Analysis of the Binding of Xenopus Transcription Factor IIIA to Oocyte 5 S rRNA and to the 5 S rRNA Gene*

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The abbreviations used are: TFIIIA, B, and C, transcription factors IIIA, IIIB, and IIIC, respectively; ICR, internal control region; RNP, ribonucleoprotein; Rh(phen), (phenanthroline)rhodium(III); CMCT, 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluene sulfonate; bp, base pair(s); DTT, dithiothreitol.

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Binding of transcription factor IIIA (TFIIIA) to site-specific mutants of Xenopus oocyte 5 S rRNA has been used to identify important recognition elements in the molecule. The putative base triple G75:U76:A100 appears used to identify important recognition elements in the cleoprotein; Rh(phen)2(phen)3IIIB, and IIIC, respectively; ICR, internal control region; RNP, ribonucleoprotein.

The factor along nearly the entire length of 5 SrRNA, the essential elements for high affinity binding are limited to the central region of the molecule. Analysis of the corresponding mutations in the gene confirm that box C and the intermediate element provide the high affinity sites for binding of the factor to the DNA. Despite the small thermodynamic contribution made by contacts to box A, mutations made in this element can cause substantial changes in the orientation of the carboxyl-terminal fingers along the 5'-end of the internal control region.

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In addition to being a potentially important aspect of the developmental regulation of 5 S rRNA synthesis, the binding of TFIIIA to both RNA and DNA is of considerable interest with respect to protein-nucleic acid recognition. TFIIIA possesses nine zinc finger domains (6) that mediate mutually exclusive interactions with the two nucleic acids (4). The binding sites for the factor on the gene and on 5 S rRNA are similar (7–9), which led to speculation that the protein associates with the two nucleic acids through common determinants. However, studies using truncated or mutated forms of TFIIIA have demonstrated that the nine zinc finger domains, while all contributing to some degree to DNA and RNA binding, are not functionally equivalent. Peptides containing the three amino-terminal finger domains bind to the gene with an affinity nearly equal to that of the intact factor (10, 11). Moreover, variants of TFIIIA in which fingers 2 or 3 have been deleted or disrupted exhibit considerably reduced DNA binding affinity without a corresponding decrease in RNA binding (12–14). Nuclease protection (11, 15–17) and missing nucleotide experiments (18) indicate that these three fingers bind in the major groove of the internal control region (ICR) from approximately nucleotide position 79 to 92. Both hydroxyl radical (16) and DNase I (15, 17) footprinting reveal a change in the orientation of the factor relative to the DNA helix in the vicinity of base pair 78 which likely signifies the exit of the protein from the major groove with finger 4 crossing the minor groove. The triplet of fingers 4–6 runs parallel to one side of the helix for nearly 20 base pairs. The factor then re-enters the major groove near base pair 60 and the three carboxyl-terminal fingers appear to be contiguously aligned in a manner similar to the amino-terminal fingers. The distinct orientation of the central three fingers on the gene may reflect their role as the primary mediators of RNA binding (12, 19). A polypeptide comprised of zinc fingers 4–7 binds with high affinity to 5 S rRNA and deletion of two or more of these fingers from the factor significantly reduces binding to RNA. Thus, a model has emerged in which two subsets of fingers within TFIIIA confer differential specificity for binding of the factor to DNA (fingers 1–3) or RNA (fingers 4–6). Viewed in this way, the interactions of TFIIIA with the 5 S gene and 5 S rRNA are quite different. However, this oversimplification minimizes the contributions that the remaining fingers clearly make to the free energy of binding in both complexes (12–14).
A variety of studies have used mutagenesis to define the interaction of TFIIA with 5 S rRNA genes and 5 S rRNA (20–32). The cumulative results of these studies establish that the binding site for TFIIA on the 5 S rRNA gene is composed of three sequence elements which seemingly reflect the disposition of the protein along the DNA described above. As a consequence of disrupting sequence-specific interactions, mutation of any of these three sequence elements was found to have a substantial effect on the binding of TFIIA. On the other hand, binding of the factor to 5 S rRNA is most notably altered by changes in the higher order structure of the molecule’s secondary structure. In particular, the integrity of loop E, which appears to be determined by a base triple interaction (34), is especially important for binding of the factor. While the results presented here support earlier work establishing that the primary determinants of binding are located in nonhomologous regions of the two nucleic acids, helix V and its corresponding sequence in the gene, the intermediate element, appear to be common sites of recognition for the factor.

