INHIBITION OF PROTEIN SYNTHESIS DIRECTED BY ADDED VIRAL AND CELLULAR MESSENGER RNAs IN EXTRACTS OF INTERFERON-TREATED EHRLICH ASCITES TUMOR CELLS. LOCATION AND DOMINANCE OF THE INHIBITOR(S)

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SUMMARY. Protein synthesis directed by exogenous (viral or cellular) messengers is impaired, but endogenous protein synthesis is not affected in an extract of interferon-treated Ehrlich ascites tumor cells (INT-extract). Protein synthesis directed by exogenous messengers is also impaired in a mixture of an INT-extract with an extract from control cells. This reveals that the impairment is due to one or more inhibitors in the INT-extract. The nondialyzability of the inhibitor(s) is probably an indication of large molecular size. In a not incubated INT-extract much of the inhibitory activity is in the high speed sediment fraction i.e., is presumably bound directly or indirectly to ribosomes. During incubation of the extract most of the inhibitory activity is released into the high speed supernatant fraction. The dose-response curve shows that in our conditions the translation of cellular messengers (from mouse L cells) is as sensitive to impairment by the inhibitor(s) as that of viral messengers (from reovirus or from encephalomyocarditis virus).

Interferons are macromolecules, presumably glycoproteins, which are formed 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. The mechanism of action of interferons has not yet been established (1).

Extracts prepared from mouse L cells which had been treated with partially purified mouse interferon (INT)*have an impaired capacity to translate various added viral and cellular mRNAs (2-5). Recently we shifted our studies on INT action from mouse L cells to mouse Ehrlich ascites tumor (EAT) cells (6). Virus replication in our EAT cell line is as sensitive to INT as in L cells. We find that the impairment of the translation of exogenous mRNA in extracts of INT-treated EAT cells is caused by one or more inhibitors. This communication deals with some of the characteristics of the inhibitor(s).

Results and discussion

The amount and the time course of endogenous protein synthesis are not impaired in an unfractionated extract (S30) prepared from EAT cells which had been treated with 60 units/ml of INT (Fig. 1). (This was the concentration of INT used in all experiments). If cells treated with this concentration of INT are infected e.g., with 5 plaque forming units of reovirus/cell, they give rise to 90% less viral progeny than untreated cells (7). It was shown earlier that a) in extracts of

*Abbreviations: EAT, Ehrlich Ascites Tumor; EMC, encephalomyocarditis; INT, interferon; VSV, vesicular stomatitis virus.

