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Additivity of Interactions of Zinc Finger Motifs in Specific Recognition of RNA

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***Xenopus* transcription factor IIIA (TFIIIA) binds 5S rRNA and the 5S rRNA gene, these interactions being mediated by nine zinc fingers. To determine the contribution of each finger to the binding to 5S rRNA we prepared a series of peptides containing different numbers of zinc fingers and analyzed their interactions with RNA. The topography of these complexes was analyzed with the specific RNase and hydroxyl radical footprinting methods. Our results show the direct contribution of each zinc finger (ZF) peptide to the specific recognition of 5S rRNA. These data clearly suggest that total binding of TFIIIA with 5S rRNA is the sum of the specific interactions of the individual zinc fingers with RNA and that they have an additive character.**

Key words: additivity of interaction, RNA protein interaction, zinc finger.

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Among various structural motifs found in nucleic acid binding proteins, a zinc finger domain is unique and defines the ability to form sequence-specific complexes with either RNA, or DNA or both (*1*). The first protein in which a zinc finger domain was found was *Xenopus laevis* transcription factor IIIA (TFIIIA) (*2, 3*). TFIIIA is a 40 kDa protein required for transcription of 5S rRNA genes by RNA polymerase III, and consists of nine motifs with similar sequences termed the zinc fingers. They have 30 amino acids with the following sequence Tyr/Phe-Xaa-Cys-Xaa-Cys-Xaa$_{2,4}$-Cys-Xaa$_3$-Phe-Xaa$_5$-Leu-Xaa$_2$-His-Xaa$_{3,4}$-His-Xaa$_5$ (Xaa = variable amino acids) (*2*). Two pairs of invariant cysteine and histidine residues coordinate a zinc ion and mediate the peptide folding into the three-dimensional structure (zinc finger) formed by two antiparallel β-sheets and an α-helix. TFIIIA zinc finger motifs are responsible for specific binding to an internal control region of the 5S rRNA gene (*3, 4*). A crystallographic study of the zinc finger peptide Zif268, Gli, and six-finger TFIIIA fragment in complexes with DNA, as well as NMR analysis of three N-terminal zinc fingers of TFIIIA with DNA, revealed an interaction of amino acids with nucleotides, and which provides a basis for the zinc finger–DNA recognition model (*5–10*). Specific contacts between nucleic acid bases and zinc finger α-helical amino acids occur in the major groove of DNA. For the last several years the specificity of protein–DNA interactions has been extensively studied and now is quite well understood (*6, 11–20*), while our knowledge about the recognition of RNA remains incomplete. It has been shown that in the case of TFIIIA, the first three fingers (ZF1–3) contribute significantly to the DNA binding affinity, while zinc fingers 4–7 are mostly involved in binding to 5S rRNA (*2, 13, 19, 21*). The polypeptide consisting of fingers 4–7 of TFIIIA showed higher affinity to 5S rRNA then TFIIIA or its fragment consisting of nine zinc fingers. The wild mode of binding is dependent on the structural integrity of finger 6 and the continuity of the sugar–phosphate backbone in loop A of 5S rRNA (*22*).

Recent analysis of the crystal structure of the 50S subunit from *Thermus thermophilus* allowed visualization of the 5S rRNA molecule, which shows a different structure to models already proposed (*23, 24*). In ribosomes, domains α and α of 5S rRNA are coaxial while domain α is perpendicular to it (Fig. 5b) (*25*). Studies on *X. laevis* 5S rRNA have shown that critical nucleotides for recognition are centered around internal loops A, B, and E, and helices II and V. Loop A is very important for the recognition of 5S rRNA, since only C12 could be mutated into every nucleotide without losing the TFIIIA binding property (*26*).

We analyzed the topology of the 5SrRNA complex with a series of zinc finger peptides derived from TFIIIA. This work was undertaken in order to determine the boundary of a particular zinc finger on the 5SrRNA surface. The results obtained hitherto concern peptides having 1–6, 4–6, or 4–7, or 4–9 fingers (*20–29*). We prepared a series of peptides having different numbers of zinc domains in order to precisely determine the boundaries of these succeeding zinc finger binding sites.