**EXPERIMENTAL PROCEDURES**

Preparation of TFIIA and 5 S rRNA—The 7 S RNP particle of TFIIA bound to 5 S rRNA was prepared from immature ovaries of Xenopus laevis (Nasco, Fort Atkinson, WI) (35). TFIIA was isolated from the RNP particle by the method of Smith et al. (36) with some modifications. The 7 S particle was digested with RNase A (15 μg/ml particle) and RNase T₁ (200 units/mg particle) for 1 h at room temperature. The sample was loaded onto a Bio-Rex-70 column equilibrated with buffer A containing 1 M KCl, and then dialyzed extensively. The concentrations of ribonucleases T₁ and A, extracting several times with phenol and phenol/chloroform, and then dialyzing extensively. The concentrations of RNA samples were determined spectrophotometrically at 260 nm using an extinction coefficient of 22.2 (mg/ml)⁻¹ cm⁻¹.

Quantitative DNase I footprinting experiments were carried out with plasmids linearized with EcoRI and end-labeled on the coding strand. The concentration of all DNA samples used in the TFIIA binding assays was determined by a fluorometric method (44) specific for DNA in order to eliminate interference from any trace contamination of residual RNA. Sonicated calf thymus DNA was used as the standard for the fluorometric assays was prepared by treating the nucleic acid with RNase-free DNase I (1 unit) and RNase A (20 mU) for 1 h at 25°C, followed by incubation at 37°C for 2 h.

The DNA was then digested with BamHI to generate the 153-bp fragment containing the oocyte 5 S rRNA gene which was purified by electrophoresis on 6% polyacrylamide gels (45). The fragment was eluted from the excised gel slices, extracted twice with phenol, once with phenol/chloroform (1:1), twice with chloroform, and then precipitated with ethanol. The assay mixture for footprinting contained 200 μM HEPES, pH 7.5, 70 mM NaCl, 7 mM MgCl₂, 15 mM DTT, 20 μM ZnCl₂, and 0.01% Nonidet P-40, 1.8 mM 5 S rDNA in the form of linearized plasmid, and the indicated concentration of TFIIA. DNase protection experiments were performed as described earlier (24, 38). The autoradiographs of the sequencing gels were scanned with a laser densitometer to determine the intensity of each band. A minimum of three bands which were the protected region were used to measure the amount of DNA bound to TFIIA and two bands outside the ICR were used to normalize each lane relative to one another. The binding activity of TFIIA was determined from Scatchard plots; however, wild type plasmid was included in every series of binding assays as a control for the activity of each sample of RNA, in which the data for each mutant was compared. The same buffer was used to determine a dissociation constant by nonlinear regression analysis using the program E.Z-Fit (46). Binding assays were performed in duplicate and repeated with a minimum of two different preparations of DNA.

Binding of TFIIA to mutant 5 S RNAs was measured by a shift in the mobility of the RNA in nondenaturing 8% polyacrylamide gels run at room temperature at 100 V (38). Binding assays were carried out in the same buffer used to measure binding of TFIIA to the 5 S gene except for the addition of ribonuclease inhibitor (RNAse, Promega; 0.4 units). The RNA samples were purified over two successive CsCl gradients. The concentration of all DNA samples used in the TFIIA binding assays was determined by a fluorometric method (44) specific for DNA in order to eliminate interference from any trace contamination of residual RNA. Sonicated calf thymus DNA was used as the standard for the fluorometric assays was prepared by treating the nucleic acid with RNase-free DNase I (1 unit) and RNase A (20 mU) for 1 h at 25°C, followed by incubation at 37°C for 2 h. One-fifth of the extension reaction was used to transform E. coli (DH5α). Mutants were identified by sequencing plasmids prepared from randomly selected colonies.

Binding Assays—Protein concentrations were determined by the method of Bradford (42) using bovine serum albumin as the standard. Plasmid samples were prepared by the method of Holmes and co-workers with some modifications and were purified over two successive CsCl gradients.

