Effect of Heparin Contained in Preparations of Small Cytoplasmic RNAs on Cell-free Translation*

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It has been reported that small cytoplasmic RNA (scRNA) from human placenta inhibits translation of most mRNAs in both wheat germ extracts and reticulocyte lysates (Lorberboum, H., Digweed, M., Erdmann, V. A., Servadio, Y., Weinstein, D., De Groot, N., and Hochberg, A. A. (1986) Eur. J. Biochem. 155, 279–287). We have investigated the mechanism by which scRNA preparations inhibit mRNA translation in vitro. We demonstrate that the inhibitory agent(s) is not sensitive to treatment with various ribonucleases but that translational inhibition is sensitive to incubation with 1 N NaOH at 95 °C or to treatment with heparinase. Based on these findings and on the ability of heparin to inhibit cell-free translation by itself, we conclude that the presence of heparin in preparations of scRNA from human placenta is responsible for effects which have previously been attributed to inhibitory RNA molecules. Since heparin is also frequently used in the isolation of translationally inhibitory scRNAs from other sources, we suggest that the sensitivity of these preparations to ribonucleases and heparinase should be examined.

Small RNA molecules are important constituents of both the nucleus and the cytoplasm of eukaryotic cells. They are usually found in association with proteins and thus form RNA-protein complexes referred to as small ribonucleoprotein particles (RNPs)1 (1). Small nuclear RNPs constitute a well known class of RNA-protein complexes which have been shown to be involved in several steps of pre-mRNA and rRNA processing (2, 3). In contrast to nuclear small RNPs, the biological functions of small cytoplasmic (sc) RNPs are largely unknown. These molecules have been isolated from numerous sources and have been suggested to play a role in tRNA processing (4), protein degradation (5, 6), and mRNA translation. With regard to the latter suggestion, several groups reported the isolation of scRNAs which can inhibit mRNA translation in cell-free extracts from wheat germ or rabbit reticulocytes (7–13). One of these inhibitory scRNAs was isolated from human placenta (11, 14) and shown to block translation of most but not all (15) polyadenylated mRNAs. The major inhibitory RNA species from human placenta, referred to as scRNA species 1, was estimated to be approximately 35 nucleotides long and further characterized by RNA sequencing (11). The physiological role(s) and the exact molecular mechanism by which this placental (and other) scRNAs inhibit translation in vitro are unknown; most experimental evidence suggests that, in vitro, the initiation step of translation is affected (11).

We have purified scRNA from human placenta as previously described (11). The purified material was added to wheat germ extract or to reticulocyte lysate and caused strong inhibition of mRNA translation in both systems. However, in contrast to previous reports, we were unable to collect convincing evidence for a functional role of scRNAs in these preparations. Our results strongly indicate that these preparations contain quantities of heparin which are sufficient to cause inhibition of translation.

MATERIALS AND METHODS

Chemicals and Reagents—[35S]Methionine and [α-32P]UTP were purchased from Du Pont-New England Nuclear; nucleotides, heparinase II, proteinase K, and micrococcal nucleases were from Sigma; RNase A, RNase T1, and DNase I were from Boehringer Mannheim; RNase T1 and RNase T2 were from Bethesda Research Laboratories; and T7 RNA polymerase was from U. S. Biochemical Corp. Heparin was purchased from Sigma and dissolved in H2O. The A260 of this solution was, when compared on a w/v basis, approximately 200-fold lower than the A260 of RNA; the A260/A230 ratio of the heparin solution was approximately 1.6.

