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Interaction of a Transcription Factor IIIA Peptide Containing Two Zinc Fingers with 5S rRNA

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The interaction of lupin ribosomal 5S RNA with a chemically synthesized peptide containing 60 amino acid, derived from *Xenopus laevis* transcription factor IIIA, is analyzed. The results show that such short fragment retains the ability of binding to 5S rRNA molecule, as shown by electrophoretic gel shift and RNase footprint assay. The peptide protects from hydrolysis with specific nucleases helix II and V of 5S rRNA.

Key words: zinc finger peptide, transcription factor IIIA, *Xenopus laevis*, ribosomal 5S RNA, protein – nucleic acid interaction

Among various structural protein motifs, which identify nucleic acid binding proteins, a zinc finger module is unique and defines an ability to form sequence specific complexes either with RNA or DNA or both [1]. One of members of the zinc finger protein superfamily is the *Xenopus laevis* transcription factor IIIA (TFIIIA). It is a 40-kDa protein required for the transcription of the 5S rRNA genes by RNA polymerase III [2,3], which consists of nine copies of a characteristic motif, termed a zinc finger. Two pairs of invariant cysteine and histidine residues mediate the three dimensional folding of two antiparallel β-sheets and an α-helix with bonded zinc ion [3]. The zinc finger motifs of TFIIIA are responsible for a specific recognition of 5S rRNA and promote sequence-specific binding to the internal control region of the 5S rRNA gene [4,5]. The crystal structures of different zinc finger protein – DNA complexes have revealed basic principles of a zinc finger peptide – DNA recognition [6–9]. Specific contacts of nucleic acid bases with the zinc finger α-helical element are localized in the major groove of the DNA. In the ZIF268 – DNA complex (ZIF268 – it is DNA binding domain from the mouse intermediate early protein), three zinc fingers of the protein contact only nine base pairs along the major groove of DNA. Each zinc domain of ZIF268 recognizes three base pairs and forms similar hydrogen bonds [6]. A different binding pattern was found in the TFIIIA – DNA complex, in which three N-terminal zinc fingers bind to the DNA major groove in a manner reminiscent of ZIF268, but in contrast to it, TFIIIA fingers cover a more extended DNA surface spanning a 13 base pairs [10]. Crystal structure of the six zinc-finger fragment of

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TFIIIA in complex with DNA shows that the domains 4–6 lie along one side of DNA. Finger 5 binds to bases in the major groove, but fingers 4 and 6 do not wrap around the double helix but traverse the minor groove [9]. In addition to binding DNA, TFIIIA binds the 5S rRNA molecule. Structural studies and systematic RNA mutational analyses identify essential elements of 5S rRNA for recognition by TFIIIA [11–13]. A wild-type TFIIIA protect loop: A,B,E and helices II, III, V and a small segment of loop C from hydrolysis. The fragment of TFIIIA, containing fingers 4–7, protects loops A, E and helix V and less loop B, but in the presence of this peptide, enhanced hydrolysis of helix III is observed [13]. The peptide containing fingers 4–9 from N-terminus of TFIIIA causes a stronger cleavage of helix IV, while the peptide having fingers 1–6 – helix III, peptide containing only three internal fingers 4–6 enhance hydrolysis of both helices III and IV [12]. Taking these results into account, a model of the 5S rRNA – TFIIIA complex was prepared: fingers 4–6 interact with helices I, II and V including loop A and E, fingers 1–3 with helix IV and fingers 7–9 with helices II/III and loop B [12,13]. Experiments made by another group show clear protection of 5S rRNA by a wild type of TFIIIA in helices II, IV and V. But the protection of the peptide containing fingers 4–9 is indistinguishable from that observed for the whole TFIIIA molecule [14]. Mutagenesis of TFIIIA provided evidence for a pivotal function of finger 6 in RNA recognition [12,13,15–17].

EXPERIMENTAL

Synthesis of polypeptide: A peptide corresponding to zinc fingers 5–6 of *Xenopus laevis* TFIIIA [TFIIIA(133–192)] was synthesized using the fluoren-9-ylmethoxycarbonyl (Fmoc) procedure on a Millipore 9050 Plus peptide synthesizer by continuous flow method with a TentaGel S AC resin substituted with Fmoc-Ala (0.15 mM/g). Following side chain protecting groups were applied: tert-butyl (tBu) for Asp, Glu, Ser and Thr, Tyr, t-butoxycarbonyl (Boc) for Lys, trityl (Trt) for Gln and His, acetamidomethyl (Acm) for Cys, 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Pmc) for Arg.

