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rRNA Cleavage as an Index of ppp(A2'p)$_n$A Activity in Interferon-Treated Encephalomyocarditis Virus-Infected Cells

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In cell-free systems, 2-5A [ppp(A2'p)$_n$A, n = 2 to ≥4] activates a latent endoribonuclease, the 2-5A-dependent RNase, which cleaves rRNA in intact ribosomes into discrete and characteristic products (D. H. Wreschner et al., Nucleic Acids Res. 9:1571–1581, 1981). Here we present Northern blots which have identified the 18S or 28S origins of the cleaved products from rRNA. In addition, identical 3' termini were observed for fragments of 18S rRNA from a HeLa cell-free system incubated with 2-5A and from interferon-treated, encephalomyocarditis virus-infected HeLa cells. The previous assumption of identity of such fragments was based only on comigration on electrophoresis in agarose gels. We conclude that appropriate patterns of cleavage found in RNA isolated from intact cells are an indicator of prior 2-5A-dependent RNase activity. The assay of rRNA cleavage is relatively convenient and unambiguous. Accordingly, in the search for situations in which the 2-5A system may be active, it provides a useful alternative to the direct assay of 2-5A.

The unusual oligonucleotide series 2-5A [ppp(A2'p)$_n$A, n = 2 to ≥4] (9) has been implicated in the mechanism of action of interferons against certain viruses (6, 12, 14, 20, 23) and on cell growth (11). At least three enzymes are involved: (i) the 2-5A synthetase, which is double-stranded RNA dependent and converts ATP to 2-5A, and whose level increases in response to interferon (9); (ii) the 2',5'-phosphodiesterase, which degrades 2-5A to ATP and AMP (19, 24); and (iii) the 2-5A-dependent RNase, a latent endoribonuclease (1, 2, 16, 24). Activation of the nuclease by nanomolar concentrations of 2-5A inhibits protein synthesis (2, 9), presumably through the degradation of mRNA (1, 2), rRNA (8, 17, 25), or both.

The addition of 2-5A to a variety of cell-free systems results in the cleavage of rRNA in intact ribosomes into discrete and characteristic products (25). Similar (25) or apparently identical (20) patterns of rRNA breakdown have been observed in RNA isolated from interferon-treated, encephalomyocarditis virus (EMCV)-infected cells. In addition, rRNA cleavage in the intact cell appeared to correlate with intracellular 2-5A levels (20). To date, however, the assumption of identity of the products formed in the intact cell with those produced in response to 2-5A in cell-free systems has been based on the limited criterion of comigration on electrophoresis in agarose gels. Here we demonstrate the validity of monitoring rRNA breakdown as an indicator of 2-5A activity in cells by (i) identifying the 28S and 18S origins of the rRNA cleavage products and (ii) establishing the identity of at least one of the cleavages observed in the intact cell with one occurring in the cell-free system in response to 2-5A, by determining the identity of 3' termini of the cleavage products.

In Fig. 1A is shown an ethidium bromide-stained gel of RNA from untreated or interferon-treated, EMCV-infected L-cells (lanes 1 and 2) and from an L-cell-free system incubated without or with added 2-5A (lanes 3 and 4). The RNA was transferred to nitrocellulose membranes and hybridized to either 28S or 18S, ^{32}P-labeled rDNA probes (Fig. 1B and C, respectively). These probes do not cross-hybridize, as is evident from Fig. 1. Although it is not clear that they are 2-5A-mediated cleavage products, it is reasonable to assume that the major bands (a through c) between the 28S and 18S rRNAs (Fig. 1A) must be of 28S origin. Accordingly, the autoradiograph in Fig. 1B was overexposed to increase the sensitivity of detection in the region below 18S. The origins of bands d and e are uncertain, but 28S and 18S rRNAs, respectively, are suggested. Below this on the gels the results

