Vol. 82, No. 1, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 15, 1978 Pages 384-391

INHIBITION OF EXOGENOUS RNA-DEPENDENT PROTEIN SYNTHESIS BY A LOW-MOLECULAR-WEIGHT RNA FROM NUCLEAR RIBONUCLEOPROTEIN PARTICLES OF ADENOVIRUS-INFECTED HELA CELLS

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Received April 3,1978

SUMMARY

Low-molecular-weight RNA (4S to >5.5S) isolated from nuclear ribonucleoprotein particles of adenovirus-infected HeLa cells inhibited cell-free protein synthesis directed by polyribosomal RNA from rabbit reticulocytes by more than 80%. In a reconstituted system inhibitory RNA did not prevent the binding of Met-tRNA$_f$-GTP-IF ternary complex to 40S subunits; however, it repressed the formation of 80S from 40S-mRNA complex and 60S subunits. In binding assays in which authentic IF-M2A and IF-M2B were present, the inhibitor competed with messenger molecules for binding site(s) in IF-M2B. The inhibitory RNA appears to be a 5.5S RNA.

INTRODUCTION

A group of low-molecular-weight RNAs (4S to > 5.5S), here designated as I-RNA, has been isolated from nuclear ribonucleoprotein particles of adenovirus-infected HeLa cells. This fraction inhibited encephalomyocarditis virus RNA-dependent cell-free protein synthesis in an S-30 extract from Ehrlich ascites cells by more than 70% (unpublished data). The inhibition appeared to result from a block in the initiation step. Since oligonucleotides and low-molecular-weight RNAs inhibit protein synthesis (1, 2) and since adenovirus-infected cells synthesize large quantities of low-molecular-weight RNAs in the nucleus (3, 4), the nature of the inhibitory RNA and its mode of inhibition are of interest.

The kinetic studies and experiments on the partial reactions of initiation reported here show that I-RNA does not inhibit the binding of Met-tRNA$_f$-GTP-IF ternary complex to 40S subunits but does significantly inhibit the formation of 80S complex. Since mRNA and I-RNA compete for one of the

0006-291X/78/0821-0384$01.00/0

initiation factors of subunit joining, IF-M2B, I-RNA evidently forms a complex with IF-M2B and inhibits the interaction of mRNA with this factor. The inhibitor appears to be a 5.5S RNA.

Initiation factors IF-M2A and IF-M2B are designated according to Adams et al. (5).

MATERIALS AND METHODS

Isolation and Purification of I-RNA from Adenovirus-Infected Cells

Growth of HeLa S-3 cells, infection with adenovirus type 2, preparation of nuclear ribonucleoprotein particles from the infected cell nuclei, and isolation of total RNA from these particles have been described (6). In this study total RNA was isolated from unlabeled cells as well as from cells labeled with 1 μCi/ml of [^{3}H]uridine (50-55 Ci/mmol) at 6-19 h after infection. The RNA, unlabeled or labeled, was denatured in 90% Me_2SO for 20 min at 37° and sedimented in a 15-30% sucrose gradient containing 20 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 0.5% sodium dodecyl sulfate for 17 h at 25° using a Spinco SW25.1 rotor. Appropriate marker RNAs were mixed, denatured, and sedimented in parallel gradients. I-RNA was isolated from fractions 26-28 (see Fig. 1) after precipitation with 2 volumes of ethanol and 150 mM NaCl at -20°. The precipitated RNA was dissolved in water.

