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REPORT

Protein–RNA interactions in the subunits of human nuclear RNase P

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ABSTRACT

A yeast three-hybrid system was employed to analyze interactions in vivo between H1 RNA, the RNA subunit of human nuclear RNase P, and eight of the protein subunits of the enzyme. The genetic analysis indicates that subunits Rpp21, Rpp29, Rpp30, and Rpp38 interact directly with H1 RNA. The results of direct UV crosslinking studies of the purified RNase P holoenzyme confirm the results of the three-hybrid assay.

Keywords: HeLa cells; precursor tRNAs; three-hybrid assay; UV crosslinking

INTRODUCTION

Unlike prokaryotic RNase P, which consists of one RNA and one protein subunits, eukaryotic nuclear RNase P (Hela nuclear RNase P) consists of many proteins associated with an essential RNA subunit. This latter RNA subunit, H1 RNA, has not been shown to be catalytic in vitro (Altman & Kirsebom, 1999). No HeLa cell protein subunits have been identified as essential participants in catalytic function in a reconstitution assay for RNase P (Nichols et al., 1988; True & Celander, 1998; Altman & Kirsebom, 1999), as has been done with the protein subunit from the enzyme of *Escherichia coli* (Kole & Altman, 1979; Guerrier-Takada et al., 1983). However, these subunits may have a direct role in the catalytic activity of the enzyme. Of course, some of the same protein subunits are also participants in the complex of eukaryotic RNase MRP (Lee et al., 1996; Pluk et al., 1999; van Eenennaam et al., 2000) and possibly in mammalian mitochondrial RNase P (Puranam & Attardi, 2001), but no function of the subunits has yet been shown for these other organisms.

A critical tool in the analysis of the complex subunit architecture of the human nuclear RNase P holoenzyme and in the function of individual subunits is the ability to reconstitute active enzymes from these subunits. Assembly of the holoenzyme may proceed through a well-defined series of steps that involve addition of particular subunits to form and then to add to a core structure. On the presumption that H1 RNA, the RNA subunit of the enzyme, would be a component of the core structure, we have employed both a genetic (three-hybrid; Sengupta et al., 1996) and a direct biochemical method (UV crosslinking) to determine which protein subunits may also be part of that core structure. The results of these analyses, in conjunction with those of protein–protein subunit interactions, have enabled us to construct a rough three-dimensional picture of the holoenzyme and to design a reconstitution assay.

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RESULTS

Interactions of protein and RNA subunits of human RNase P as determined by three-hybrid analysis

To test interactions between the human nuclear RNase P H1 RNA and its protein subunits, we utilized a three-hybrid system (Sengupta et al., 1996) by employing the commercially available RNA–Protein Hybrid Hunter™ Kit. The host strain, *Saccharomyces cerevisiae* L40*ura*MS2, was cotransformed with pYESTrp2/Rppx and pRH3′/H1 RNA, pYESTrp2/Rppx and pRH3′, and pYESTrp2 and pRH3′/H1 RNA. The parent plasmids, pRH3′/IRE (pRH3′) and pYESTrp2/IRP (pYESTrp2), which respectively express IRE RNA and IRP protein and are known to interact, were also cotransformed in the host strain as controls. Transformed cells were then

tested for activation of lac Z reporter gene by a filter lift assay of β-galactosidase activity determined by visual inspection and activation of a HIS3 reporter gene on His-deficient medium. As shown in Table 1, transformants carrying the plasmid pRH3′/H1 RNA with each of the four plasmids pYESTrp2/Rpp*x* (*x* = 21, 29, 30, and 38) showed readily detectable β-galactosidase activity and growth on yeast synthetic medium (SC-Trp-Ura-His) that included 5 mM 3-AT (3-amino-1,2,4-triazole), as did the commercially provided positive control strain carrying pRH3′/IRE (pRH3′) and pYESTrp2/IRP (pYESTrp2). Other cotransformants displayed little β-galactosidase activity or no significant growth of colonies. We conclude that H1RNA interacts with Rpp21, Rpp29, Rpp30, and Rpp38 specifically in a yeast three-hybrid analysis.

