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4.5S RNA forms with Ffh protein the prokaryotic signal recognition particle (SRP), a highly conserved ribonucleoprotein complex essential for protein secretion. It also independently binds to elongation factor G (EF-G) in the ribosome and has a function in a subset of translocation events that is transient but required for viability. Crystals of three different constructs encompassing the conserved domain IV of 4.5S RNA, containing the recognition elements for both Ffh and EF-G, were obtained. Native X-ray diffraction data were collected for two crystal forms under cryogenic cooling conditions. The best crystals are of a 45 nt construct, diffract anisotropically to 2.6 Å resolution using synchrotron radiation and belong to space group $P3_221$, with unit-cell parameters $a = b = 69.1$, $c = 84.6$ Å and a single RNA molecule per asymmetric unit.

1. Introduction

Bacterial 4.5S RNA has the peculiarity among structural RNA molecules of having two completely independent functions in the cell metabolism. In association with Ffh protein, it constitutes the prokaryotic signal recognition particle, a ribonucleoprotein complex essential for GTP-dependent translocation of signal peptide-bearing proteins across the periplasmic membrane (Walter & Johnson, 1994; Lütcke, 1995). 4.5S RNA increases the affinity of Ffh protein for signal peptides (Luirink *et al.*, 1992; Luirink & Dobberstein, 1994) and is required for the interaction of SRP with its membrane receptor FtsY (Miller *et al.*, 1994; Powers & Walter, 1995; Macao *et al.*, 1997). 4.5S RNA also transiently binds to elongation factor G in the ribosome, exerting a function which is not required for every protein elongation cycle but is likely to be responsible for the observed lethality of 4.5S RNA depletion (Brown & Fournier, 1984; Bourgaize & Fournier, 1987; Jensen *et al.*, 1994; Jensen & Pedersen, 1994). Complementation analyses and identification of a ten-nucleotide sequence identical in 4.5S RNA and the 23S rRNA region to which EF-G binds suggest that 4.5S RNA might facilitate the release of EF-G from ribosomes in the post-translocation state by competing for its binding site in 23S rRNA (Brown, 1987, 1989, 1991). This hypothesis is supported by recent biochemical and *in vivo* experiments (Shibata *et al.*, 1996; Nakamura *et al.*, 1999; Suzuma *et al.*, 1999).