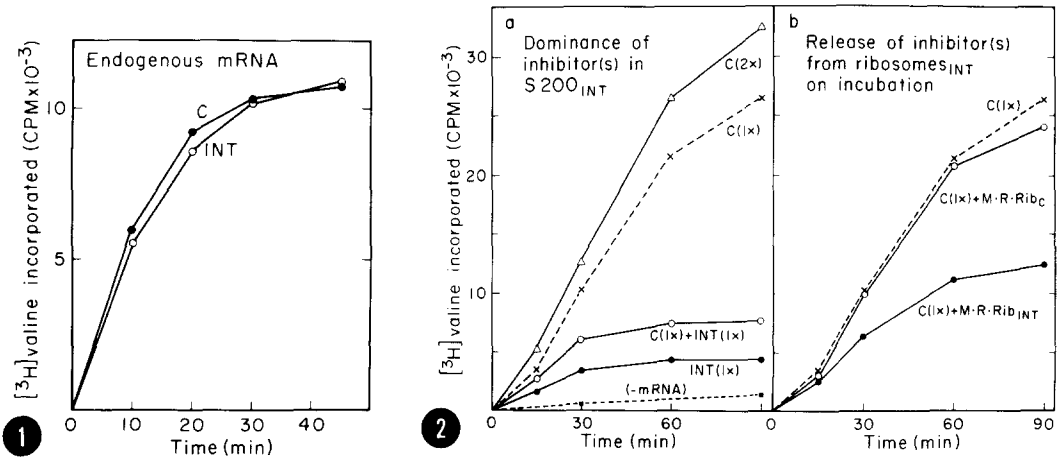

Fig. 1. Similarity in the kinetics of endogenous protein synthesis between nonpreinc. extracts from INT-treated and from control cells. The sp. act. of the mouse INT preparation used was 2 x 10^7 NIH mouse reference strandard units/mg protein. This corresponds to 8 x 10^6 vesicular stomatitis virus (VSV) plaque reduction units/mg protein in an assay used in this laboratory (7). The units throughout this paper are VSV plaque reduction units. The procedure for purifying INT from EAT cells will be published elsewhere (Weideli et al., in preparation). Preparation of the S30 extract from EAT cells. EAT cells (6) were grown in suspension cultures in Eagle's MEM (from Grand Island Biological) with 10% fetal calf serum at 37°. The growing culture was diluted to a density of 4 x 10^5 cells/ml and was distributed into two flasks. One part was treated with 60 units/ml of INT for 18 hours the other served as a control. Thereafter S30 extracts were prepared according to the procedure in ref. (9) except that the solution in which the cells were washed was supplemented with 12 mM glucose. Each S30 extract was passed through a column of Sephadex G25 (medium) equilibrated with TKM buffer (25 mM Tris-Cl (pH 7.5), 80 mM KCl, 4 mM Mg acetate, 6 mM 2-mercaptoethanol). The product nonpreinc. S30 (30 A_{260} units/ml) was stored in liquid nitrogen. Amino acid incorporation in vitro and the processing of the samples was performed as in ref. (9) except that no tRNA was added. Each reaction mixture (50 µl) contained 0.88 A_{260} unit of nonpreinc. S30 from either INT-treated cells (curve INT) or control cells (curve C). The sp. act. of the [^{3}H] valine used in all experiments was 6.7 Ci/mmole. The incubation was at 37°.

Fig. 2. a) Dominance of the inhibitor(s) in the $S200_{INT}$. b) Release of the inhibitor(s) from ribosomes into the S200 during incubation. The cell extracts ($S30_C$ and $S30_{INT}$ nonpreinc. and preinc.), the ribosomal fractions (Rib_C and Rib_{INT} nonpreinc. and preinc.) and the S200 fractions ($S200_C$ and $S200_{INT}$ nonpreinc. and preinc.) were prepared according to the procedures in the legend to Fig. 1 and Table I. Protein synthesis was directed by 1 µg EMC mRNA/30 µl reaction mixture in all cases except those designated (-mRNA). The ionic conditions of translation were as described in Table I for the case of EMC mRNA. The incubation was at 30°. At the times indicated 20 µl aliquots were taken for determining the amount of valine incorporated. a) Each reaction mixture (120 µl) contained 3 A_{260} units of Rib_C preinc. and 4 µg EMC mRNA unless otherwise indicated. Moreover C(1X) contained 0.3 A_{260} unit of $S200_C$ preinc., C(2X) 0.6 A_{260} unit of $S200_C$ preinc., INT(1X) 0.3 A_{260} unit of $S200_{INT}$ preinc. and C(1X)+INT(1X), 0.3 A_{260} unit of $S200_C$ preinc. and 0.3 A_{260} unit of $S200_{INT}$ preinc. (-mRNA) incubations were performed with all the above reaction mixtures. The actual curve shown is that with the components of C(2X) which gave the highest incorporation. b) Preparation of material released from ribosomes (M·R·Rib): 11 A_{260} units of Rib_C nonpreinc. and 11 A_{260} units of Rib_{INT} nonpreinc. were incubated in separate tubes each with