Interactions of protein and RNA subunits of human nuclear RNase P as determined by direct UV-crosslinking studies

To determine which proteins from partially purified Hela nuclear RNase P holoenzyme bind to H1 RNA (Bartkiewicz et al., 1989) or to a fragment of H1 RNA (H1/P3 RNA: nt 1–74) containing the P3 domain (Chen & Pace, 1997), we carried out crosslinking experiments as generally described in Materials and Methods. The first kind of UV-crosslinking design was used only to show what kind of results we could obtain. The results revealed some complexes of a higher molecular weight than the RNAs that we added after transcription in vitro, which was composed of RNA (H1 or H1/P3) and protein(s). These complexes were subjected to a mild treatment with RNase T1 and reanalyzed on 9% polyacrylamide SDS gel: The apparent molecular weight of the resulting complex was about 70 kDa (data not shown). Similar experiments, done with M1 RNA, did not show any crosslinked complex, indicating perhaps that the interaction observed with transcribed in vitro H1 RNA was specific (data not shown). These experiments were then carried out with nonradioactive biotinylated C in the RNA as a means of identifying the proteins crosslinked with the RNA.

To identify which proteins interact directly with H1 RNA, we carried out the same UV-crosslinking experiments but we used internally labeled H1 (and H1/P3) that was transcribed using ATP, GTP, UTP, and CTP: bioCTP (1:1). Reactions were carried out as described in Materials and Methods. After UV irradiation, 100 μL aliquots were mixed with 20 μL streptavidin agarose beads that had been equilibrated with HMNG buffer. As a control, a 100-μL sample with RNase P, but no RNA, was also mixed with 20 μL streptavidin agarose beads. Samples were processed as described in Materials and Methods and then separated in 9%/15% polyacrylamide SDS gels. From these gels, the membrane filters were prepared for western blotting. The blots were reprobed several times using antibodies to all the available protein subunits.

The results of the western blots (Eder et al., 1997; Jarrous et al., 1998) are summarized in Figure 1 and Table 2. Only proteins interacting with the RNA containing biotinylated CTP should bind to the streptavidin agarose beads. The fraction that did not bind to the beads was collected and compared to the sample prior to mixing with beads to see whether depletion in any proteins tested was observed (see Materials and Methods). The membrane was first probed with antibodies against Rpp38 and Rpp21 and the results indicated that Rpp21 binds with H1 RNA and also with the P3 domain, whereas Rpp38 binds only with H1 RNA (Fig. 1). Similar results obtained by UV-crosslinking experiments identified Rpp38 to bind to a 3′ domain of RNase MRP RNA (van Eenennaam et al., 2000). The same membrane was stripped and reprobed sequentially with all other antibodies available, and finally they were reprobed again with Rpp38 and Rpp21, to ascertain that the initial results were reproducible. Although the intensity of the signal obtained was weaker than before (protein loss occurred with the several rounds of hybridization), we observed the same results, indicating that qualitatively the results were valid (data not shown).

TABLE 1. Yeast three-hybrid system detects human nuclear RNase P RNA (H1 RNA) interactions with the other protein subunits.

Hybrid proteins	Hybrid RNAs	β-gal analysis (filter lift assay)	Colonies growth on medium lacking histidine
pYESTrp	pRH3′	−	−
	pRH3′/H1RNA	−	−
pYESTrp/Rpp14	pRH3′	−	−
	pRH3′/H1RNA	−	−
pYESTrp/Rpp20	pRH3′	−	−
	pRH3′/H1RNA	−	−
pYESTrp/Rpp21	pRH3′	−	−
	pRH3′/H1RNA	+	+
pYESTrp/Rpp29	pRH3′	−	−
	pRH3′/H1RNA	+	+
pYESTrp/Rpp30	pRH3′	−	−
	pRH3′/H1RNA	+	+
pYESTrp/Rpp38	pRH3′	−	−
	pRH3′/H1RNA	+	+
pYESTrp/Rpp40	pRH3′	−	−
	pRH3′/H1RNA	−	−
pYESTrp/hpop1	pRH3′	−	−
	pRH3′/H1RNA	−	−

Two plasmids, encoding the proteins and RNAs indicated, were introduced to the *S. cerevisiae* L40*ura*MS2. The transformants were plated on the medium lacking tryptophan and uracil. RNA–protein interactions were confirmed by restreaking three colonies of each transformants on the medium lacking tryptophan, uracil, and histidine and including 5 mM 3-AT. The assay of β-galactosidase activity was performed by the filter lift assay. −: no visible color or significant growth; +: visible blue or growth.

