The Use of Chemical Nucleases to Analyze RNA-Protein Interactions

THE TFIIIA-5 S rRNA COMPLEX*

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The complex of Xenopus transcription factor IIIA (TFIIIA) with 5 S rRNA was analyzed in nuclease protection experiments using hydroxyl radical. The protection pattern reveals that TFIIIA interacts with a substantial amount of the RNA molecule, with close association between the factor and the arm of the RNA composed of helix IV-loop E-helix V. Additional sites of protection punctuate the other arm of the molecule. Important contact sites within the complex were identified in "missing nucleoside" experiments. Random single-nucleoside gaps were introduced into 5 S rRNA using either Fe[EDTA]-/H2O2 or bis(1,10-phenanthroline)copper(I). This modified RNA was allowed to bind to TFIIIA in an exchange reaction, and, afterward, bound and unbound fractions were separated on nondenaturing polyacrylamide gels. Missing nucleoside positions specifically enriched in the unbound fraction of RNA are located in the two strands that comprise loop E. These are not necessarily sites of sequence-specific contacts, but rather may constitute a region of secondary structure essential to recognition and binding of TFIIIA to 5 S rRNA.

Nuclease protection analysis or footprinting is a mainstay of molecular biology (1-4). The binding site for a protein on a nucleic acid can be located in a simple experiment requiring only small amounts of material. Furthermore, the protein sample need not be pure, but rather can be a mixture as complex as a nuclear extract. The potential of the technique exceeds the simple identification of a nucleic acid binding site for a factor in that the procedure can also be used to acquire thermodynamic information from binding isotherms constructed in titration experiments (5).

Since the first description of this method by Gallas and Schmitt (1), several modifications to the procedure have been introduced; certainly, one of the most important has been the use of chemical nucleases as cutting reagents (6-8). The advantages of these reagents include their small size, which minimizes steric effects, and their general insensitivity to the sequence and secondary structure of DNA. These small molecules can provide a description of the site along a segment of DNA that is protected by a protein in greater detail than is possible with enzymatic probes.

The adaptation of the footprinting technique to RNA-protein complexes has been hampered by the lack of appropriate nucleases that can generate sufficient digestion ladders of this nucleic acid. The higher order structure of RNA is much more complex than that of DNA, and, if not all, ribonucleases are sensitive to this aspect of structure. In addition to being structure-specific, ribonucleases are generally nucleoside-specific as well. In native conditions, most will hydrolyze only a small number of sites within a particular RNA molecule. Some of these problems were overcome with the use of the ribonuclease α-sarcin which cuts 3' of purines in both single- and double-stranded RNA (9). This enzyme can generate an acceptable digestion ladder for many RNAs and has been used to map the binding sites for a number of proteins (10-14). There are two aspects of the nucleolytic activity of α-sarcin, however, that limit its usefulness as a reagent in footprinting experiments (4). The most serious problem is a severe inhibition by divalent cations, which usually are necessary for the formation and stability of RNP complexes. Second, the digestion pattern depends on the RNA itself and seems to be affected by the tertiary structure of the nucleic acid. Chemical cleavers such as copper phenanthroline (OP-Cu) and hydroxyl radicals generated from the EDTA complex of iron(II) can cut RNA as effectively as DNA and should provide useful reagents for footprinting RNA-protein complexes (15-17). Unlike enzymatic ribonucleases, these complexes are not adversely affected by the presence of monovalent or divalent cations, and, because of their small size, should not be constrained by the secondary structure of the substrate RNA.

We have used Fe[EDTA]- and OP-Cu to analyze the binding site for Xenopus transcription factor IIIA (TFIIIA) on 5 S rRNA. The analysis of this interaction has been refractory to site-directed mutagenesis (18-21), suggesting that secondary structure, rather than sequence, is the principal determinant for binding. In the present experiments, we find that, while the protein protects a substantial amount of the 5 S rRNA molecule, nucleotides within and immediately flanking loop E (nucleotides 73-77 and 99-102) appear to provide the most important contact sites for the factor. These procedures utilizing chemical nucleases should be generally useful for the analysis of RNA-protein interactions.

EXPERIMENTAL PROCEDURES

Preparation of TFIIIA—The 7 S RNP particle of TFIIIA bound to 5 S rRNA was prepared from immature ovaries of Xenopus laevis (Nasco, Fort Atkinson, WI) (22, 23). TFIIIA free of 5 S rRNA was prepared by two precipitations of the 7 S RNP particle with (NH4)2SO4 as described by Shang et al. (24).