MATERIALS AND METHODS

Recombinant peptides with different numbers of zinc fingers of the TFIIIA *X. laevis* sequence were prepared by site-directed mutagenesis: a fragment of a TFIIIA cDNA clone was modified by PCR in order to produce a construct coding for a factor Xa cleavable peptide, consisting of an N-terminal 6×His-tag. Each construct was cloned into the *Nde*I and *Hin*dIII site of expression vector pT7-PL. The prepared expression plasmids, pT7-ZF3, pT7-ZF4, pT7-ZF6, pT7-ZF7, pT7-ZF8, and pT7-ZF9, were transfected into *Escherichia coli* BL21 cells. The overproduced peptide was extracted from the inclusion body pellet in neutralized phenol, precipitated by the addition of ethanol and then redissolved in the reducing buffer (6 M GuHCl, 50 mM Tris-HCl, pH 8). Peptides reduced with excess thiopyridyl glutathione disul-

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phide were further purified by gel filtration and a single step on a nitrilotriacetic acid–Ni^{2+} agarose column in the non-denaturing buffer (50 mM Tris-HCl, pH 8, 0.5 M NaCl), in which the required peptide was released through cleavage with the Xa factor. Metal reconstitution and folding were carried out in buffer containing excess zinc-mercaptoethanol complex at pH 5, followed by gel filtration (*30, 31*).

5S rRNA was isolated from *Lupinus luteus* seeds by phenol extraction, fractionated on Sephadex G-75, and repurified on a 15% polyacrylamide gel containing 7 M urea, 50 mM Tris/borate buffer, pH 8.3, and 1 mM EDTA (TBE), as described previously (*32*). RNA was labeled with [γ-^{32}P]ATP and T4 kinase at the 5′ end, and [^{32}P]pCp and RNA ligase at the 3′ end, respectively. 5S rRNA–peptide complex formation was carried out in a total volume of 10 μl for 40 min at 22°C in buffer containing 1 nM labeled 5S rRNA, 2 μg crude tRNA, 100 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM $MgCl_2$, 5 μM $ZnCl_2$, 5 mM DTT, and appropriate amounts of different peptides. The concentrations of peptides are indicated in the legends to the figures.

Analysis of complex formation was carried on a 0.7% agarose gel in 0.09 Tris/borate. The samples were loaded on the gel in buffer containing 20% glycerol and 0 02% bromophenol blue Electrophoresis was performed at 30 mA. Analysis of the influence of mono- and divalent cations as well as DTT and EDTA was performed in buffer containing 1 nM 5S rRNA, 2 μg crude tRNA, and 7 5 mM Tris HCl, supplemented with appropriate concentrations of the analyzed compounds

Footprinting of the complexes was carried out, in total volume of 15 μl, with 200,000 cpm of 5S rRNA ^{32}P labeled at 3′ or 5′, 1nM 5S rRNA, 2 μg crude tRNA, 100 mM Tris HCl, pH 7.5, 100 mM KCl, 2 mM $MgCl_2$, 5 μM $ZnCl_2$, 5 mM DTT, and appropriate amounts of ZF peptides. After 40 min incubation, the samples were divided to three parts and 1 μl of each of T1 (1×10^{-3} u) and V1 (0.06 u) RNases was added. After 10 min incubation at 22°C, 5 μl of loading buffer (7 M urea, 0.02% xylene cyanol, 0.02% bromophenol blue) was added, followed by chilling on ice, and then 5 μl of each probe was loaded onto 10 and 20% polyacrylamide denaturing gels. A ladder was prepared by boiling the 5S rRNA (80,000 cpm) in 50 mM NaOH and 1 mM EDTA for 1 min. The reaction was stopped by the addition of loading buffer and cooling on ice A G-ladder was prepared as follows: 80,000 cpm of 5S rRNA, 2 μg of crude tRNA in buffer containing 7 M urea, 0.02 M CH_3COONa, pH 4.5, and 0.001 M EDTA were incubated for 10 min at 55°C with 1×10^{-4} u of RNase T1.

The cleavage of 5S RNA with hydroxyl radicals was performed as follows: labeled 5S RNA (about 200,000 cpm) was dissolved in 160 μl of 50 mM Tris-HCl, pH 7 4, and 10 mM $MgCl_2$, and then 20 μl of a freshly prepared 10 mM solution of $Fe[EDTA]^{2-}$ and 20 μl of 50 mM DTT were added, followed by incubation for 80 min at 37°C. The reaction was quenched by the addition of 2 μl of 1 M thiourea. Samples were extracted with phenol, and the pellet was dissolved in 200 μl of 0.3 M CH_3COONa and precipitated with ethanol. This protocol was repeated three times (*35*). Such modified 5S rRNA was used for complex formation experiments. For this purpose an appropriate amount of modified 5S rRNA was dissolved in binding buffer and the zinc finger peptide was added. Samples were incubated for 30 min at room temperature, and then loaded onto a 6% nondenaturing polyacrylamide gel and run at 100V. After autoradiography the bands corresponding to free 5S rRNA and the complex were excised from the gel, eluted overnight, precipitated, and dissolved in loading solution. The recovered samples were analyzed on sequencing gels.

