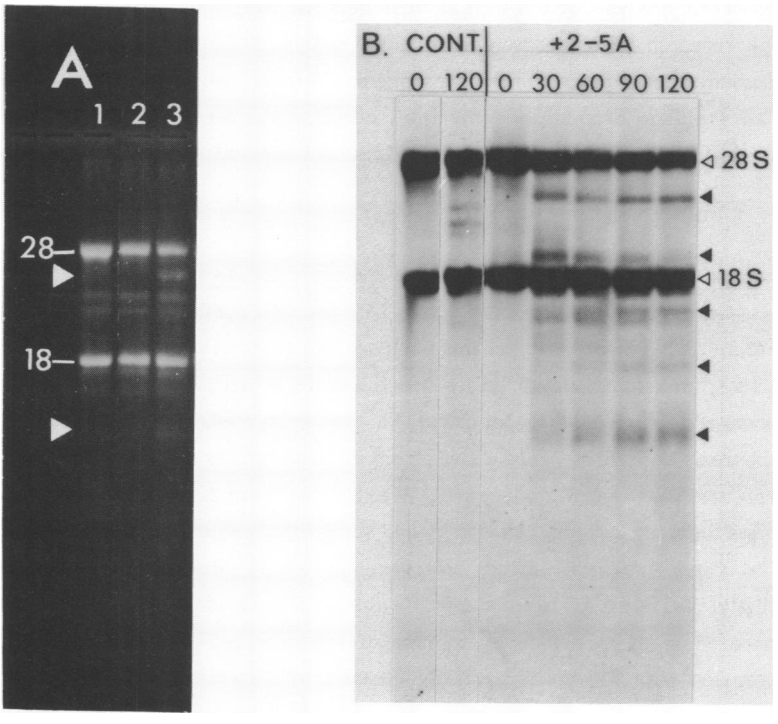


Figure 1. 2-5A induced rRNA cleavages in reticulocyte lysates.

Lysates were incubated under protein synthesis conditions but without creatine phosphokinase : (A) for 120 min at 0°C (1) or 30°C (2,3) in the absence (1,2) or presence (3) of 100nM ppp(A2'p)$_3$A. (B) With $^{14}$C-labelled L-cell ribosomes (4µg per 50µl assay, equivalent to 35,000cpm) for the times indicated (min) at 30°C with (2-5A) or without (CONT) 100nM ppp(A2'p)$_3$A. Aliquots (50µl) were taken, the RNA extracted and analysed by electrophoresis on glyoxal-agarose gels (15). (A) is a photograph of an ethidium bromide stained gel (15) taken under ultraviolet light (302 nmetres). (B) is a fluorograph of the dried gel. The position to which 18S and 28S rRNAs migrated are indicated to the left and right of the Figure.

systems addition of tetramer 2-5A induced breakdown of the labelled rRNA (Fig.3. The RNA analysis was in urea-agarose gels (16) and the positions of the bands are, therefore, different from that observed in the glyoxal-agarose gels used in Figs.1,2,4, and 5). The extent of cleavage and the cleavage patterns obtained were, however, significantly different in the two cell-free systems. The systems also differed in that the 2-5A analogue ppp(A2'p)$_3$A3'pCp was as efficient as tetramer 2-5A in activating the reticulocyte but not the L-cell enzyme (18, Fig.3, lane 4). The greater activity of the analogue in the reticulocyte system does not, incidentally, simply reflect its rapid conversion to tetramer 2-5A (18).

