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IN VITRO ASSOCIATION OF SELECTIVE tRNA SPECIES WITH 28S RNA OF MOUSE CELLS*

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Summary — 28S RNA prepared either from the poly(A) RNA-depleted fraction of mouse embryo culture cells or from 60S ribosome subunits of adult mouse liver is able to bind selective species of tRNAs in an in vitro hybridization reaction. The bound tRNA consists predominantly of proline tRNA and, in minor amounts, glycine, alanine, and aspartic acid tRNAs. Quantitative analysis revealed that the hybridization of tRNA may involve a 28S RNA subpopulation, which is present in higher quantity in embryo cells than in adult liver of the mouse.

Approximately half the mass of ribosomes is RNA; the precise function of ribosomal RNA is still unknown. Recent experimental evidence has indicated a possibility that the functional properties of rRNA molecules may reside in their nucleotide-sequence characteristics. A sequence at the 3' end of 18S rRNA is complementary to a 5'-end sequence of messenger RNAs, suggesting a role of 18S rRNA in the initiation process of protein synthesis (1-3). Likewise, 5S rRNA contains a sequence which is complementary to the TψC loop portion of tRNAs, except the initiator $tRNA^{met}$, suggesting a role of 5S RNA in the elongation process of protein synthesis (4). No such finding has yet been reported with 28S rRNA. In unpublished control experiments for the study of "primer" tRNA binding to genomic RNA of murine leukemia viruses (5, 6), one of us observed that 28S rRNA preparations appeared to bind tRNA. In this communication, we describe the characterization of this tRNA-binding property of mouse 28S RNA. A striking similarity exists between the mouse 28S RNA and the genomic RNA of murine leukemia viruses in that both RNA molecules are able to hybridize with proline tRNA.

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Materials and Methods

This study utilized cells from (i) the livers of 6- to 10-week-old BALB/c (Cumberland View Farm, Clinton, TN) and NIH Swiss (Life Science Laboratories, St. Petersburg, FL) male mice, (ii) secondary embryo cultures of these mice, and (iii) an established cell line, NIH 3T3. The cell culture method has been described (7).

Preparations of tRNA and aminoacyl tRNA synthetases were isolated from mouse liver according to the procedures of Yang and Novelli (8). Labeled tRNA was prepared from NIH 3T3 cells exposed for 48 hr to [^{32}P]phosphate (50 μCi/ml) or [5'-^{3}H]uridine (25 μCi/ml) in the tissue culture medium.

28S rRNA was prepared by two procedures: (i) To a postmitochondrial fraction of cell homogenate was added sodium dodecyl sulfate to a final concentration of 0.5% and then extracted three times with two volumes of phenol:chloroform (1:1) mixture (9). RNA in the aqueous phase, collected by precipitation from 70% ethanol at -20°C overnight, was subjected to chromatography in oligo-dT cellulose (T-2, Collaborative Research Lab., Mass.) according to a published procedure (10). The 0.5 M NaCl breakthrough fraction was adjusted to contain 1 M NaCl, with subsequent standing in an ice bath for 3–4 hr to precipitate rRNA. The precipitated rRNA, dissolved in a solution of 10 mM TrisCl (pH 7.6), 10 mM NaCl, and 1 mM EDTA, was separated in a 10–30% sucrose gradient (containing the solution) by centrifugation in a Spinco SW 41 rotor at 40,000 rpm for 8 hr. (ii) Separation of ribosomal subunits from mouse liver was performed as described by Martin and Wool (11). The 60S subunits were subjected to phenol extraction, as described, and the isolated RNA preparation was directly separated by sucrose gradient sedimentation.