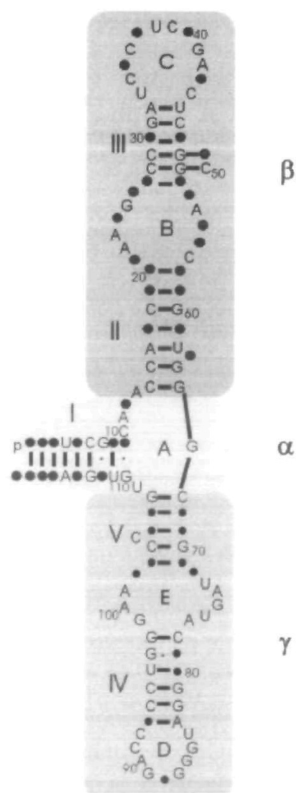

Fig 1 **The secondary structure model of *Lupinus luteus* 5S rRNA.** Nucleotides in black circles are identical to those of *X. laevis* 5S rRNA In the model there are 5 stems I, II, III, IV, and V, and 5 loops. A, B, C, D, and E, which form 3 domains: α, β, and γ

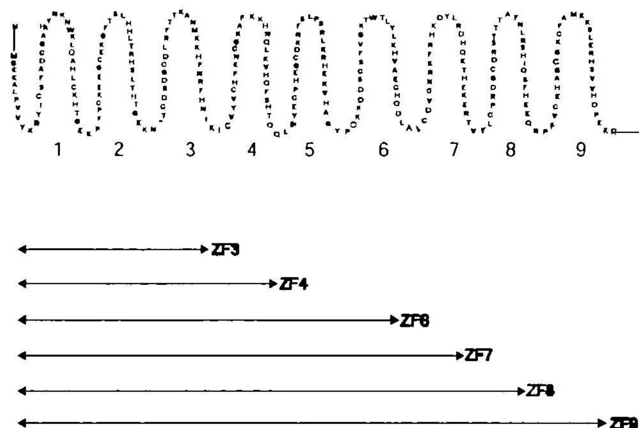

Fig 2 **Amino acid sequence of TFIIIA of *Xenopus laevis* and fragments of it containing various numbers of zinc fingers (ZF) motifs.** All of them have the same N-terminal part and were synthesized *in vitro* (*30, 31*)

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RESULTS

As a Model of Protein—RNA recognition in our study we used the TFIIIA-5S rRNA complex. We would like to identify the binding sites of particular zinc finger peptides on the 5S rRNA molecule. For this purpose we used recombinant peptides having different numbers of N-terminal zinc fingers of TFIIIA from *X. laevis* (Fig. 2). Their ability to form complexes with 5S rRNA was checked on agarose gels. In order to determine the optimal molar ratio of RNA: peptide for complex formation, separate analysis was performed (Fig. 3, a–e). The molar ratio of 5S rRNA to peptide was 1 to 5, 12.5, 7.5, 5.0, and 7.5 for ZF3, ZF4, ZF6, ZF7, ZF8, and ZF9, respectively The effects of Mg^{2+}, Zn^{2+}, K^{+}, DTT, and EDTA were also analyzed (data not shown). The results indicated that the concentrations of 0.1 mM for Mg^{2+} and 5 μM for Zn^{2+} ions are absolutely required for complex formation. Monovalent cations like K^{+} are not necessary, but the presence of 1 mM EDTA or 100 mM DTT disrupts the complex. In order to check the specificity of the complexes, we used 5.8 S rRNA of *L. luteus* and $tRNA^{Phe}$ of *Saccharomyces cerevisiae* instead of 5S rRNA (data not shown). We did not observe complex formation between the peptides and either RNA.

