ENDONUCLEASE OF HIGH SPECIFIC ACTIVITY IN A PURIFIED MOUSE INTERFERON PREPARATION

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SUMMARY. We find an endonuclease of high specific activity in a purified mouse interferon preparation. The interferon was purified from Ehrlich ascites tumor cultures which were induced with Newcastle disease virus. It has a higher specific activity (1.5×10^9 NIH mouse reference standard interferon units/mg protein) than reported for any interferon preparation but is not homogeneous. We do not know if the endonuclease activity is due to a contaminating protein or to interferon. The endonuclease does not degrade in our conditions polyuridylic acid or double stranded reovirus RNA and does not inactivate the $tRNA_2^{Gln}$ species from E. coli, or $tRNA^{Val}$ species or polysomes from mouse L cells. Endonuclease in as little as 0.5 ng protein of the interferon preparation degrades µg quantities of messenger RNA from mouse L cells, of encephalomyocarditis virus RNA and of in vitro-synthesized reovirus messenger RNA at 37° in 1 hour. Further characteristics of the endonuclease and its possible relationship (if any) to interferon remain to be established.

Interferons are macromolecules, presumably glycoproteins, which are synthesized in a variety of vertebrate cells upon viral infection or some other stimuli. They are excreted, are bound to other cells and make these inefficient in supporting the growth of a broad range of viruses. No interferon has been purified to homogeneity and the mechanism of interferon action has not been definitely established (1). Taylor-Papadimitriou et al. reported an endonuclease activity in a chick interferon preparation with a specific interferon activity of 9×10^3 vaccinia plaque reduction units/mg protein (2). The endonuclease activity was not separated from interferon during chromatography on CM Sephadex using a pH gradient for elution (2). We have been purifying mouse interferon from Ehrlich ascites tumor cells induced with live Newcastle disease virus. Our best preparation has a specific activity of 1.5×10^9 NIH mouse reference standard units/mg protein. In this communication we describe some of the characteristics of a potent endonuclease in our interferon preparation. It remains to be established if the endonuclease activity in this preparation is due to a contaminating protein or to interferon.

Materials and methods

The procedure for purifying interferon will be published elsewhere (Weideli et al. in preparation).

The specific activity of the interferon preparation used in this study was 1.5×10^9 NIH mouse reference standard units/mg protein. This corresponds to 6×10^8 vesicular stomatitis virus (VSV) plaque reduction units/mg protein in an assay used in this laboratory (3). Electrophoresis through polyacrylamide gels at pH 7.2 in the presence of urea and sodium dodecylsulfate fractionates the preparation into a major and a minor band. As described in detail elsewhere (4-6): L cells (mouse L929 fibroblasts) were grown in suspension culture.

Labeled reo mRNA was synthesized in vitro with the transcriptase in chymotrypsin-treated reovirus cores. Encephalomyocarditis (EMC) mRNA was extracted from labeled EMC virions. L cell polysomes were obtained by fractionating an extract by centrifugation through a sucrose gradient. L cell$_C$ mRNA (a mixture of those