1,3-Diisopropylcarbodiimide/1-hydroxybenzotriazole coupling method in a DMF – dichloromethane-1-methyl-2-pyrrolidone (1:1:1 v/v/v) solution was used (1.5 h). After completion of the synthesis (60 h), the peptide was cleaved from the resin with a trifluoroacetic acid (TFA) – phenol – triisopropylsilane – water (88:5:2:5 v/v/v/v) mixture for 2 h. During this procedure all protecting groups except S-acetamidomethyl (Acm) were removed. Crude Acm-peptide was lyophilized and desalted on a Sephadex G-25 column (2.8×105 cm) in 30% acetic acid (AcOH) and chromatographed twice on a preparative RP-HPLC Vydac C-18 column (32×240 mm, 15–20 μm particle size) with 30–42% and 5–42% acetonitrile in 0.1% TFA linear gradients. Each step was monitored by an analytical RP-HPLC System Gold Beckman chromatograph with Ultrasphere ODS column (5 μm particle size, 4.6×195 mm) in 0.1% TFA (A) and acetonitrile – 0.08% TFA (80:20 v/v) (B) with linear gradient 40–60% of B for 30 min, (flow rate 1 ml/min), A_{226}. The purified Acm-peptide was characterized by the mass spectra measured with a Finningan MAT 95Q mass spectrometer by electrospray ionization method (ESI) (Fig. 1).

The Acm protecting groups were removed using mercury(II) acetate for 4 h and mercury(II) was removed by precipitation with thioethanol (2-sulfanylethan-1-ol). The supernatant was separated and treated with EDTA. In order to obtain a reduced peptide sample was treated with 50 mM 2,3-dihydroxybutane-1,4-dithiol (DTT) at 90°C for 30 min [18]. The mixture containing 60aa peptide with free thiol groups was desalted on a Sephadex G-10 column in 1 M AcOH. Free thiol groups of cysteine residues were quantitatively determined by the Ellman method [19,20]. Amino acid analysis was performed on a Beckman model 121 M analyzer. The peptide was hydrolyzed with constantly boiling hydrochloric acid containing 1% phenol at 110°C for 24 h.

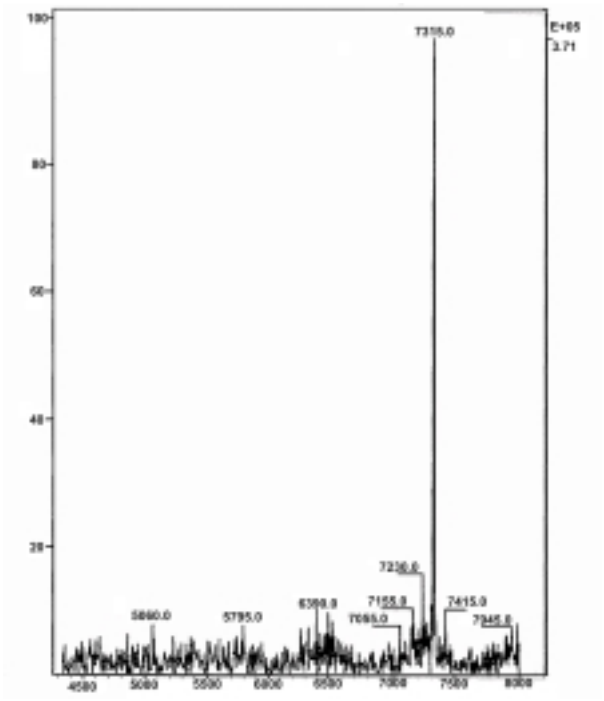

Figure 1a. ESI spectrum of Acm-protected TFIIIA(133–192) (deconvoluted of the molecular mass).