The complexes were probed with specific ribonucleases, T1 and V1. One example gel is presented in Fig. 4. The digestion patterns obtained for free 5S rRNA and that in the complex were analyzed, and the results are summarized in Fig. 5, a and b In the presence of ZF3, 5S rRNA was protected from hydrolysis within helices IV and V (nucleotides 93–98 and 67–71) by RNase V1. There are no differences in the hydrolysis pattern with RNase T1 between free 5S rRNA and that bound to ZF3. The ZF4 peptide prevents hydrolysis by RNase V1 in the same part of 5S rRNA as for peptide ZF3 and the 3′ part of helix V (nucleotides 103–108), and additionally protects guanosines 56, 58, 60, 65, 66, 85, 108, and 111 from hydrolysis by RNase T1 (Fig. 5a). Elongation of ZF4 with fingers 5 and 6 affects the protection of helices I and II from hydrolysis by

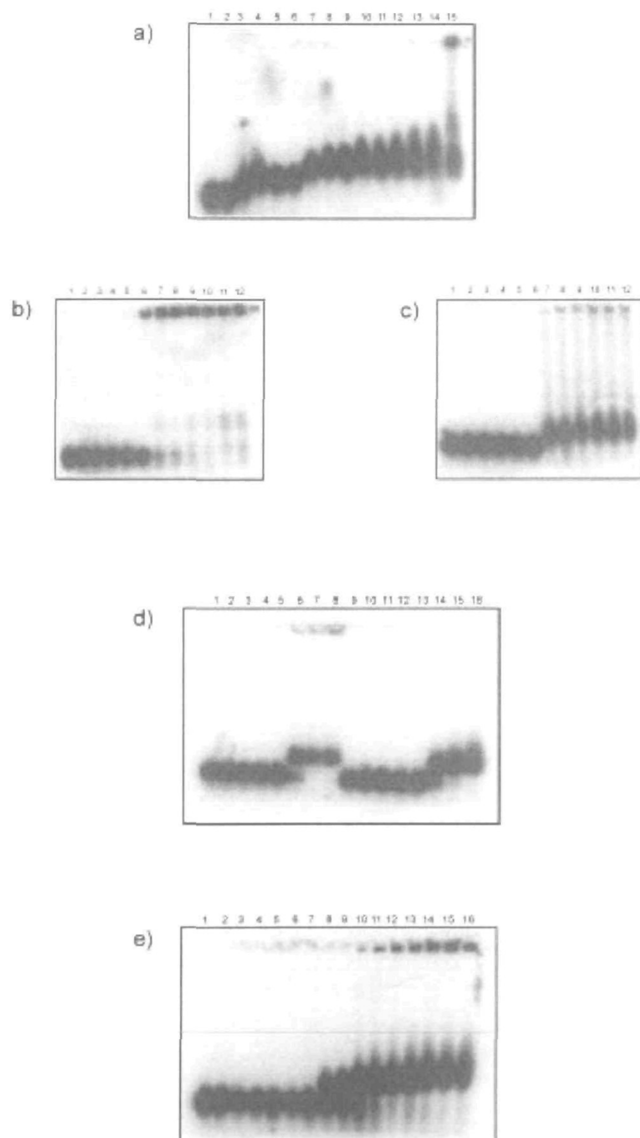

Fig 3 **0.7% agarose gel mobility shift assaying of [3′-³²P] 5S rRNA-zinc finger peptide complexes.** Formation of the complexes was performed as described under "MATERIALS AND METHODS." (a) Concentrations of ZF-3 0.0, 1 0, 2 5, 5.0, 7 5, 10 0, 12.5, 15 0 17 5, 20 0, 22 5, 25 0, 27 5, 30 0, 35 0 nM. (b) ZF-4: 0 0, 0 5, 0.7, 1.0, 2 5, 5.0, 7.5, 10 0, 12 5, 15 0, 17 5, 20 0. (c) ZF-6: 0 0, 0.5, 0 7, 1.0, 2 5, 5 0, 7 5, 10 0, 12 5, 15 0, 17 5, 20 0 nM (d) ZF-7 (lanes 1–8) and ZF-8 (lanes 9–16) 0 0, 0.5, 0 7, 1 0, 2 5, 5 0, 7 5, 0 0, 0 5, 0 7 5 nM. (e) ZF-9: 0.0, 0 02, 0 05, 0.1, 0 15, 0.18, 0.2, 0 6, 1 0, 2 5, 5.0, 7 5, 10 0, 12 0, 15 0, 20 0 nM.