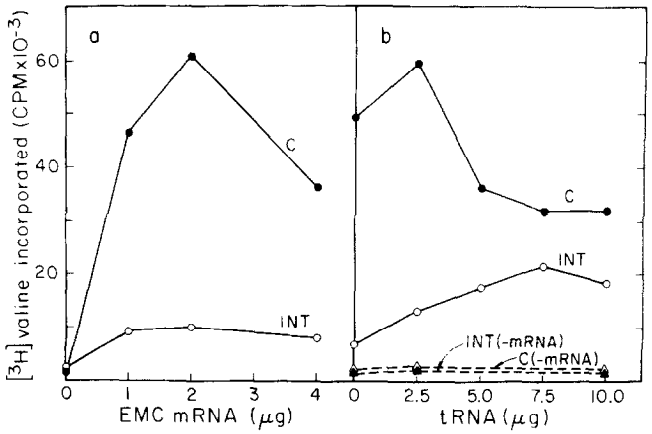

Fig. 3. Protein synthesis directed by EMC mRNA in preinc. extracts of INT-treated and control cells. Dependence on the concentration of a) EMC mRNA, and b) tRNA. Each reaction mixture (30 µl) contained 0.52 A_{260} unit of $S30_C$ preinc. (curve C) or $S30_{INT}$ preinc. (curve INT) and in a) EMC mRNA in the amounts indicated, in b) 1 µg EMC mRNA (unless otherwise indicated) and in the amount indicated tRNA from mouse L929 fibroblasts (for the preparation of this see (10). Other conditions were as described for translation in Table I.

INT-treated L cells the translation of various viral and cellular mRNAs is impaired and b) the impairment is not due to the lack of required components but to the presence of inhibitor(s) (4,5). This conclusion is based on the fact that the translation of added mRNA is also impaired in a mixture of extracts from INT-treated and from control cells (5).

Distribution of the inhibitor(s) between the ribosomal and the high speed supernatant fractions in preincubated and nonpreincubated extracts. Before testing the promotion of protein synthesis by exogenous mRNA in cell extracts the extracts are usually incubated ("preincubation") to lower protein synthesis resulting from the translation of endogenous mRNA. The data in Table I may indicate that in such preincubated (preinc.) extracts (from INT-treated cells) which had been fractionated into ribosomal and high speed supernatant fractions (S200) by centrifugation, more of the inhibitory activity is located in the S200 than in the ribosomal fraction. The same data are also consistent with a deficiency of a component required for protein synthesis in the S200 from INT-treated cells ($S200_{INT}$). However, supplementing a mixture of preinc. ribosomes and preinc. S200 from control cells

0.7 A_{260} unit of protein of $S200_C$ nonpreinc. at 37° for 45 minutes under conditions for preincubation (Table I). Thereafter the reaction mixtures were centrifuged at 200,000 g for 2-1/2 hours and the resulting supernatant fractions were dialyzed against TKM buffer for 8 hours. The dialyzed supernatant fractions were designated $M{\cdot}R{\cdot}Rib_C$ and $M{\cdot}R{\cdot}Rib_{INT}$. The composition of C(1X) was described in a). 120 µl of the reaction mixture contained, if so indicated, 0.24 A_{260} unit of $M{\cdot}R{\cdot}Rib_C$ or $M{\cdot}R{\cdot}Rib_{INT}$.

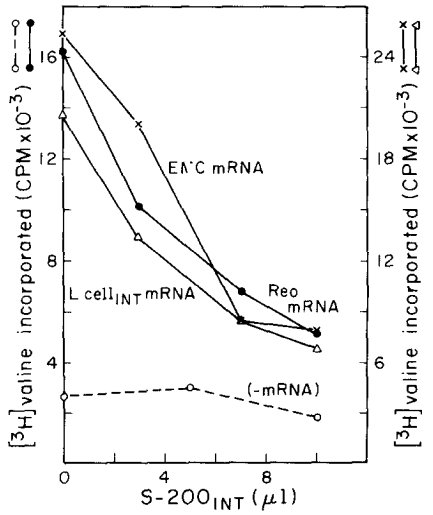

Fig. 4. Comparison of the sensitivity of the translation of viral and cellular mRNAs to inhibition by the inhibitor(s) in the S200 of preinc. extracts from INT-treated cells. Each reaction mixture (30 μl) contained either no mRNA or 1.4 μg reo mRNA (for the preparation see (9)) or 1.2 μg of L cell$_{INT}$ mRNA (from mouse L929 fibroblasts which had been treated with 40 units/ml of INT, for the preparation see (5)), as well as 0.52 A_{260} unit of S30$_C$ preinc. and S200$_{INT}$ preinc. (7.8 A_{260} unit/ml, from INT-treated EAT cells) in the amounts shown. Translation was carried out at 80 mM KCl (the optimal concentration for the translation of L cell mRNA and reo mRNA). All other conditions were as described for translation in Table I.