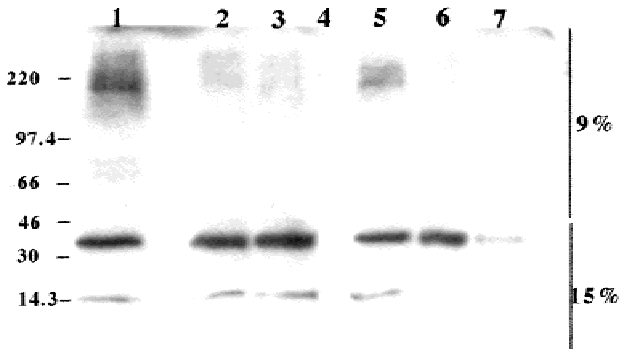

FIGURE 1. Immunoblot using polyclonal antibodies raised in rabbits against Rpp38 and Rpp21. The enzyme is HeLa nuclear RNase P purified through glycerol gradients as described in Materials and Methods and loaded onto a composite 9%/15% polyacrylamide SDS gel and subsequently transferred to a nitrocellulose membrane. Lanes 1–3: Samples before incubation with streptavidin agarose beads. Lanes 5–7: Samples after incubation with streptavidin agarose beads (not bound). Lane 1: Control RNase P sample, no RNA added. Lane 2: RNase P sample crosslinked to P3 domain of H1 RNA. Lane 3: RNase P sample crosslinked to H1 RNA. Lane 4: Protein size marker lane. Lane 5: Control RNase P sample. Lane 6: RNase P sample crosslinked to P3 domain of H1 RNA. Lane 7: RNase P sample crosslinked to H1 RNA. The relative position and size of proteins loaded on lane 4 is indicated. Some nonspecific, high molecular weight bands can be seen.

Rpp14, Rpp20, Rpp40, hPop1, and hPop5 (the latter a subunit of RNase MRP; H. van Eenennaam, unpubl. observations) failed to interact with either H1 or P3 domain as shown in Table 2. Earlier results (Yuan et al., 1991) indicated that a 40-kDa protein was associated with the P3 domain, and that hPop1 protein did not show binding to RNase MRP RNA (van Eenennaam et al., 2000). Signals obtained for Rpp29 and Rpp30, though weaker, were similar to the one obtained for Rpp38, that is, the relative signal in the not bound sample was much weaker in H1 and P3 lanes, when compared with the control lane.

A number of control experiments were carried out separately (data not shown). Rpp21, Rpp29, Rpp30, and Rpp38 did not bind to the streptavidin beads if the same procedure was carried out as described above except that the RNA and proteins were not UV irradiated. Similarly, if another RNA was included rather than H1 RNA, a fragment of chloramphenical acetyl transferase mRNA (265 nt), then no binding to the beads was observed after UV irradiation of the mixture by any of the proteins mentioned above. The absence of the aforementioned proteins in the supernatant not bound to the streptavidin beads is a direct result of the specific interaction of these proteins with H1 RNA.

TABLE 2. Summary of western blot analysis.

	Total (before strept. agarose)			Not bound (after strept. agarose)		
Proteins	(XL) GG-H1	(XL) GG-P3	GG	(XL) GG-H1	(XL) GG-P3	GG
Rpp14	(+)	(+)	(+)	(+)	(+)	(+)
Rpp20*	−	−	−	−	−	−
Rpp21	+	+	+	−	−	+
Rpp29	+	+	+	(+)	(+)	+
Rpp30	+	+	+	(+)	(+)	+
Rpp38	++	++	++	(+)	++	++
Rpp40	+	+	+	+	+	+
hPop5	+	+	+	+	+	+
hPop1	+	+	+	+	+	+

Abbreviations used in the table: (XL): UV crosslinked; GG-H1: glycerol gradient purified RNase P with H1 RNA added; GG-P3: glycerol gradient purified RNase P with RNA P3 added; GG: glycerol gradient purified RNase P. hpop5 is another nuclear protein thought to be involved in the complex of RNase MRP and possibly RNase P (H. van Eenennaam, unpubl. observations).

*The concentrations of Rpp20 may be too dilute to give active antigen–antibodies results in these samples.

++: strongest signal; −: no signal; (+) much weaker than +.

DISCUSSION

We show that for the eight proteins on nuclear RNase P in HeLa cells that have been cloned and sequenced, four of these proteins interact directly with H1 RNA, the RNA subunit of the enzyme. These proteins, Rpp21, Rpp29, Rpp30, and Rpp38, both genetically and biochemically are intimately in contact with the RNA subunit. Although we cannot be certain that these are the only proteins involved in abetting the reaction with substrates, further tests of a reconstitution assay will prove this point (see below). There may be other proteins that are part of the enzyme and that we have not characterized yet as part of our characterization. Certainly, Rpp25, identified in an early study of the enzyme (Eder et al., 1997), has yet to be tested in the manner described here. Furthermore, both RNase MRP (see also hpop5; van Eenennaam et al., 2000; H. van Eenennaam, pers. comm.) and mitochondrial RNase P (Puranam & Attardi, 2001) have proteins that may be similar to the ones we report here and that should be tested in the ways we describe here.

