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Crosslinking of transcription factor TFIIIA to ribosomal 5S RNA from *X.laevis* by *trans*-diamminedichloroplatinum (II)

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ABSTRACT

Trans-diamminnedichloroplatinum (II) was used to induce reversible crosslinks between 5S rRNA and TFIIIA within the 7S RNP particle from *X. laevis* immature oocyte. The crosslinked fragments have been unambiguously identified. These fragments exclusively arise from three RNA regions centered around the hinge region at the junction of the three helical domains. Major crosslinking sites are located in region 9-21 (comprising loops *A* and helix *II*) and region 54-71 (comprising loop *B*, helices *II* and *IV*). A minor site is also found in the 3' part of helix *I* and helix *V* (region 100-120). Our results point to the crucial role of the junction region and of the three-dimensional folding of the RNA in the recognition of the 5S rRNA by TFIIIA.

INTRODUCTION

In *Xenopus laevis* oocytes, transcription factor TFIIIA binds to the internal control region of the 5S RNA gene and modulates the expression of the gene during oogenesis (e.g. 1, 2). After transcription in immature oocytes, about 50% of the 5S rRNA is found associated with TFIIIA in a stable complex that sequesters the 5S rRNA until its incorporation into the ribosome (3, 4). Several studies have been performed in order to understand how the protein can recognize specifically two different nucleic acids targets. Analysis of the primary sequence of TFIIIA has revealed the presence of nine tandem repeats which contain two highly conserved pairs of cysteine and histidine residues (5-7). It has been proposed that in each repeat, the coordination of these residues to a Zn^{2+} ion folds the protein into nine "finger" domains, each of which is thought to act independently in binding to DNA (7). A model for the DNA-TFIIIA interaction has been proposed (8). In this model, the protein lies on one face of the DNA but the alternate "finger" domains lie in two different planes at an angle to each other in order to maintain contact with the major groove (8).

The formation of the binary TFIIIA/5S rRNA complex from *X. laevis* has been studied by kinetic studies (9). Numerous investigations have been attempted to determine the regions of the 5S rRNA molecule in close contact with TFIIIA and to study the conformational changes of the RNA induced by the protein (9, 10-17). Binding studies with deletion mutants of 5S rRNA suggest that the minimum TFIIIA binding site is located within nucleotides 52 to 108 (14-15). It

was also shown that none of the conserved residues in single-stranded regions or in a bulged out conformation are essential for the binding, with the exception of nucleotides in loop *A* (16-17).

In the present paper, we used the ability of *trans*-diamminedichloroplatinum (II) (*trans*-DDP) to induce reversible RNA-protein crosslinks, to determine the 5S rRNA region in close contact with TFIIIA. This reagent has been used to promote RNA-protein crosslinks in the following complexes : tRNA/aminoacyl-tRNA synthetase (18), tRNA/EF-Tu (19), ribosomal protein-16S rRNA within the ribosome (18, 20), and IF3/16S rRNA within the specific IF3/30S complex (21). *Trans*-DDP has a square planar geometry where the two chlorines span a 7 Å long distance. These chlorines can easily be substituted by stronger nucleophilic groups. In ribonucleoprotein complexes, platinum coordinates on the RNA side primarily to position N7 of guanines and to a lesser extent to position N1 of adenines and N3 of cytosines. On the protein side, it binds to the sulfur atom of cysteines and methionines and to the unprotonated imidazole ring of histidines (18). These crosslinks can be reversed by stronger nucleophilic groups. Two major crosslinks within the TFIIIA/5S rRNA complex have been induced by *trans*-DDP. They are mainly centered on loop *A*, and the helices *II* and *V*.

MATERIALS AND METHODS

1. Materials

Trans-DDP was from Sigma. [γ^{32}P]ATP (3000 Ci/mmol) was from Amersham. RNase T1 was from Sankyo, T4 polynucleotide kinase and RNase kits for oligonucleotide sequencing were from PL Biochemicals.