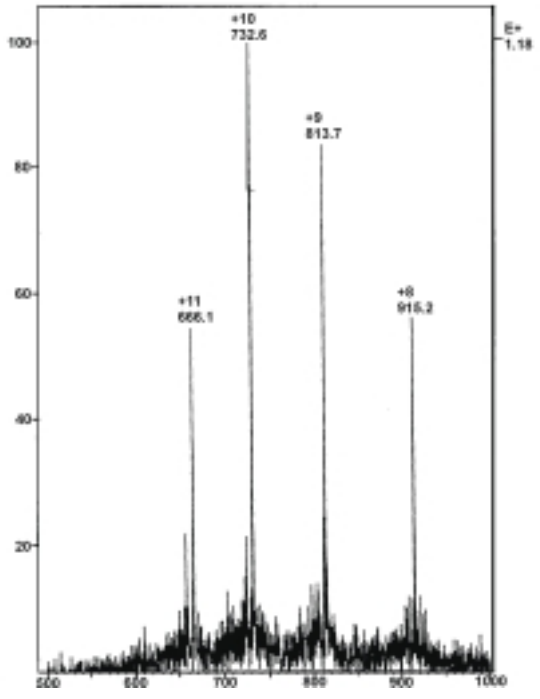

Figure 1b. ESI spectrum of multiple charged ions (unconvoluted).

Capillary electrophoresis (CE) analysis was performed with a Beckman P/ACE System 2100 instrument with cathode on the detection side. All solutions and samples were filtered through an 0.22 μm teflon membrane filter prior to use. Electrophoresis was performed in 100 mM phosphate buffer at pH 7.0. The capillary cassette was fitted with 75 μm i. d. uncoated fused-silica capillary, 57 cm in length (50 cm to the detector). Runs were made at a constant voltage of 18 kV. The temperature of the analysis was maintained at 25°C± 0.1°. The separation effect was monitored at 214 nm. The sample injection time was 3 s (Fig. 2). All the determined physicochemical properties of TFIIIA (133–192) fragment are summarized in Table 1.

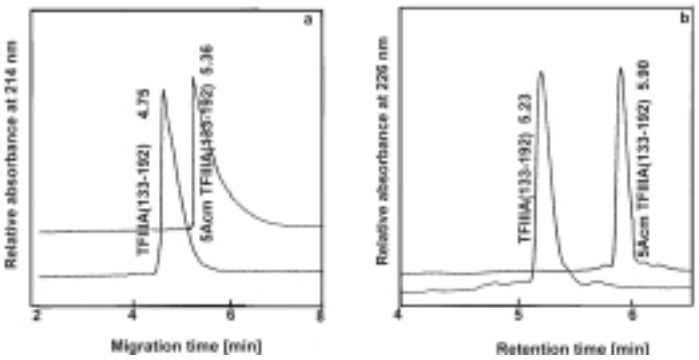

Figure 2. Capillary electrophoresis (**a**) and HPLC (**b**) of the Acm-protected and deprotected TFIIIA (133–192) peptide.

Table 1. Physicochemical properties of TFIIIA (133–192) fragment.

Compound	ESI-MS Molecular weight $M+H^+$ calc.	found	CE T_m[min]	RP-HPLC R_T[min]	Amino acid analysis *
TFIIIA(133–192) ×5 Acm	$C_{383}H_{497}N_{93}O_{92}S_5$ +H^+ 7316.4	7315.0	5.36	5.90	nd
TFIIIA(133–192)	$C_{308}H_{467}N_{88}O_{87}S_5$+$H^+$ 6951.3	nd	4.75	5.23	Asp 3.96(4), Thr 1.71(2), Ser 3.69(4), Glu 4.78(5), Pro 3.79(4), Gly 2.89(3), Ala 3.00(3), Val 2.67(3), Leu 5.77(6), Tyr 2.90(3), Phe 1.88(2), Lys 6.89(7), His 4.72(5), Arg 2.92(3)

*Tryptophan and cysteine were not determined, nd – not determined.

Immediately before use the lyophilized samples were dissolved in a buffer containing 20 mM Tris HCl (pH 7.5), 70 mM KCl, 5 mM dithiothreitol (DTT) supplemented with 1.2 molar excess of $ZnCl_2$.