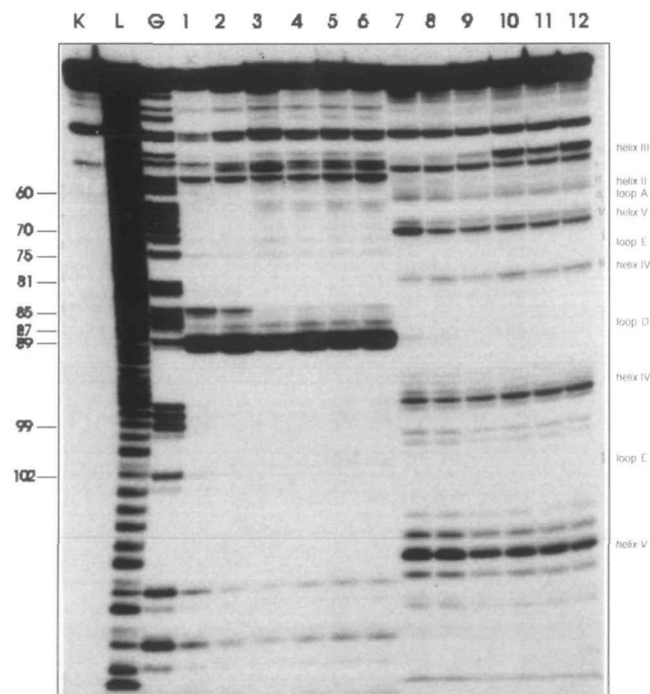

Fig. 4 **Autoradiogram of a 20% polyacrylamide gel with 7 M urea showing the hydrolysis products of 5S rRNA within the ZF complexes.** Analysis was performed as described under "MATERIALS AND METHODS." K, control, L, alkaline ladder; G, ladder; 1–6, hydrolysis of the complex with RNase T1; 7–12, hydrolysis of the complex with RNase V1, 1, 7 control, 5S rRNA in reaction mixture without a peptide; 2, 8, 5S rRNA+ZF3, 3, 9, 5S rRNA+ZF6, 4, 10, 5S rRNA+ZF7; 5, 11, 5S rRNA+ZF8, 6, 12, 5S rRNA +ZF9

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RNases V1 and T1 (Fig. 5a) The addition of the next zinc finger domain to peptide ZF6 causes the protection of nucleotides 14–20 of helix II and G25, but strong digestion was observed at nucleotides 48–51 in helix III (Fig. 5a). ZF8 and ZF9 shield all helices from hydrolysis by RNase V1 and T1, but G31 in loop C was only protected by ZF9 (Fig. 5a). Another approach for checking the direct interaction of a protein with chemically modified RNA is based on that hydroxyl radicals cause oxidative damage to ribose residues and cleavage of polynucleotide chains (*33–36*). This method has been successfully applied to studies on RNA/DNA-protein complexes (*15*) and the RNA tertiary structure (*34, 35–37*). In our experiments, complex formation was carried out using 5S rRNA modified with Fe-[EDTA]$^{2-}$, and then samples were separated on a non-denaturing gel, eluted and finally analyzed on a denaturing gel (Fig. 6). The differences observed in the intensities of bands of free 5S rRNA and that in the complex suggest that modifications of the nucleotides at positions 24, 36, 52, 55, 72, 75, 78, and 83 of 5S rRNA do not influence the complex for-