2. Preparation of the 7S particle and crosslinking conditions

The 7S RNP particle was isolated from immature oocytes of juvenile *X. laevis* by a standard procedure (3, 22) Crosslinking was performed on the 7S particle at a concentration of 2 µM in 20 mM sodium phosphate (pH 7.4), 1.5 mM magnesium acetate, 100 mM potassium acetate in the presence of *trans*-DDP at a final concentration of 0.1 mM for 1 h at 20°C in the dark.

3. Isolation of the crosslinked oligonucleotide-protein complexes

After the platination, the crosslinked 5S rRNA-TFIIIA complexes were precipitated in the presence of 0.3 M sodium acetate and three volumes of ethanol. The complexes were then subjected to RNase T1 digestion (1.5 U/µg 5S rRNA, 30 min at 37°C) in 20 mM sodium phosphate (pH 7.5), 1 mM EDTA and 2 M potassium acetate. The resulting covalent TFIIIA/oligonucleotide complexes were retained on nitrocellulose filters (Millipore type HA, 45 µm pore size, 25 mm diameter) previously soaked in the above buffer, and non-crosslinked oligonucleotides were eliminated by extensive washing (20 ml of the same buffer).

4. Identification of the crosslinked material

After filtration, the nitrocellulose filters were soaked in 500 µl 2 M thiourea in order to reverse the crosslinks. The supernatants were removed and the filters washed with 200 µl 2 M thiourea. The liberated 5S rRNA fragments were precipitated with three volumes of ethanol in the

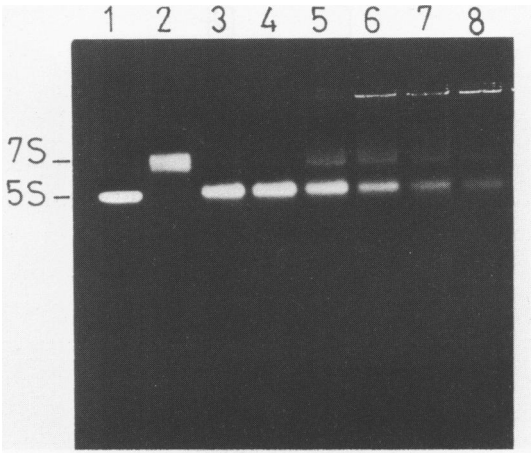


Figure 1: *Agarose gel electrophoresis of the 7S RNP particle after crosslinking reaction.* (lane 1) 5S rRNA control; (lane 2) 7S RNP particle control; (Lane 3) the 7S RNP particle after deproteinization in 20 mM sodium phosphate (pH 7.5), 100 mM lithium chloride, 0.5 % SDS and 1 mM EDTA for 30 min at 20°C; (lane 4) control experiment in the absence of *trans*-DDP and after deproteinization of the 7S RNP particle; (lanes 5-8) before deproteinization, platination was performed on 2 μM 7S RNP in the presence of 0.1, 0.2, 0.3 and 0.4 mM of *trans*-DDP, respectively. The RNA was stained with ethidium bromide. All subsequent crosslinking experiments were conducted under the conditions presented in lane 5.

presence of 0.3 M sodium acetate. The oligonucleotides were then labeled at their 5' end in the presence of T4 polynucleotide kinase and 100 μCi [γ^{32}P]ATP according to Silberklang *et al.* (23). The 5'-labeled fragments were fractionated by electrophoresis on a 20% polyacrylamide (1/20 bis)/8 M urea slab gel. After autoradiography, the fragments were excised, eluted according to Maxam & Gilbert (24), and repurified by a second electrophoresis on a 22% polyacrylamide/8 M urea gel. The fragments were eluted, precipitated with ethanol in the presence of 10 μg of tRNA as carrier, dissolved in 10 μl water, incubated for 5 min at 55°C and sequenced using several ribonucleases (25). Digestion was with RNase T1 (0.005-0.5 U/μg RNA), RNase U2 (0.5-1 U/μg RNA), RNase *Phy*M (0.5 U/μg RNA) and *B. cereus* RNase (0.5 U/μg RNA). Incubation was at 55°C for 15 min in a citrate buffer (0.02 M, pH 7.5) containing 1 mM EDTA, in the presence of 8 M urea for RNases T1, U2, *Phy*M and in the absence of urea for *B. cereus* RNase. The ladder was performed in 4 μl of deionized formamide at 90°C for 30 min. Analysis of the digests was carried out by electrophoresis on a polyacrylamide/8 M urea slab gel using a concentration of acrylamide appropriate for the size of the RNA fragment (usually between 15 and 25%).

