Note

Protein–Protein Interactions in the Subunits of Ribonuclease P in the Hyperthermophilic Archaeon *Pyrococcus horikoshii* OT3

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Ribonuclease P (RNase P) is a ribonucleoprotein complex involved in the processing of the 5′ leader sequence of precursor tRNA (pre-tRNA). RNase P in the hyperthermophilic archaeon *Pyrococcus horikoshii* OT3 consists of RNA and five protein subunits (Ph1481p, Ph1496p, Ph1601p, Ph1771p, and Ph1877p). *In vivo* interactions among five protein subunits of RNase P in *P. horikoshii* OT3 were examined using a yeast two-hybrid system. The analysis indicates that proteins Ph1481p and Ph1601p interact strongly with Ph1877p and Ph1771p respectively, whereas Ph1481p interacts moderately with Ph1601p. In contrast, no interaction was detected between Ph1496p and the other four proteins. Co-immunoprecipitation analysis confirmed the interactions obtained by yeast two-hybrid assay.

Ribonuclease P (RNase P) is a ubiquitous cellular endoribonuclease responsible for the removal of 5′ leader sequences from tRNA precursors in all known phylogenetic kingdoms.[1,2)] Although the functionality of this endoribonuclease remains almost the same from bacteria to humans, the chemical composition and enzymatic properties of this enzyme differ in various organisms. Eubacterial RNase P is composed of a catalytic RNA and a single protein subunit. In the presence of a high concentration of Mg^{2+}, the RNA itself can hydrolyze tRNA precursors (pre-tRNA) *in vitro*; hence the RNA is a ribozyme.[3)] Because of this characteristic feature, biochemical and structural studies have been extensively carried out on eubacterial RNase Ps.[4)] These studies defined crucial nucleotides at helix P4 in RNase P RNA,[5)] and the three-dimensional structures of the eubacterial protein subunit were determined.[6,7)] In addition, high resolution crystal structures of the specificity domains of the *Bacillus subtilis* and *Thermus thermophilus* RNase P RNAs were established.[8,9)]

In contrast, only a few studies have thus far been pursued on eukaryotic and archaeal RNase Ps because of the difficulty of obtaining a large amount and the complexity of their subunit components. Eukaryotic RNase Ps, such as nuclear RNase Ps from humans and *Saccharomyces cerevisiae*, are composed of at least nine protein subunits associated with an RNA subunit, and in the absence of protein subunits, the RNA subunit alone does not exhibit enzyme activity *in vitro*.[10–12)] These findings suggest that the eukaryotic RNA and protein subunits work in an interdependent manner and fulfill the requirements for substrate recognition and/or catalysis. Recently, Mann *et al.* reported that human proteins Rpp21 and Rpp29, together with the RNA subunit H1 RNA, are sufficient for 5′ cleavage of pre-tRNA.[13)] In addition, the spatial organization was reported for human and yeast RNase Ps.[14–16)] But, a common arrangement of protein subunits in the eukaryotic RNase P has not yet emerged.

As for archaeal RNase P, we found earlier in reconstitution experiments that RNase P RNA and four proteins, Ph1481p, Ph1601p, Ph1771p, and Ph1877p, are essential for the RNase P activity of the hyperthermophilic archaeon *Pyrococcus horikoshii* OT3.[17)] Subsequently, a fifth protein, Ph1496p, was found to be involved in elevation of the optimum temperature of the reconstituted RNase P (Fukuhara *et al.*, unpublished data). Thus the *P. horikoshii* RNase P RNA, like its eukaryotic counterparts, is deficient in functions and functions cooperatively with the five protein subunits in substrate recognition and/or catalysis. It is thus of interest to understand how the protein subunits in eukaryotic and archaeal RNase Ps perform their functions in catalytic activity. These investigations ought to be of help in establishing the structure and function relationships of the ribonucleoprotein ribozyme, and the resulting information should ultimately shed light on the transition from the proposed RNA world to the modern protein world.

To this end, we have been studying the structures of individual proteins from *P. horikoshii* RNase P, and have determined the crystal structures of Ph1771p and Ph1877p at 2.0 Å and 1.8 Å resolution respectively.[18,19)]