Isolation of scRNA from Human Placenta—The preparation of scRNA from human placenta was performed as described by Lorberboum et al. (11) and is summarized in Fig. 1. Full term placenta was kept in ice-cold phosphate-buffered saline and processed within 30 min of delivery. All purification steps were carried out at 4 °C. First, the placenta (400–600 g) was washed three times in phosphate-buffered saline and once in buffer A (50 mM Tris-HCl, pH 7.4, 25 mM KCl, 5 mM MgCl2, 100 mM NH4Cl, 0.5 mM EDTA, 2 mM dithiothreitol, 0.25 M sucrose, and 0.5 mg/ml heparin), all in diethyl pyrocarbonate-treated H2O. Connective tissue was removed, and the cleaned placenta (200–300 g) was homogenized in buffer A (1:1, w/v) using a Polytron and a Dounce tissue homogenizer. The homogenate was cleared twice by centrifugation for 10 min at 9,000 × g. After this step, heparin was either consistently present in or omitted from all the buffers used (see Fig. 1). Aliquots (out of a total of approximately 400 ml) of the postmitochondrial supernatant were separated on a two-step sucrose gradient (1.55 and 2.0 M sucrose in buffer A) by centrifugation at 82,000 × g for 3 h. Postmitochondrial supernatant was collected and pelleted by centrifugation for 13 h at 165,000 × g. The pellet (termed mRNP fraction I) was rinsed carefully 3 times with buffer A and then resuspended in 8 ml of buffer A. Next, this fraction was layered onto a linear 10–40% sucrose gradient in buffer A and centrifuged for 20 h at 82,000 × g. The A260 was determined for 1-ml aliquots collected after the centrifugation and peak fractions were pooled (see Fig. 1). The pooled fractions were mixed with an equal volume of 1.0 M KCl in buffer B (10 mM Tris-HCl, pH 7.4, 5 mM MgCl2, and 0.5 mg/ml heparin), incubated for 30 min with gentle agitation, and diluted with another 4 volumes of buffer B containing 0.5 M KCl. After a final centrifugation on a cushion of 1.0 M sucrose and 0.5 M KCl in buffer B for 20 h at 82,000 × g, the supernatant was extensively dialyzed against buffer B and lyophilized. The lyophilized scRNPs were gently dissolved in buffer A and then extracted

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1 The abbreviations used are: RNPs, ribonucleoprotein particles; sc, small cytoplasmic; m, messenger; IRE, iron-responsive element; SDS, sodium dodecyl sulfate; DEPC, diethyl pyrocarbonate.
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**Preparation of Synthetic RNA Transcripts—In vitro transcription of a 34-nucleotide-long IRE motif, a 35-nucleotide-long mutated version of the IRE (ΔC-IRE), and a 34-nucleotide-long so-called unrelated control transcript was performed as described by Milligan et al. (16) using cloned T7 RNA polymerase. The DNA template for the unrelated control transcript was 5'-GGGTACGAC-CAACTTCCCTGA CAACTTCTCTCAGCTTATCATGAGTCC-ATT A-3'; the template for the IRE motif was 5'-GGGTACCGTGCACAAGCAGTCTGTAAGAGCAGGTCCCCATAGTGGAGTCGATT A-3', and the template for the ΔC-IRE was identical to the IRE template except that the G residue underlined above was omitted. Synthetic DNA oligonucleotides and in vitro transcription products were purified by gel electrophoresis (15% polyacrylamide:bisacrylamide (20:1), 8 M urea) and eluted according to standard procedures (17). The RNA was finally dissolved in DEPC-treated H2O and stored at -70 °C.

**Enzymatic and Chemical Characterization of scRNA Preparations**—The scRNA preparations (30-150 ng) were treated with DNase I (1.3 units/μl), RNase A (0.1-0.5 mg/ml), micrococcal nuclease (0.25 mg/ml), RNase T1 (70 units/μl), or with a mixture consisting of 500 units/ml RNase T1, 300 units/ml RNase T2, and 0.05 mg/ml RNase A in 0.05 M NH4CH3COO, pH 4.5, for 1 h at 37 °C. Subsequently, these enzymes were inactivated by treatment with 2 μg/ml heparin to 1 N NaOH under identical conditions (even in the presence of RNase-free heparinase (0.3 units/ml) in a buffer consisting of 10 mM potassium phosphate, pH 7.0, and 100 mM NaCl (18) for 40 min at 56 °C. Subsequently, samples were phenol/chloroform (1:1)-extracted, ethanol-precipitated using glycogen as a carrier, taken up into equal volumes of DEPC-treated H2O, and added to the in vitro translation systems. 3P-Labeled RNA transcripts (the IRE motif described above) were completely degraded when incubated with ribonuclease or 1 N NaOH under identical conditions (even in the presence of scRNA preparations), whereas they remained fully intact when treated with heparinase.

**In Vitro Translation in Rabbit Reticulocyte Lysate or Wheat Germ Extract**—Total RNA from HeLa cells was isolated by the guanidinium isothiocyanate method and subsequently enriched for poly(A)+ RNA by a batch procedure with oligo(dT)-cellulose from Boehringer Mannheim (17). Cell-free translations were performed essentially as described by Clemens (19) in the presence of [35S]methionine (0.5 μCi/μl) and poly(A)+-enriched RNA from HeLa cells (20-25 μg/μl). Inhibitory substances were added as described in the figure legends. Rabbit reticulocyte lysate was purchased from Promega and adjusted to 63 mM K+ and 0.68 mM Mg2+. All samples were maintained on ice before incubation for 1 h at 35 °C (wheat germ) or 30 °C (rabbit reticulocyte). Aliquots of [35S]-labeled translation products were analyzed by electrophoretic separation on SDS-polyacrylamide gels and autoradiography, or by trichloroacetic acid precipitation and scintillation counting as described by Mans and Novelli (21).