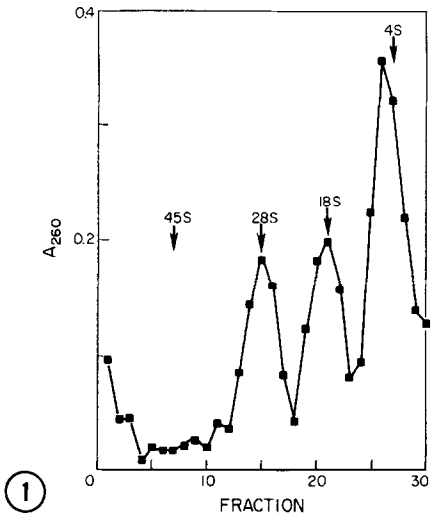
Inhibition experiments

Total and poly(A)-containing polyribosomal RNA (7) and partially purified initiation factors (8) were prepared from rabbit reticulocytes. Cell-free protein synthesis in the S-30 extract of Ehrlich ascites cells has been described (6). The following changes were made in the present study: (i) KCl and Mg^{2+} ion concentrations were 112 mM and 4.4 mM respectively. (ii) The incubation mixture (60 μl) contained 20 μg of partially purified initiation factors, 0.09 A_{260} unit of poly(A)-containing or 1 A_{260} unit of polyribosomal RNA from rabbit reticulocytes, varying amounts of I-RNA, and 2.5 μCi of [^{3}H]leucine (40-50 Ci/mmol). After incubation for 45 min at 37° radioactivity precipitable with hot acid was measured.

Binding Experiments

Three binding experiments were performed:

(1) Binding of [^{35}S]Met-tRNA$_f$ to ribosomal subunits. The 60S and 40S ribosomal subunits (9), pH 5 enzyme (10) and S-30 extract (11) were prepared from Ehrlich ascites cells following standard procedures. In addition to the components of protein synthesis, the incubation mixtures (120 μl) contained 22 μg of unfractionated rabbit liver tRNA (Grand Island Biological Co.), 5 μCi of [^{35}S]methionine (200-300 Ci/mmol), 450 μg of pH 5 enzyme, 0.68 A_{260} unit of 40S subunits, and in some cases (Fig. 3C, D) 1.7 A_{260} units of 60S subunits. I-RNA (9 μg) was also added to some incubation mixtures, as indicated in Fig. 3. After incubation for 20 min at 30°, the incubation mixtures were diluted to 800 μl with a cold buffer (10 mM NaCl, 1.5 mM $MgCl_2$, and 10 mM Tris-HCl, pH 7.5) and sedimented in 15-30% linear sucrose gradients in the buffer at 22,000 rpm for 16 h at 2° using a Spinco SW 27.1 rotor. Gradient fractions (0.6 ml) were collected, and cold trichloroacetic acid-precipitable radioactivity and absorbance at 260 nm were measured.



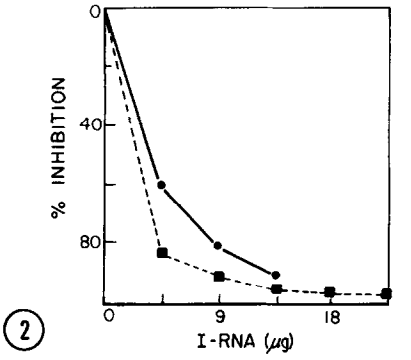


Figure 1: Sedimentation analysis (Materials and Methods) of unlabeled total RNA from nuclear ribonucleoprotein particles.

Figure 2: Inhibition of [^{3}H]leucine incorporation by I-RNA when protein synthesis is directed by poly(A)-containing (●) or total (■) RNA. Radioactivity corresponding to 0% inhibition is 47,000 cpm for poly(A)-containing RNA and 174,000 cpm for total polyribosomal RNA (in 60 μl). Experimental details are in Materials and Methods.

(2) Binding of [^{3}H]uridine-labeled mRNAs and I-RNA with reticulocyte initiation factors. The binding assay of Hellerman and Shafritz (12) was performed with histone mRNA and authentic IF-M2A and IF-M2B, kindly provided by Dr. C. Baglioni (State University of New York at Albany) and Dr. W. C. Merrick (NIH) respectively. To prepare labeled poly(A)-containing adenovirus mRNA from cytoplasmic polyribosomes (7), a suspension of cells which had been infected with the virus 16 h previously was concentrated to 2 x 10^7 cells/ml, and the suspension was incubated for 2-3 h with 15-20 μCi/ml of [^{3}H]uridine. The incubation mixtures (50 μl) contained 20 mM Tris-HCl (pH 7.5), 100 mM dithiothreitol, 15 μg of partially purified reticulocyte initiation factors, and either 1-2.2 μg of IF-M2A or 1 μg of IF-M2B. Each mixture was reacted with either 26,000 cpm of labeled poly(A)-containing adenovirus mRNA, 4,000 cpm of histone mRNA, or 2,000 cpm of I-RNA. After incubation for 5 min at 23°, 3 ml of cold buffer (20 mM Tris-HCl, pH 7.5 and 100 mM KCl) were added. The resulting protein-RNA complex was collected on a nitrocellulose filter, washed, and counted for radioactivity.