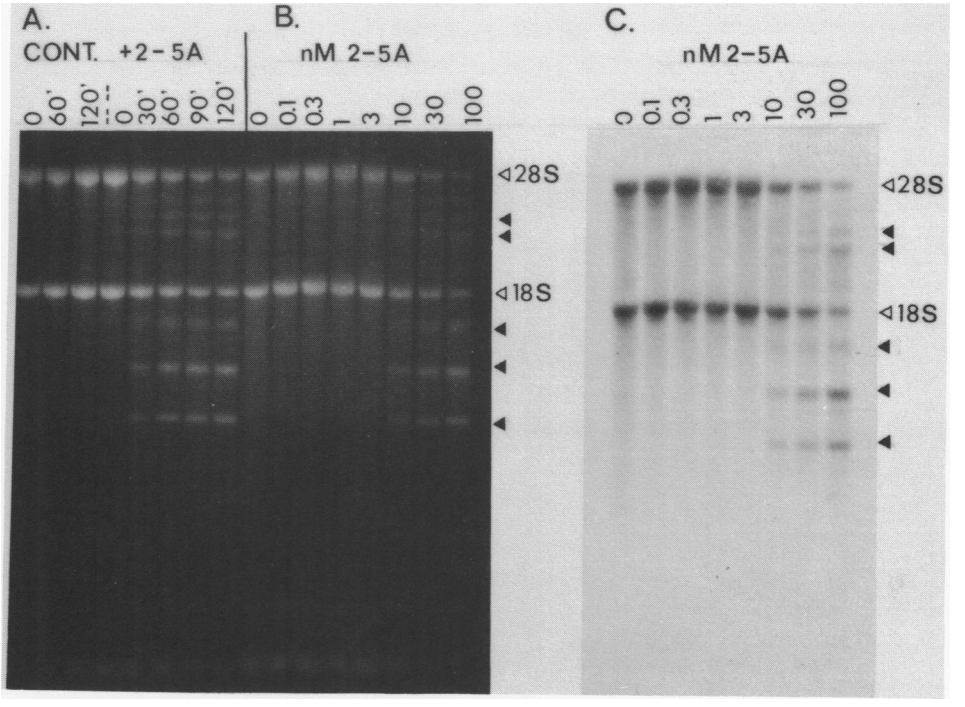


Figure 2. 2-5A induced rRNA cleavages in L-cell extracts.

L-cell extracts were incubated with (B) and (C) and without (A) $^{14}$C-labelled L-cell ribosomes (4µg per 50µl assay equivalent to 35,000 cpm). (A) With or without (CONT) 100nM tetramer 2-5A. Samples (50µl) were taken at the indicated times (min). (B) and (C) for 120 min with the indicated concentrations of tetramer 2-5A. The incubations, RNA extraction and analysis were as in Figure 1. (A) and (B) are photographs of ethidium bromide stained gels, (C) is a fluorograph of (B).

Characterisation of ribosomal RNA cleavages occurring in interferon-treated cells. Naturally occurring 2-5A has been found in interferon-treated, EMC-infected L-cells (10,11). It was of interest, therefore, to determine whether cleavage of rRNA similar to that observed on incubation of L-cell extracts with 2-5A (Fig.2) also occurs in the intact interferon-treated, EMC-infected cell. To investigate this, L-cells were grown in suspension culture without any treatment (C-control), with EMC infection (CE), interferon treatment (IF) or both interferon treatment and EMC infection (IFE). The RNA was extracted and analysed under denaturing conditions on agarose gels in comparison with RNA from a control L-cell extract incubated with tetramer 2-5A (Fig.4). The 28S and 18S rRNAs (arrowed Fig.4) from C or IF cells (Fig.4, tracks 1 and 3)

