Energetically Unfavorable Interactions among the Zinc Fingers of Transcription Factor IIIA When Bound to the 5 S rRNA Gene*  

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Xenopus transcription factor IIIA (TFIIIA) binds to over 50 base pairs in the internal control region of the 5 S rRNA gene, yet the binding energy for this interaction (ΔG° = -12.8 kcal/mol) is no greater than that exhibited by many proteins that occupy much smaller DNA targets. Despite considerable study, the distribution of the DNA binding energy among the various zinc fingers of TFIIIA remains poorly understood. By analyzing TFIIIA mutants with disruptions of individual zinc fingers, we have previously shown that each finger contributes favorably to binding (Del Rio, S., Menezes, S. R., and Setzer, D. R. (1993) J. Mol. Biol. 233, 567–579). Those results also suggested, however, that simultaneous binding by all nine zinc fingers of TFIIIA may involve a substantial energetic cost. Using complementary N- and C-terminal fragments and full-length proteins containing pairs of disrupted fingers, we now show that energetic interference indeed occurs between zinc fingers when TFIIIA binds to the 5 S rRNA gene and that the greatest interference occurs between fingers at opposite ends of the protein in the TFIIIA-5 S rRNA gene complex. Some, but not all, of the thermodynamically unfavorable strain in the TFIIIA-5 S rRNA gene complex may be derived from bending of the DNA that is necessary to accommodate simultaneous binding by all nine zinc fingers of TFIIIA. The energetics of DNA binding by TFIIIA thus emerges as a compromise between individual favorable contacts of importance along the length of the internal control region and long range strain or distortion in the protein, the 5 S rRNA gene, or both that is necessary to accommodate the various local interactions.

Xenopus transcription factor IIIA (TFIIIA) is a multifunctional protein that recognizes the major cis-acting transcriptional control element in 5 S rRNA genes (the internal control region, or ICR) and thereby nucleates the formation of transcription complexes that result in the synthesis of 5 S rRNA (1–3). It also binds to 5 S rRNA itself to form a ribonucleoprotein storage particle that accumulates in Xenopus oocytes (4, 5) and that may mediate feedback regulation of 5 S rRNA gene expression in somatic cells (5, 6). As the first sequence-specific DNA-binding protein to be purified from eukaryotic cells (1) and the archetypal zinc finger protein (7), TFIIIA has been an influential model protein for understanding the mechanisms of sequence-specific DNA and RNA recognition.

The nine zinc fingers of TFIIIA define the sequence features that can be used to recognize zinc finger motifs in other proteins (7). These include two cysteine and two histidine residues with conserved spacing; these four amino acids coordinate a Zn2+ ion (8) that contributes substantially to the stability of the folded domain (9–11). Three conserved hydrophobic residues are also likely to stabilize TFIIIA-like zinc finger domains through interactions in a hydrophobic pocket. The large number of zinc fingers in TFIIIA has made its structural analysis difficult, and structural studies of zinc finger proteins have therefore focused on peptide fragments containing 1–3 zinc fingers from TFIIIA or other proteins (10, 12–18) or on proteins containing a smaller number of consecutive zinc finger motifs (19–21). These studies have confirmed an earlier model of zinc finger structure (22) in which the N terminus of the domain folds into a pair of antiparallel β strands containing the Zn2+-coordinating cysteines and the C terminus into a helix containing the conserved histidines. Crystallographic analyses of complexes between the zinc finger proteins zif268 (19), GLI (20), or tramtrack (21) and the specific DNA sequences recognized by each protein have revealed that specific DNA binding is mediated largely through major groove and phosphate contacts with the helical region of the zinc finger. The initial solution of the zif268-DNA structure raised hopes that a simple code for zinc finger recognition of DNA might be possible, but later structures have suggested that a multiplicity of recognition modes are used by different zinc finger proteins, even when the overall structures of the zinc finger domains exhibit considerable similarity. This observation, coupled with much biochemical data as well as theoretical considerations, suggests that extrapolating from the properties of known zinc fingers to the structure of TFIIIA in a complex with the 5 S rRNA gene may be misleading.