(S200$_C$) with preinc. S200$_{INT}$ results in the impairment of viral RNA directed protein synthesis (Fig. 2a). This reveals that we are dealing with one or more inhibitors in the preinc. S200$_{INT}$.

The S200$_{INT}$ from a nonpreincubated (nonpreinc.) extract contains little inhibitory activity if reconstituted with ribosomes from preinc. extracts of cells treated with INT (Table II, expt. I). Most of the inhibitory activity in nonpreinc. extracts seems to be located in the ribosomal fraction. The incubation of nonpreinc. S200$_C$ with nonpreinc. ribosomes from INT-treated cells (first incubation of expt. II in Table II) and the fractionation of this reaction mixture results in an S200 (designated in the table as M.R.Rib 4) with an impaired capacity in promoting the translation of mRNA in a system reconstituted with preinc. ribosomes from control cells (second incubation of the same expt.). The curves in Fig. 2b reveal that this impairment is apparently due to the release during incubation of an inhibitory activity from the nonpreinc. ribosomes from INT-treated cells into S200$_C$. Mixing of an S200 obtained from this incubation (designated as M.R.Rib$_{INT}$ in Fig. 2b and as M.R.Rib 4 in Table II) into preinc. extracts of untreated cells inhibits the translation of added mRNA.

Table I. Distribution of the inhibitor(s) between the ribosomal and the high speed supernatant fractions (S200) in a preinc. extract. The extracts from INT-treated and from control cells ($S30_{INT}$ and $S30_C$) were preinc. (except that the Mg^{++} was 5 mM during the process) and Sephadexed and stored according to the procedure in ref. (9). Fractionation of S30 extracts into a ribosomal and a high speed supernatant fraction (S200). The extracts were centrifuged at 200,000 g and 2° for 150 minutes. The S200 was collected and the pellet (containing ribosomes) was rinsed with TKM buffer and resuspended in the same buffer at a concentration of 300 A_{260} units/ml. The suspension was centrifuged at 3000 g for 5 minutes and the resulting supernatant fraction (ribosomes) was collected. Aliquots of the S200 and the ribosomal fractions were stored in liquid nitrogen. Translation of exogenous mRNA. Protein synthesis was promoted by 1 µg of RNA from encephalomyocarditis (EMC) virus (prepared according to ref. (10)) in reaction mixtures (30 µl) containing 1 A_{260} unit of ribosomes and 0.08 A_{260} unit of S200 from either INT-treated (INT) or control (C) cells. The conditions of the incubation were as described in ref. (9) except that no tRNA was added and the final concentration of KCl was 120 mM (ref. 11), 5 mM phosphoenolpyruvate was added and the incubation was performed at 30° for 90 minutes. In these conditions viral mRNA-promoted amino acid incorporation proceeds at close to a linear rate for at least 90 minutes. The numbers in parentheses are pmoles of Valyl residues incorporated in reaction mixtures with no added mRNA. These were subtracted for calculating the percent inhibition.

Type of preinc. ribosomes	Type of preinc. S200	[^{3}H] Valine incorporated (pmoles) into protein	INT-mediated inhibition of amino acid incorporation (percent)
C	C	7.2 (0.085)	-
INT	INT	1.25(0.094)	83
C	INT	2.37(0.064)	68
INT	C	6.7 (0.105)	7

Characteristics of the inhibitor(s). The fact that the inhibitor(s) are present in S200 fractions which had been dialyzed or passed through Sephadex G25 (to remove small molecules) reveals that the inhibitors are large molecules or are at least attached tightly to large molecules. Heating at 95° for 10 minutes caused the loss of inhibitory activity. This makes it likely that the inhibitors are thermolabile but does not prove it. A large precipitate did form during heating and it is conceivable that the inhibitors became adsorbed to the precipitate.