Preparation and Labeling of 5 S rRNA—Samples of 5 S RNA were isolated by extraction of the 7 S RNP particle with phenol or from the supernatants of the particle precipitated with (NH4)2SO4 that

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1 The abbreviations used are: RNP, ribonucleoprotein; Fe [EDTA]-, (ethylenediaminetetraacetate)iron(II); OP-Cu, bis(1,10-phenanthroline)copper(I); pCP, cytidine 3',5'-bisphosphate; TFIIIA, transcription factor IIIA.
were also extracted with phenol. This RNA was purified by preparative gel electrophoresis and renatured (10). The RNA was labeled either at the 3' end with cytidine 3',5'-[5-32P]phosphate, synthesized according to England et al. (25), using T4 RNA ligase, or at the 5' end with [γ-32P]ATP using T4 polynucleotide kinase (26). Radioactive RNA was purified by electrophoresis through polyacrylamide gels containing urea and renatured (10).

RNA Exchange Reaction—A trace amount of radioactive, renatured 5 S rRNA was incubated for 30 min at room temperature in a solution containing 10 μM 7 S RNP particle, 10 mM Tris-Cl, pH 7.5, 3 mM dithiothreitol, 15 μg/ml nucleic acid-free bovine serum albumin (27). In order to remove glycerol from samples of the RNP complex used for hydroxyl radical digestions, the products of the exchange reaction were passed through Sephadex G-50 spin columns equilibrated with exchange buffer.

Cleavage of RNA with α-Sarcin—Footprinting experiments with α-sarcin were carried out directly in exchange buffer which lacks divalent cations. Samples containing the indicated amounts of RNA were incubated for 15 min at 30 °C as described before (10). Reactions were terminated by extraction with phenol, followed by precipitation of the nucleic acid with ethanol. Digestion products were analyzed by electrophoresis on sequencing gels (10).

Cleavage of RNA with Fe/EDTA—Cleavage reactions were carried out essentially as described by Tullius et al. (28) for the scission of DNA. Solutions of (NH4)2Fe(SO4)2 and Na2EDTA were prepared immediately before use and passed through 0.45-μm nitrocellulose filters. Three reaction conditions were used to cut RNA that differ in the final concentrations of iron(II), EDTA, hydrogen peroxide, and ascorbic acid: 20 μM, 20 μM, and 1 mM, respectively. Reaction II, 100 μM, 300 μM, 0.0035%, and 1 mM, respectively; Reaction III, 1 mM, 2 mM, 0.3%, and 2 mM, respectively. Reactions were carried out at room temperature in a volume of 20 μl for the indicated amount of time and were quenched by the addition of thiourea and EDTA. Samples were extracted twice with phenol and then precipitated with ethanol. The resulting pellets were washed at least three times with 70% ethanol to remove salts from the cutting reactions and then suspended in loading solution containing urea. Alternatively, after hydroxyl radical cleavage, the reactions were quenched by the addition of glycerol to 10% and loaded directly on a nondenaturing polyacrylamide gel (8% acrylamide, 0.4% bisacrylamide, 100 mM Tris and 3 mM mercaptopropionic acid). Reactions were incubated at room temperature initiated by addition of the cutting reagents to the following final concentrations: 50 μM 1,10-phenanthroline, 12 μM CuSO4, 4.8 mM 3-mercaptopyrrolic acid. Reactions were incubated at room temperature for the indicated times and stopped by the addition of 2.5-30 μl of 2.3 M HCl, 1.3 M ethylenediaminetetraacetic acid (EDTA), and 1.3 M NaOH. The samples were then extracted with water-saturated 1-butanol to remove the (OP)3Cu complex, and the nucleic acid in the aqueous phase was precipitated with ethanol. These precipitates, likewise, were washed several times with 70% ethanol to remove traces of salt before analysis on sequencing gels.

Missing Nucleoside Experiment—This is an adaptation of the procedure devised by Hayes and Tullius (29). Samples of end-labeled 5 S rRNA in 10 mM Tris-Cl, pH 7.5, containing 2 μg of Escherichia coli tRNA were cleaved with hydroxyl radical using one of the three conditions described above or with OP-Cu. The RNA was precipitated with ethanol and resuspended in exchange buffer. The 7 S RNP particle was added to 10 μM, and the samples were incubated for 30 min at room temperature in a volume of 10 μl. Glycerol, without tracking dyes, was added to the exchange mixture which was then loaded onto 8% nondenaturing polyacrylamide gels run at 100 V. After autoradiography, the bands corresponding to free 5 S RNA and 7 S RNP complex were excised from the gel and eluted overnight (10). The eluants were extracted twice with phenol and twice with ether, precipitated with ethanol, and resuspended in loading solution containing urea. The recovered products were analyzed on sequencing gels.