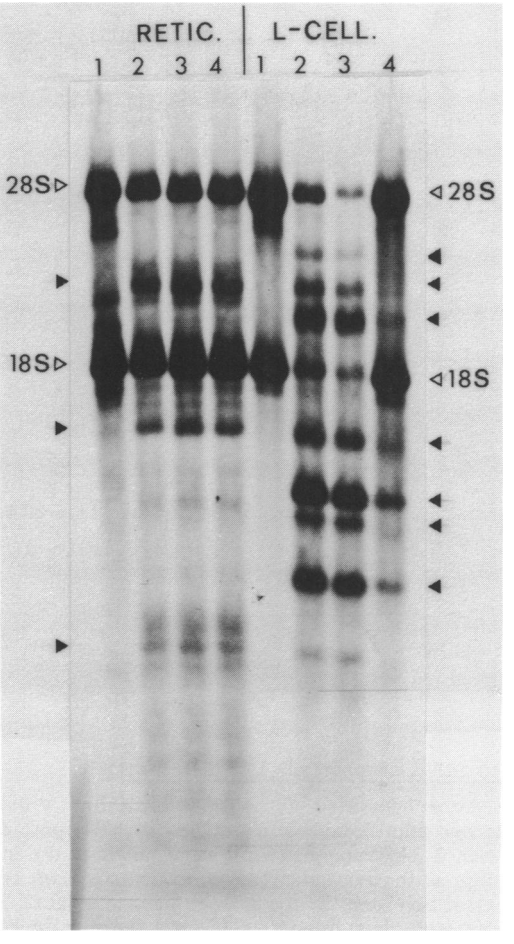


Figure 3. Comparison of 2-5A-dependent nuclease activity in reticulocyte lysates and L-cell extracts.

Reticulocyte lysates (RETIC) or L-cell extracts (L-CELL) supplemented with $^{14}C$-labelled L-cell ribosomes (4µg per 50µl assay, equivalent to 35,000cpm) were incubated as in Figures 1 and 2 for 150 min at 30°C in the absence of 2-5A (track 1), with 100nM tetramer 2-5A (track 2), with 100nM tetramer 2-5A added every 30min (track 3) and with 100nM ppp(A2'p)$_3$ApCp (track 4). The RNA was extracted and analysed by electrophoresis on a urea agarose gel (16). A fluorograph of the dried gel is presented.

or from a control L-cell-free system (Fig.4, track 5) were essentially intact. There was some evidence for degradation of the rRNA from CE cells (Fig.4, track 2). This was much more obvious, however, with the RNA from IFE cells in which there are three

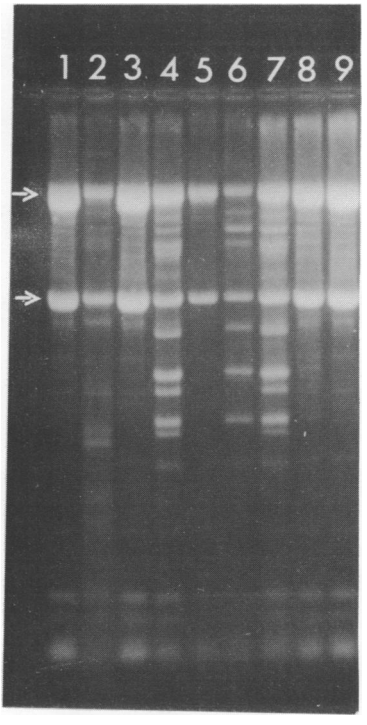


Figure 4. Analysis of RNA extracted from L-cells after interferon treatment with or without subsequent EMC infection.

Mouse L-cells were either treated with interferon (3,8,9) for 20hr EMC-infected (2) for 4.5hr, both interferon-treated and EMC-infected (4) or grown under control conditions (1). RNA was extracted and analysed by electrophoresis on glyoxal-agarose gels with (8,9) or without (1-4) the addition of 1μM 2-5A at the time of cell lysis, in parallel with RNA from an L-cell-free system incubated (120 min) in the absence (5) or presence (6) of 100nM 2-5A. 7 is a mixture of 4 and 6. In 8 and 9 the times taken from cell lysis to the addition of phenol were 13 (as for 1 to 4) and 18 min respectively. A photograph of the ethidium bromide stained gel is presented.

additional characteristic and highly reproducible major cleavage products which migrate faster than the 18S rRNA on the gel (Fig.4, track 4). It is these three additional products, in particular, which appear to coincide with those mediated by 2-5A in the cell-free system (Fig.4, tracks 4 and 6 and a mixture of these, 7). This similarity in cleavage pattern suggests that the same enzyme may be involved.