For hybridization, a solution containing 0.5 mg/ml 28S rRNA, 0.5–1.5 mg/ml tRNA, 10 mM TrisCl (pH 7.6), 10 mM NaCl, 1 mM EDTA, and 0.1% sodium dodecyl sulfate was heated at 80°C for 5 min, brought to 65°C, adjusted to contain 0.5 M NaCl, and then incubated for 18–24 hr at 60°C. The incubated mixture was separated either directly by sucrose gradient sedimentation or by initial 1 M NaCl precipitation followed by sucrose gradient sedimentation. The fractions corresponding to 28S regions were pooled and the RNA content was precipitated from 70% ethanol. Reversed phase (RPC-5) chromatography of tRNA was performed according to Pearson et al. (12). To determine the amino-acid-accepting properties, a tRNA sample was included in a reaction mixture which contained 30 mM TrisCl (pH 7.6); 12 mM Mg^{2+}; 4 mM ATP; 1 mM dithiothreitol; 17 ^{3}H-labeled amino acids (all except Asn, Cys, and Gln), each at 2 μM with a specific activity of 10 Ci/mmol (New England Nuclear Corp.); and an enzyme preparation containing all 20 aminoacyl-tRNA synthetases. After incubation at 37°C for 15 min, the reaction was chilled and directly chromatographed on DEAE-cellulose to isolate the aminoacylated tRNAs from free amino acids (9). ^{3}H-labeled amino acids were subsequently discharged from the tRNA and analyzed (13, 14). The analysis was made on a Beckman 121 M amino acid analyzer with the single column system according to the manufacturer's instructions. The effluent from the analyzer was collected in vials (1-min fractions) to which 10 ml of cocktail (15) was then added. Radioactivity in each fraction was measured in a liquid scintillation spectrometer.

Results

When isolated 28S rRNA and excess radioactive total tRNA are mixed, heat-denatured, and subsequently incubated at 60°C in a buffered solution containing 0.5 M NaCl [the hybridization conditions which allow binding of primer $tRNA^{Pro}$ to 35S genomic RNA of murine leukemia viruses (6)], a considerable amount of the radioactivity becomes associated

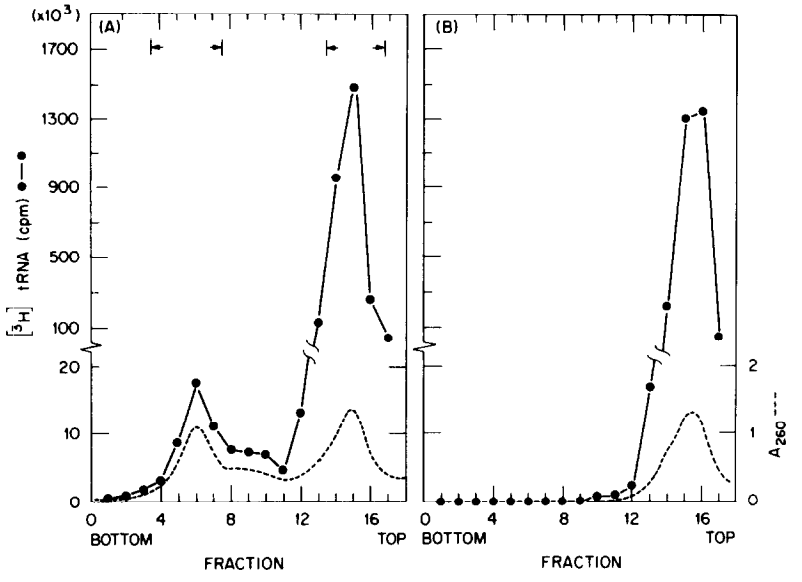

Figure 1. Sucrose gradient sedimentation of a mixture of 28S RNA and [^{32}P]tRNA (A panel) and [^{32}P]tRNA alone (B panel) after in vitro hybridization reaction. The reaction mixture (0.2 ml) of 4.8 A_{260} units of 28S RNA and 5.0 A_{260} units of [^{32}P]tRNA (both from NIH 3T3 cells) was layered onto a 3.8-ml 10–30% sucrose linear gradient containing 1.0 mM TrisCl (pH 7.6), 10 mM NaCl, 1 mM EDTA, and 0.05% sodium dodecyl sulfate. After centrifugation in a Spinco SW 56 rotor at 50,000 rpm for 4.5 hr, 0.2 ml fractions were collected from the bottom of the gradient. The fractions were diluted to 1 ml for determination of absorbance at 260 nm and of radioactivity by Cerenkov counting.