Sequencing Gels—Nucleic acid digestions were analyzed along side ribonuclease T1, and alkaline hydrolyses on 10% or 20% polyacrylamide gels containing 7 M urea. Digestion with ribonuclease T1, or alkali yields products with 3'-phosphates, whereas Fe/EDTA- and OP-Cu eliminate nucleoside moieties to generate products with both 5' and 3'-phosphorylated termini. Consequently, the fragments produced by these two chemical nucleases migrate faster relative to corresponding fragments in the two sequencing lanes. Nucleotide positions denoted in the figures have been corrected for this difference. The autoradiographs of sequencing gels were scanned with an LKB Ultrascan XL laser densitometer to quantitate the intensity of bands in the missing nucleoside experiments.

RESULTS

RNA Exchange Reaction of the TFIIIA-5 S RNA Complex—Typically, in a footprinting experiment, end-labeled nucleic acid is incubated with an excess of protein to saturate the binding site in order to achieve maximum protection from a nuclease. In our attempts to reconstitute the 7 S complex in vitro using free 5 S rRNA and TFIIIA, we always observed a significant amount of high molecular weight aggregate on nondenaturing polyacrylamide (mobility shift) gels (Fig. 1A). Although the severity of the problem varied with the particular preparation of 7 S RNP complex, we were not successful in eliminating this complication. Reconstitution in the presence of nonspecific nucleic acid (poly(I)·poly(C)), heparin, Nonidet P-40, or high concentrations of monovalent cation did not significantly decrease the formation of aggregated material. Moreover, preparation of free TFIIIA either by the method of Smith et al. (22) or Shang et al. (24) yielded the same results. Experiments reported by Wu and co-workers have demonstrated the tendency of TFIIIA to interact with either DNA (30) or RNA (31) as an aggregate mediated by protein-protein interactions. In the case of 5 S RNA, sedimentation velocity revealed formation of a 1:1 complex of factor and RNA that then proceeds to dimerize (31). In our work, we did observe a putative dimer complex (Fig. 1A); however, much of the sample aggregates into a high molecular weight structure that is retained in the pockets of the mobility shift gels.

Andersen and Delilas (27) have characterized an RNA exchange reaction in which exogenous radioactive 5 S rRNA is incorporated into samples of the 7 S complex. The reaction appears to represent a specific interaction, since denatured Xenopus 5 S RNA or deletion fragments of this nucleic acid do not undergo exchange into the 7 S complex; moreover, prokaryotic 5 S RNA and tRNAs exchange poorly or not at all. In order to circumvent the problems associated with the direct reconstitution of the TFIIIA-5 S RNA particle, we have used this exchange reaction to generate 7 S complex containing RNA labeled at a specific end. We tested several fractions of the 7 S complex and used only those that gave complete exchange of the radioactive RNA into the 1:1 complex.

![Fig. 1. Products of the direct reconstitution of the TFIIIA-5 S RNA complex and of the RNA exchange reaction.](image-url)
plex without any evidence of higher molecular weight aggregates (Fig. 1B). We presume that complete incorporation of trace amounts of exogenous 5 S rRNA into the complex is possible due to the presence of some free TFIIIA in the sample. In agreement with this suggestion, the molar ratio of TFIIIA to 5 S rRNA in the samples used for the exchange reactions was slightly greater than 1.

Earlier experiments to define the binding site for TFIIIA on 5 S rRNA by footprinting with α-sarcin used native particle that had been labeled directly at the 3' terminus with [32P]pCp using RNA ligase (11). The site protected by the factor extended from nucleotide 64 to 116. In order to demonstrate that the exchange reaction yields an RNP complex that corresponds to the native TFIIIA-5 S rRNA particle, we used α-sarcin to analyze the product of the RNA exchange reaction (Fig. 2). Oocyte 5 S rRNA labeled at either the 5' or 3' end was incubated in exchange buffer in the presence or absence of 7 S particle for 30 min at room temperature, the samples were then digested with the indicated amounts of α-sarcin for 15 min at 30 °C. The digestion products were analyzed on sequencing gels alongside ribonuclease T₁ and alkaline hydrolysates of the same RNA sample. The footprint observed with 3' end-labeled 5 S rRNA in the complex formed by exchange is nearly identical with that reported for the native particle labeled directly. The two notable differences are that G₂₀, which appeared to be exposed in the earlier experiment, is protected in the complex formed by exchange, and that protection of weakly cut positions extends the 5' end of the footprint from nucleotide 64 to 49. The 5' edge of the footprint is protected in the complex formed by exchange, and that protection of weakly cut positions extends the 5' end of the footprint from nucleotide 64 to 49.