The 5' terminal nucleotide of each fragment was identified by total hydrolysis of the 5' labeled fragments for 12 h at 37°C with RNase P1 (0.5 μg) in the presence of 5 μg tRNA as

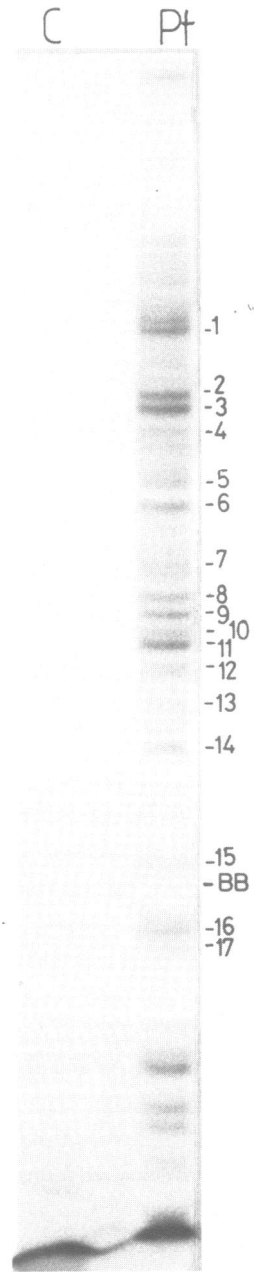


Figure 2: *Fractionation of the crosslinked oligonucleotides on polyacrylamide-urea gel electrophoresis.* The crosslinked TFIIIA/T1 oligonucleotides complexes were subjected to 5' end labeling and to reversion of the crosslinks before fractionation. (C) Control experiment in the

carrier. The liberated nucleotides were separated by chromatography on thin-layer cellulose plate in the presence of HCl/2-propanol/H_2O (17.6/68/14.4) as solvent.

RESULTS AND DISCUSSION

1. Formation of the crosslinked complex and isolation of the crosslinked oligonucleotides

The 7S RNP particles were treated with increasing amounts of *trans*-DDP for 1 h, subjected to deproteinization by SDS treatment and fractionated by non-denaturing agarose gel electrophoresis (see Figure 1). Under all of the tested conditions, the crosslinked 7S particle migrates exactly at the same position as the native 7S RNP particle, indicating that the 1:1 stoichiometry of the TFIIIA/5S rRNA complex is maintained. However, increasing the concentration of *trans*-DDP leads to an increase the amount of the material unable to enter the gel. Thus, conditions for subsequent crosslinking experiments were selected in which the aggregation of the crosslinked 7S particle was negligible. Such conditions were met for a *trans*-DDP concentration of 0.1 mg, corresponding to a *trans*-DDP/7S particle molar ratio of 50 (Figure 1, lane 5). These conditions are very similar to that described by Tukalo *et al.* (18) and Wikman *et al.* (19) who could demonstrate that tRNA was specifically crosslinked to aminoacyl-tRNA synthetase and EF-Tu, respectively and that no RNA aggregates or degradations occured.