Control experiments were performed to verify that the cleavage patterns obtained in the RNA from IFE cells did not result from activation of the 2-5A-dependent nuclease during the extraction or processing of the samples prior to RNA

analysis. Mixing of EMC-infected cells with interferon-treated cells prior to the preparation of extracts, did not result in additional RNA cleavage. More importantly, RNA prepared from experiments in which 2-5A (1μM) was added to interferon-treated cells at the time of cell lysis (Fig.4, tracks 8 and 9), was found to be identical to that obtained in the absence of these oligonucleotides (Fig.4, track 1).

Comparison of the 2-5A-mediated cleavage of rRNA with that produced by other endonucleases. The similarity of the RNA cleavage patterns obtained in the above experiment suggested that the 2-5A-dependent endonuclease is active in interferon-treated, EMC-infected L-cells. It remained possible nevertheless that the patterns observed reflected exposed sites on the rRNA rather than RNase specificity. Very different patterns, however, were obtained with TI and pancreatic RNases. TI RNase at low concentrations (0.1 to 10 units/ml) induced a series of rRNA cleavage products (Fig.5, lanes 8-10). In this and a further more extensive analysis, a sufficient spectrum of products was obtained that some apparent correspondence of individual bands cannot be excluded, but in no case did the overall pattern of cleavage even approximate that obtained with the 2-5A-dependent endonuclease (Fig.5, track 12). No specific cleavage products were resolved in similar experiments with pancreatic RNase (0.01 to 100μg/ml, lanes 2-6, Fig.5). This was true even when a very narrow range of concentrations producing intermediate levels of breakdown (between those in lanes 4 and 5, Fig.5) was employed.

## DISCUSSION

We have shown that 2-5A can induce cleavage of rRNA in intact ribosomes to yield a characteristic pattern of discrete products. Such cleavage may prove useful as a simple assay for exogenous 2-5A or for the presence of a 2-5A-dependent endonuclease in crude extracts.

A 2-5A-activatable nuclease capable of inducing rRNA breakdown is present in both reticulocyte and L-cell extracts. A different pattern of products was obtained, however, when $^{14}$C-labelled L-cell ribosomes were added to the two systems (Fig.3). The specificity of the cleavage, therefore, lies with the system and does not seem to simply represent non-specific cleavage at exposed portions of the RNA molecule in the ribosome structure. It is possible that the different specificities reflect differences in the 2-5A-dependent nucleases per se, but secondary cleavages (see below) cannot yet be excluded. That the two enzymes are in some way different is clear: that from reticulocytes is unusual in requiring the tetramer or higher oligomers of 2-5A rather than the trimer for full activity (11) and is fully activated by the 2-5A analogue ppp(A2'p)A3'pCp (Fig. 3 and reference 18).