The extensive interaction of the factor with 5 S RNA in the oocyte complex cannot be labeled directly with radioisotope. The earlier experiments with α-sarcin provide no evidence for binding of TFIIIA to the 5'-half of the RNA, but the exchange reaction now enabled us to use RNA labeled at this terminus to analyze the interaction more carefully. As expected, due to primary versus secondary sites of hydrolysis, the digestion pattern of the 5' end-labeled 5 S rRNA is somewhat different from that of the 3' end-labeled nucleic acid. The analysis using α-sarcin and 5' end-labeled RNA also exhibits strong protection in the 3' half of the nucleic acid, but some additional sites of protection are evident in the remainder of the RNA (Fig. 2, lane 10). These positions include nucleotides 21–24, 26–27, and 31–32. The results with α-sarcin indicate that a continuous, close interaction of TFIIIA with 5 S rRNA occurs in the regions that encompass helices I, IV, and V, and loops D and E (Fig. 3), with additional contacts in helices II and III and in loop B. During the course of these experiments, we have observed that high concentrations of α-sarcin seem to compete effectively with TFIIIA for binding to the latter sequences, diminishing protection, and suggesting a weak interaction of the factor with these sites in the molecule. This would account for why they were not identified in the earlier experiments. The extensive interaction of the factor with 5 S RNA is in accord with the putative role of the 7 S particle as a storage complex for the nucleic acid.

Footprinting RNA Using Fe[EDTA]²⁺—Hydroxyl radical is generated at uniform concentrations in solution by the reaction of hydrogen peroxide with the EDTA complex of iron(II) (8, 28). In the presence of duplex DNA, this small, uncharged...
species initially abstracts a hydrogen atom from a deoxyribose position in the minor groove (probably a 4′-hydrogen), with subsequent scission of the DNA backbone in a sequence-insensitive manner. Because a ladder of bands of uniform intensity can be generated with Fe[EDTA]$^{3-}$, it is an ideal reagent for protection experiments. This complex cleaves RNA equally well (16, 17), and we have used it here to further characterize the interaction of TFIIIA with 5 S rRNA. Cleavage of RNA with Fe[EDTA]$^{3-}$ appears to be indifferent to secondary structure, but can be used to detect the three-dimensional folding of the nucleic acid; in some cases, ribose residues in the interior of an RNA molecule are protected from strand scission (17). The Fe[EDTA]$^{3-}$-catalyzed digestion of renatured 5 S rRNA exhibits only minor modulation in cleavage intensity along the molecule, indicating that this RNA possesses very little, if any, tertiary structure.

The RNA exchange reaction was used to produce labeled 7 S particle which then was passed through a spun column equilibrated with exchange buffer in order to remove glycerol contained in the storage buffer for the particle which acts as an effective radical scavenger. Since the exchange buffer contains dithiothreitol and a low concentration of Tris buffer, it is particularly suitable for the Fe[EDTA]$^{3-}$ reaction (28). The autoradiographs from a series of footprinting experiments using Fe[EDTA]$^{3-}$ are presented in Fig. 4. The digested samples were prepared for analysis by one of two methods. In one procedure, strand scission was stopped by the addition of thiouracil, and the digested nucleic acid was isolated by extraction with phenol and precipitation with ethanol. Alternatively, glycerol was added to the mixture to quench the cutting reaction, and the sample was loaded onto a nondenaturing polyacrylamide gel to separate any free RNA that arose from the complex during the cleavage reaction. The section of the gel containing the intact RNP particle was excised; and the sample was eluted, extracted with phenol, and precipitated with ethanol. Processing the samples in this way greatly improved the detection of protected regions of the 5 S rRNA.

Lanes 8 and 9 in Fig. 4 contain the products obtained by the latter procedure and can be compared to the profiles in lanes 10 and 11 which represent samples analyzed directly after digestion with the chemical nuclease.

The instability of the 7 S RNP particle during digestion with hydroxyl radical may be due to the reported inactivation of TFIIIA by H$_2$O$_2$ observed during footprinting experiments with the DNA complex (28). Recently, Rana and Meares (32) covalently attached an EDTA complex of iron(II) to bovine serum albumin and observed site-specific cleavage at two distinct positions in the protein upon addition of the moderate amount of dissociation of the 7 S RNP complex that occurs during footprinting experiments. Samples of the particle were incubated with various concentrations of hydroxyl radical reagents (I, II, or III) or with the OP-Cu reagents for 20 min at room temperature. Analysis of the protein by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels revealed no detectable degradation of TFIIIA (data not shown).