The concentrations of all DNA samples used in the TFIIA binding assays was determined by a fluorometric method (44) specific for DNA in order to eliminate interference from any trace contamination of residual RNA. Sonicated calf thymus DNA was used as the standard for the fluorometric assays was prepared by treating the nucleic acid with RNase-free DNase I (1 unit) and RNase A (20 mU) for 1 h at 25°C, followed by incubation at 37°C for 2 h. One-fifth of the extension reaction was used to transform E. coli (DH5α). Mutants were identified by sequencing plasmids prepared from randomly selected colonies.
wild type 5 S rRNA into the native 7 S RNP particle was measured in competition assays. The exchange of radiolabeled 42 site-specific mutations were made in the same experiment in order to control for any differences in the presented relative to that for wild type 5 S rRNA measured in the experiment the dissociation constant so of the mutants are presented relative to that for wild type 5 S rRNA measured in the same experiment in order to control for any differences in the activity of TFIIIA from one series of assays to another. The dissociation constant measured here for the TFIIIA-5 S rRNA complex is 2 nM which is in good agreement with values reported elsewhere (49, 50). In addition, we found no difference in the affinity of TFIIIA for 5 S rRNA synthesized in vitro and native 5 S rRNA purified from 7 S RNP particle. The binding of TFIIIA to the 5 S rRNA variants was also measured in competition assays. The exchange of radiolabeled wild type 5 S rRNA into the native 7 S RNP particle was measured in the presence of increasing concentrations of each mutant. Samples were analyzed by electrophoresis followed by autoradiography. The competition binding data, likewise obtained by densitometry, were processed using the program LIGAND (48). The relative dissociation constants measured in these assays were very similar to those determined in the direct binding assays, providing an independent determination of binding strength.

Quantitative DNase I footprinting was used to measure the binding of the factor to the mutant 5 S rRNA genes in the same conditions used above for assays with RNA. Autoradiographs of sequencing gels were scanned with a densitometer and binding isotherms were again constructed using the EZ-Fit program (Fig. 4). The value of the Hill number was not constrained, which resulted in nonhyperbolic binding curves giving the best fit to the data. Only a single molecule of TFIIIA binds to the 5 S rRNA gene (36, 51); however, sigmoidal binding isotherms for TFIIIA have been observed in other studies (35, 52). In our experiments this behavior may reflect the limitations of the footprinting method to detect small amounts of complex at low concentrations of protein. The dissociation constant measured...
for TFIIIA and the oocyte gene in these experiments is 3 nM. The dissociation constants for each mutant relative to wild type are presented in Table I.

Helix IV-Loop D—There is accumulating evidence that specific features of secondary structure such as bulged nucleotides, non-Watson-Crick or mismatched base pairs, and base triples are important for the recognition of RNA by proteins (53, 54). These elements can distort the usual A conformation of RNA helices, making the major groove accessible to proteins. Helix IV contains a U:U mismatch and a bulged adenosine flanked by a G:U pair that could be potentially utilized by TFIIIA. Experiments with chemical and enzymatic probes indicate that the bulged nucleotide at position 83 is external to helix IV, as are the other bulged nucleotides at positions 49, 50, and 63 (8,55). Additionally, the metal complex Rh(phen)2(phen)(phi)3+, which targets widened major grooves in RNA helices such as occurs at base triples and mismatched pairs, does not cleave at A83 (56). This demonstrates that the bulged nucleotide is not involved in a base triple interaction with the adjacent G:U pair, but rather is in accord with the nucleoside being external to the helix. Baudin and Romaniuk (28) have shown that deletion of any of the bulged residues in Xenopus oocyte 5 S rRNA has no effect on the binding of TFIIIA. We also observe no appreciable effect upon deletion of A83 or substitution by G or C. However, a transversion to U at position 83 results in a 2-fold increase in the affinity of TFIIIA for the RNA.