**RESULTS AND DISCUSSION**

Cell-free translation systems were programmed with poly(A)+-enriched RNA from HeLa cells, and 2.0 μg/ml scRNA or a similar quantity of control transcripts was added before translation reactions were started. Analysis of [35S]methionine-labeled in vitro translation products by SDS-polyacrylamide gel electrophoresis revealed essentially complete inhibition of protein biosynthesis by the scRNA preparation in both rabbit reticulocyte lysates (Fig. 2A, lane d) and wheat germ extract (Fig. 2B, lane d). In contrast, no inhibition of translation was seen with the same amount (based on A260 determination) of three different synthetic RNA transcripts of similar length and predicted secondary structure as the scRNA species 1 (Fig. 2, A and B, lanes c, e, and f). Interestingly, we noticed that the addition of a short transcript corresponding to the IRE from human ferritin H-chain mRNA induced the translation of a protein similar in size to human ferritin in rabbit reticulocyte lysate (Fig. 2A, lane e), but not in wheat germ extract (Fig. 2B, lane e). This induction

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**Figure 1. Purification scheme for scRNA from human placentas.** The purification was performed as described in detail under "Materials and Methods." Buffers which contain heparin are indicated as such (+/− heparin); buffers which should contain heparin according to the original protocol (11) but where it was omitted to serve as a control (see text) are indicated by (+/− heparin).

Twice with Tris-EDTA-saturated phenol (pH 7) and once with chloroform to remove the scRNP proteins. The scRNA was precipitated with 2.5 volumes of 99.5% ethanol without carrier, washed twice with 80% ethanol, and resuspended in DEPC-treated H2O. The apparent concentration of scRNA was determined by measuring the absorbance at 260 nm; the ratio of A260/A340 was generally 1.6-1.8.
Fig. 2. Inhibition of protein biosynthesis in rabbit reticulocyte lysates (A and C) and wheat germ extracts (B) by scRNA preparations from human placenta. Cell-free translation systems were programmed with 20 ng/µl poly(A)\(^\pm\) enriched HeLa cell mRNA (panels A and B, lanes b-f, panel C). A and B, translation products were labeled with \(^{35}\)S)methionine and analyzed by SDS-polyacrylamide gel electrophoresis. The migration of molecular weight markers and the position of human ferritin are indicated. Lanes a, no mRNA added; lanes b, mRNA but no small RNA added; lanes c, +2.0 ng/µl unrelated control transcript; lanes d, +2.0 ng/µl scRNA; lanes e, +2.0 ng/µl IRE transcript; lanes f, +2.0 ng/µl ΔC-IRE transcript. C, increasing quantities of two scRNA preparations made independently in Jerusalem (filled diamonds) or Heidelberg (dotted squares) were added simultaneously with mRNA to rabbit reticulocyte lysate. The inhibition of protein biosynthesis was determined by trichloroacetic acid precipitation and scintillation counting and was expressed as a percentage of controls to which no scRNA had been added. The open squares represent a titration curve which was previously obtained by Kloppstech et al. (15) for placental scRNA in wheat germ extract. Two of the three titration curves include determinations at scRNA concentrations of 0, 5.0 ng/µl which are not shown, but which are indicated by the extension of the graph beyond the highest point of the determination that is shown.
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**FIG. 3.** Effect of proteinase, nuclease, and NaOH treatment on the inhibitory activity of scRNA preparations. Panel A, equal aliquots of either a control RNA transcript (lanes c–h) or of an scRNA preparation from human placenta (lanes i–n) were treated with proteinase K (lanes d and j), RNase A and subsequently proteinase K (lanes e and k), 1 N NaOH at 95 °C (lanes g and m), 1 N NaOH at 25 °C (lanes h and n) and remained untreated at 95 °C (lanes f and l) or 25 °C (lanes c and e) as described under "Materials and Methods." Samples were extracted with phenol/chloroform and precipitated with ethanol before aliquots corresponding to 2.0 ng/μl final concentration were added to rabbit reticulocyte lysate as described in Fig. 2. Molecular mass standards are indicated on the left. Lane a, no mRNA added; lane b, no unrelated control RNA transcript added. Panel B, the sensitivity of an scRNA preparation (s, striped bars) to DNase I, RNase T1, and micrococcal nuclease (micrococ.) was tested as described above. Translational activity was monitored in rabbit reticulocyte lysate and determined by trichloroacetic acid precipitation and scintillation counting. Translational activity was expressed as a percentage of control, where the unrelated control transcript (c, black bars) was added without prior treatment (indicated with *). The lane labeled mRNA shows the endogenous translational activity of the extract without exogenously added HeLa mRNA. Addition of untreated scRNA (s, striped bars) reduces the translational activity to levels at or below the endogenous activity of the extract. The scRNA is insensitive to pretreatment with DNase I, RNase T1, and micrococcal nuclease. Proteinase K (prot. K) and NaOH treatment at 95 °C were done as described above and are shown as controls.