The results with Fe[EDTA]$^{3-}$ closely parallel those with $\alpha$-sarcin; however, the regions weakly protected from $\alpha$-sarcin are better identified using the chemical nuclease. The 3′-half of 5 S rRNA is markedly protected by the factor, just as was observed with $\alpha$-sarcin, and this region is enclosed by brackets in Fig. 4. Additional sites of protection in the other arm of the RNA molecule are indicated by dotted lines on the autoradiographs and include positions 5–9, 16–24, 31–35, 43–46, 49–54, and 58–61. Protection of the 3′ strand of helix I by TFIIIA is very obvious using $\alpha$-sarcin; however, because the 5′ strand of this helix is poorly cut by the nuclease, it was not clear in the earlier experiments whether the factor interacted with this segment of the nucleic acid. Since the terminal five or six nucleotides from either end of the RNA cut with Fe[EDTA]$^{3-}$ are partially lost during precipitation and washing with ethanol, which is required to remove residual salts from the samples, we can make only qualified comments on helix I. However, despite the reduced intensity of these bands in the autoradiographs, the hydroxyl radical footprint does reveal an interaction of TFIIIA with both strands of helix I.

**Missing Nucleoside Experiments**—The transcription factor interacts with a substantial amount of 5 S rRNA, but with an apparent disparity in binding between the two arms of the molecule. Although protection analysis is invaluable for locating the binding site for a protein on a nucleic acid, the technique is incapable of identifying the specific nucleotides in contact with the protein. A method for determining these positions was devised by Brunelle and Schleif (33) and later modified by Hayes and Tullius (29). In the “missing nucleoside” experiment, Fe[EDTA]$^{3-}$ is used to introduce single-
nucleoside gaps randomly into a DNA molecule. This modified DNA is incubated with its respective binding protein; then, bound and free DNA are separated by electrophoresis on nondenaturing gels. The populations of DNA in the two fractions are analyzed on sequencing gels. Bands that are enriched in the lane corresponding to the free DNA represent positions where a missing nucleoside interferes with binding of the protein. We have used this method to identify contact sites on 5 S rRNA for TFIIIA.

We treated end-labeled 5 S rRNA with either Fe[EDTA]$^{2-}$ or OP-Cu; the former will remove nucleosides randomly throughout the RNA, while the latter, which also removes nucleosides, reacts primarily with single-stranded segments. These gapped RNAs, which possess less than one modified position per molecule, were used in the RNA exchange reaction with the 7 S RNP particle. After incubation in standard conditions, the mixtures were applied to nondenaturing polyacrylamide gels (Fig. 5). The untreated 5 S rRNA exchanged completely into the RNP complex (Fig. 5, lane 2); however, RNA that was reacted either with Fe[EDTA]$^{2-}$ or OP-Cu (Fig. 5, lanes 3 and 4, respectively) exhibits only partial exchange. Bands representing free and bound RNA were excised from the mobility shift gels, and the nucleic acid eluted from the gel slices. These samples were analyzed on sequencing gels (Fig. 6) to visualize the contact sites for TFIIIA.

The autoradiographs presented in Fig. 6 are examples of missing nucleoside experiments used to survey the 5 S rRNA molecule. Each experiment is represented by three lanes which display: 1) the gapped RNA substrate used in the exchange reaction, 2) RNA recovered from the bound fraction, and 3) free RNA that did not exchange into the 7 S RNP complex. The two experiments shown in lanes 1–3 and 4–6 represent 5 S rRNA cut using two different concentrations of the hydroxyl radical reagents. The former reaction contained reduced amounts of hydrogen peroxide (II), while the latter contained increased amounts of all the cutting reagents (III). The autoradiographs for lanes 10–12 (Fe[EDTA]$^{2-}$) and 13–15 (OP-Cu) were underexposed to accentuate the differential intensity of bands enriched in the lanes representing the unbound fraction of RNA. Regardless of these experimental variations or the chemical nucl ease used to cut the 5 S rRNA, two segments of the nucleic acid, derived from the strands that form loop E, were always enriched in the unbound fraction (bracketed regions of the autoradiographs).