The N3 positions of both U80 and U96 do not react with CMCT (8,55), suggesting a 2-carbonyl-N3, 4-carbonyl-N3 mismatched pair between these two bases. Cleavage by Rh(phen)2(phen)(phi)3+ at positions U80 and G97 establishes that the major groove is accessible at the site of this mismatch (56). Mutations that convert this site to either a Watson-Crick pair (U96A) or a wobble pair (U96G) have no effect on the binding of TFIIIA; however, cleavage by the rhodium probe is eliminated by these substitutions, indicating that the helix now approximates a canonical A-type conformation with a major groove that is inaccessible to the metal complex. Inversion of the G83:C97 pair that flanks the U:U mismatch to C83:G97 has no influence on binding of the factor, despite having some subtle effect on the geometry of the helix (56). Substitution of C97 by G, which creates a second mismatch adjacent to the U:U pair and should have an appreciable effect on the structure of helix IV, also binds TFIIIA with wild type affinity. The only single nucleotide substitution that we have made in helix IV that has a notable effect on binding is proximal to loop E at C79. A transversion to G, which generates a G:G mismatch, increases the Kd 3-fold relative to wild type; however, a transition to U, which generates a G:U wobble pair in place of G97:C79, does not change binding affinity.

Loop D of Xenopus 5 S rRNA belongs to the family of tetranucleotide loops having the consensus sequence GNRA (57). Structures of these loops determined by NMR spectroscopy reveal that their exceptional stability is a consequence of base pairing between the first and fourth bases in the loop, stacking of the bases in the loop, and putative base-phosphate hydrogen bonds (57, 58). There are examples, particularly in ribosomal RNAs, where the GNRA loop is an essential component of a protein binding site (57). However, substitutions made at position 86, which will enlarge the size of the loop D, or at position 87, which will eliminate the stabilizing hydrogen bonds between the first and fourth positions of the tetraloop, do not alter binding of TFIIIA.

Numerous chemical and enzymatic protection experiments indicate that TFIIIA is in close proximity to nucleotides within the helix IV-loop D region of 5 S rRNA (7–9, 49, 50, 59). Nonetheless, we do not detect any significant thermodynamic contributions to binding in this region of the RNA upon making substitutions that will, in most cases, change local secondary structure in addition to sequence. The fortuitous increase in affinity seen with A83U does indicate that the protein is contiguous with this segment of the nucleic acid. However, our results are in accord with those from experiments in which

![TFIIIA-Nucleic Acid Interactions](image-url)
helix IV was disrupted by block mutations or was truncated without appreciable decreases in binding TFIIIA (30, 60). The mutation C79G, however, does decrease TFIIIA binding. This substitution creates two adjacent mismatched pairs proximal to loop E and may express its influence on binding through the latter structure (see later).

The binding of the factor to the corresponding mutations in the 5 S rRNA gene clearly establishes not only the differences between the two TFIIIA-nucleic acid complexes, but also the importance of bp 81–96 (box C) for binding to the DNA. Whereas the deletion of A83 has no impact on the binding of TFIIIA to 5 S rRNA, there is a significant 3.5-fold decrease in binding to the equivalent mutation in the DNA. Moreover, substitutions made at this position affect RNA and DNA binding differentially. This position is not important for factor binding to the RNA; however, all three possible substitutions have a negative effect on binding of TFIIIA to the gene. Mutations at G86 and G87 which are silent in 5 S rRNA, likewise, have a negative effect on binding of TFIIIA to the gene. Mutations at G81C, C95G, which simply invert a base pair in helix IV, has little impact on binding of the factor to 5 S rRNA; however, it has a very marked effect on binding to the gene. This is most likely due to the change at residue 81, since the single mutation, G81C, has already been shown to significantly decrease binding of TFIIIA to the gene (23). Zinc fingers 1 through 3 associate with box C of the ICR (11, 15–18) and the helix IV-loop D region of 5 S rRNA (12). The effects of mutations characterized here support previous data that this subset of fingers is of primary importance in mediating binding to DNA and makes only a small contribution to binding to 5 S rRNA (10, 12, 19, 62).

Loop E—In "missing nucleoside" experiments we determined that loop E provides a critical structure for recognition and binding of TFIIIA to 5 S rRNA (9). Studies with the structural probe Rh(phen)2(phen)3+ demonstrated that loop E possesses a helical structure, due to base stacking interactions, with an opened major groove (56). The conformation of a 27-nucleotide duplex which represents loop E has been determined by NMR spectroscopy (34) and reveals that this domain is comprised of several non-Watson-Crick pairings as well as a reverse-Hoogsteen pair forming a base triple with G75 (Fig. 1A). The structure, which closely resembles an A-form helix, is stabilized by significant base-stacking interactions and, perhaps, interstrand hydrogen bonding. Of the single nucleotide mutants tested in the present experiments, those in loop E have the greatest negative effect on binding of TFIIIA to the RNA.