In order to identify the regions of the 5S rRNA molecule involved in the crosslinking reaction, the crosslinked TFIIIA/5S rRNA complex was subjected to a limited RNase T1 hydrolysis. The resulting crosslinked TFIIIA/oligonucleotide complexes were separated from the non-crosslinked fragments by nitrocellulose filtration in the presence of a high concentration of a monovalent salt. The retention of RNA fragments on nitrocellulose filters requires these fragments to be crosslinked to TFIIIA. However, it cannot be totally excluded that some fraction of the oligonucleotides can be retained by TFIIIA-RNA-RNA crosslinks, especially where segments from both sides of a helix are retained. Nevertheless, since only those RNA-RNA crosslinks which coincide with a RNA-protein crosslink on the same molecule can be detected, such crosslinked fragments are expected to be found at a low and variable yield. Control experiments were conducted in the absence of *trans*-DDP, to verify that non-crosslinked RNA fragments were not unspecifically retained under these conditions. The presence of 2 M potassium acetate was found to be sufficient to prevent such non-specific binding of RNA fragments.

2. Sequence analysis of the crosslinked oligonucleotides

After reversion of the crosslinks by thiourea, the crosslinked oligonucleotides were 5' end labeled. Gel electrophoresis of the resulting labeled products is shown in Figure 2. A control experiment in the absence of *trans*-DDP indicates that only RNA fragments crosslinked to the protein were retained on the filters. All crosslinked fragments were repurified on polyacrylamide-urea gel electrophoresis. Several examples of sequence analysis are shown in Figure 3. The 5'

absence of *trans*-DDP; (Pt) crosslinking experiment as described in Material and Methods; (BB) bromophenol blue position.