A notable difficulty in applying the missing nucleoside methodology to the TFIIIA-5 S rRNA complex was the uneven recovery of cleavage fragments from the nondenaturing polyacrylamide gels. At several positions in the autoradiographs of Fig. 6, the sum of the fragments in the bound and unbound fractions is significantly less than the corresponding fragment in the starting material. This problem is not encountered in experiments with DNA since the elimination of a single nucleoside does not markedly affect the stability of the duplex during electrophoresis. On the other hand, RNA, because of its more complex secondary structure, is sensitive to the introduction of single nucleoside gaps into the molecule. It is likely that cleavage at some sites destabilizes the molecule, reducing the recovery of intact nucleic acid. Consequently, these positions are under-represented in the autoradiographs. In support of this suggestion, we have observed a small degree of smearing below the bands corresponding to free 5 S rRNA on the nondenaturing gels used to separate free and bound RNA. These truncated fragments, which are not recovered from the gel, would account for the losses manifested in the sequencing gels. Despite this drawback, the technique was used successfully because much of the radioactivity in the unbound 5 S rRNA is confined to a small number of fragments specifically enriched in this fraction and easily identified.

The missing nucleoside data are summarized on the secondary structure of 5 S rRNA presented in Fig. 7. Notwithstanding the different specificity of the two chemical nucleases, the identified nucleosides are quite similar, seemingly because most of the positions occur in single-stranded loop E, which is susceptible to scission by either complex. Although the two segments encompassing these contact sites lie across from one another in the secondary structure of the RNA, they are not of equal length and there appears to be a 3' stagger of one relative to the other. One region includes nucleosides 73–77 with positions 73 and 76 being particularly intense in the lanes representing unbound RNA. On the opposite side of the loop, sites identified using RNA digested with Fe[EDTA]$^{2-}$ extend from position 98 to 106, while those found with RNA treated with OP-Cu extend from 99 to 108. Within these sequences, RNA containing missing nucleosides at positions 100, 101, 102, and 105 are the predominant bands in the unbound fractions.

The contacts in loop E were detected using 5 S rRNA labeled at either the 5' or 3' terminus which ensures that these were sites of primary and not secondary cuts. The results of the missing nucleoside experiments establish the importance of this region for recognition and binding of TFIIIA and support the conclusion drawn from footprinting experiments that the protein makes close contact with this arm of 5 S rRNA.

**DISCUSSION**

Wang and Padgett (16) used Fe[EDTA]$^{2-}$-catalyzed cleavage to study the formation of splicing complexes at the 3' splice site of precursor messenger RNAs. Protection at positions that included the intron/exon junction, the polypurine tract, and the branch site could be correlated with the formation of intermediary complexes of the spliceosome. The protected regions of the pre-mRNA in those experiments likely were due to the binding of several protein factors and/or RNP complexes. In the experiments reported here, we have demonstrated that it is also possible to characterize the RNA binding site for a single protein using hydroxyl radical footprinting techniques.

The binding site for TFIIIA on 5 S rRNA determined with α-sarcin and native 7 S RNP particle labeled directly at the
Fig. 6. Autoradiographs of sequencing gels used to analyze products from missing nucleoside experiments. Bands representing bound and unbound fractions of RNA treated with Fe[EDTA]$^{2-}$ or OP-Cu were excised from nondenaturing gels; the eluted nucleic acids were then subjected to electrophoresis in sequencing gels. Each experiment is represented by three lanes with the first containing the gapped RNA added to the exchange reaction, the second containing the fraction of RNA bound by TFIIIA, and the third containing the unbound fraction. Lanes 1–3, 10–12, 16–18, and 22–24, RNA cleaved in hydroxyl radical conditions II; lanes 4–6, RNA cleaved in hydroxyl radical conditions III; lanes 7–9, 13–15, 19–21, and 25–27, RNA cleaved with OP-Cu. Brackets enclose nucleotides that have specifically accumulated in the fractions of unbound RNA.

Fig. 7. The secondary structure of Xenopus 5 S rRNA with designations of the sites of protection by TFIIIA determined with hydroxyl radical and contact sites identified in missing nucleoside experiments. Sequences enclosed with boxes designate nucleotides protected from hydroxyl radical digestion by TFIIIA. Arrows point to positions identified in the unbound fractions of RNA in missing nucleoside experiments, and the lengths are proportional to the intensity of their respective bands determined by scanning the autoradiographs with a densitometer. Open arrowheads denote results with Fe[EDTA]$^{2-}$, and filled arrowheads denote results with OP-Cu.