RNA extracted from interferon-treated, EMC-infected cells and from control cell

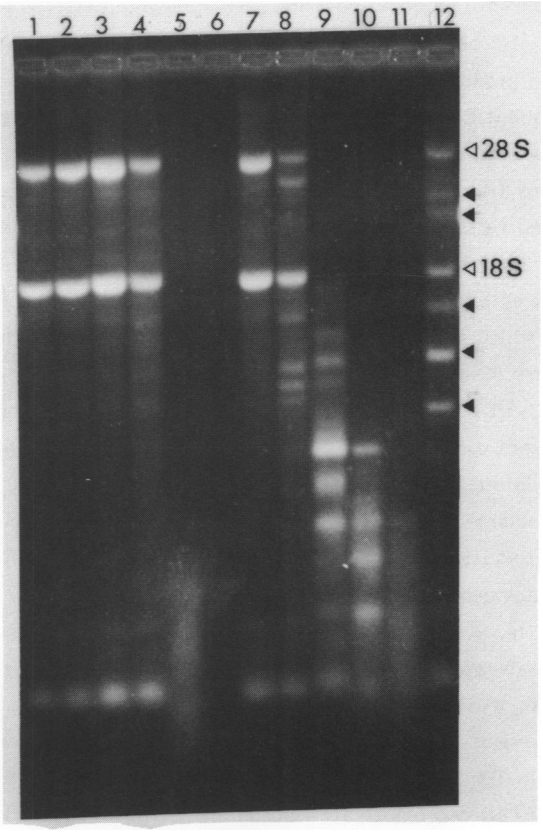


Figure 5. Comparison of the rRNA cleavages induced by pancreatic RNase, T1 RNase and the 2-5A-dependent endonuclease.

L-cell extracts were incubated as in Methods in the absence (track 1) or presence, tracks 2-6, of 0.01, 0.1, 1, 10 and 100µg/ml of pancreatic RNase; tracks 7-11 of 0.01, 0.1, 1, 10 and 100units/ml of T1 RNase; track 12 of 100nM tetramer 2-5A. RNA was extracted and electrophoresed on glyoxal-agarose gels. A photograph of the ethidium bromide stained gel is presented.

extracts incubated with 2-5A showed similar patterns of cleavage products (Fig.4, tracks 4, 6 and 7). Cleavage of the RNA from intact cells (track 4) was the more extensive, however, suggesting that additional non-2-5A-mediated, EMC-induced breakdown was occurring. In accord with this RNA from interferon-treated, EMC-infected cells taken at slightly earlier times (2.5 to 3hr) post-infection showed a pattern of cleavage essentially identical to that obtained with 2-5A in the cell-free system (R.H.S. unpublished data) In addition, variable results have been obtained on interferon treatment alone. In the vast majority of cases no significant cleavage was observed

(e.g. Fig.4, track 3). On two occasions, however, cleavage patterns essentially identical to those characteristic of 2-5A in the cell-free system were obtained. In one case the cleavage was more pronounced than the other. In neither case was it as great as that observed on subsequent EMC infection. The reason for this variability is not known. It is reminiscent, however, of the low and variable inhibition of protein synthesis observed in cell-free systems from such cells (Table 3 reference 19) and may well reflect the presence of low and variable levels of 2-5A. Taken together these results suggest that the 2-5A-dependent nuclease is responsible, in part at least, for rRNA cleavage in intact interferon-treated cells. It is possible, however, that some or all of the rRNA cleavage products which occur in the intact cell or in cell extracts in response to 2-5A are due to secondary RNase activity on the ribosome following only one or a few initial cleavages by the 2-5A-dependent enzyme. We have recently reported that the 2-5A-dependent cleavage of different RNAs in a variety of crude cell extracts occurs on the 3'-side of UpN doublets with a high preference for UpU or UpA sequences (20). It remains to be determined whether this specificity is that of the purified enzyme and whether this same specificity is observed for any, or all, of the rRNA cleavage products observed in the intact cell.

Revel et al. have shown that rRNA cleavages similar to those reported here, occur in interferon-treated, SV40-infected monkey cells (9). It is possible, therefore, that limited rRNA cleavage (whether 2-5A mediated or not) is a feature of certain interferon-treated, virus-infected cells. Since specific rRNA cleavages such as those produced by colicin E3 in prokaryotes (21) or by the reticulocyte membrane-bound endoribonuclease M (22) can inactivate ribosomes, it will be of considerable interest to determine the effect of the 2-5A-mediated rRNA cleavages and those occurring in the intact cell on ribosome function.

Levy and Riley (23) did not detect any change in rRNA following interferon treatment of mouse and rabbit cells, but it is not clear whether a low level of cleavage would have been detected under the experimental conditions employed. Nor is it clear what relation, if any, Maroun's observation (24,25) of a decreased de novo synthesis of rRNA in response to interferon has to the results reported here. It will be intriguing, therefore, to determine whether the quantitatively less extensive cleavage of rRNA observed on only rare occasions to date on interferon treatment alone has any significance and if so what part it plays in the cell growth inhibitory activity of interferon.