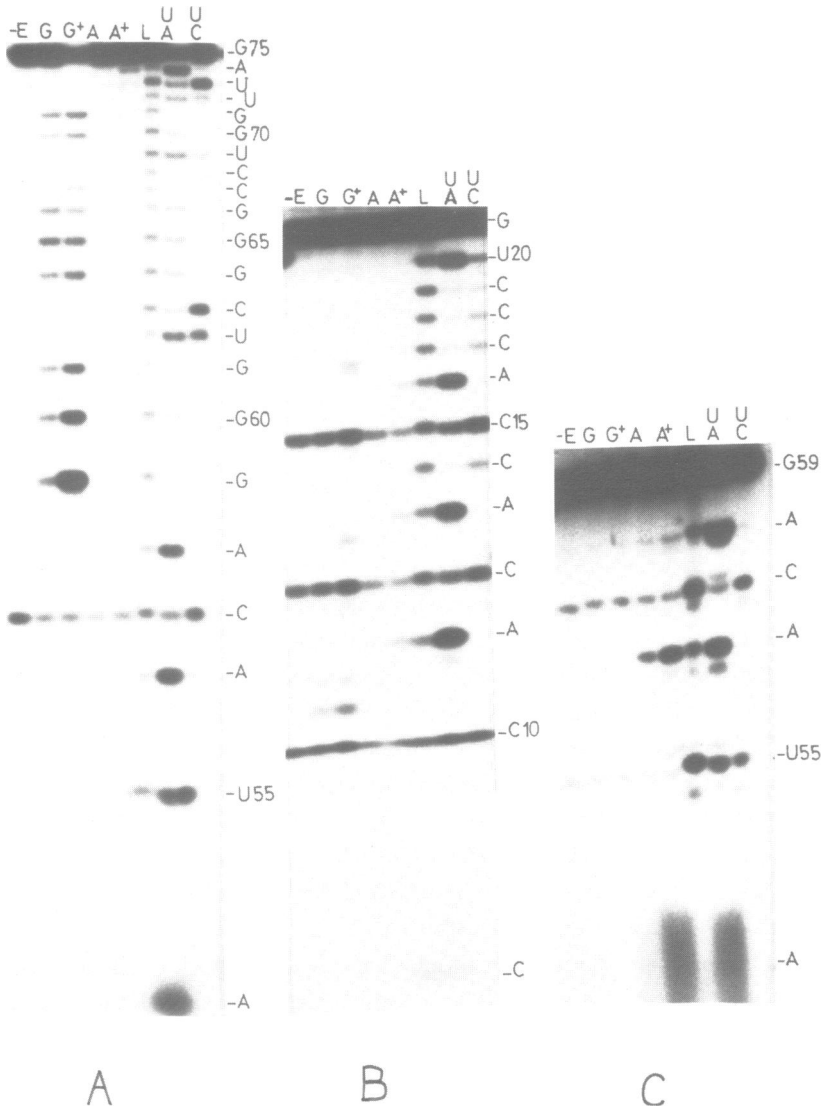


Figure 3: *Sequence analysis of the crosslinked 5S rRNA fragments from Figure 1.* (A, B, C) correspond to fragments *3*, *11* and *15*, respectively. Lane (-E) : control in the absence of enzyme. Lanes (G), (A), (AU), (CU) : hydrolysis with RNases T1, U2, *Phy*M, from *B. cereus*, respectively. Lane (L) : formamide ladder. Conditions of the digestions are given in Material and Methods.

end labeled nucleotide of the different fragments was further identified by a total RNase P1 digestion followed by thin-layer plate chromatography. However, this methodology does not accurately estimate the relative amount of crosslinking, since the intensity of labeling does not

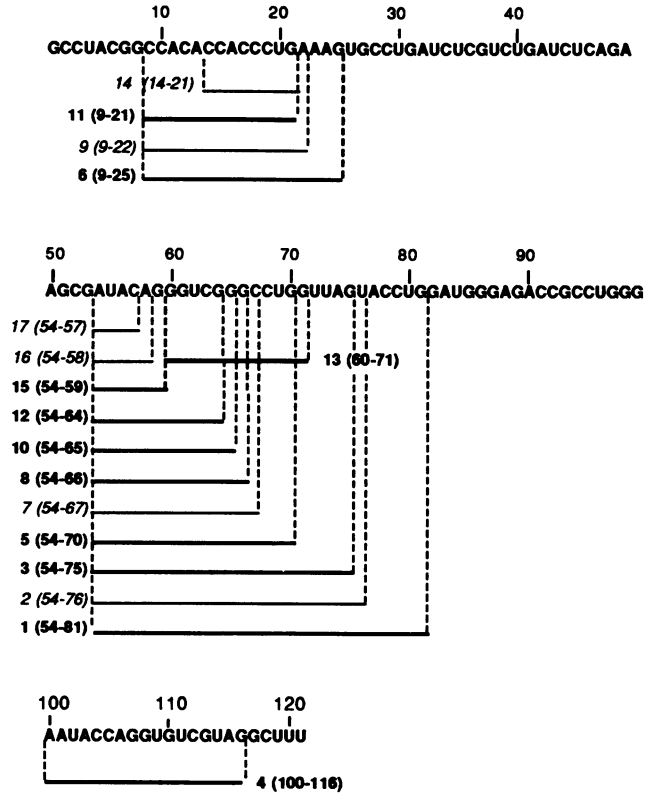
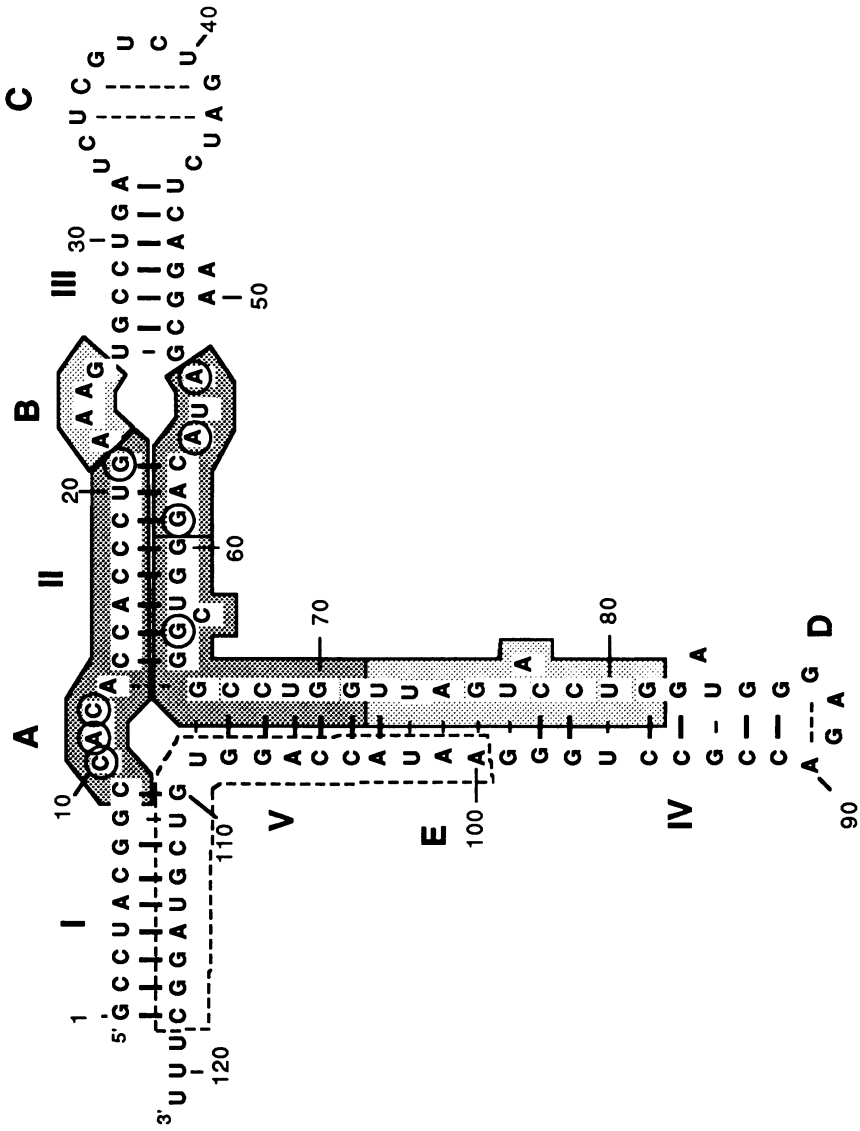