In particular, the three nucleotides (G75, U76, and A100) of the putative base-triple comprise an important element for recognition by TFIIIA. The conversion of the reverse-Hoogsteen A:U pair to a G:U pair (mutant A100G) has a modest influence on binding; however, a change to a C:U mismatch (mutant A100C) has a much more pronounced effect. Alternatively, when the Hoogsteen pair is disrupted by a substitution at U76, the consequences are far more acute; quantitatively, the 9-fold effect of the mutant U76G is significantly greater than the many block mutations that have been used to characterize the TFIIIA-5 S rRNA complex (31). The greater impact of a substitution at nucleotide 76 relative to its partner at 100 may reflect the fact that the former is also paired to G75 in the base triple and the latter is not. The U76G mutation engenders an alternative conformation in 5 S rRNA that is in equilibrium with the wild-type structure and this accounts for the exceptionally large effect on binding of the protein. The two forms are resolved on the nondenaturing polyacrylamide gels used for the binding assay (Fig. 2) with the alternative structure migrating ahead of the wild-type conformation. Although the two conformations are in equilibrium, their distinct mobility suggests that the global higher-order structures of the two forms are considerably different. The identity elements in 5 S rRNA for ribosomal protein L5 are confined to the hairpin structure composed of helix III-loop C. No mutations in the helix IV-loop E-helix V arm, including the quadruple mutation G70C, G71C, G81C, G82C, alter binding of L5; the only exception is U76G which has a greatly reduced affinity for L5. 2 These results indicate that structural changes in U76G occur in regions of the RNA distal to the site of the mutation. In "missing nucleoside" experiments removal of U76 had the most deleterious impact on binding of TFIIIA to 5 S rRNA (9). Additionally, U76 becomes cross-linked to G90 upon irradiation of 5 S rRNA with ultraviolet light (63) and the strong NOEs between these two bases (34) indicate that the helix is greatly overwound at the step between the Hoogsteen pair and the flanking G90,A77 mismatched pair. This is borne out by the absence of cleavage by Rh(phen)2(phen)3+ at this end of the loop (56).

2J. B. Scripture and P. W. Huber, unpublished results.
have shown that deletion of the bulged G from 5 S rRNA causes a severe decrease in the binding of a four-fingered peptide derived from TFIIIA. This result further supports the contention that the base triple in loop E is either used directly by TFIIIA or creates a higher order structure essential for recognition.

The A101:A74 mismatch pair immediately flanking the base triple structure is unexpectedly insensitive to mutagenesis; substitutions at either position do not disturb binding of TFIIIA. NMR studies indicate that the ribose of A74 (and G75) is predominantly C2'-endo and that this residue is in a reversed conformation, so that the direction of the two strands is parallel at this position. If substitutions at A74 change this local conformational perturbation, it does not influence the binding of TFIIIA. The structure generated at the Hoogsteen base pair, however, may be favored strongly enough to put any nucleotide at position 74 into a reversed conformation, maintaining this distortion in the helical structure of this strand.

The mutation C79G, which results in a 3-fold increase in $K_D$, creates consecutive mismatched pairs in helix IV, whereas conversion to a G:U wobble pair (C79T) has no effect. The consequence of the former substitution may be direct; however, it is equally possible that this disruption in the structure of the helix propagates itself into loop E. Indeed, a quadruple mutant G70C,G71C,G81C,G82C that will alter the structures of both helices IV and V binds TFIIIA with an affinity comparable to E. coli 5 S rRNA. This result attests to the importance of this arm of the molecule for high affinity binding and at the same time provides evidence that the intricate structure of loop E is critically dependent on the integrity of the flanking stem structures.

None of the mutations corresponding to the loop E region has an effect on the binding of TFIIIA to the 5 S rRNA gene, indicating again that the primary determinants for binding to the two nucleic acids are different.