The predicted secondary structure of 4.5S RNA is that of an extended highly base-paired hairpin with five internal loops (Fig. 1*a*). All highly phylogenetically conserved nucleotides are found within internal loops A and B and the apical tetraloop. Together with their flanking helices, these loops constitute a region highly homologous both in sequence and predicted secondary structure to the domain IV of all SRP RNAs (Poritz *et al.*, 1988; Struck *et al.*, 1988; Zwieb, 1988; Althoff *et al.*, 1994; Zwieb & Samuelsson, 2000). This conservation reflects a common biological role, as it has been shown that 4.5S RNA and its eukaryotic homolog 7S RNA are functionally interchangeable (Poritz *et al.*, 1990; Ribes *et al.*, 1990; Patel & Austen, 1996; Powers & Walter, 1997). In the bacterial system, domain IV of 4.5S RNA can substitute for the full-length molecule both *in vitro* and *in vivo* (Schmitz *et al.*, 1996; Jovine, 1998; Batey *et al.*, 2000). Furthermore, it includes the ten-nucleotide sequence indentical in 4.5S RNA and 23S rRNA and required for interaction of EF-G with both RNAs (Brown, 1991; Shibata *et al.*, 1996). It is clear from all these data that 4.5S RNA domain IV must possess unique structural features that allows it to independently and specifically bind two different proteins both crucial to their respective cellular pathways. NMR studies of unbound 4.5S RNA domain IV, as well as its crystal structure in complex with the RNA-binding domain of Ffh protein, have been recently reported (Schmitz, Behrens *et al.*, 1999; Schmitz, James *et al.*, 1999; Batey *et al.*, 2000). As a first step towards a high-resolution structural investigation of the free RNA fragment, we report in this paper the crystallization and preliminary X-ray analysis of the domain IV of *E. coli* 4.5S RNA.
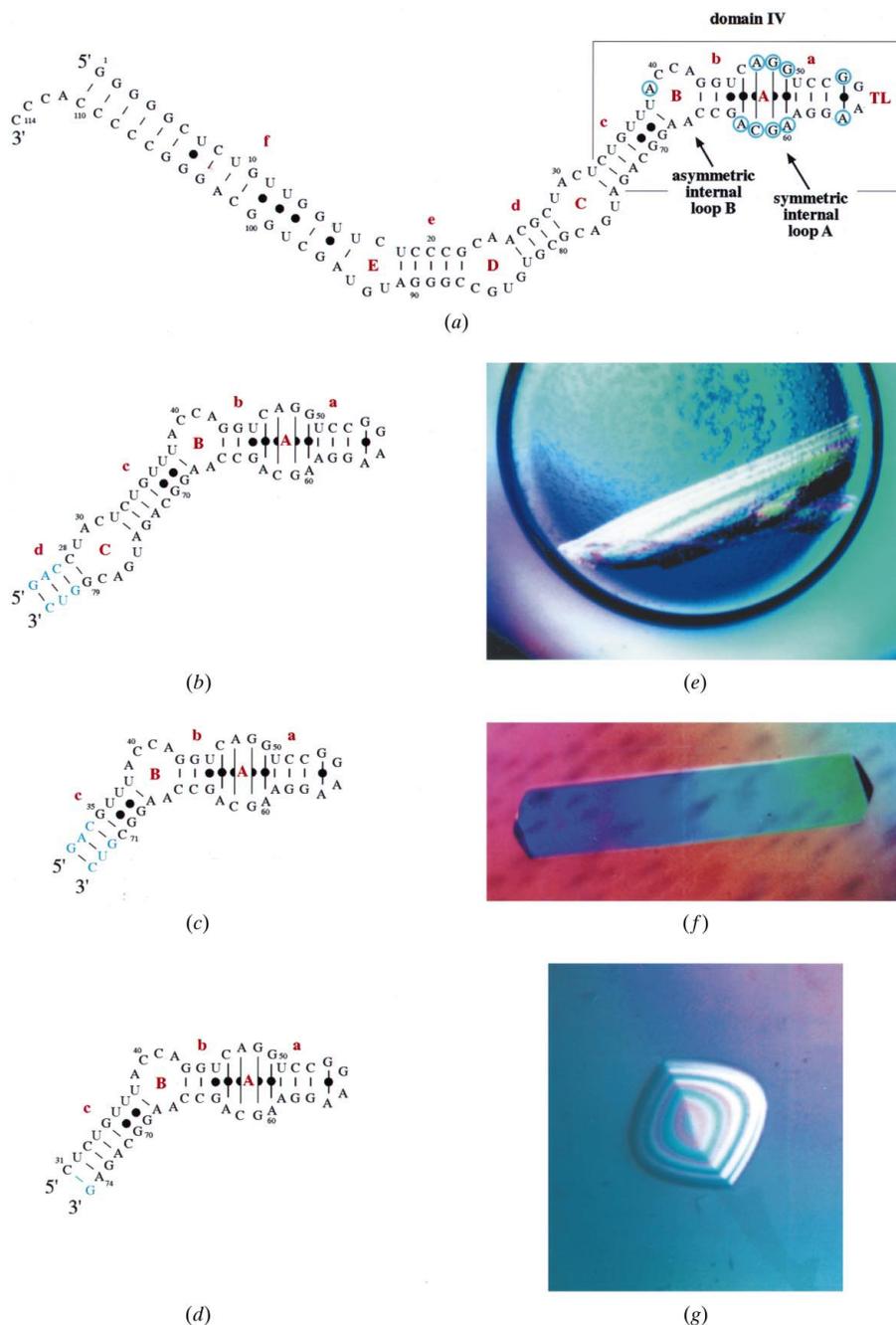
crystallization papers