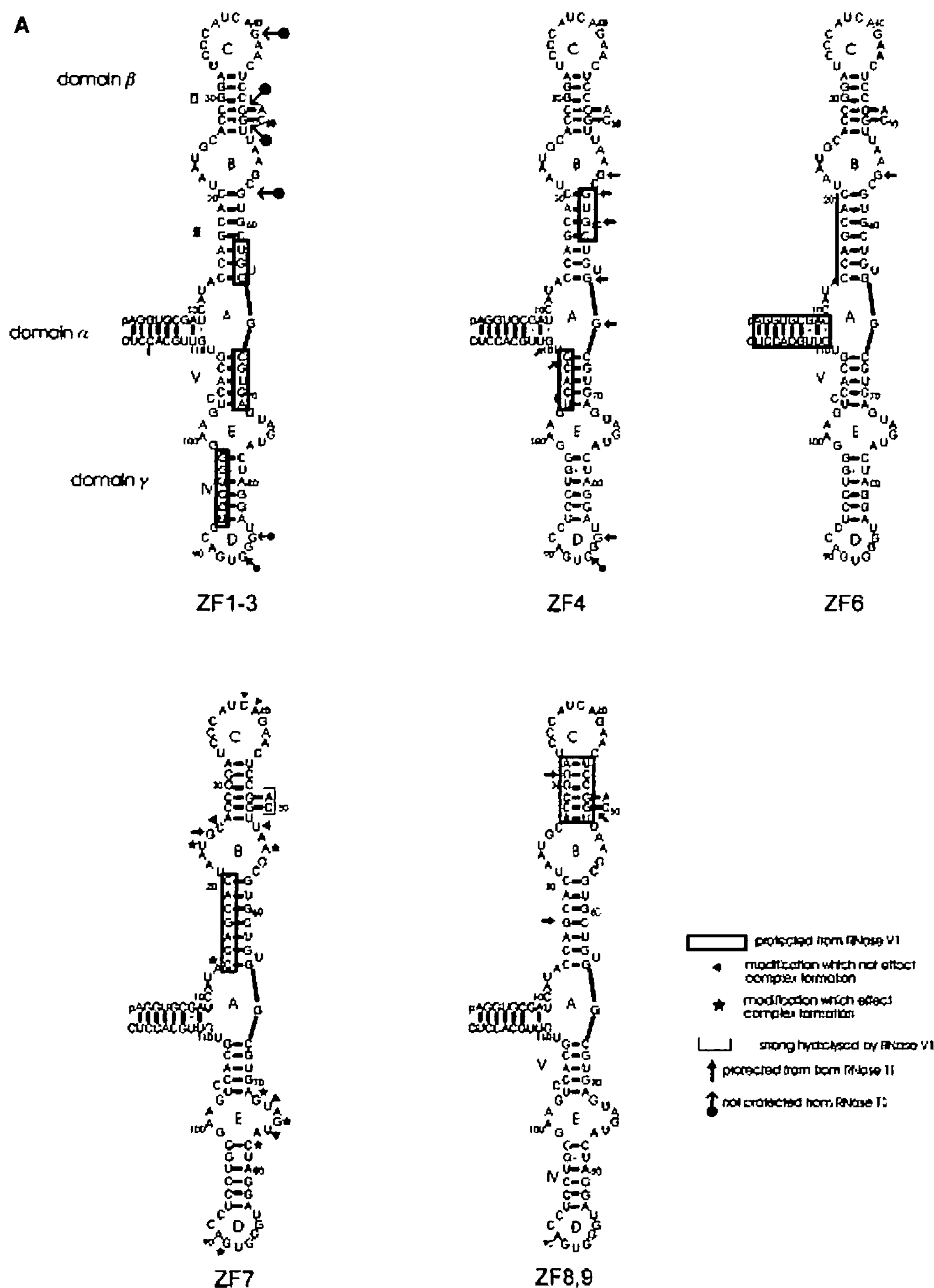

Fig. 5 A

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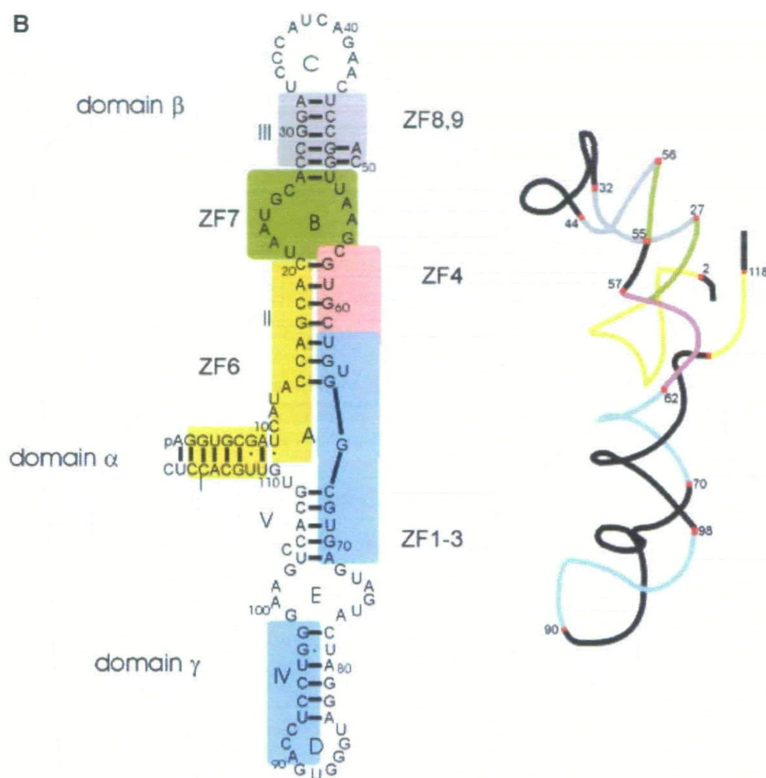

Fig 5 Secondary structure of 5S rRNA from *Lupinus luteus* (a) with the indicated nucleotides protected by peptides: ZF1-3, finger 4 (ZF4), fingers 5 and 6 (ZF6), finger 7 (ZF7), fingers 8 and 9 (ZF8,9), fingers 8 and 9 (grey), (b) summary of protection of 5S rRNA by zinc finger peptides shown on the secondary and tertiary structures found in ribosome fingers 1-3 (blue), finger 4 (pink), fingers 5 and 6 (yellow), finger 7 (green), and fingers 8 and 9 (grey) (*25*).