TFIIIA binds to the 5 S rRNA gene with an equilibrium binding affinity (Kd) of about 0.4 nM (23, 24), which is comparable to the affinity exhibited by many other sequence-specific DNA-binding proteins that occupy much smaller recognition surfaces than that represented by the 52 base pairs bound by TFIIIA (positions 45–96 of the Xenopus borealis somatic-type 5 S rRNA gene). A variety of analyses has demonstrated that the zinc fingers of TFIIIA are aligned more or less in order along the length of the internal control region of the 5 S rRNA gene, with the N-terminal fingers near the 3′ end of the control region and the C-terminal fingers near the 5′ end (9, 25–28). Results obtained with truncation mutants have been interpreted to mean that almost all the free energy of DNA binding can be attributed to interactions between the first three zinc fingers of TFIIIA and the C box of the internal control region, whereas fingers 4–6 or 4–7 perform an analogous function in providing most of the specificity and free energy for 5 S rRNA.
recognition (29–31). In fact, numerous studies have confirmed that a fragment containing only zinc fingers 1–3 binds with high affinity and specificity to the 3′ end of the 5 S rRNA gene’s ICR (26, 32–35), whereas the central 3–4 zinc fingers of TFIIIA suffice for high affinity 5 S rRNA binding (29, 35, 36). Thus, the surprising dual specificity of TFIIIA, which permits recognition of both 5 S rRNA and the 5 S rRNA gene, has been explained by proposing that zinc fingers are specialized either for DNA binding (fingers 1–3) or RNA binding (4–7) (29, 30, 35). The results of various footprinting studies with intact TFIIIA and a nested set of TFIIIA truncation mutants have also been used to support models for the alignment of the zinc fingers of TFIIIA relative to the underlying sequence of the 5 S rRNA gene (27, 28, 30, 37). In these models, zinc fingers 1–3 and 7–9 assume zif268-like conformations at the 3′ and 5′ ends of the ICR, respectively, whereas finger 5 interacts in the major groove of the so-called intermediate element. Fingers 4 and 6 are proposed to bind on a single face and cross the minor groove on either side of the intermediate element.

Although studies of truncation mutants of TFIIIA have been most informative, it is important to realize that the interpretation of such analyses is strongly dependent on the assumption that functional interactions between zinc fingers are absent or unimportant and that the structure and function of a protein fragment reflect those of the same moiety in the context of the full-length protein. As a complement to the study of TFIIIA fragments, we have therefore taken an alternative approach in which individual zinc fingers have been structurally disrupted in the context of an otherwise normal, full-length protein. These “broken finger” mutants have been generated by the mutation of one of the Zn²⁺-coordinating residues of a single zinc finger, and their structural and functional analysis has also been described previously (9). Two points are worthy of comment in the context of the current study. First, footprinting analyses of the broken finger mutants on the 5 S rRNA gene suggested that existing models for alignment of the various zinc fingers of TFIIIA with respect to the underlying DNA sequence are unlikely to be correct in detail, particularly for the C-terminal fingers, since footprint alterations observed with the relevant broken finger mutants occurred up to 10 base pairs away from their putative sites of interaction in models based on the study of truncation mutants. Second, quantitative analysis of the DNA binding affinity of broken finger mutants suggested that all the zinc fingers of TFIIIA contribute favorably to the free energy of binding of the intact protein to the 5 S rRNA gene, with little indication of thermodynamic dominance by the first three zinc fingers. Furthermore, the data suggested that thermodynamically unfavorable interactions between zinc fingers occurred during the DNA binding reaction, resulting in a “compensatory” mode of binding. This mode of binding would result in a kind of “thermodynamic buffering” in which loss of binding by a single zinc finger would be partially compensated by relief of thermodynamically unfavorable strain in the complex. This interpretation, however, was dependent on the assumption that the mutations used to disrupt zinc finger structure resulted in complete loss of DNA binding activity by the relevant finger.