**FIG. 4.** Effect of scRNA preparations and heparin on cell-free translation in rabbit reticulocyte lysate. A, increasing quantities (determined by A₂₆₀) of heparin (dotted squares) or scRNA (filled diamonds) were added, and translational activity was estimated by trichloroacetic acid precipitation and scintillation counting, and expressed as a percentage of controls to which H₂O was added. B, 22 ng/μl heparin (h) or 0.06 ng/μl scRNA (s) was added to rabbit reticulocyte lysates essentially as described above after preincubation with heparinase (+) or buffer alone (heparinase, −) and phenol/chloroform extraction followed by ethanol precipitation.

significant proportion of the A₂₆₀ in the scRNA preparations, their inhibitory activity could be fully explained by the presence of heparin. Furthermore, a mock preparation of scRNA using only buffers without placental tissue also inhibited cell-free translation with *). The lane labeled mRNA- shows the endogenous translational activity of the extract without exogenously added HeLa mRNA. Addition of untreated scRNA (s, striped bars) reduces the translational activity to levels at or below the endogenous activity of the extract. The scRNA is insensitive to pretreatment with DNase I, RNase T1, and micrococcal nuclease. Proteinase K (prot. K) and NaOH treatment at 95 °C were done as described above and are shown as controls.
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free translation (data not shown). Preparation of placental scRNA with heparin present only during the homogenization step but absent from all subsequent steps yielded a sample with a 100-fold lower inhibitory activity (based on A\textsubscript{260} quantitation). We attribute this difference to a lower concentration of heparin relative to RNA in this preparation. Moreover, this step but absent from all subsequent steps yielded a sample because heparin can resemble small RNAs in many physical characteristics as the scRNA preparations were confirmed by labeling of 3' termini with [5'-\textsuperscript{32}P]pCp and T4 RNA ligase (data not shown).

Finally, we tested the prediction that treatment of an scRNA preparation with heparinase (from Flavobacterium heparinum) should decrease the inhibition of translation in rabbit reticulocyte lysates. Fig. 4B shows that both heparin (h) and an scRNA preparation inhibit translation to levels <35% of control translations. When either one of these two inhibitors was pretreated with heparinase, we observed reproducible recovery of translation activity to approximately 70% of control values. Different experimental conditions of heparinase treatment consistently resulted in similar responses for heparin and scRNA-mediated translational inhibition, but a complete recovery of translational activity was not reproducibly obtained (data not shown). The heparinase used in our studies was shown to be free of contamination. RNase activities, ruling out the possibility that the recovery of translational activity after treatment of the scRNA preparation could be attributed to the activity of a ribonuclease other than RNase T\textsubscript{1}, RNase T\textsubscript{2}, RNase A, or micrococcal nuclease, which were directly tested (Fig. 3 and data not shown).

We conclude that the presence of heparin is primarily responsible for the translational inhibition caused by scRNA preparations from human placenta.

In this study we have attempted to characterize the molecular mechanism by which scRNA preparations from human placenta inhibit mRNA translation in cell-free extracts. Surprisingly, we did not obtain any evidence which implicated the scRNA species 1 (or any other RNA molecule) as the molecular effector for this translational inhibition. The findings reported here provide strong support for an alternative explanation of previous results. We conclude that heparin (which is added as an anticoagulant and RNase inhibitor to the scRNA preparations and which may also be a physiological component of placental tissue) co-purifies with scRNAs and subsequently gives rise to the translational inhibition which has been attributed to the scRNA. Heparin is also contained in the purification protocols for several other translational inhibitory RNAs (7, 9, 10). We suggest exclusion of these cases. The example of the placental scRNA species 1 illustrates that even gel purification, solvent extraction, and ethanol precipitation of a small RNA molecule may not sufficiently safeguard against possible heparin contamination, because heparin can resemble small RNAs in many physical and chemical properties such as molecular weight, electrophoretic migration, and solubility. While the involvement of scRNAs and scRNPs in translational regulation in vivo remains an exciting prospect, this communication illustrates a potential misinterpretation that may confuse such analyses.

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