Helix V—Disruption of this helix by block substitutions can increase the dissociation constant of the complex up to 3-fold; however, in most cases a second mutation that restores a base paired helical structure, but not the wild type sequence, can restore the binding affinity. These results indicate that recognition occurs primarily through the higher order structure of the helix. Interestingly, we find that a transition at position 70 (G70A), which results in an A:C pair in helix V, is well tolerated by TFIIIA. An A:C pair, in which the imino nitrogen of adenine is protonated, has been shown to fit quite well into an A-form RNA double helix with virtually no distortion of the backbone. The geometry of this non-Watson-Crick pair is similar to a G:U base pair and, indeed, we find that conversion to a G:U pair (C105T) at this position of helix V also has no detrimental effect on binding. The transversion mutant G70C, however, produces a 3-fold increase in $K_D$. The magnitude of the effect of this point mutation equals or exceeds

![Quantitative DNase I footprint assays for binding of TFIIIA to mutant 5 S rRNA genes.](image)
those of block mutants designed to disrupt the entire helix, suggesting that G70C, rather than simply disrupting the secondary structure of the helix, removes an important site for a sequence-specific contact with the protein. The fact that substitution by adenosine (G70A) has no effect supports this contention and provides evidence that contact at position 70 could occur through the N7 position of the purine base.

Methylation interference experiments indicate that G70 is also a critical contact point in the gene (61). The consequences of substitutions made at this position are similar for both the RNA and DNA, indicating that this could be a common site of recognition shared between the two nucleic acids. A transition to A has no effect on the binding of TFIIIA to either nucleic acid, while transversion to C causes a decrease for both. The slightly greater effect of G70C on binding of the factor to RNA relative to DNA can be explained by the pyrimidine:pyrimidine apposition resulting in the former. Various mapping experiments indicate that finger 5 is contiguous to base pairs centered around position 70 both in the gene (11, 13, 15, 16, 18) and 5 S rRNA (19, 64). Results from interference (61) and missing contact (18) experiments as well as the differences noted here between transition and transversion mutations all point to an interaction at this site through the major groove (possibly at the N7 of the purine) of both nucleic acids. Zinc fingers 4–6 of TFIIA run parallel to the helical axis of the DNA, rather than following the trajectory of the major groove (15, 18). Although finger 5 appears to bind through the major groove of the DNA, missing nucleoside experiments indicate that its orientation relative to the helix is different from the other major groove fingers, i.e. fingers 1–3 and 7–9 (18). This distinct alignment could reflect the fact that this finger may also be required to penetrate the less sterically accessible major groove of an A-form RNA helix. Thus, contacts to the nucleic acids may occur through amino acid side chains of the &-sheet or tip region rather than the residues of the &-helix that dominate the interactions seen in the Zif 268 (66), GLI (67), and Tramtrack (68) co-crystal structures. In this instance it is relevant that the structure of the DNA helix at the binding site for individual zinc finger domains frequently has a deep, but wide, major groove that can be characterized as intermediate between canonical A and B conformations (67, 69). We have shown that the helical structure of the Xenopus S S RNA gene is highly polymorphic, containing elements with A-like conformation (38, 70). This feature in the structure of the DNA may also explain how the protein could make an equivalent contact to sites on both DNA and RNA.

Loop A—A model for octoyt S S RNA based on chemical reactivity data has helices II and V nearly co-axial; this orientation is dependent on a triple interaction among nucleotides A13, G66, and U109 (71). This model has been tested by an exhaustive set of mutations in loop A which show that substitutions that decrease the flexibility of this “hinge” region often have a negative effect on the affinity of TFIIA (27). We have changed U109 to a C which can then form a Watson-Crick pair with G66, extending helix V toward loop A. This change results in a 2-fold increase in Kd, which has been reported by Baudin et al. (27). It is clear from these studies that the specific three-dimensional structure, dictated by the hydrogen bonding pattern within loop A, is an essential recognition element for this interaction. Zinc fingers 4 and 6 are especially important for binding of TFIIA to S S RNA (12, 19) and appear to be positioned at loops E and A, respectively (64). It has been proposed that these two fingers, unlike the others, extend across the minor groove of the ICR when the factor is bound to the gene (15, 17, 18), reflecting their functional difference relative to the other fingers. The only portion of loop A that has corresponding nucleotides in the ICR is positions 65–67. There is no data from the many mutagenesis or chemical and enzymatic probing experiments that indicate these nucleotides are important for the binding of TFIIA to the gene.