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A

B

	DNA binding domain fusion proteins					
Activation domain fusion protein	none	Ph1481p	Ph1496p	Ph1601p	Ph1771p	Ph1877p
none	- / -	- / -	- / -	- / -	- / -	- / -
Ph1481p	- / -	+ / +	- / -	- / +	- / -	+ / +
Ph1496p	- / -	- / -	- / -	- / -	- / -	- / -
Ph1601p	- / -	+ / -	- / -	+ / -	+ / -	- / -
Ph1771p	- / -	- / -	- / -	+ / +	+ / -	- / -
Ph1877p	- / -	+ / -	- / -	- / -	- / -	- / -
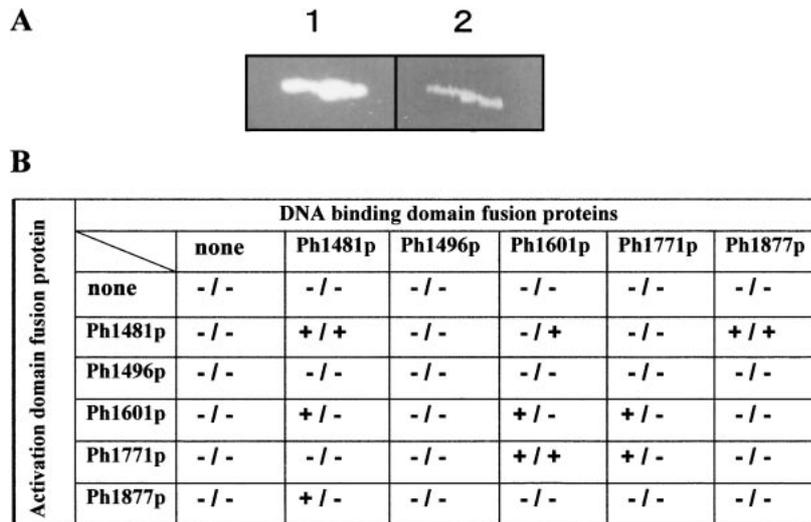
Fig. 1. Protein–Protein Interaction Determined Using Yeast Two-Hybrid Assay.

A, a typical two-hybrid assay on SD-Leu–Trp–His (lane 1) and SD-Leu–Trp–His–Ade (lane 2), where the Ph1481p and Ph1877p genes were in the prey and bait vectors respectively. B, summary of protein–protein interaction data determined by the yeast two-hybrid assay. Plus and minus signs shown at the left side of a slash indicate the degree of growth or absence of growth on SD-Leu–Trp–His respectively, and at the right side, the degree on SD-Leu–Trp–His–Ade.

Furthermore, the essential amino acid residues in Ph1877p for *P. horikoshii* RNase P activity were assigned by site-directed mutagenesis.[19] In this study, to gain more structural insight into *P. horikoshii* RNase P, we examined the spatial organization of the protein subunits in *P. horikoshii* RNase P using a yeast two-hybrid assay as well as co-immunoprecipitation assays.

Two-hybrid assays were done essentially as described in the Clonetech product protocol for the Matchmaker Two-Hybrid System 3. The genes encoding five protein subunits (Ph1481p, Ph1496p, Ph1601p, Ph1771p, and Ph1877p) in the *P. horikoshii* RNase P were cloned in both the pGBKT7 (bait vector) and pGADT7 (prey vector) in-frame with the sequences encoding the GAL4 DNA binding domain and activating domain respectively. All combinations of bait and prey plasmids were sequentially transformed into the tester strain AH109, which has three reporter genes for two-hybrid interactions, *HIS3*, *ADE2*, and *MEL1*.[20] The transformed cells were plated onto synthetic medium lacking Leu and Trp (SD-Leu–Trp) and incubated for 3 to 4 d at 30 °C. Simultaneously, the transformants were plated onto media lacking Leu, Trp, and His (SD-Leu–Trp–His) and Leu, Trp, His, and adenine (SD-Leu–Trp–His–Ade) containing X-α-gal to evaluate moderate and tight protein–protein interactions, respectively. This procedure was performed twice, with a reverse order of transformation of the bait and prey vectors into the tester cells. A typical two-hybrid assay is shown in Fig. 1A, and an overview of the results is given in Fig. 1B. Four transformants carrying combinations of Ph1481p and Ph1877p, and Ph1601p and Ph1771p, showed growth on SD-Leu–Trp–His, and two of four transformants containing the Ph1771p and Ph1661p genes or Ph1481p and

Ph1877p genes in the prey and bait vectors respectively showed detectable α-galactosidase activity. Although physical interactions observed on SD-Leu–Trp–His–Ade were not reciprocal, the result suggests strong interactions of Ph1481p with Ph1877p, and of Ph1601p with Ph1771p. In addition, transformants carrying the Ph1601 and Ph1481 genes alternatively as prey and as bait grew on either SD-Leu–Trp–His or SD-Leu–Trp–His–Ade, suggesting a possible interaction of Ph1481p with Ph1601p. No interacting partners were observed for Ph1496p, which is involved in elevation of the optimum temperature for the reconstituted RNase P. Moreover, one clear self-interaction was identified for Ph1481p, and two possible self-interactions were detected in two proteins, Ph1601p and Ph1771p.