with 28S RNA. Figure 1 shows that, in sucrose gradient centrifugation, the associated radioactivity sedimented mainly to the 28S region and some to the less than 28S region; the latter was presumably heat-fragmented 28S RNA. By heating the gradient fractions of the 28S region at 90°C for 3 min, quickly chilling, and subsequently subjecting the reheated RNA to another sucrose gradient centrifugation, the radioactivity was found to be dissociated and migrated to the 4S region. To determine whether the binding involved total tRNA species or selective tRNA species, the 28S-associated 4S radioactivity (^{32}P) was mixed with a total tRNA preparation of the same source (^{3}H) and chromatographed in a reversed-phase (RPC-5) column. Figure 2 shows that, whereas the total tRNA preparation gave a complex peak pattern as expected, the 28S-associated 4S RNA was resolved into a peak pattern suggesting selective tRNA species. Similar analysis, made with the 4S radioactivity of the less than 28S region, showed the same peak migration but different peak height when compared with the 28S-associated 4S RNA.

The RPC-5 chromatographic peaks may represent individual amino acid tRNAs, or

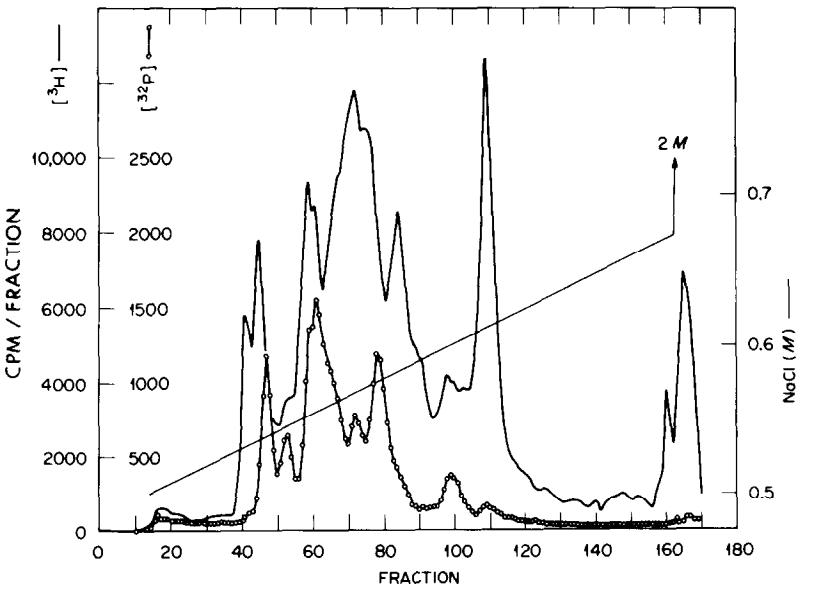

Figure 2. Reversed-phase chromatography of the 28S-associated 4S RNA (^{32}P) and the total tRNA (^{3}H) preparations derived from NIH 3T3 cells. The sample containing approximately 10 μg of tRNA was applied to a 0.6 X 30-cm RPC-5 column and eluted at 20°C with 200 ml of 0.5–0.7 M NaCl linear gradient containing 10 mM Na-acetate (pH 4.5), 10 mM $MgCl_2$, 1 mM EDTA, and 2 mM 2-mercaptoethanol. Acid precipitable radioactivities of 1-ml fractions were measured (8).

composites of isoaccepting tRNAs, or contaminant polynucleotides in the tRNA preparations. To differentiate these possibilities, we examined the amino-acid-accepting activity of the 28S RNA-associated 4S RNA in an aminoacylation reaction containing 17 ^{3}H-labeled amino acids (without Asn, Cys, and Gln). The aminoacylated tRNA was isolated, and the esterified amino acids were then discharged and identified by use of an amino acid analyzer. Figure 3 shows the results of such a study with NIH Swiss mouse liver samples. Whereas a total tRNA preparation, after being subjected to the hybridization condition, but without 28S RNA, was able to accept all 17 amino acids (Fig. 3A), the 28S RNA-associated 4S RNA derived from this preparation accepted predominantly proline and small amounts of other amino acids (Fig. 3B). Repeated experiments with 28S RNA preparations from embryos, secondary embryo cultures, and adult livers of the mice have revealed that the 28S-associated 4S RNA contains chiefly $tRNA^{pro}$ and, subordinately but consistently, $tRNA^{gly}$, $tRNA^{asp}$, and $tRNA^{ala}$. No accepting activity for asparagine, cysteine, and glutamine was detected in the associated 4S RNA, as determined by individual reactions with a filter-paper-disc assay (8). To determine whether or not bound tRNA molecules are present