Figure 4: *Diagram of fragments found to be crosslinked to TFIIIA.* Fragment are numbered as in Figure 1. Their nucleotide content is indicated in brackets. RNase T1 products are indicated by thick lines, while fragments resulting from non enzymatic clevage are indicated in italic and by thin lines.

necessarily reflect the absolute amount of RNA, because 5' phosphorylation is sequence and structure dependent. A diagram showing the sequence of the identified fragments is presented in Figure 4.

The set of isolated products is rather complicated, but a good reproducibility was observed among the resulting fractionation patterns. Despite this apparent complexity, two major groups of fragments were reproducibly isolated from three independent experiments. The first one covers nucleotides 9-25 and the second one nucleotides 54-81. In one experiment, an additional fragment including nucleotides 100-118 was obtained. No crosslinked fragments have been isolated from other regions of the RNA molecule. Inside each of the two major groups, a set of subfragments was generated as the result of partial RNase T1 digestion. However, some fragments arise from non enzymatic cuts (see fragments *2, 7, 9, 14, 16* and *17* in Figure 4). This type of cleavage


A
B
C
D
E
I
II
III
IV
V
5'
3'
1
10
20
30
40
50
60
70
80
90
100
110
120

essentially occurs in pyrimidine-adenine phosphodiester bonds and reflects the intrinsic fragility of the RNA chain (26). Such non-enzymatic cleavages were also observed in the crosslinked [EF-Tu/tRNA^{Phe}] complex (19). Since these cuts might have been produced after reversion of the crosslinks, the resulting fragments have not been taken into consideration. The location of the major crosslinking regions is represented on the secondary structure of the 5S rRNA (Figure 5).