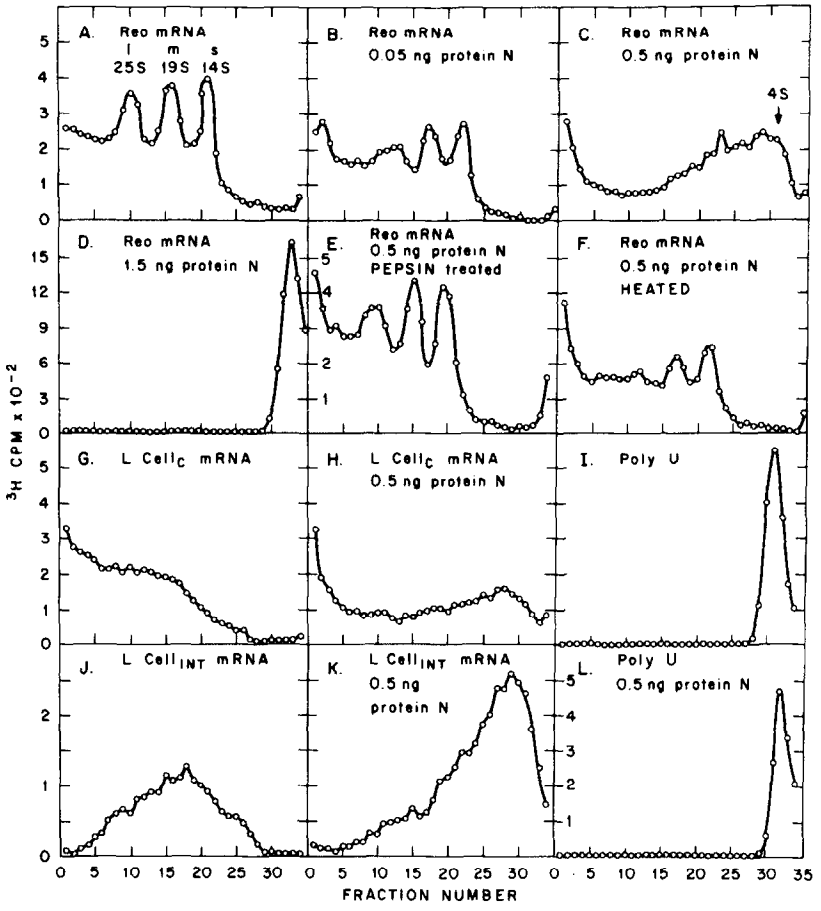

Fig. 1. Effect of protein N on the sedimentation patterns of single stranded ribonucleic acids from viruses and L cells and of polyuridylic acid. Each of the various RNAs (i.e., reo mRNA (7.5 µg, 8600 cpm), L cell$_C$ mRNA (7.5 µg, 8800 cpm), L cell$_{INT}$ mRNA (5 µg, 8800 cpm), poly U (27 µg, 16,000 cpm) was (unless otherwise indicated) incubated with or without protein N at 37° for 60 min in 20 µl reaction mixtures in solution I (20 mM Tris-Cl (pH 7.6), 10 mM magnesium acetate, 40 mM NaCl, 50 µM ATP, 5 µM SAM). Thereafter each RNA was analyzed by centrifugation through a 7 to 25% w/v sucrose gradient in 10 mM Tris-Cl (pH 7.6), 10 mM NaCl, 1 mM ethylenediaminetetraacetate in the SB283 rotor of the IEC B60 ultracentrifuge at 2° and 100,000 g for 13 hours and counting of the fractions of the gradient in Brays solution (17). The nature of the RNA and the amount and pretreatment of protein N with which it was incubated are indicated in the figure. In C) the arrow indicates the position in the gradient of E. coli B tRNA marker, in E) protein N was previously treated with 50 µg pepsin at pH 1.9 at 37° for 2 hours and neutralized to pH 7 with NaOH two hours before adding to the RNA. In F) protein N was previously heated at 95° for 6 min in 2 mM sodium phosphate (pH 7.0). The RNAs in G), I), and J) were not incubated.