Our results indicate a possible path to the reconstitution reaction. Clearly, if there is a required order of reactions to develop this assay, it might very well be the formation of a complex between H1 RNA and these four proteins (Rpp21, Rpp29, Rpp30, Rpp38) with the final proteins to be added later (Fig. 2). In fact, Figure 2 does represent a speculative model of the two- and three-dimensional scheme of the organization of the subunits as determined here and elsewhere (Jiang & Altman, 2001). Because most work on the isolated proteins has been done with his-tagged derivatives, only the purification of large scale amounts of native enzyme complete with untagged subunits might be suitable for reconstitution.

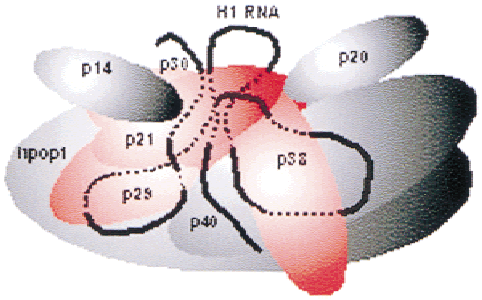

FIGURE 2. Schematic model of RNase P showing the relative arrangements of the H1 RNA subunit (red line) and the protein subunits (red for those in contact with H1 RNA and black for other subunits).

MATERIALS AND METHODS

Strains, plasmids, and media

All yeast three-hybrid tests were performed with components of the RNA-Protein Hybrid Hunter™ Kit (Invitrogen). The *S. cerevisiae* yeast strain is L40*ura*MS2 [*MATa, ura3-52, leu2-3112, his3Δ200, trp 1Δ1, ade2, LYS2::(LexA op)4-HIS3, ura3::(LexA-op)8-lacZ pLexA/MS2/Zeo(Zeocin^R)*]. The L40*ura*MS2 produces a protein hybrid consisting of the LexA DNA binding domain fused to the bacteriophage MS2 coat protein. The LexA/MS2 fusion protein is stably integrated in L40*ura*MS2. Human RNase P protein subunit coding sequences (Rpp14, Rpp20, Rpp21, Rpp29, Rpp30, Rpp38, Rpp40, and hpop1; Eder et al., 1997; Jarrous et al., 1998) codons were fused to B42 transcription activation domain (AC) in pYESTrp2 forming the "prey" protein, designated as pYESTrp2/Rpp*x* (Rpp*x* represents any of the RNase P protein subunits including hpop1).

To construct the "prey" protein, pDB-Rpp14, pDB-Rpp20, pDB-Rpp21, pDB-Rpp29, pDB-Rpp30, pDB-Rpp38, and pDB-hpop1 (Jiang & Altman, 2001) were digested with *Sal*I, filled in with DNA Polymerase I (Klenow) and, then digested with *Not*I to make one blunt and one sticky end of DNA fragments encoding HeLa nuclear RNase P protein subunits Rpp14, Rpp20, Rpp21, Rpp29, Rpp30, Rpp38, and hpop1. These DNA fragments were then subcloned into pYESTrp2 digested with *Eco*RI, filled in with DNA Polymerase I (Klenow) to generate the blunt end and then digested with *Not*I to generate the sticky end. The Rpp40 DNA fragment excised from pHTT7K-Rpp40 (Jarrous et al., 1998) by *Nde*I (followed by treatment with Klenow enzyme) and by *Bam*HI was ligated to pYESTrp2 digested with *Hin*dIII, (followed by treatment with Klenow enzyme) and with *Bam*HI. The subunits in frame with the B42 activation domain sequence in pYESTrp2, designated as pYESTrp2/Rpp*x*, were confirmed by DNA sequencing. A fragment of HeLa nuclear RNase P RNA subunit, H1 RNA excised from pGEM-H1 RNA by *Eco*RI, treatment with Klenow enzyme, was fused to MS2 RNA in pRH3′ digested with *Sma*I for creating an RNA hybrid, designated as pRH3′/H1 RNA, which was also confirmed by DNA sequencing.

All the media used in the study were prepared according to the RNA-Protein Hybrid Hunter™ Kit (Catalog no. K5100-01).