(3) Competition between mRNA and I-RNA for specific initiation factor. A mixture containing a specific initiation factor was preincubated with unlabeled I-RNA (or mRNA) for 5 min at 23°. Labeled mRNA (or I-RNA) was then added, and the mixture was incubated for an additional 5 min and filtered on a nitrocellulose filter as described above.

Polyacrylamide Gel Electrophoresis

An aliquot of I-RNA was reacted with IF-M2B as before, and the I-RNA

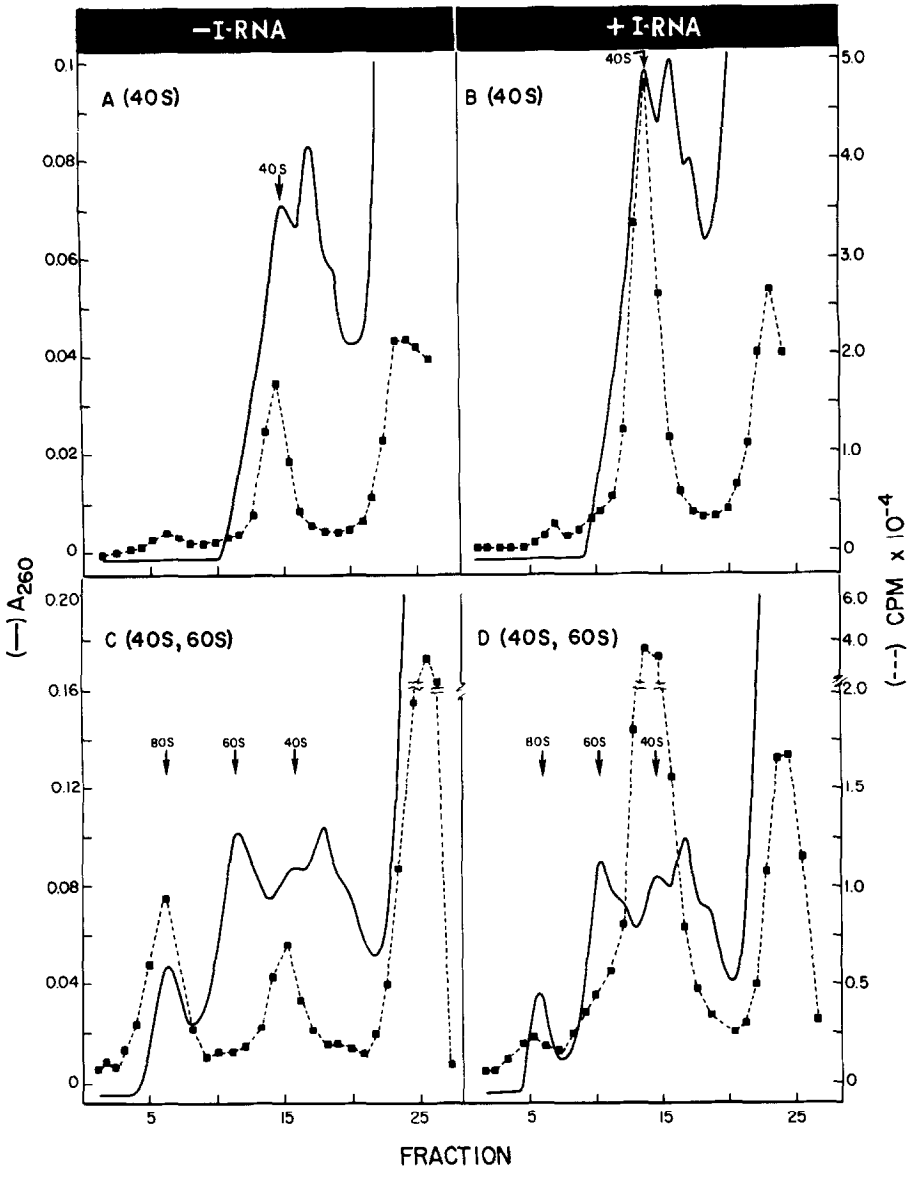


Figure 3: Binding of [^{35}S]Met-tRNA$_f$ to 40S subunits (A, B) and 80S ribosomes (C, D) in the absence and presence of I-RNA. Experimental details are in Materials and Methods.

bound to the nitrocellulose filter was extracted with 1% sodium dodecyl sulfate (13) and electrophoresed, along with total unreacted I-RNA and appropriate markers, in parallel gels. The procedure of gel electrophoresis in 12.5% acrylamide containing 1M urea has been described (14). For each marker the relationship between the distance migrated and the S value was linear.

RESULTS AND DISCUSSION

The sedimentation analysis of total RNA isolated from nuclear particles of adenovirus-infected HeLa cells is shown in Fig. 1. Fractions 26-28 are the I-RNA used in this study. Increasing concentrations of I-RNA inhibited the incorporation of leucine by more than 80% when protein synthesis was directed by either total or poly(A)-containing rabbit reticulocyte polyribosomal RNA (Fig. 2). This inhibition occured within 5 min of incubation (data not shown). Leucine incorporation by total RNA and poly(A)-containing RNA was inhibited by 80% and 50% respectively with 4.5 μg of I-RNA (Fig. 2). The kinetics of inhibition were similar with both