Isolation of 5S rRNA: 5S rRNA was isolated from *Lupinus luteus* seeds by phenol extraction, fractionated on Sephadex G-75 and repurified on 15% polyacrylamide gel containing 7 M urea, 50 mM Tris/borate buffer (pH 8.3) and 1 mM EDTA as described previously [21]. RNA samples were labeled with [γ-^{32}P]ATP and T4 kinase or [^{32}P]pCp and RNA ligase at 5′ or 3′ end respectively.

5S rRNA binding and electrophoretic gel shift assay: 5S rRNA-peptide complex formation was carried out in a total volume of 10 μl for 40 min at 22°C in 100 mM Tris HCl buffer (pH 7.5) containing: 1 nM 5S rRNA, 5000 cpm of ^{32}P labeled 5S rRNA, 2 μg crude tRNA, 100 mM KCl and different amounts of peptides as shown in the legend to the figures. The analysis of the complex formation was carried on 0.7% agarose gel in 0.09 M Tris/borate buffer. The samples were loaded on the gel in a buffer containing 20% of glycerol and 0.02% of bromophenol blue.

RNase footprint assay: RNase footprint of the 5S rRNA-peptide complex was carried out in total volume of 5 μl using 1 nM 5S rRNA (50 000 cpm of ^{32}P labeled 5S rRNA), 2 μg crude tRNA, 20 mM Tris

HCl pH 7.5, 70 mM KCl, 1 mM $MgCl_2$, 5 μM $ZnCl_2$, 5 mM DTT 0.1% Nonidet P-40 and different amount of peptides. After 40 min of incubation, 1 μl of RNase was added (T1 RNase 1×10^{-3}U, V1 RNase 0.03 and 0.06U). After 10 min of incubation at 22°C, 5 μl of loading buffer (7 M urea 0.02% Xylene Cyanol, 0.02% Bromophenol Blue) was added, chilled on ice and 5 μl of each probe was loaded on 10% and 20% polyacrylamide denaturing gel. The alkali ladder was prepared by boiling for 1 min the 5S rRNA (80 000 cpm) in 50 mM NaOH and 1 mM EDTA. The reaction was stopped by adding of loading buffer and chilling. For G-ladder preparation 80 000 cpm of 5S rRNA was incubated with 1×10^{-4}U of RNase T1 for 10 min at 55°C in a buffer containing 7 M urea, 0.02 M CH_3COONa (pH 4.5), 0.001 M EDTA and 2 μg/probe of crude tRNA.

RESULTS AND DISCUSSION

The TFIIIA – 5S rRNA complex was extensively investigated for several years. Big effort was focused on precise localization of binding sites of particular zinc fingers in the 5S rRNA molecule. The evidence gathered up to the present time shows that fingers 4–6 or 7 bind to helices II, V and loop A. The margins of binding of TFIIIA fingers 4–6 determined by analysis of footprint experiments are U96 on helix IV and C18 on helix II [16]. We attempted to find boundaries for the binding site of a single zinc finger. We did not observe a complex with the single zinc finger domain TFIIIA-2 and 5S rRNA [22]. Although in case of single C_2H_2 domain isolated from phage display zinc finger library and recombined the complex with Rev responsive element was observed with dissociation constant similar to native protein [23]. That is why in the next step we used the synthetic peptide containing 60-amino acids corresponding to zinc fingers 5 and 6 of TFIIIA (133–192) from *Xenopus laevis* (Fig. 3). The polypeptide was synthesized by the Fmoc procedure using a continuous flow method. Four-fold molar excess of a protected amino acid was sufficient to obtain a satisfactory coupling degree. To avoid side reactions, full protection of all amino acid side chains was used. No coupling problems were detected, although a problem of Nα-amino Fmoc deprotection group was noticed in Gly^{164} and Pro^{187} positions. An efficiency of Fmoc group deprotection was 20–35% lower, compared to other positions. This problem was monitored but not corrected during the synthesis.