2. RNA construct design, synthesis and purification

Because of the presence of several internal loops within its predicted secondary structure (Fig. 1a), native 4.5S RNA is likely to be a relatively flexible molecule in solution. Based on the structural and functional conservation of its domain IV region, shorter RNA constructs for structural studies were designed in which the natural sequence was truncated at the junctions between helices c, d and e and loops C, D and E, respectively (Fig. 1a). By reducing the number of flexibility centres, we were hoping to improve the conformational homogeneity of the RNA and thus favour its crystallization. Previous studies of RNA–protein complexes (Oubridge et al., 1995; Price, Evans et al., 1998) and of isolated RNA molecules (Scott et al., 1995; Golden et al., 1997) have shown that subtle sequence changes at the RNA termini can dramatically affect the ability of a given construct to crystallize, as well as the quality of the resulting crystals. Several sequence variants of each construct were therefore tested; the predicted secondary structures of three constructs which produced diffracting crystals are shown in Figs. 1(b)–1(d).

In order to ensure sequence homogeneity, synthetic DNA templates for RNA transcription were assembled in which the genes encoding the RNA sequences were preceded by hammerhead ribozyme sequences and followed by either hammerhead or hepatitis δ virus (HDV) ribozyme sequences (Fig. 2), as described in Price, Oubridge et al. (1998). The sequence of the 58 and 43 RNA constructs (Figs. 1b and 1c) was restricted by the GUC sequence for hammerhead ribozyme cleavage at the 3′-end, whereas the sequence of 45 RNA (Fig. 1d) is authentic up to the very end of helix c, as sequences cleaved by hepatitis δ virus (HDV) ribozyme are less restricted (Price, Oubridge et al., 1998). After in vitro transcription with T7 RNA polymerase and ribozyme-catalysed cleavage of the resulting precursor RNAs, constructs were purified by denaturing gel electrophoresis essentially as detailed in Price, Oubridge et al. (1998). They were then concentrated by both rotary and SpeedVac evaporation to 5–15 mg ml^{-1} in 5 mM sodium cacodylate pH 6.5 and stored at 203 K.

3. RNA annealing and functional characterization

In several cases, RNAs that adopt an hairpin structure in solution have been shown to dimerize under specific crystallization conditions (Holbrook et al., 1991; Ennifar et al., 1999; Wild et al., 1999). Since the biologically relevant fold of 4.5S RNA is that of a monomeric hairpin (Bourgaize et al., 1984; Poritz et al., 1988; Lentzen et al., 1996), an extensive survey of annealing conditions was performed in order to minimize this potential problem (Jovine, 1998). Although shorter constructs consistently displayed a higher tendency to dimerize than longer ones, an annealing protocol was developed which effectively prevented dimerization of even the shortest construct, 43 RNA (Fig. 1c), as shown by native gel electrophoresis (0.5 mg ml^{-1} annealed RNA in

Figure 1
(a)–(d) Predicted secondary structure of 4.5S RNA constructs, with helices and internal loops indicated as a–f and A–E, respectively, according to Lentzen et al. (1996). The secondary structure of domain IV (Fig. 1a, box) is based on the crystal structures of its free and bound forms (Jovine et al., 2000; Batey et al., 2000). The apical GNRA tetraloop is marked with 'TL'. Watson–Crick base pairs and non-canonical pairs are represented as lines and dots, respectively. (a) Native E. coli 4.5S RNA. Highly conserved nucleotides are circled in cyan. (b)–(d) 58, 43 and 45 RNA constructs. Nucleotides and base pairs not found in the native sequence are highlighted in blue. (e)–(g) Crystals of the 58, 43 and 45 RNA constructs. Dimensions are as reported in the main text.

10% glycerol, 10% polyacrylamide gels in $1\times$ TB buffer). Each construct was diluted to 0.370 mM concentration in 20 mM sodium cacodylate buffer pH 6.5 and incubated on ice for 1 h. RNAs were then heated at 338 K for 10 min, immediately centrifuged at 13 000 rev min^{-1} for 10 s and finally snap-cooled on ice for a further hour before crystallization setup. All constructs annealed using this procedure bound to Ffh with the same affinity as full-length 4.5S RNA; furthermore, they could promote reciprocal stimulation of GTP hydrolysis by Ffh and its receptor FtsY within a ternary complex (T. Hainzl, unpublished data; Jovine, 1998).