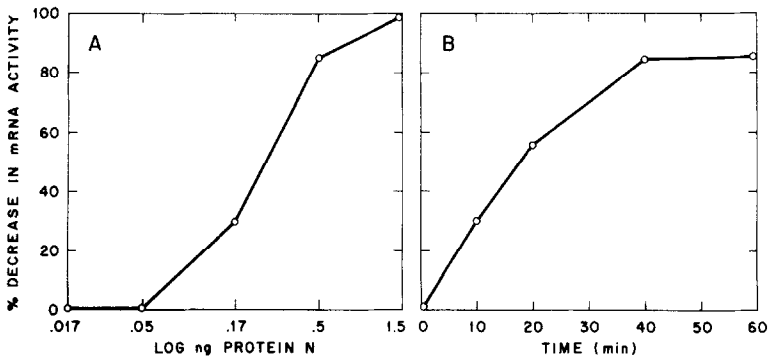

Fig. 2. Inactivation of reovirus mRNA by protein N: A) Dependence on the concentration of protein N, B) time course of the inactivation. Protein synthesis was performed in "preincubated S30 extracts" of L cells (final volume 30 µl) containing ^{3}H-valine (2 µCi) at 37° for 60 min. A) 7.5 µg samples of reo mRNA were incubated with the amount of protein N shown in the figure for 60 min in the conditions of Fig. 1. Thereafter aliquots of the incubated reo mRNA (containing 1.5 µg of RNA) were tested for activity in promoting protein synthesis. The amount of ^{3}H-valine incorporated in the presence of reo mRNA (incubated without protein N) from which that incorporated in the absence of reo mRNA was subtracted serves as the basis of the calculations. B) 7.5 µg samples of the reo mRNA were incubated with 0.5 ng protein N in the conditions of Fig. 1 but for the times indicated in the figure. Thereafter aliquots of the incubated reo mRNA (containing 1.5 µg RNA) were tested for activity in promoting protein synthesis.

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mRNAs in polysomes which contain polyadenylate sequences) was prepared by fractionation on oligo dT-cellulose of the RNA extracted from polysomes of labeled L cells. L cell$_{INT}$ mRNA was prepared in the same way except that the cells were first treated with 300 (VSV plaque reduction) units of mouse interferon/ml at a cell density of 10^{6} cells/ml for 3 hours then one volume of growth medium was added to the culture and it was incubated for an additional 15 hours. Labeled, double stranded reovirus RNA (ds reo RNA) was extracted from reo virions, grown in L cells in a medium containing 1 mc of ^{3}H uridine/l. The activity of various mRNAs and L cell polysomes (before and after treatment with protein N) in promoting protein synthesis (i.e., incorporation of labeled amino acids into hot acid insoluble products) was tested in "preincubated S30 extracts" from L cells. ^{3}H-uridine (38.5 Ci/mmole), ^{3}H-valine (7 Ci/mmole), ^{14}C-glutamine (0.269 Ci/mmole), and ^{14}C-phenylalanine (0.46 Ci/mmole) were obtained from New England Nuclear, S-adenosyl methionine (SAM) from Calbiochem, pepsin (2X cryst.) from Worthington, and polyuridylic acid (poly U) from Miles Laboratories.

Results

We will designate the endonuclease in our interferon preparation as protein N. The effect of treating various mRNAs with protein N at 37° for 1 hour on the sedimentation patterns of the RNAs is shown in Fig. 1. The unfractionated reo mRNA preparation consists of ten mRNA species which are distributed among three

size classes: long (l), medium (m), short (s) (A). Incubating the reo mRNA preparation with as little as 0.05 ng of protein N causes partial degradation (B), incubation with 0.5 ng leads to the disappearance of all three peaks (C) and incubation with 1.5 ng results in the cleavage into fragments smaller than 4S (D). Treatment of protein N with pepsin at pH 1.9 inactivates the endonuclease almost completely (E); the effect of incubation at pH 1.9 with no pepsin was not tested. Heating at 95° for 6 min results in the loss of much of the activity of protein N (F). The mRNA fraction from L cells or from L cells treated with interferon is cleaved by protein N (G, H, J, and K). Poly U is either not cleaved by protein N in our conditions, or is cleaved only at a very slow rate (I, L). The same conclusion was drawn from an experiment in which the electrophoretic pattern of poly U on a 10% polyacrylamide gel was not noticeably affected by treatment of poly U with protein N (not shown). The sedimentation pattern of ds reo RNAs is not affected by treatment with 0.5 ng of protein N at either high salt concentration (in solution I of Fig. 1 except containing 300 mM NaCl instead of 40 mM NaCl) under which conditions the double stranded RNA structure is retained or low salt concentrations (in solution I of Fig. 1) under which conditions the interaction between the two strands of the double stranded RNA is looser and reo mRNA is cleaved by the enzyme (not shown).