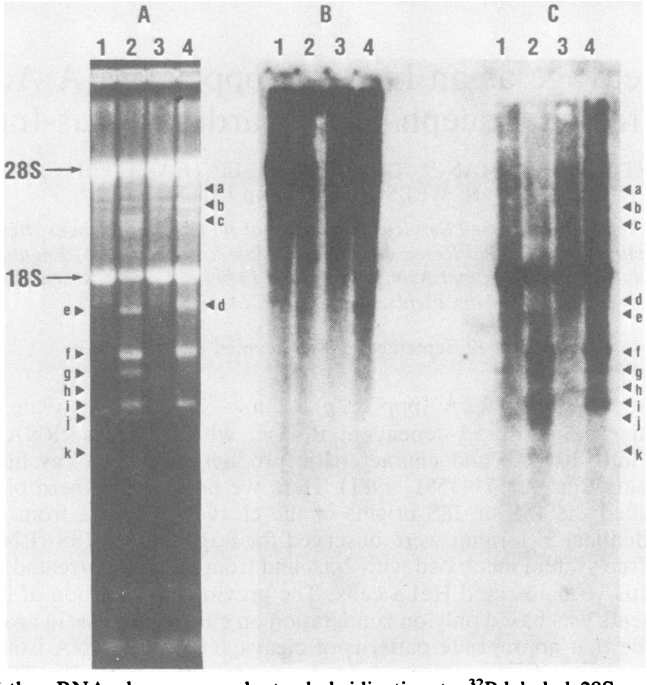

FIG. 1. Origin of the rRNA cleavage products: hybridization to ^{32}P-labeled 28S and 18S rDNA probes. Mouse L-cells were grown in Spinner culture in Eagle minimal essential medium supplemented with glutamine, antibiotics, and 10% heat-inactivated newborn calf or fetal calf serum. Interferon treatment was with 200 to 400 reference (10 to 20 effective) units of mouse L (α and β) interferon per ml (1×10^7 to 5×10^7 reference units per mg of protein). Infection with purified (10) EMCV was at 20 PFU per cell. (A) RNA (about 10 μg) from post-mitochondrial supernatant fractions of: (lane 1) control and (lane 2) interferon-treated mouse L-cells, both at 5 h postinfection with EMCV, and from L-cell-free systems incubated (lane 3) without or (lane 4) with 200 nM trimer 2-5A. This RNA was separated by glyoxal-agarose gel electrophoresis as described previously (13, 20, 25). A photograph of the ethidium bromide-stained gel is shown. The RNA from the gel shown in (A) was transferred to nitrocellulose membranes (Schleicher and Schuell, 0.1 μm, code no. PH79) according to Thomas (22) and hybridized with (B) 28S and (C) 18S ^{32}P-labeled rDNA probes as follows. The filters were prehybridized for 18 h at 42°C in 50% deionized formamide, 50 mM phosphate (pH 6.8), 5× SSC (1× SSC is 15 mM sodium citrate plus 150 mM sodium chloride, pH 7.0), 0.2% bovine serum albumin, 0.2% Ficoll 400, 0.2% polyvinylpyrrolidine 360, and 100 μg of *Saccharomyces cerevisiae* tRNA per ml. Cloned *Neurospora crassa* L-rDNA and S-rDNA (3) were nick-translated to a specific activity of 5×10^8 cpm/μg by the method of Rigby et al. (18) for use as probes for 28S and 18S rRNA sequences, respectively. The hybridizations were for 24 h with fresh prehybridization buffer containing 10% (wt/vol) dextran sulfate and heat-denatured (10 min, 100°C), nick-translated probe (10^7 cpm). The filters were washed twice in 2× SSC–0.2% sodium dodecyl sulfate at room temperature for 15 min and twice at 50°C for 15 min. Preflashed X-ray film was exposed to the filters at −70°C for 3 days. Autoradiographs of the nitrocellulose filters are shown. The arrows indicate the positions of the 28S and 18S rRNAs and of the rRNA cleavage products (a through k).

are more clear cut. All of the remaining products, f to k, including the major bands f and i, characteristic of 2-5A-mediated cleavage, are clearly 18S in origin. The similarities between the major 28S and 18S rRNA cleavage products formed in the intact cell and those from the cell-free system are apparent (Fig. 1A).