4. Crystallization

Preliminary screening of crystallization conditions was perfomed using a 72-condition sparse matrix which combined solutions from the screen of Scott *et al.* (1995), solutions of the commercial NATRIX screen (Hampton Research, USA) and a few additional PEG conditions (Jovine, 1998). For each construct, precipitant and salt concentrations were increased so that a comparable ratio to the number of nucleotides was maintained, as described by Golden *et al.* (1997). Because of the importance of polyamines in the crystallization of transfer RNAs (Brown *et al.*, 1972; Ladner *et*
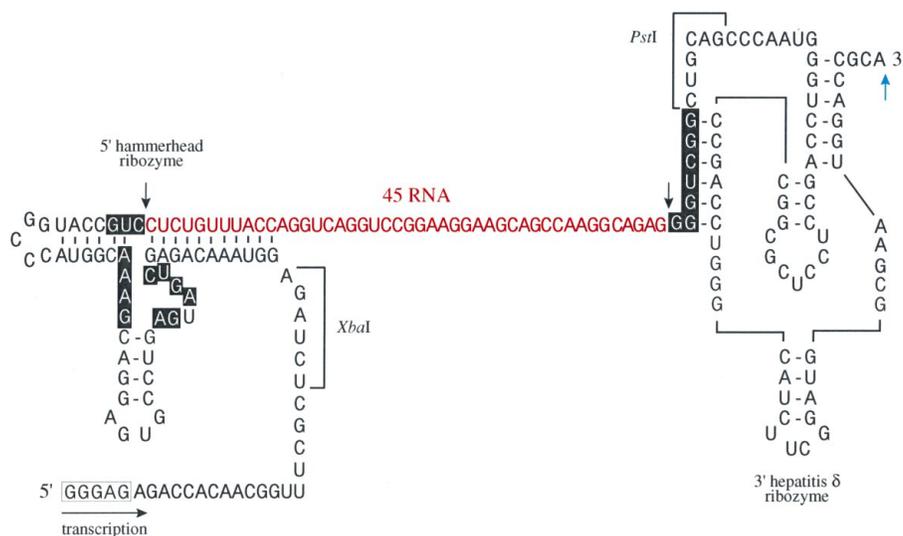
al., 1972; Kim *et al.*, 1973), hammerhead ribozyme (Pley *et al.*, 1993; Scott *et al.*, 1995) and of a P4-P6 group I intron fragment (Doudna *et al.*, 1993), parallel screens were performed both at 293 and 277 K in the presence of spermine (2:1 molar ratio to RNA) or in its absence. Crystallization trials were carried out by sitting-drop vapour diffusion (1 µl RNA plus 1 µl crystallization solution against 750 µl reservoir), using non-siliconized microbridges in Linbro plates. Between 1 and 5 mg of RNA, depending on the length of the construct, were required for a complete screen of the 72 conditions.

Prismatic crystals of the 58 RNA construct (Fig. 1*b*) grew in ~14 d at 293 K in 200 mM KCl, 100 mM Mg(OAc)$_2$, 50 mM sodium cacodylate pH 6.5 and 6% PEG 8000 to maximum dimensions of $1.50 \times 0.30 \times 0.10$ mm (Fig. 1*e*).

Crystals of the 43 RNA construct (Fig. 1*c*) appeared in 2–10 d in several solutions of the screen; the most promising condition [200 mM NH$_4$Cl, 150 mM Mg(OAc)$_2$, 50 mM Na HEPES pH 7.0, 5% PEG 4000] was optimized to 120–170 mM MgCl$_2$, 50 mM Na HEPES pH 7.0 [or 80–130 mM MgCl$_2$, 4 mM Co(NH$_3$)$_6$Cl$_3$, 50 mM Na HEPES pH 7.0] and reproducibly yielded rectangular plates within ~12 h, which in rare cases grew to dimensions of $1.20 \times 0.25 \times 0.07$ mm at 293 K (Fig. 1*f*). Nucleation and