Characterization of RNA–protein interactions in human RNase P by yeast three-hybrid assay

The entire procedure was performed according to instructions in the RNA-Protein Hybrid Hunter™ Kit with some modifications indicated: The self-activation of subunits was tested by cotransformation of pYESTrp2/Rpp*x* and pRH3′. The cotransformants were plated and restreaked at 30 °C for 3 or 4 days on SC-Trp-Ura-His media that included 5 mM 3-AT. To test RNA–protein interactions within RNase P subunits, 18 cotransformants of pYESTrp2/Rpp*x* (including pYESTrp2) and pRH3′/H1RNA, or pYESTrp2/Rpp*x* in pRH3′, were plated on SC-Trp-Ura. After incubation at 30 °C for 2 or 3 days, three of each of the transformants were patched onto SC-Trp-Ura, grown at 30 °C for 3 or 4 days, and were assayed for β-galactosidase activity by a filter lift assay. Three of each of the yeast transformants were restreaked onto SC-Trp-Ura-His media that included 5 mM 3-AT for 4 or 5 days.

Preparation of RNase P holoenzyme, RNA subunits, and substrates

The HeLa nuclear RNase P and its activity were prepared and assayed as indicated previously (Eder et al., 1997). The fraction purified through the glycerol gradient step was used in most of our experiments. This fraction loses subunits as a matter of time and can be complemented by the addition of subunits. The period of time of 5 min that is allowed for preincubation with biotin labeled H1 RNA is enough to facilitate exchange of the added RNA with subunits to make functional enzyme. The RNA subunit and fragments thereof of RNase P were prepared by transcription (Vioque et al., 1988). The substrate was the precursor to *E. coli* tRNA^{Tyr} (pTyr) and was also prepared by transcription and labeling with [^{32}P-α]-GTP (Guerrier-Takada et al., 1989).

UV crosslinking

HeLa nuclear RNase P purified through the glycerol gradient or Mono Q step was incubated at 37 °C alone or in the presence of RNAs (H1 [36 nM] and H1/P3 [55 nM], in 20 mM HEPES-KOH, pH 8.0, 1 mM $MgCl_2$, 200 mM NaCl, 5% glycerol [HMNG buffer]) that had been transcribed in the presence of biotinylated CTP (Gibco/BRL). After 5 min, the samples were placed on a sheet of saran wrap over a short-wave UV transilluminator, and irradiated for 4 min at room temperature. For samples that were used in the western blots, the RNA–protein subunits photochemically crosslinked were recovered and 100-μL aliquots were mixed with streptavidin agarose beads (Gibco/BRL) preequilibrated in the same buffer. Samples were placed at 4 °C on a nutator, and mixed for about 4 h. Only proteins interacting with the RNA containing biotinylated CTP should bind to the streptavidin agarose beads. The fraction that did not bind to the beads was collected and compared to the sample prior to mixing with beads to see whether depletion in any of the proteins tested was observed. This flow through, or not bound fraction, was obtained by centrifugation, and 20-μL aliquots were loaded on to a 9%/15% polyacrylamide/SDS gel. For proteins not interacting with H1 (or P3) RNA, the signal after the streptavi-

din agarose binding step is about the same as before. If the signal is weaker, therefore showing depletion of the protein in question to binding the streptavidin beads, the protein interacts with the RNA as was expected. Proteins were electrotransferred to nitrocellulose membrane and western blot analyses were performed.

Western blot analysis

After electrophoretic separation in 9/15% polyacrylamide/SDS gels, proteins were transferred overnight to nitrocellulose membranes in 10 mM CAPS (pH 11), 10% methanol, at 50 mA, and at 4 °C. The membrane was blocked with TNT buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) and 5% nonfat dry milk for 30–40 min at room temperature. Polyclonal antibodies (raised against proteins that copurify with RNase P activity, Rpp14, Rpp20, Rpp21, Rpp29, Rpp30, Rpp38, Rpp40, hPop1, and hPop5) were used at a 1:100 dilution in TNT/5% nonfat dry milk, and incubated for 90–120 min. As a secondary antibody, a 1:5,000 dilution of anti-rabbit mouse IgG antibody (Vector Laboratories) was made in the same buffer and incubated for about 60 min. Blots were washed with TNT and TN (TNT without Tween 20) buffer and antibody–antigen complexes were visualized using the ECL-Plus detection system (Amersham-Pharmacia), following manufacturer's instructions.

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NOTE ADDED IN PROOF

Rpp25, now cloned and characterized, does interact directly with H1 RNA as determined by the UV crosslinking study (C.G.-T. and S.A., unpublished).

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