The peptide was cleaved from the resin as Acm protected peptide to avoid any problems with the disulfide bridge formation or the thiol group oxidation during purification. Acm groups were removed in the last purification step, just before complexation of the peptide with zinc ions. The purity of the peptide was checked

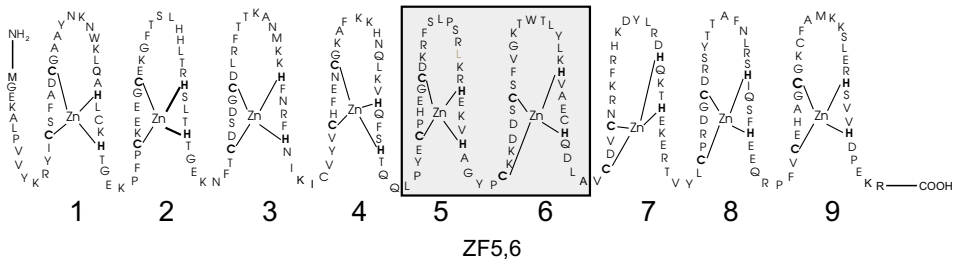

Figure 3. Sequence of the N-terminal part of TFIIIA from *Xenopus laevis* containing 9 zinc fingers. The synthesized polypeptide TFIIIA(133–192) containing zinc fingers 5 and 6 is boxed.

using mass spectrometry (Fig. 1 a,b), capillary elctrophoresis and HPLC (Fig. 2 a,b). This procedure is very useful for synthesis of peptides containing thiol groups especially for long peptides as TFIIIA (133–192).

We showed that native 5S rRNA forms a complex with the peptide (Fig. 4), although the 100% complex saturation was not obtained. In order to find a 5S rRNA polypeptide binding site, its limited RNase hydrolysis was carried out. The peptide protects nucleotides 14–20, 58–65 and 67–69 from hydrolysis with RNase V1 (Fig. 5).

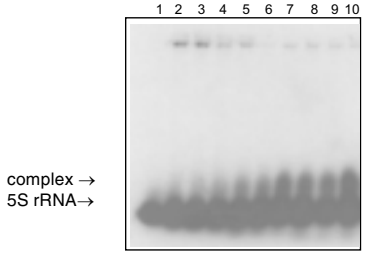

Figure 4. Analysis of the [3′-^{32}P]5S rRNA – TFIIIA (133–192) complex on 0.7% agarose. Formation of the complex was performed as described in Experimental, concentration of peptides are as follows: 1) control without peptide; 2) 2.5 nM of peptide; 3) 5.0; 4) 7.5; 5) 10; 6) 12.5; 7) 15; 8) 17.5; 9) 22.8; 10) 40 nM of TFIIIA (133-192) peptide.

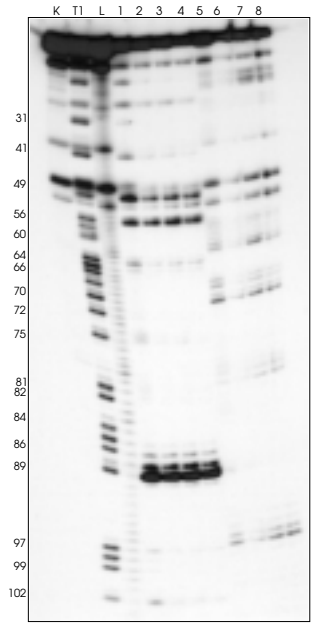

Figure 5a. Autoradiogram of 20% polyacrylamide gel with 7 M urea showing hydrolysis products of the 5S rRNA – TFIIIA (133–192) complex. Analysis was performed as described in Experimental. K – control, T1 – G-ladder, L – alkali ladder, lanes 1–4 hydrolysis of complex with RNase T1 (0.001 units), 5–8 hydrolysis of complex with RNase V1 (0.03 units); lanes 1,5:5S rRNA control in the reaction mixture without peptides; lanes 2,6:1nM 5S rRNA, 22, 8nM TFIIIA (133–192); lanes 3,7:1nM 5S rRNA, 11.4nM TFIIIA (133–192); lanes 4,8:1nM 5S rRNA, 5.7nM TFIIIA (133-192).