The first group essentially contains two RNase T1 fragments, differing by their 3' extremity. One or more crosslinking sites are most probably located within the shortest fragment (nucleotides 9-21). Since the crosslinks were reversed before sequencing of the coordinated oligonucleotides, the platinated residues could not be identified. Nevertheless, potential candidates can be deduced from the known reaction specificity of *trans*-DDP and chemical probing data on *X. laevis* 5S rRNA (27) and 7S complex (13). Thus, C10(N3), A11(N1), C12(N3) in loop *A* and G21(N7) in the G-C base pair closing loop *B*, are the only potential crosslinking sites which are fully reactive in both the naked RNA and in the 7S particle (Figure 5).

In the second group, most fragments are found to extend in the 3' direction from a short RNA region corresponding to nucleotides 54-59 (fragment *15*, Figure 4), thus revealing the presence of a crosslinking site in these nucleotides. Another short fragment (fragment *13*) containing nucleotides 60 to 71, directly adjacent to the previously defined region, has also been isolated, suggesting a second crosslinking site in this area. In region 54-59, nucleotides A54(N1) and A56(N1) in loop B, and G59(N7) in helix II are good candidates for being crosslinking sites. Indeed, A54 and A56 present a high reactivity at their N1 position towards dimethylsulfate in the naked RNA and are found partially protected by TFIIIA (13). In fact, the coordination between TFIIIA and a position which is protected by the protein should be able to take place, since the time of incubation with *trans*-DDP (1h.) exceeds the half life of the complex (approximately 45 min., ref. 9). Also, it has been demonstrated that the somatic substitutions in loop *B* (at positions 53, 55 and 56) would be responsible for the increased affinity of somatic 5S rRNA towards TFIIIA as compared to the oocyte 5S rRNA (15). In region 60-71, G64 is a potential crosslinking site. This residue is highly reactive at its N7 position (13, 27). Note that the presence of other crosslinking sites in the region extending from nucleotides 72 to 81 cannot be excluded. The recovery in one experiment of fragment 100-116, involving nucleotides from helices I and II, may suggest the presence of a minor crosslinking site in this area. However, the possibility that this fragment could be retained through a double TFIIIA-RNA-RNA crosslink cannot be ruled out.

Figure 5: *Secondary structure of* X. laevis *oocyte 5S rRNA*. Helices and loops are numbered according to Romaniuk (9). Regions reproducibly crosslinked are boxed and regions crosslinked at a variable extent are delimited by a broken line. The dark shadowed boxes correspond to the shortest crosslinked fragments and the sequence extension of these fragments are shown by the clear shadowed boxes. Potential crosslinking sites are encircled.