The inhibition in extracts is not overcome by increasing the concentration of either $S200_{INT}$ up to fivefold above the optimal (not shown), or of ribosomes from control cells up to threefold above the optimal (not shown), or of mRNA twofold above the optimal (Fig. 3a). Added transfer RNA overcomes the inhibition, but only partially (Fig. 3b). Amounts of a partially purified initiation factor preparation M_3 from reticulocytes (8) which increase the rate of translation of reo mRNA in an $S30_C$ three fold, do not increase its rate of translation in an $S30_{INT}$ (not shown).

The translation of cellular mRNAs is apparently as sensitive to impairment by the inhibitor(s) in the preinc. $S200_{INT}$ as that of two viral mRNAs

Table II. Distribution of the inhibitor(s) between the ribosomal and the high speed supernatant fractions (S200) in a nonpreinc. extract. Expt. I. Each reaction mixture (30 µl) contained 0.8 A_{260} unit of ribosomes and 0.08 A_{260} unit of S200 of the type indicated as well as 1 µg of EMC mRNA if so indicated. Other conditions were as described for translation in Table I. Expt. II. The FIRST INCUBATION served as a source of four types of M·R·Rib (1, 2, 3, and 4). These were prepared as described in the legend for Fig. 2 except that the indicated types of nonpreinc. ribosomes and S200 were used. In the SECOND INCUBATION each reaction mixture (30 µl) contained 0.8 A_{260} unit of Rib_C preinc. and 0.06 A_{260} unit of M·R·Rib of the type indicated. M·R·Rib_1 was prepared from sample No. 1 of the FIRST INCUBATION, etc. Other conditions were as described for translation in Table I.

Expt.	Type of ribosomes	Type of S200	[^{3}H] Valine incorporated (pmoles) into protein	INT-mediated inhibition of valine incorporation (percent)
I	INT, preinc.	C, nonpreinc.	5.4 (0.10)	-
	INT, preinc.	INT, nonpreinc.	4.9 (0.11)	10

II	Sample No.	FIRST INCUBATION: Type of nonpreinc. ribosomes	FIRST INCUBATION: Type of nonpreinc. S200	SECOND INCUBATION: Type of preinc. ribosomes	SECOND INCUBATION: Type of M·R·Rib	[^{3}H] Valine incorporated (pmoles) into protein	INT-mediated inhibition of valine incorporation (percent)
	1	C	C	C	1	9.0 (0.65)	-
	2	INT	INT	C	2	1.1 (0.63)	94
	3	C	INT	C	3	6.5 (0.63)	30
	4	INT	C	C	4	3.4 (0.65)	67

(Fig. 4). (Addition of $S200_C$ in place of $S200_{INT}$ did not cause any impairment.) The cellular mRNA used was purified on oligo dT-cellulose from polysomal RNA of INT-treated L cells (5). (We found earlier that in an extract of INT-treated L cells the translation of mRNA from INT-treated and from untreated L cells was impaired to the same extent; Gupta et al., unpublished). The nondiscriminate inhibition of the translation of exogenous viral and cellular mRNAs in the extract from INT-treated cells does not appear to reflect faithfully upon the events occurring in intact INT-treated cells. In these the accumulation of viral proteins is blocked but that of host protein is not affected (5). The resolution of this apparent discrepancy and the elucidation of further characteristics of the inhibitor(s) should hopefully contribute to our knowledge of INT action.

It should be noted that in these and all other studies heretofore partially purified preparations of INT were used. Pure preparations of INT will be needed to establish if the inhibitor(s) described in this communication are induced by INT itself or by some other component of the INT preparation.

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