To characterize further the 2-5A-mediated rRNA products, we chose to compare RNA from HeLa cells and cell-free systems. This was because there were fewer cleavages in the HeLa cell than in the L-cell systems, and there was an apparently identical pattern of products (X, Y, and Z, Fig. 2A) in interferon-treated, EMCV-infected HeLa cells and in HeLa cell-free systems incubated with 2-5A (Fig. 2A, lanes 1 and 2, respectively; reference 20). The RNAs shown in Fig. 2A, lanes 1 and 2, were labeled at their 3′ termini by the ligation of [5′-^{32}P]pCp and applied to a 3% polyacrylamide gel containing 7 M urea. The 18S rRNA and rRNA products (Y) were eluted from the gel and electrophoresed on a second gel after partial digestion with *Physarum* RNase (Fig. 2B, lanes 1, 3, and 5) or with pancreatic RNase (lanes 2, 4, and 6). Identical digestion products were found whether Y was isolated from the cell-free system (Fig. 2B, lanes 1 and 2) or from intact cells (lanes 3 and 4). This

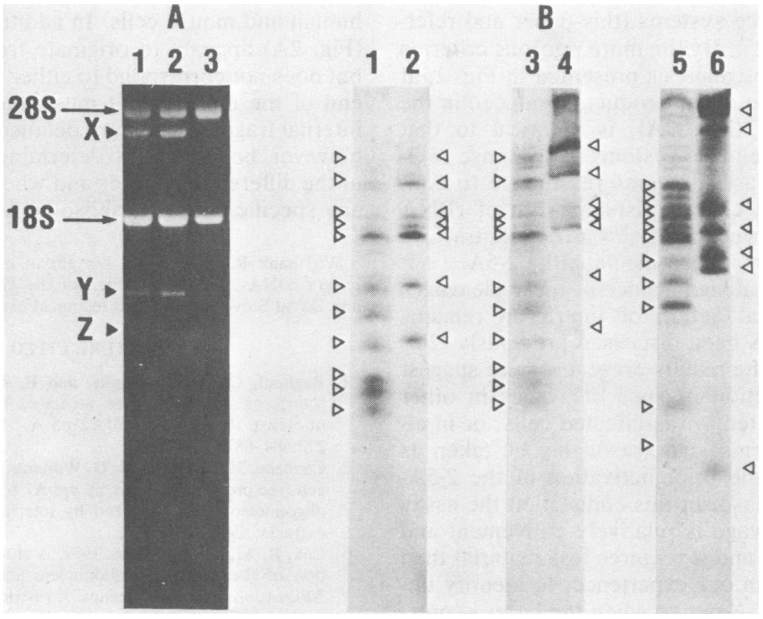

FIG. 2. Identity of one of the rRNA cleavage products from interferon-treated, EMCV-infected HeLa cells with one mediated by 2-5A in a HeLa cell-free system: comparison of partial RNase digests of the [^{32}P]pCp 3′-labeled products from the cell-free system and intact cells by electrophoresis on polyacrylamide gels. The growth, infection with EMCV, and interferon treatment of HeLa (S3) cells were as described previously (20). (A) RNA from post-mitochondrial supernatant extracts from (lane 1) interferon-treated HeLa cells at 6 h postinfection with EMCV, or from cell-free extracts from control HeLa cells incubated with (lane 2) and without (lane 3) 100 nM trimer 2-5A. A photograph of the ethidium bromide-stained gel is shown. The arrows indicate the positions of 28S and 18S rRNA and of the 2-5A-mediated rRNA cleavage products (X, Y, and Z). (B) Separate samples (about 500 μg) of the RNAs analyzed in (A) were ^{32}P-labeled at their 3′ termini with [5′-^{32}P]pCp (0.2 mCi) and T4 RNA ligase (5) and applied to a 3% polyacrylamide–7 M urea gel at pH 3.5. The ^{32}P-labeled RNA products (Y) from the HeLa cell-free system incubated with 2-5A (lanes 1 and 2) or from the interferon-treated EMCV-infected HeLa cells (lanes 3 and 4), and the 18S RNA (lanes 5 and 6) from the latter, were isolated from the gel as described previously (21). The eluted RNAs were ethanol precipitated, dissolved, and reprecipitated with cetyltrimethylammonium bromide (15). The labeled RNAs were then partially digested according to Donis-Keller et al. (4) with *Physarum* RNase (lanes 1, 3, and 5) or with pancreatic RNase (lanes 2, 4, and 6), and the digests were applied to 20% polyacrylamide gels and electrophoresed for 16 h at 5 V/cm. Autoradiograms of the gels are shown. Arrows indicate the sites of nucleolytic degradation.