The effect of treatment of reo mRNAs with protein N on their activity in promoting amino acid incorporation was tested in S30 extracts of mouse L cells (Fig. 2 and Table I). The dependence of the extent of inactivation of the mRNAs after 1 hour of incubation on the concentration of protein N in the incubation mixture is shown in Fig. 2A: 0.3 ng protein N causes about 50% inactivation of 7.5 µg of the reo mRNAs. The time course of the inactivation of the mRNAs resulting from treatment with 0.5 ng protein N is shown in Fig. 2B. Protein N inactivates reo mRNAs in the absence of SAM and ATP as well as in their presence (SAM and ATP were included in the reaction mixtures because certain endonucleases from *E. coli* require these compounds as cofactors (7) (Table I, Expt. 1). Incubation of reo mRNAs with 0.5 ng of protein N for 1 hour causes an 85% decrease in the mRNA activity, but 0.5 ng of protein N does not impair the translation of reo mRNAs if added to the S30 extract at the same time as the reo mRNAs. Treatment of protein N with 1 mM dithiothreitol (DTT) does not cause inactivation; whereas heating at 95° for 6 min results in the loss of most of its activity (Expt. 1). The mRNAs from L cells, interferon-treated L cells and EMC virus, are also inactivated by treatment with protein N (Expts. 1,2). The activity of poly U and of the mRNAs in L cell polysomes is, however, not affected (Expts. 3,4). The valine acceptor capacity of a crude L cell tRNA and the glutamine acceptor capacity of a purified glutamine specific *E. coli* tRNA species are not decreased by incubation with 0.5 ng of protein N for 1 hour (Table II).

Table I. Inactivation of various viral and L cell mRNAs by protein N. Lack of effect of protein N on the messenger activity of polyuridylic acid and of L cell polysomes. The various mRNAs (reo mRNA (7.5 µg), L $cell_C$ mRNA (7.5 µg), L $cell_{INT}$ mRNA (5 µg), poly U (27 µg) and L cell polysomes (0.78 A_{260} unit) were incubated with or without protein N in the amounts shown at 37° for 60 min in the conditions of Fig. 1 unless otherwise indicated (i.e., SAM and ATP were omitted from one reaction mixture, 1 mM DTT was added to a second and the protein N used in a third had been treated at 95° for 6 min in 2 mM sodium phosphate (pH 7.0) before the experiment). Protein synthesis was performed in "preincubated S30 extracts" from L cells (final volume 30 µl) containing either ^{3}H-valine (2 µCi) (in experiments 1, 2, and 4) or ^{14}C-phenylalanine (0.15 µCi) (in experiment 3) with or without pretreated or untreated reo mRNA (1.5 µg), L $cell_C$ mRNA (1.5 µg), L $cell_{INT}$ mRNA (1.5 µg), EMC RNA (2.4 µg), poly U (5 µg) or L cell polysomes (0.2 A_{260} unit). The conditions of the translation of the reo mRNA, the L $cell_C$ and L $cell_{INT}$ mRNAs and the L cell polysomes was as in reference 4. However, the concentration of KCl was 120 mM for EMC RNA translation and that of Mg^{++} was 10 mM for poly U translation. The net amount of amino acids incorporated was calculated by subtracting the amount incorporated when no mRNA was added from the amount when mRNA was added. The calculation of the net inhibition was based whenever the data were available on the incorporation promoted by mRNAs preincubated at 37° for 60 min without protein N. In other cases it was based on incorporation promoted by not preincubated mRNA. * indicates mRNA not incubated at 37° for 60 min prior to addition to the incubation mixture in which protein synthesis was performed. + indicates 1.5 µg protein N added to the incubation mixture when protein synthesis was started.