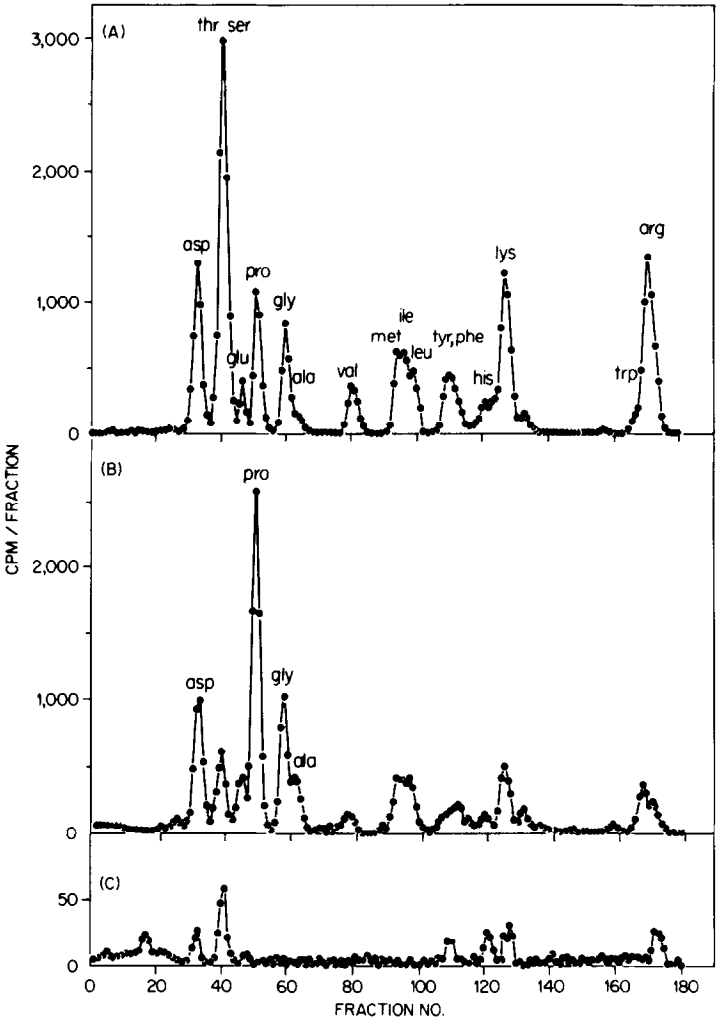

Figure 3. Analysis of amino acids accepted by NIH Swiss mouse liver tRNAs (A panel), NIH Swiss mouse liver 28S RNA-associated tRNA from in vitro hybridization (B panel), and 4S size RNA heat-generated from NIH Swiss mouse liver native 28S rRNA (C panel).

in vivo, native 28S RNA was heat-dissociated and examined for the release of amino-acid-accepting activity; the experiment detected no such tRNA–28S RNA complex (Figs. 3C).

Based on the associated tRNA radioactivity and the amino-acid-accepting activities, it was found that not all 28S RNA molecules had the capacity to bind tRNA (Table 1). Also, there is a marked difference in the tRNA-binding capacity of 28S RNA isolated from adult mouse liver, the ratios of tRNA bound per 28S RNA being 0.5 to 0.7 and 0.1 to 0.2,

TABLE 1

Association of tRNA and 28S RNA preparation of mouse cells in in vitro hybridization reaction