3' end of the RNA encompassed helices IV and V, loops D and E, and the 3' strand of helix I (11). Using the RNA exchange reaction with 3'-end-labeled 5 S rRNA, we obtain a similar footprint, supporting other evidence that the exchange reaction yields a complex identical with the native particle (27). However, using 5'-end-labeled RNA, we have detected some additional sites of apparently weaker protection in the other arm of the molecule (Fig. 3). The difficulty in observing these latter positions may be a consequence of the high concentrations of α-sarcin used to generate sufficient digestion ladders of the nucleic acid. The molar ratio of α-sarcin to RNA in footprinting experiments typically ranges from 5 to 20 because of the extremely low turnover of the nuclease (4, 9). It is possible that the nuclease can effectively compete with and disrupt weak interactions in the complex at these high concentrations.

The results of protection experiments using hydroxyl radical are in agreement with those obtained from experiments using α-sarcin; however, because the chemical nuclease cleaves at every position in the nucleic acid, the binding site is better defined. Moreover, because Fe[EDTA]$^{2-}$ does not bind to the nucleic acid, sites weakly protected from α-sarcin are more distinct in the hydroxyl radical footprint. The protection pattern obtained with the chemical nuclease reveals that TFIIIA interacts with a substantial amount of the 5 S rRNA molecule (Fig. 7) which is reasonable, given that this storage particle has a lifetime of several months in the oocyte (34). The continuous protection that extends throughout the 3'-half of the RNA indicates that the factor is closely associated with this region of 5 S rRNA. On the other hand, contact between the protein and the other arm of 5 S rRNA is intermittent, suggesting that the interaction of TFIIIA differs qualitatively between these two domains of the RNA.

The TFIIIA-5 S rRNA complex has been studied in a variety of protection experiments (11, 35–38). Most of these data are in fair agreement and implicate sequences within helix II-loop B and helix IV-loop E-helix V as the sites of primary contact between the protein and the RNA. Christiansen et al. (38) used several enzymatic and chemical probes to measure the accessibility of nucleotides of 5 S rRNA bound to TFIIIA. Protection effects of varying degrees could be
detected at sites dispersed throughout most of the nucleic acid in accord with the extended interaction defined here by the hydroxyl radical footprint. A model of the 7 S complex (39) based on neutron scattering and hydrodynamic measurements is a highly elongated cylinder 140 Å in length and 59 Å in diameter, which can be compared to the cylindrical dimensions of 5 S rRNA (122 Å × 34 Å). Thus, the shape of the particle is somewhat more elongated than the RNA and clearly indicates an extended interaction of the factor with 5 S rRNA. Moreover, the scattering data suggest a complex arrangement of the two component molecules which possibly relates to the differences in protection seen for the two arms of 5 S rRNA in the footprinting experiments.

Sites of protection cannot be equated with sites of contact between a protein and a nucleic acid. We used the missing nucleoside procedure to determine where important interactions occur within the broad region of protection determined with Fe[EDTA]^{2-}. Nucleosides in the two strands of loop E constitute a major determinant for the association of TFIIIA with 5 S rRNA. Removal of single nucleosides from other positions in the molecule do not have a detectable effect on binding.

The missing nucleoside methodology was developed by mapping λ repressor and cro sites on O_{1,2}, since there is ample information available from crystallographic and mutagenesis studies of these complexes (29). Nearly all effects detected by this procedure could be correlated with known interactions between specific sites in the operator and residues of the proteins. However, there was evidence that at least one positive signal may have resulted not from a missing contact to the repressor, but from a change in the secondary structure of the DNA as a consequence of the one nucleoside gap. This points out a very important consideration when applying this procedure to interactions involving RNA which possesses a higher order structure that is much more complex than that of DNA, and that appears to play a substantial role in directing the binding of proteins. It is quite possible that removal of a nucleoside from the regions detected in these experiments disrupts some aspect of secondary structure that is indispensable to recognition and binding of TFIIIA and does not represent loss of a sequence-specific contact.

TFIIIA can bind to a variety of heterologous eukaryotic 5 S rRNA molecules; however, it has very little affinity for prokaryotic 5 S rRNA molecules, indicating that conserved elements of secondary structure rather than sequence may serve as the primary determinants of the interaction (27, 37, 40). Alternative models for the structure of loop E have been presented based on experiments using chemical and enzymatic probes and from NMR studies (38, 41–43). The question is whether non-Watson-Crick base pairs occur within loop E, allowing formation of a continuous double-stranded segment that connects helices IV and V. The constituent strands of loop E are moderately resistant to single strand-specific ribonucleases, and one group (41) has reported weak hydrolysis by the double strand-specific ribonuclease, V_{1}, at positions U_{52} and U_{76}. Likewise, chemical probes that target base-pairing positions (N-1 of purines and N-3 of pyrimidines) are unreactive with several nucleotides in loop E under native conditions (38, 42). All of these results have been taken as evidence that non-Watson-Crick base pairs are present, and alternative base-pairing schemes for the loop have been proposed (38, 41, 42). Conversely, the structure of a duplex RNA oligonucleotide similar in sequence to loop E has been studied by one- and two-dimensional NMR and provides no evidence either for noncanonical base pairs or for the syn conformation nucleotides suggested by chemical modification studies (43).