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mation with ZF7, while modification at positions 14, 23, 26, 53, 56 73, and 76 prevent 5S rRNA peptide binding (Figs. 5a and 6).

DISCUSSION

We are interested in the mechanism of protein–nucleic acid specific recognition. As a model for such study we used peptides of different lengths containing zinc fingers of TFIIIA and 5S rRNA. The details of zinc finger interactions come from several X-ray structures of DNA-Zif complexes (*5–10*). The most elegant example is the Zif268-DNA complex, in which three fingers of this protein wrap around the major groove and each of them forms a hydrogen bond between arginine and guanosine (*5, 7*). A similar interaction was found in the Gli-DNA (*6*) and TFIIIA-DNA complexes (*8–10*). In the last case, fingers 1-2-3 wrap smoothly around the major groove (like in Zif268), while 4-5-6 run along one side of the DNA helix. Finger 5 is in contact with bases in the major groove while 4 and 6 straddle the minor groove. Extending these results to the model proposed for whole TFIIIA, fingers 7-8-9 interact with DNA in the same way as the first three (*11, 14, 16*). It seems that all zinc fingers specifically recognize DNA in its major groove through arginine–guanosine hydrogen bonds (*38–40*). Fingers 4-5-6 of TFIIIA do not interact specifically but they help in their precise docking (*10*). Such successive binding of zinc fingers to the specific site of nucleic acid can be called an additive interaction In this study we tried to answer the question of how zinc fingers contribute to the binding and recognition of 5S RNA. To answer this question we prepared a set of polypeptides consisting of different numbers of zinc fingers derived from TFIIIA (Fig. 2). We used native *L. luteus* 5S rRNA, which can be regarded as a mutant of *X. laevis* 5S rRNA. From previous studies we know that *X. laevis* TFIIIA forms a complex with *L. luteus* 5S rRNA with K_a 2.7 × 10^9 M^{-1} (*41*). A nucleotide sequence of *X. laevis* and *L luteus* 5S rRNA exhibits 70% homology (Fig. 1). Because the secondary structures of these 5S rRNAs seem to be very similar, if not identical, one can suspect that their tertiary structures may be similar as well.

The complex formation experiment involving gel retardation assays showed that peptides ZF3 and ZF4 bind *L. luteus* 5S rRNA (Fig. 3, a and b), while experiments carried out with the *Xenopus* system suggests that peptides lacking finger 6 do not bind 5S rRNA (*20*). We also tried to obtain a complex of 5S rRNA with a single and double zinc finger peptide, but we did not observe any complex with a single domain (*42*), and only weak binding with peptide corresponding to fingers 5 and 6 of TFIIIA. This suggests that the addition of successive zinc finger domain improves the binding specificity (*21*). Divalent cations such as Zn^{2+} and Mg^{2+} influence the complex with polypeptides similarly to as in the case of a complex with full length TFIIIA (*43*). Analysis of the ZF-5S rRNA complexes with specific RNases provided a picture of the boundary of the interaction sites of different peptides with the 5S rRNA molecule (Figs. 4, a and b, and 5). The peptide having three N-terminal zinc fingers of TFIIIA protects helices V and IV, and a part of helix II (nucleotides 62–65) of 5S rRNA from hydrolysis by RNase V1. Simultaneously, helix II, G75 and G89 are accessible to RNase T1 (Fig. 5, a and b). RNase T1 specifically recognizes guanosine moieties forming hydrogen bonds between amino acids and O6 and N1 of the base, while nuclease V1 interacts mainly with the phosphate backbone (*44, 45*) It may suggest that due to steric hin-