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[3] The 2-5A-dependent nuclease in rabbit reticulocyte lysates is unusual in requiring the tetramer or higher oligomers rather than the trimer or higher oligomers of 2-5A for activity (11).

REFERENCES

1 Kerr, I.M. and Brown, R.E. (1977) Proc. Natl. Acad. Sci. U.S.A. 75, 256-260.
2 Clemens, M.J. and Williams, B.R.G. (1978) Cell 13, 565-572
3 Baglioni, C., Minks, M.A. and Maroney, P.A. (1978) Nature (Lond.) 273, 684-687.
4 Schmidt, A., Zilberstein, A., Shulman, L., Federman, P., Berissi, H. and Revel, M. (1978) FEBS Lett. 95, 257-264.
5 Slattery, E., Ghosh, N., Samanta, H and Lengyel, P. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 4778-4782.
6 Williams, B.R.G., Kerr, I.M., Gilbert, C.S., White, C.N. and Ball, L.A. (1978) Eur. J. Biochem. 92, 455-462.
7 Stark, G.R., Dower, W.J., Schimke, R.J., Brown, R.E. and Kerr, I.M. (1979) Nature (Lond.) 278, 471-473.
8 Hovanessian, A.G., Wood, J., Meurs, E. and Montagnier, L. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 3269-3265.
9 Revel, M., Kimchi, A., Schmidt, A., Shulman, L., Chernajovsky, Y., Rapoport, S. and Lapidot, Y. (1979). In, Regulation of Macromolecular Synthesis (Koch, G. and Richter, D. eds.) pp.284-302, Academic Press, New York.
10 Knight, M., Cayley, P.J., Silverman, R.H., Wreschner, D.H., Gilbert, C.S., Brown, R.E. and Kerr, I.M. (1980) Nature (Lond.) 288, 189-192.
11 Williams, B.R.G., Golgher, R.R., Brown, R.E., Gilbert, C.S. and Kerr, I.M. (1979) Nature (Lond.) 282, 582-586.
12 Clemens, M.J., Henshaw, E.C., Rahamimoff, H. and London, I.M. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 2946-2950.
13 Hunt, T., Vanderhoff, G. and London, I.M. (1972) J. Mol. Biol. 66, 471-481.
14 Martin, E.M., Birdsall, N.J.M., Brown, R.E. and Kerr, I.M. (1979) Eur. J. Biochem. 95, 295-307.
15 McMaster, G.K. and Carmichael, G.G. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 4835-4838.
16 Smith, R.E. and Furuichi, Y. (1980) Virology 103, 279-290.
17 Laskey, R.A. (1980) In, Methods in Enzymology (Grossman, L. and Moldave, K. eds.) Vol.65, pp.363-371 Academic Press, New York.
18 Silverman, R.H., Wreschner, D.H., Gilbert, C.S. and Kerr, I.M. (1981) Eur. J. Biochem. in press.
19 Kerr, I.M., Brown, R.E., Clemens, M.J. and Gilbert, C.S. (1976) Eur. J. Biochem. 69, 551-561.
20 Wreschner, D.H., McCauley, J.W., Skehel, J.J. and Kerr, I.M. (1981) Nature (Lond.) 289, 414-417.
21 Bowman, C.B., Sidikaro, J. and Nomura, M. (1971) Nature (New Biol.) 234, 133-137.
22 Wreschner, D., Melloul, D. and Herzberg, M. (1978) Eur. J. Biochem. 89, 341-352.
23 Levy, H.B. and Riley, R.L. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 3815-3819.
24 Maroun, L.E. (1978) Biochim. et Biophys. Acta 517, 109-114.
25 Maroun, L.E. (1974) Biochem. J. 179, 221-225.