The extensive base stacking in the internal loop detected in these experiments, however, permits the continuation of an A-type helix from one flanking stem structure to the other with little distortion. The sequence of the duplex used in the NMR experiments was not identical with that of loop E. In order to favor formation of putative G:A base pairs, the nucleotide corresponding to U_{72} was deleted, and some base pairs within the flanking stems were changed (to G:C) to improve stability. Thus, the data cannot be used to describe the actual structure of the loop in 5 S rRNA. It is likely, nonetheless, that loop E possesses a distinct structure that closely resembles an A-form helix; however, the absence of interstrand hydrogen bonding may change the dimensions of the major and minor grooves enabling contacts with the factor that would be sterically less favorable if the helix were in a canonical, base-paired A conformation.

The structure of loop E and its influence on the binding of TFIIIA to 5 S rRNA has been studied in several mutagenesis experiments. Romanik (20) created three block mutations centered in loop E; two simply changed the sequence of the strands on either side of the loop without specifically altering secondary structure, while the third mutant introduced three replacements that converted the loop to a completely base-paired helix. The affinity for TFIIIA was decreased only about 2-fold for all three mutants. In agreement with the data from mapping experiments with structure-specific probes and from the NMR experiments, these results indicate that loop E is very close in structure to an A-form helix, and conversion to a base-paired structure only modestly affects binding of the factor. In contrast, two linker mutations that spanned loop E and continued into either one of the flanking helices severely inhibited binding of TFIIIA (44). Unfortunately, it is not known how these substantial nucleotide substitutions (6 and 11 nucleotides changed) affected the secondary structure of the RNA, but the disruption of either helix IV or V presumably will transform the secondary structure of loop E as well. The results from these mutagenesis experiments indicate that the association of TFIIIA with loop E is not sequence-dependent, in which case the sites of single nucleoside gaps identified in the unbound fraction of RNA must alter the interaction either directly by elimination of moieties in the ribose-phosphate backbone that make specific contact with amino acid residues in the factor, or indirectly by changes in the local secondary structure that result from disruption of base-stacking interactions. It is important to note that the decreased affinity of TFIIIA for RNA modified at one of these positions may represent the cumulative loss of more than one contact, since the interruption of base-stacking, brought about by the missing nucleoside, could propagate structural changes throughout most or all of the loop.

The one region of 5 S rRNA where binding of TFIIIA appears to be sensitive to nucleotide substitutions made by mutagenesis is loop A (20). However, hydroxyl radical and α-sarcin footprints do not include this region, nor are any positions in this loop implicated in the missing nucleoside experiments. The structure of loop A controls the co-axial stacking arrangements possible between helices I, II, and V. The results presented here support the interpretation (20) that reduced binding of TFIIIA to mutants in this hinge region is a consequence of changes in the relative orientation of the two arms of the RNA. The nucleoside substitutions that produce this indirect effect on binding of the factor create base pairs which favor alternate stacking arrangements (45). Apparently, single nucleoside gaps in this region do not constrain the configuration of the 5 S rRNA molecule to the degree that effects on binding of TFIIIA can be detected in
the missing nucleoside experiment.

The use of chemical nucleases as reagents in footprinting and missing contact experiments provides an effective methodology for the analysis of RNA-protein complexes. In particular, cleavage by hydroxyl radical is much less sensitive to the higher order structure of RNA than cleavage by enzyme nucleases, can tolerate a wide range of buffer conditions, and provides a probe for each nucleotide along the RNA backbone with little dependence on sequence. It is exceptionally well suited for protection experiments. The analysis of the TFIIIA-5 S rRNA complex has been difficult because the interaction is chiefly determined by the higher order structure of the nucleic acid rather than sequence-dependent contacts (45). The production of gapped RNA substrates with Fe[EDTA]− or OP-Cu for missing nucleoside experiments has allowed the identification of essential contact sites that could not be determined by other techniques. In combination, these two procedures should permit a detailed characterization of any RNA-protein structure.

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