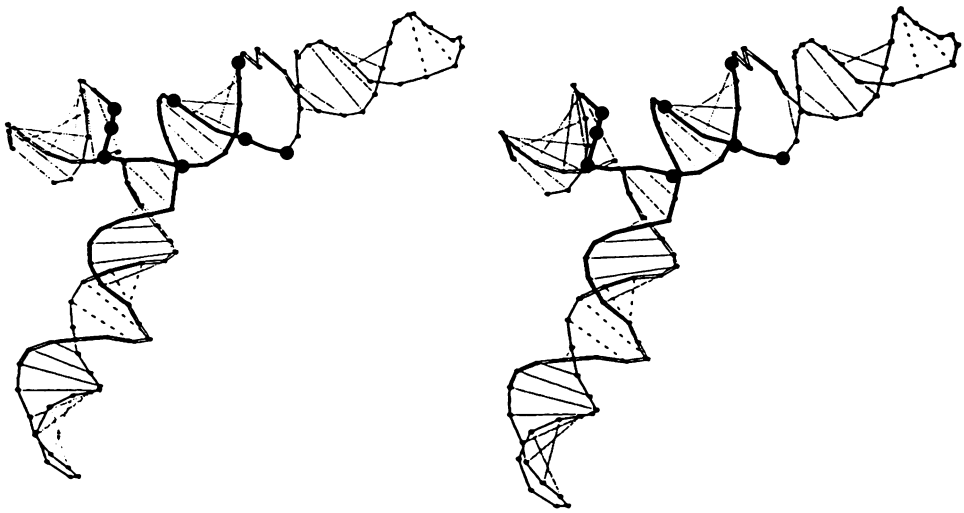


Figure 6: *Three-dimensional stereo models of the phosphate backbone of the* X. laevis *oocyte 5S rRNA*. The model is taken from Westhof *et al.* (20). The Watson-Crick base pairs are joined by continuous lines and the non-canonical or tertiary base pairs are joined by broken lines. The major crosslinking regions are drawn in heavy lines and those crosslinked at a variable extent are shown by a double line. Potential crosslinking sites are shown by circles. The stereoscopic views were drawn with the program PLUTO (S. Motherwell & P. Evans, MRC Cambridge).

CONCLUSION

In the present work, we have used *trans*-DDP as a reversible crosslinking reagent to study the 5S rRNA regions in close contact with TFIIIA. The high yield of crosslinking (up to 20%) has greatly facilitated the isolation and the identification of the crosslinked 5S rRNA regions. Remarkably, the crosslinked fragments are not spread along the molecule but are clustered around the junction of the three helical domains. The major crosslinking domains cover loops *A* and *B*, and helices *II* and *V* : at least one crosslinking site is found in nucleotides 9-21 (loop *A* and the 5' strand of helix *II*), in nucleotides 54-59 (loop *B*) and 60-71 (the other strand of helix *II* and the 5' strand of helix *V*). A minor crosslinking site may also occur in nucleotides 100-118 (3' strands of helices *I* and *V*, and loop *E*).

Our results confirm earlier footprinting experiments which strongly indicated the involvement of helix *II*/loop *B* and helix *V*/loop *E*/helix *IV* in the interaction with TFIIIA (9-13). On the other hand, studies on several deletion mutants of *X. laevis* 5S rRNA have revealed that the helix *II*/loop *B* domain represents the primary interaction site of TFIIIA (15). More recently, it was shown that substitutions of most of the highly conserved nucleotides in loops *C, D, E* and *B* (5' side) or in a bulged out conformation of *X. laevis* 5S rRNA had little effect on the binding affinity of TFIIIA (16, 17). In contrast, the importance of nucleotides in loop *A* is supported by

recent findings that substitutions of residues in this loop have a drastic effect on the binding activity of TFIIIA (16). However, no protection induced by TFIIIA binding could be detected in loop *A* (e.g. 9, 10, 12, 13). It is possible to rationalize this apparent discrepancy by assuming that TFIIIA may recognize the unique tertiary struture of 5S rRNA and form a number of relatively weak sequence-specific contacts with single-stranded regions (16). The coaxial stacking of helices was proposed to be an element of recognition, in particular the relative orientation of helices *II* and *V*. Indeed, studies on deletion mutants indicate that helix *I* is not important for the binding of TFIIIA and that alteration of helices *II* and *III* results in a more dramatic effect on the TFIIIA binding than the deletion of helices *IV* and *V* of the *X. laevis* 5S rRNA (15). Our results are in total agreement with this observation and show that TFIIIA is indeed in a close vicinity of helix *II*, loop *A* and *B*. Otherwise, a model based on footprinting experiments was proposed, in which TFIIIA contacts mainly occur in the major groove of the 5S rRNA, extending over helices *II/III* and *IV/V*, and loops *B* and *E*, organized in a colinear conformation (13).

Recently, a three-dimensional model of *X. laevis* oocyte 5S rRNA has been proposed (28) which integrates stereochemical constraints and experimental probing data on the reactivity of each base and phosphate towards several structure-specific probes (27). According to this model, the 5S rRNA adopts a Y-shape structure with helices *II* and *V* not far from colinearity. By comparing 5S rRNA from *X. laevis* and spinach chloroplast, it was shown that the tertiary fold at the fork depends essentially on the conformation of loop *A* (28). The two major crosslinked domains are located in this crucial fork region (Figure 6). Our results stress the fundamental role of the hinge region and are fully consistent with the view that the coaxial stacking of helices II and V of the 5S rRNA is an element of recognition for the protein.

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