growth of the 43 RNA crystals were completely dependent on the presence of magnesium ions and were extremely sensitive to their exact concentration. To the best of our knowledge, this is the first reported case of diffracting crystals of a ribonucleic acid obtained by simple addition of Mg^{2+} ions. A closely related crystallization condition, including both magnesium ions and spermine, was described for *E. coli* tRNA^{Tyr} (Brown *et al.*, 1972) and crystals of nucleosome core could also be grown by using various divalent ions as precipitant, with Mn^{2+} ions yielding the best results when combined with KCl (Rhodes *et al.*, 1989). In the latter case, nevertheless, crystallization probably occurred by non-specific neutralization of the negative charges of DNA wrapped around the histones, while this effect is almost certainly combined with a specific structural stabilization in the case of the 4.5S RNA domain IV fragments. Biochemical (Bourgaize *et al.*, 1984; Lentzen *et al.*, 1996) and NMR (Schmitz *et al.*, 1996) studies have in fact demonstrated a marked stabilization of 4.5S RNA in the presence of magnesium and suggested that a strong binding site for this ion might be present within the symmetric loop A of its domain IV. In agreement with these data, it was not possible to grow crystals of domain IV constructs with any divalent ion other than Mg^{2+} (Jovine, 1998).

The 45 RNA construct (Fig. 1*d*) initially crystallized in 10 mM Mg$_2$SO$_4$, 50 mM sodium cacodylate pH 6.5, 2 M (NH$_4$)$_2$SO$_4$. These crystals appeared at 293 K after ~14 weeks and were often split. Optimization of the condition to 90 mM Mg(OAc)$_2$, 50 mM sodium cacodylate pH 6.0, 1.7–1.9 M (NH$_4$)$_2$SO$_4$, 303 K allowed us to obtain conical-shaped single crystals, which appeared within ~8 weeks and grew to maximum dimensions of ~$0.20 \times 0.20 \times 0.20$ mm (Fig. 1*g*). It is interesting to note that the refined crystallization conditions of 45 RNA are extremely similar to those reported for crystal form III of the 46 nt RNA/DNA hammerhead ribozyme (Pley *et al.*, 1993), suggesting that variations around these conditions might represent a good starting point when screening for crystals of other RNA molecules of similar size.

5. Crystallographic data collection and analysis

Crystals of 58 RNA were cryoprotected by 5 min stepwise soaks in solutions equivalent to 80% of the mother liquor plus 5, 10, 15, 20 and finally 25% PEG 400 and were then flash-frozen in a nitrogen stream at 100 K.

Figure 2
Predicted folding of a generic 5′ hammerhead ribozyme-SRP RNA-3′ hepatitis δ virus ribozyme transcript (in this case, the sequence of the 45 RNA construct is shown in red). Bases of the transcription plasmid insert important for efficient T7 RNA polymerase transcription and ribozyme activity are boxed in white and black, respectively. Cloning sites are in brackets and 5′ and 3′ ribozyme cleavage sites are indicated by black arrows. Constructs of this type were cloned into the *Xba*I/*Pst*I sites of a pUC18-based vector carrying a 5′ T7 promoter and a 3′ fragment of an hepatitis δ virus ribozyme sequence (pUC18T7*Pst*δV; Dr Sandra Searles, MRC-LMB, personal communication). This created a new 5′ hammerhead ribozyme sequence and reconstituted a complete 3′ hepatitis δ virus ribozyme sequence. After digestion of the resulting plasmid at the 3′ *Hin*dIII site (indicated by a blue arrow) and *in vitro* run-off transcription/co-transcriptional RNA 5′-end cleavage by hammerhead ribozyme (Price, Oubridge *et al.*, 1998), 3′-end cleavage of the RNA product by the hepatitis δ virus ribozyme required a 2 min annealing step at 338 K followed by a 4 h incubation at 328 K.

Table 1
Crystallographic data collection and statistics.

Construct	43 RNA	45 RNA
Number of crystals	1	1
Temperature (K)	100	100
Space group	$C222/C222_1$	$P3_221$
Unit-cell parameters (Å)	$a = 56.5, b = 185.0,$	$a = b = 69.1,$
	$c = 45.8$	$c = 84.6$
Resolution range (Å)	20.0–3.2	34.5–2.6
Wavelength (Å)	1.5418†	0.9430‡
Number of reflections	44260	362846
Number of unique reflections	4072	7425
Completeness§ (%)	93.6 (81.1)	98.8 (90.7)
Redundancy§	4.2 (3.7)	13.7 (8.6)
$\langle I/\sigma(I)\rangle$§	10.0 (4.9)	17.5 (2.0)
R_{sym}§¶ (%)	11.0 (23.5)	10.0 (65.1††)