Helix II-Loop B-Helix III—This arm of S S RNA encompasses the binding site for fingers 7–9 which make corresponding contacts to the box A element (bp 50–64) of the ICR. These three fingers are not essential for binding of the factor to either nucleic acid (12). Although dispensable, these fingers still make thermodynamic contributions to the free energy of binding to both S S RNA (19, 64) and the gene (13, 72). Contacts made by TFIIA to this region of the RNA appear to utilize the secondary structure of the nucleic acid. Disruption of helix II with block mutations lowers affinity for TFIIA as much as 3-fold; restoration of duplex structure, but not sequence, with complimentary changes in the opposite strand returns binding to wild-type affinity (30). Likewise, point mutations at several positions in helix II lower binding of the factor (12), but these effects can also be reversed by secondary mutations that restore Watson-Crick pairing. Linker (25, 29, 30) and point (12)
mutations in the remaining domains of this arm (i.e. loop B, helix III, and loop C) have only small effects, if any, on the affinity of TFIIIA for 5 S rRNA. Of the mutants we have made in this arm of the RNA, many, including the deletion of the two bulged adenosine residues in helix III, have no impact on binding of TFIIIA. However, substitutions at positions 43/44, 53, and 56, which occur at stem-loop junctions, do have modest effects. These results are in accord with chemical and enzymatic protection data (8) which suggest that TFIIIA utilizes the accessible, widened major groove that is known to occur at stem-loop junctions in RNA (73).

Similarly, the binding of TFIIIA to box A is not significantly influenced by point mutations made in this element. Both DNase I and hydroxyl radical footprinting experiments (74) as well as missing nuclease experiments (18) demonstrate that TFIIIA is closely associated with this region of the ICR. In contrast, no essential contacts within box A were identified in chemical interference experiments (61). Studies using polypeptides representing subsets of zinc fingers derived from TFIIIA have demonstrated that binding affinity is not strictly correlated with the loss of contacts made to this region of the ICR (72). The interaction of fingers 7–9 with box A appears to be complex and it has been proposed that the free energy of binding may be counterbalanced by other processes such as bending of the DNA (72) or unfavorable interactions between certain finger domains (13). The apparently small energetic contribution made by fingers 7–9 is well illustrated by mutations made at position 56. A transversion at this site from A (major oocyte) to C (trace oocyte) abolishes over one-third of the TFIIIA footprint, yielding a protection pattern similar to that found on the trace-oocyte gene (bp 62–94). We have measured a 2-fold decrease in binding for the A to C transversion made at this site. The alternative transversion, A56T, has even less effect on the affinity for TFIIIA; yet, this mutant also exhibits this site. The alternative transversion, A56T, has even less effect on the affinity for TFIIIA; yet, this mutant also exhibits this site for TFIIIA. Our results, however, cannot be used to conclude whether contacts with finger 4 occur through the major or minor groove of this structure. Fingers 4 and 6 appear to be specifically designed for recognition of the unique geometries presented by loops E and A, explaining both their importance for binding of the factor to 5 S rRNA and their relatively subordinate interaction with the gene.

Helix V of 5 S rRNA corresponds to the intermediate element of the ICR. These are the respective binding sites for finger 5. The various mutations we have made at nucleotides 70 and 105 affect binding to RNA and DNA similarly and provide evidence that this finger makes comparable interactions through the major grooves of both nucleic acids.

The three carboxyl-terminal fingers of TFIIIA associate with the helix I-loop B-helix III region of 5 S rRNA or the corresponding box A element of the ICR. We find that only substitutions made at stem-loop junctions in this arm of 5 S rRNA have any detectable effect on binding and these are rather small. Likewise, these mutations have little effect on the affinity of TFIIIA for the gene; yet, as demonstrated by substitutions made at A46, they can cause considerable disruptions between the 5′-end of the ICR and the carboxyl-terminal fingers of the factor. Thus, although the apparent thermodynamic contribution of these three fingers to binding is not large, these interactions are important for properly orienting the factor on the ICR.

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