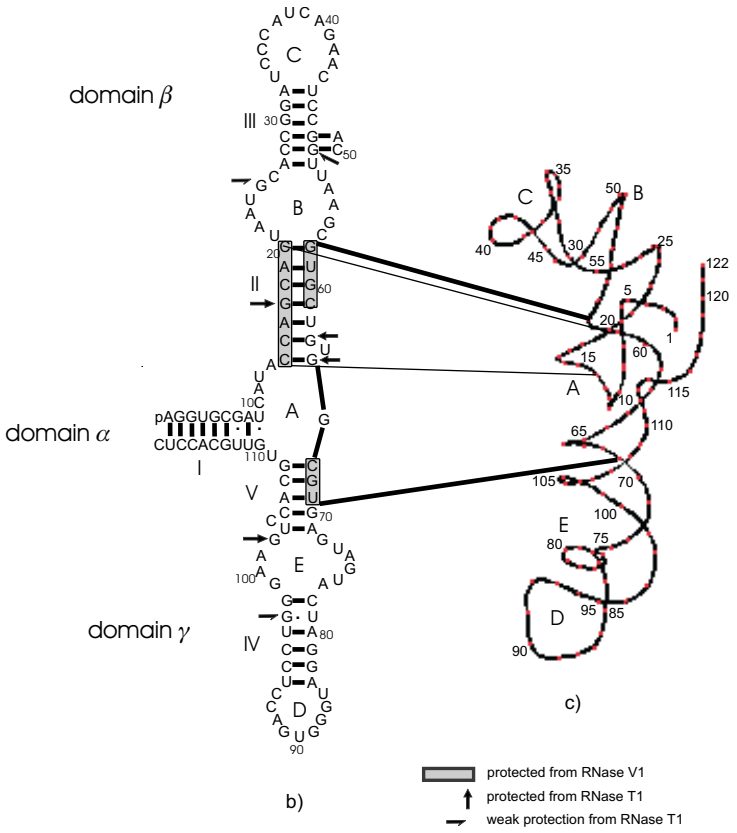

Figure 5. **b)** Secondary structure of 5S rRNA from *Lupinus luteus*.
c) Three-dimensional model of plant 5S rRNA. The lines between secondary and tertiary structure show the part of 5S rRNA protected by peptide.

RNase T1 does not hydrolyze nucleotides 17, 25, 64, 65, 102, 51 and 97 in the presence of TFIIIA (133–192). This suggests that the synthetic peptide binds to helix II and part of helix V. Generally, enzymatic footprints of RNA – protein complexes suffers from a number of limitations. One of them is overestimation of the protected region of RNA due to steric hindrance. The evidence for that can be an unsuccessful attempt at simultaneous binding of two peptides containing fingers 1–3 and 4–9 from the N-terminal end of TFIIIA to 5S rRNA, which means that the binding sites for both of them overlap [14]. This observation was supported by a footprint reaction of 5S rRNA bound to a wild type of TFIIIA and its fragment containing fingers 4–7, which showed that there was no difference between them [14]. Nevertheless, taking together results of enzymatic hydrolysis of 5S rRNA in a complex with the whole TFIIIA molecule and its fragments, it was possible to build the model of 7S particle presented in the introduction. However, there is no answer where the binding sites of particular zinc fingers on the surface of 5S rRNA are and how many of nucleotides do interact with a single zinc finger. Our data obtained for peptides having 1–3 or 1–4 zinc fingers (zf) respectively (manuscript submitted), show that zf 1–3 protect helices

IV and V and addition of another domain causes that helix II becomes inaccessible. The result obtained for zf 4–6 and 4–7 [12–14,16] showed that helix V and loop E are protected by these peptides. Since the data are different, we somehow used the smallest fragment of TFIIIA that retained the binding activity to 5S rRNA. We used the 60-amino-acid peptide corresponding to finger 5 and 6 of *Xenopus* TFIIIA. Comparing the intensity of bands corresponding to hydrolysis product of 5S rRNA in the presence and absence of peptide, we found that TFIIIA-5,6 peptide protects fragment of helix II and less helix V. We did not see any difference in the hydrolysis pattern obtained with RNase S1. Our results are slightly different from previously obtained data according to which finger 6 interacts with loop A and finger 7 with helix II [15,16]. We realize that our complex may be partly unspecific, because we use big excess of protein but a lower amount did not show any results similarly to experiment, which was done by Frenkel *et al*. [18]. It confirms previous observation that tight specific binding require a set of cooperative contacts. In order to better visualize the binding sites of fingers 5 and 6, we projected data using, as a model, three-dimensional structure of 5S rRNA obtained from the crystal structure of 50S ribosomal subunit (Fig. 5) [24]. It can be seen that the binding site for finger 5 and 6 is located in the cleft formed between loop A and helix V.

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