To corroborate the interaction obtained by two-hybrid analysis, we examined the interaction of the protein subunits by co-immunoprecipitation assay. In this analysis, two proteins Ph1481p and Ph1601p or Ph1481p and Ph1877p were mixed, and then immunoprecipitated with rabbit sera directed to either Ph1601p or Ph1877p. The resulting precipitates were subsequently analyzed by Western blotting using rabbit sera directed to Ph1481p. As Fig. 2 shows, Ph1481p was efficiently co-precipitated with both proteins Ph1601p and Ph1877p, indicating Ph1481p–Ph1601p and Ph1481p–Ph1877p interactions. It should, however, be noted that the immunoprecipitation for Ph1771p could not be carried out due to its insolubility in the solution. Taken together, a possible interaction for the *P. horikoshii* RNase P proteins derived from the present study is presented in Fig. 3, where four proteins form a linear heterocomplex with flanking Ph1877p and Ph1771p on the sides, while Ph1496p has no protein partners.
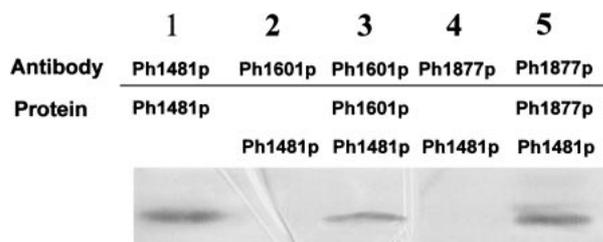
Fig. 2. Immunoprecipitation Assay for Ph1481p, Ph1601p, and Ph1877p.

Protein or protein mixtures were immunoprecipitated using rabbit sera containing anti-Ph1481p (lane 1), anti-Ph1601p (lanes 2 and 3), or anti-Ph1877p (lanes 4 and 5) antibodies which were immobilized to Protein G Sepharose (Amercham Pharmacia). The proteins analyzed were Ph1481p (lane 1), Ph1481p (lane 2), Ph1481p and Ph1601p (lane 3), Ph1481p (lane 4), and Ph1481p and Ph1877p (lane 5). Co-precipitated proteins were separated on SDS–PAGE and analyzed by Western blotting using rabbit sera directed to Ph1481p.[22]
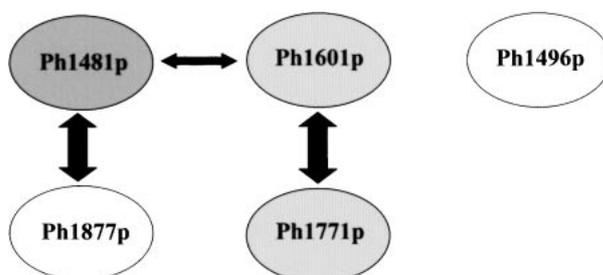
Fig. 3. A Model of Interactions between Protein Subunits in *P. horikoshii* RNase P.

Strong interactions are indicated by heavy lines and a weak interaction by a thin line. Arrowheads at both ends indicate that interactions are reciprocal. *i.e.*, observed in both directions.

Yeast two- and three-hybrid analyses have been reported on human and yeast RNase P holoenzymes.[14–16] Human RNase P proteins hpop1, Rpp21, Rpp29, Rpp30, Rpp38, and Rpp40 were found to be involved in extensive protein–protein interaction.[14] Yeast protein Pop4 was perhaps a core protein, interacting with seven protein subunits, and the remaining proteins showed one or more specific protein–protein interactions with the other protein subunits.[16] Comparing these results with that obtained in the present study, one of the interactions Ph1601p–Ph1771p in *P. horikoshii* is conserved in both humans and yeast RNase Ps. This finding suggests that the protein–protein interaction might be an essential interaction in both archaeal and eukaryotic RNase Ps. This assumption is consistent with a recent study in which it was reported that Rpp21 and Rpp29, human homologs of Ph1601p and Ph1771p respectively, are essential for 5′ cleavage of pre-tRNA.[13] Furthermore, interaction between Pop5 and Rpp1, human homologs of Ph1481p and Ph1601p respectively, was observed in human RNase P, whereas the corresponding interaction was undetectable in yeast RNase P.

During preparation of this manuscript, protein–protein interaction in the four protein subunits in the archaeal RNase P in *Methanothermobacter thermoautotrophicus* was reported.[21] The tight interactions of Ph1481p–Ph1877p and Ph1601p–Ph1771p observed in the present study were detected in corresponding proteins in *M. thermoautotrophicus* RNase P (Mth687–Mth688 and Mth1618–Mth11 respectively). Thus, it is likely that the two protein–protein interactions are conserved in archaeal RNase Ps. In contrast, the moderate interaction Ph1481p–Ph1601p observed in *P. horikoshii* RNase P was not detectable in *M. thermoautotrophicus* RNase P, where two additional interactions Mth11–Mth688 and Mth11–Mth687 were observed instead.

High resolution structure is prerequisite for a detailed understanding of the molecular mechanism of eukaryotic and archaeal RNase Ps. It is, however, still difficult to prepare them in a sufficient amount to be crystallized for X-ray crystallography. An alternative approach that is now advancing is to incorporate high resolution models of isolated proteins into a model of RNase P RNA based on biochemical and physicochemical experiments. Although the present result obtained for *P. horikoshii* RNase P proteins has some discrepancies with those obtained for eukaryotic and *M. thermoautotrophicus* RNase Ps, it definitely provides a framework for this approach and also gives a clue to making a protein–protein complex for crystallization. Indeed, the present result allowed us to determine the crystal structure of the complex Ph1481p–Ph1877p at 2.0 Å resolution (Kawano *et al.*, unpublished data).

Acknowledgments

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