was obvious for the *Physarum* RNase digests (Fig. 2B, lanes 1 and 3), but was less so for the pancreatic RNase digests (Fig. 2B, lanes 2 and 4), as the partial digestion was less extensive in lane 4 than in lane 2. On close examination, however, it is clear that the same series of products (arrows to the right of lanes 2 and 4, Fig. 2B) are present in the two extracts, though in very different amounts. Identical patterns of products were also found after partial T_1 RNase digestion of product Y from the intact cells and cell-free system (data not shown). The intact-cell and cell-free system Y products, therefore, have identical 3′ termini. Very different patterns of products were obtained by digestion of [^{32}P]pCp-labeled 18S rRNA (Fig. 2B, lanes 5 and 6). The 3′ terminus of product Y is not, therefore, that of 18S rRNA.

To implicate the 2-5A system in interferon action it is necessary, although not sufficient, to demonstrate that 2-5A is not only present but also active in intact cells. The development of specific patterns of rRNA cleavage is shown here to be a valid index of such activity. The cleavages characteristic of the 2-5A-dependent nuclease observed with rRNA from interferon-treated, EMCV-infected cells do not occur during the extraction of the RNA (20, 25). Nor, incidentally, do they reflect the presence of contaminating double-stranded RNA in the infecting virus. For example, RNA isolated from the EMCV failed to result in rRNA cleavage when added to control or interferon-treated L cells and incubated under the conditions for virus growth (data not shown). More particularly, the cleavage of the rRNA correlates with the intracellular levels of 2-5A (20). On the basis of migration during electrophoresis in glyoxal-agarose gels, cells form rRNA products which appear identical to those produced in response to

2-5A in cell-free systems (this paper and references 20 and 25). By the more rigorous criterion provided by the analysis presented in Fig. 2, at least one of the major products produced in the intact cell, Y (Fig. 2A), is identical to that produced in cell-free systems in response to 2-5A. On this basis it seems reasonable to conclude that the characteristic pattern of rRNA cleavage observed in interferon-treated, EMCV-infected cells is indeed mediated by 2-5A.

The biological significance of these cleavages in the antiviral action of interferon remains unclear, as has been discussed previously (20). Despite this, the results presented here suggest that the detection of such cleavages in other interferon-treated, virus-infected cells, or in alternative systems, can reasonably be taken as indicative of the prior activation of the 2-5A-dependent RNase. In this connection the assay of rRNA cleavage is relatively convenient and unambiguous, and it requires less material than is necessary, in our experience, to identify unequivocally 2-5A per se when the latter is present in cells at a concentration of <20 nM (20). Accordingly, it may prove a very useful initial alternative to the direct assay of 2-5A in the search for other systems in which 2-5A may be involved.

The 2-5A-dependent RNase is known to cleave RNA added to cell-free systems on the 3′ side of UN sequences, liberating UpNp-terminated products; this cleavage occurs predominantly at UA or UU (7, 26). It is not known, however, whether the same specificity applies to naturally occurring ribonucleoproteins in the intact cell. The techniques used for Fig. 2 were of the type employed in RNA sequencing. However, the resolution on the gels was not adequate to obtain an unequivocal 3′-terminal sequence for product Y (Fig. 2). It is not possible, therefore, to say whether the 2-5A-dependent nuclease shows the same specificity with rRNA in intact ribosomes as it does with the different added RNAs. In the original experiments with added RNAs (7, 26), no evidence was obtained for any preference for particular sequences 3′ to the cleavage sites, i.e., at the 5′ termini generated by cleavage. Interestingly, in a series of experiments similar to those described in Fig. 2, but involving the comparison of 5′-^{32}P-labeled HeLa product Y with a similarly labeled 18S rRNA product from a mouse L-cell-free system incubated with 2-5A, an extensive identity of sequence was observed. This sequence did not correspond to the 5′ terminus of the 18S rRNA. It can, therefore, be concluded that, regardless of how this is determined, the 2-5A-dependent nuclease cleaves rRNA in intact ribosomes at highly specific sites, at least one of which appears to be common to ribosomes from both human and mouse cells. In addition, product Y (Fig. 2A) appears to originate from 18S rRNA but does not correspond to either the 3′ or the 5′ end of the molecule. It must, therefore, be an internal fragment. A more detailed analysis will, however, be required to determine the exact site of the different cleavages and whether they have any specific effect on ribosome function.

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