preparations. Total RNA directed incorporation of somewhat more leucine than the poly(A)-containing RNA, probably because the ribosomal RNA protected messenger molecules from the ribonuclease activity of the extract. Therefore the remaining experiments were performed with the total RNA.

To investigate whether I-RNA-dependent inhibition occurs at the level of chain initiation or elongation, the kinetics of this inhibition were compared with those by aurintricarboxylic acid, a known inhibitor of initiation. The results (not shown) indicate that I-RNA-dependent inhibition occurs predominantly at the level of chain initiation. Those experiments will be described in detail in a subsequent paper.

Initiation of protein synthesis occurs in a number of steps, each of which is controlled by a combination of initiation factors (see ref. 5 for general discussion). In this study I-RNA-dependent inhibition could be partially relieved (about 30%) by adding an excess of initiation factors (results not shown), which suggests that I-RNA interacts directly with some of these factors. Consequently, the effect of I-RNA on the formation of 80S initiation complex was tested.

Two steps of 80S initiation complex formation are of interest here: (i) the formation of a complex of Met-$tRNA_f$, 40S, and messenger RNA in the presence of GTP and a number of initiation factors, and (ii) the association of

TABLE 1

Binding of [^{3}H]uridine-labeled mRNAs and I-RNA with reticulocyte initiation factors

	Adenovirus mRNA		Histone mRNA		I-RNA	
Factor	Input cpm	% of input bound	Input cpm	% of input bound	Input cpm	% of input bound
Partially purified IF	16,200	62	--	--	1,373	70
IF-M2A	6,300	24	210	5	91	4
IF-M2B	18,455	71	3,112	75	732	36