† This data set was collected on an MSC R-AXIS II image-plate area detector using a Rigaku RU-200 rotating-anode source at the Protein Structure Group of Glaxo Wellcome R&D, UK. ‡ This data set was collected at ESRF synchrotron beamline ID14-4 using an ADSC Quantum-4 CCD detector. § Values in parentheses refer to the highest resolution shell (43 RNA, 3.31–3.20 Å; 45 RNA, 2.69–2.60 Å). ¶ $R_{sym} = \sum_h \sum_i |I_i(h) - \langle I(h)\rangle|/\sum_h \sum_i I_i(h)$, where $I_i(h)$ is the ith measurement and $\langle I(h)\rangle$ is the mean of all measurements of $I(h)$ for Miller indices h. †† The high R_{sym} of the highest resolution shell of the 45 RNA data is a consequence of the significant anisotropy of diffraction, so that although data coverage is 90.7% complete in this shell, a significant proportion of reflections within it are extinct. This effect was much less pronounced to 2.9 Å resolution (R_{sym} of the 3.08–2.93 Å shell is 18.5%).

Analysis of partial diffraction data collected on a rotating-anode X-ray source identified the crystals lattice as primitive orthorhombic and approximately determined the unit-cell parameters to be $a = 44.9$, $b = 116.6$, $c = 125.5$ Å. Assuming a density of ~ 1.635 g cm^{-3} for the RNA (Sober, 1970), these crystals probably contain three or four molecules of 58 RNA per asymmetric unit. Because they did not diffract isotropically beyond ~ 6 Å resolution even at synchrotron sources, the 58 RNA crystals were not pursued. In contrast, diffraction to 3.0 Å resolution was observed from crystals of the 43 RNA construct cryoprotected in a solution of 50 mM Na HEPES pH 7.0, 20 mM sodium cacodylate pH 6.5, 600 mM MgCl$_2$, 4 mM Co(NH$_3$)$_6$Cl$_3$, 30%(v/v) saturated glucose, 35%(v/v) saturated sucrose and flash-frozen in liquid propane. The crystals belong to space group $C222_1$ (or $C222$) and have unit-cell parameters $a = 56.5$, $b = 185.0$, $c = 45.8$ Å, with either one or two molecules per asymmetric unit. Following overnight stabilization in 2.2 M (NH$_4$)$_2$SO$_4$, 90 mM Mg(OAc)$_2$, 50 mM sodium cacodylate pH 6.0 at 292 K, crystals of 45 RNA can be cryoprotected by stepwise [+5%(v/v) in 2 min] addition of glycerol to a final concentration of 20%(v/v) in stabilization solution. After flash-freezing in liquid nitrogen, these crystals diffract anisotropically to 2.6 Å resolution at synchrotron sources. They are trigonal, with space group $P3_221$, unit-cell parameters $a = b = 69.1$, $c = 84.6$ Å and one RNA molecule per

asymmetric unit. Data sets were indexed and integrated with *DENZO* and scaled and reduced with *SCALEPACK* (Otwinowski & Minor, 1997); statistics for representative native data sets collected from both 43 RNA and 45 RNA crystals are given in Table 1.

Loss of isomorphism or severe reduction of diffraction power was observed upon heavy-atom soaking of both 43 and 45 RNA crystals. We therefore attempted to substitute either all or single uridine nucleotides with their modified analogue 5-Br-dU by *in vitro* transcription or solid-phase phosphoramidite chemical synthesis (Gait *et al.*, 1991), respectively. While the former method lead to extensive cross-linking of the completely modified RNAs, constructs produced by the latter were significantly less soluble than their unmodified counterparts and did not yield diffracting crystals. Luminescence spectroscopy measurements of Tb^{3+} ion binding to domain IV RNA constructs in solution (Horrocks, 1993; Feig *et al.*, 1998) have recently allowed us to produce a single-site Lu^{3+} derivative of 45 RNA, with which we have now solved the structure by multiple anomalous dispersion (MAD; Jovine *et al.*, 2000).

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