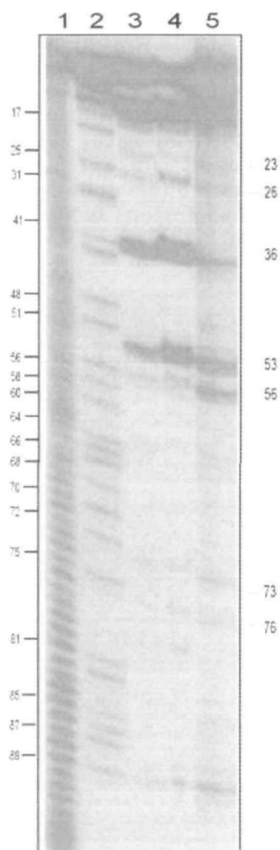

Fig 6 **Autoradiography of the sequencing gels used to analyze the products in chemical modification experiments.** Bound and unbound fractions of 5S rRNA were treated with Fe-[EDTA]$^{2-}$ 1, alkali ladder, 2, G-ladder; 3, 5S rRNA not bound to ZF7, 4, 5S rRNA bound to peptide, 5, control, 5S rRNA modified

drance, this part of the molecule, which was hydrolyzed by T1, was not accessible for RNase V1. In our case, the fragment of 5S rRNA protected by ZF3 is longer then determined in experiments with peptides having fingers 4-6 or 4-7, where the boundary of finger 4 was around U96 (*20, 27, 33, 46, 47*). Further elongation of ZF3 with finger 4 shields a part of helix II from RNase T1, supporting our observation that the inaccessibility to RNase V1 is caused by steric hindrance. The next two zinc fingers, 5 and 6, protected helices I and II (including the 5′ part). Additionally, G87 became accessible, which in the free 5S rRNA molecule was not digested by RNase T1. The hypersensitivity of nucleotides 48–51 of helix III for hydrolysis by RNase V1, while ZF7 was bound to 5S rRNA, was not observed for other peptides. Finally, we can say that the ZF 1-3 peptide interacts with a part of domain γ, finger 4 with helix II, fingers 5 and 6 with helices I and II, and loop A, finger 7 with a part of helix II and loop B, and fingers 8 and 9 with helix III (Fig. 5). The binding sites determined in our experiment comprise a bigger fragment of the 5S rRNA molecule than was proposed previously by others (*20, 22, 26, 27, 29, 47*). The results obtained in experiments with the peptide having ZF 4-7 showed that finger 4 interacts with loop E, finger 5 with helix V, finger 6 with loop A, and finger 7 with helix II (*46*). But the footprint experiment performed with polypeptides having 4-9, 1-6, and 4-6 zinc fingers showed that they protect the internal region containing loops A, B, and E, and helices I, II and V. The addition of fingers 7-9 to the ZF4-6 peptide caused the protection of helix III and loop C. Fingers 1–3 added to ZF4-6 prevented the digestion of helix IV and loop D (*20*).

Taking into account the 70% sequence homology between *X. laevis* and *L. luteus* 5S rRNA (Fig. 1), the results presented here strongly support the hypothesis that a protein recognizes the tertiary rather than the primary structure of RNA (*27*), although sequence-dependent recognition could not be excluded. It has been reported that DNA bound by a zinc finger protein has properties intermediate between those of canonical forms A and B, which means a deep and narrow major groove but also a wide minor groove what resembles the RNA helix (*47*). We suggest a certain similarity between the interactions of zinc finger peptides with DNA and RNA. Even if not every finger interacts directly with a nucleotide, they can facilitate the localization of others. In order to determine which fragments of the 5S rRNA molecule are the most important for the recognition process, we carried out experiments with modified samples.

The use of chemical cleavage reagents is one of the methods for studying the three-dimensional structures of RNA molecules. The Fe[EDTA]$^{2-}$ complex and reducing agents generate reactive oxygen species, mostly neutral hydroxyl radicals (*34*), which initiate the oxidative degradation of the ribose ring but not base residues in the nucleic acids (*34, 35*). Because the EDTA complex of iron possesses a negative charge, the metal complex does not bind to the polyanionic RNA molecule. This radical reaction thus offers an extremely delicate probe for the surface analysis of nucleic acids. We used it to introduce small conformational changes randomly through the molecule (Fig 6). The results obtained for finger 7 show that the complex is disrupted mainly through modification of loops E and B. The data obtained for peptides are in agreement with those for the wild type TFIIIA (*43*). The RNA recognition core found by Theunissen *et al.* (*26*) is located in one strand of helix V and in the part of helix II flanking loop A. Weaker effects were observed at positions G71 and U72 with the N456C protein and G70-U73 for full length TFIIIA. Our results show that modification of positions 14, 23, 26, 53, 73, and 76 prevents complex formation with both peptides (Fig. 5, a and 6).

In summary, we propose that in the case of a protein built of domain-like zinc fingers additivity of binding of particular modules occurs, even if the thermodynamic contribution in energy of binding is not significant, *e.g.* the k_D values for TF(4-7) and TF(4-6) are the same (*48*). Modules which do not significantly influence the dissociation constant might play an important role in the precise orientation of the recognizing domain

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