Sources of RNA		Mole tRNA bound/mole 28S RNA	
28S RNA	tRNA	tRNA radioactivity	Amino acid acceptance
NIH 3T3	NIH 3T3	0.50	—
BALB/c embryo secondary culture	NIH 3T3	0.66	—
NIH Swiss liver	NIH 3T3	0.150	0.135
BALB/c liver 60S ribosome subunits	BALB/c liver	—	0.183

Hybridization conditions were as described in Materials and Methods. The radioactivity method is based on an assumption that the specific radioactivity of tRNA bound equals that of the total tRNA. For the calculation, molecular weights of 28S RNA and tRNA were assumed to be 1,750,000 and 25,000, respectively.

respectively. To further ascertain the ribosomal origin of the tRNA-binding 28S RNA, we isolated the 60S subunits of ribosomes from mouse liver as a source for making a 28S RNA preparation. This preparation was found to have the same tRNA-binding specificity.

Discussion

The present study reveals that there exist, in certain mouse 28S RNA molecules, some nucleotide sequences which are complementary to nucleotide sequences present in $tRNA^{pro}$ and in a few other amino acid tRNAs. Since by molar ratio less than one tRNA molecule was bound per 28S molecule in all preparations, we initially considered that the hybridization activity of 28S rRNA preparations might be due to containment RNA of 28S size, which could have lost poly(A) stretches during phenol extraction (9). However, poly(A) RNA of 28S size isolated by oligo-dT cellulose chromatography showed no enrichment of the tRNA-hybridizing activity. Also, non-poly(A) 28S RNA preparations of mouse embryo cells hybridized up to 0.6 tRNA molecule per 28S molecule, and it is difficult to conceive of this as being due to a contaminant. Furthermore, 28S RNA derived from isolated liver 60S ribosome subunits showed the same extent of tRNA hybridization as the 28S non-poly(A) RNA of the same tissue. Based on these results, we tentatively conclude that the novel finding of a unique tRNA-hybridizing property has been made with a subpopulation of 28S ribosomal

RNA. Further investigations are needed to establish the biochemical nature of this 28S rRNA–tRNA interaction, such as nucleotide sequences involved, location of the hybridizing site on the 28S molecule, and properties of the hybridized tRNA. Regarding the last point, the present data would predict that the multiple isoacceptor proline tRNAs could all hybridize, since the 28S RNA-associated 4S RNA accepted only a few amino acids but yielded a greater number of peaks in RPC-5 chromatography.

The biological significance of the existence of tRNA-hybridizing sequences in the 28S RNA is still unknown. Three points should be noted. (i) Different cell sources, such as embryos and livers, give 28S RNA of different $tRNA^{pro}$-hybridizing capabilities. (ii) Selectivity of specific tRNAs by 28S RNA may vary from species to species. Our preliminary results indicated that human 28S RNA hybridized mainly $tRNA^{glu}$, whereas avian 28S RNA hybridized mainly $tRNA^{trp}$. (iii) The specific tRNAs which the 28S RNA can hybridize are the same tRNAs shown to serve as primer molecules for reverse transcription of oncornavirus genomes — $tRNA^{trp}$ for avian leukemia-sarcoma viruses and $tRNA^{pro}$ for murine leukemia viruses (6, 16, 17). Thus, these sequence properties of 28S RNA might not be related to protein synthesis, or, if related, they might belong to a unique and specific rather than a basic and general category of ribosome function. On the other hand, ribosomal RNA synthesis and processing have been found to be intimately associated with cell-growth phenomena (e.g., see refs. 18, 19). Our preliminary results indicated that the tRNA-hybridizing capacity of 28S RNA appeared to correlate with the state of cell growth and differentiation. In this regard, we are interested in the fact that ribosomal genes of eukaryotes are heterogeneous (e.g., see ref. 20). It is possible that mouse genome may contain within the 28S gene an interrupting sequence, "the ribosomal insertion," as reported for Drosophila melanogaster (21-24), which is speculated to have regulatory function (25), and that the tRNA hybridizing property is related to this insertion sequence. It is also possible that the tRNA-hybridizing sequence of 28S RNA is the result of incomplete or different rRNA maturation processing (26). All these possibilities can be clarified by further investigation.

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