No. of Expt.	mRNA	Protein N (ng) used in treating mRNA	^{3}H amino acid incorporated (CPM) Total	Net	Net inhibition (%)
1	None	--	1492	--	--
	Reo	*--	9220	7728	--
	Reo	--	6932	5440	--
	Reo	2.3(-SAM,-ATP)	590	-902	> 100
	Reo	2.3	622	-870	> 100
	Reo	0.5	2305	813	85
	Reo+	*--	9988	8496	0
	Reo	0.5(1mM DTT)	2160	668	88
	Reo	0.5(Heated)	6424	4932	9
	L $cell_C$	*--	4542	3050	--
	L $cell_C$	0.5	2097	605	80
	L $cell_{INT}$	*--	6161	4669	--
	L $cell_{INT}$	0.5	1899	407	91
2	None	--	534	--	--
	EMC	--	6568	6034	--
	EMC	0.5	630	96	98
3	None	--	288	--	--
	Poly U	*--	14,540	14,252	--
	Poly U	0.5	13,269	12,981	9
4	None	--	1021	--	--
	L cell polysomes	*--	25,547	24,526	--
	L cell polysomes	--	14,666	13,645	--
	L cell polysomes	0.5	15,751	14,730	0

Discussion

When partial digests of various natural mRNAs with protein N are centrifuged through sucrose gradients only a small amount of material remains near the top.

Table II. Lack of effect of protein N treatment on the amino acid acceptor capacity of $tRNA^{Val}$ from mouse L cells and of the $tRNA_2^{Gln}$ species from E. coli.
Crude L cell tRNA (0.5 A_{260} units) prepared from L cells by phenol extraction according to Aviv et al. (18) and E. coli K12 $tRNA_2^{Gln}$, 80% pure, (0.1 A_{260} unit) were treated with 0.5 ng of protein N at 37° for 60 min in the conditions of Fig. 1. Thereafter identical aliquots (0.125 A_{260} unit) of pretreated and untreated L cell tRNA were separately charged with ^{3}H-valine (2 µCi) under protein synthesis conditions using "preincubated S30" at 37°C for 10 min and identical aliquots (0.024 A_{260} units) of pretreated and untreated E. coli $tRNA_2^{Gln}$ were separately charged with ^{14}C-glutamine (0.2 µCi) using an about 15 fold purified Gln-tRNA synthetase from E. coli according to Lapointe and Söll (19) except that sodium cacodylate was substituted for Hepes at 37°C for 10 min. The reactions were stopped by adding 1 ml of 5% cold trichloroacetic acid containing 0.1% of casamino acids. The resulting precipitates were collected on nitrocellulose filters, washed and counted in a toluene based scintillator. The net amount of amino acid charged was calculated by subtracting the amount of amino acid charged when no tRNA was added to the incubation mixture for charging from the amount charged when tRNA was added.

No. of expt.	tRNA	Protein N (ng) used in treating tRNA	Amino acid charged (CPM) Valine Total	Valine Net	Glutamine Total	Glutamine Net	Net inhibition (%)
1	None	--	4605	--	--	--	--
	tRNA (L cell)	--	9283	4678	--	--	--
	tRNA (L cell)	0.5	9061	4456	--	--	5
2	None	--	--	--	54	--	--
	$tRNA_2^{Gln}$(E. coli)	--	--	--	4062	4008	--
	$tRNA_2^{Gln}$(E. coli)	0.5	--	--	3794	3740	7

This and the characteristics of the RNA cleavage products analyzed by two dimensional paper electrophoreses (to be reported elsewhere) reveal that protein N is an endonuclease. The fact that it does not cleave (or at least cleaves only slowly) poly U, double stranded RNA, and several species of transfer RNAs seems to indicate the base and/or shape specificity (or at least preference) of protein N. The resistance of polysomes to protein N may be due to steric problems or to inactivation of protein N by enzymes associated with the polysomes.

The determination of the relationship of protein N to the various endonucleases of mammalian tissues (8) and specifically Ehrlich ascites tumor cells (9) will require further studies. Whether protein N is related to interferon or not remains to be seen. This could be established by testing for protein N activity interferons purified through different procedures and even better homogeneous interferon preparations (whenever these become available). Nevertheless, it is a curious thought that an endonuclease of proper specificity could in principle account for at least two of the reported manifestations of interferon treatment of cells: a) the decreased accumulation of viral mRNAs (3,10-14) and b) the impaired capacity of the cell extracts to translate exogenous messengers (6,15,16).

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