this complex with a 60S subunit in the presence of GTP, IF-M2A, and IF-M2B to form 80S. In the absence of I-RNA (Fig. 3A) about 50% of the recovered radioactivity from [^{35}S]Met-tRNA$_f$ was associated with 40S, which is consistent with the complex formation. Addition of enough I-RNA to reduce leucine incorporation by 80% (see Fig. 2) tripled the amount of radioactivity bound to 40S (Fig. 3B), which indicated that the inhibition is not occurring at this step of 80S formation. On the other hand, in the absence of I-RNA, 20% and 17.5% of the recovered radioactivity were associated with the 80S and 40S regions respectively (Fig. 3C). Incubation with I-RNA reduced the radioactivity bound to the 80S regions by about 75% and increased that bound to 40S by 3.5-fold (Fig. 3D). I-RNA also inhibited binding of [^{35}S]Met-tRNA$_f$ to polyribosomes by 80% (data not shown). The small amount of radioactivity still bound to the polyribosomes and the 80S region could be due to the residual initiation (since I-RNA-dependent inhibition was 80%) and to transfer of [^{35}S]methionine from Met-tRNA$_m$ into internal positions.

These results indicate that I-RNA inhibits the association of the complex with the 60S subunit. Since the association of the 40S complex with 60S subunits is catalyzed by IF-M2A and IF-M2B, we investigated whether the inhib-

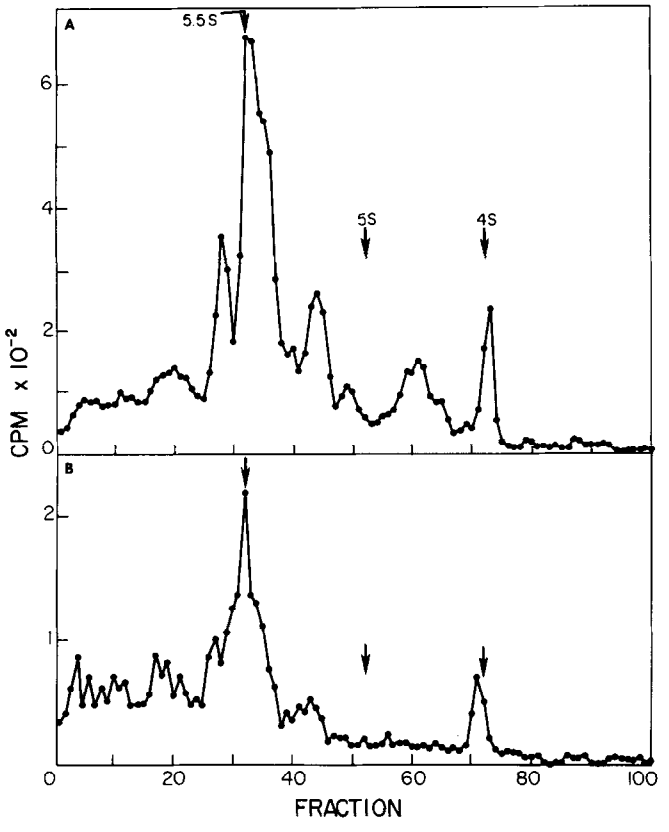


Figure 4: Polyacrylamide gel electrophoresis of [^{3}H]uridine-labeled total I-RNA before (A) and after (B) binding with IF-M2B. The binding of I-RNA with IF-M2B, extraction of the bound RNA from nitrocellulose filter, and the procedure of gel electrophoresis are described in Materials and Methods.

itor binds with either or both of these factors. Considerable radioactivity from I-RNA or from the messengers used was bound both to partially purified initiation factors and to IF-M2B (Table 1). The results of a competition experiment between I-RNA and the messengers (data not shown) indicated that the inhibitor competes with the messenger for common binding site(s) in IF-M2B. Since this factor is essential for subunit association, binding of I-RNA with IF-M2B appears to prevent the formation of 80S.

Four or five low-molecular-weight species ranging from 4S to > 5.5S were found in a polyacrylamide gel electrophoresis profile of I-RNA (Fig. 4A).

The 5.5S RNA is thought to be the inhibitor, since a major fraction of labeled I-RNA bound to IF-M2B migrated in a parallel gel (Fig. 4B) to the same position. Experiments are in progress to investigate further the nature of this molecule and to find out whether it is virus-coded or is induced after virus infection.

ACKNOWLEDGMENTS

This work was supported by NIH research grant number 1RO1-CA 19707 awarded by the National Cancer Institute/DHEW. We also wish to thank L. Mitchell for her technical assistance.

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