Based on the binding properties of two complementary N- and C-terminal fragments of TFIIIA, and of a set of full-length proteins that contain pairs of disrupted zinc fingers, we now document that thermodynamic interference indeed occurs in the TFIIIA-ICR complex and that the degree of interference is greatest between zinc fingers at opposite ends of the protein. The fact that energetic strain is built into the TFIIIA-5 S rRNA gene complex has interesting implications for the assembly and function of the 5 S rRNA transcription complex, for the evolutionary divergence of TFIIIA from different species, and for the interpretation of data derived from the analysis of TFIIIA fragments.

**EXPERIMENTAL PROCEDURES**

**Plasmids—**E. coli expression plasmids pTA1–100 and pTA100–344 were designed to produce complementary N- and C-terminal fragments of TFIIIA. pTA1–100 encodes the polypeptide A1–100, which contains amino acids 1–100 of TFIIIA, whereas pTA100–344 encodes the polypeptide A100–344, containing amino acids 100–344, preceded by a methionine residue. The boundaries of these fragments lie in the linker between histidine 98, the most C-terminal Zn²⁺-binding residue of zinc finger 3, and cysteine 105, the first Zn²⁺-binding residue of finger 4.

pTA1–100 was constructed by inserting a polymerase chain reaction (PCR)-generated fragment containing the first 100 codons of TFIIIA between the NdeI and BamHI sites of pET11b (38). Specifically, the relevant DNA fragment was synthesized from the plasmid pTA102 using the 5′ primer 5′-CTTTAAGAAGGAGATA and the 3′ primer 5′-TGAACGGGTACCTCAGATGTTATGGAACAT. The PCR product was double cut with NdeI and BamHI, and the cut product was gel-purified, concentrated by precipitation in ethanol, and ligated into the large NdeI/BamHI vector fragment of pET11b. Plasmids from resulting colonies were screened by digestion with PstI, and a positive clone sequenced throughout the A1–100-coding region to ensure that no unanticipated mutations were introduced by the PCR process.

To construct pTA100–344, an NdeI site was introduced into the plasmid pGA13 (see below) in place of TFIIIA codons 98 and 99 by site-directed mutagenesis (39), using the anticyodon strand primer CCTCTTTCTATCTGCTGTTAAGG. Potential positives were screened for introduction of the new NdeI site, and a single clone was chosen and sequenced throughout the TFIIIA-coding region to verify the expected alteration. An NdeI/BamHI fragment from this plasmid was ligated into NdeI/BamHI-cleaved pET11b to generate pTA100–344.

Plasmids encoding mutant TFIIIA proteins with two-finger disruptions were generated in the plasmid pTA105. pTA105 was constructed by insertion of the NdeI-BamHI fragment of pGA13–894 between the NdeI and BamHI sites of pET11b. N-terminal to codons 93–894 was a site for directed mutagenesis of pGA13 and contains a substitution of the codons GAG AAG for codons 309–311 of TFIIIA, which are GAA AAA AAT in the wild type. These alterations do not affect the sequence of TFIIIA encoded and were made for reasons that are irrelevant in the context of this paper. pGA13 was derived from pGA11–NdeI (23) and contains the EP (300–304) sequence changes that have been described previously in the generation of pTA102 (23) but is otherwise identical to pTA11–NdeI. pET11b-150 was prepared by excising the Alu/N1-PerI fragment of pZ150 (40) that contains the M13 replication origin and using it to replace the corresponding Alu/N1-PerI fragment of pET11b. pTA105 therefore can be used to express wild-type TFIIIA protein from the T7 promoter in pET11b and also can be used to obtain single-stranded DNA for purposes of directed mutagenesis or sequencing. In pTA105, the single-strand packaged in phage particles corresponds to the anti-codon-sense strand of TFIIIA. Five codon-sense primers were used for introduction of the histidine to asparagine substitutions in the mutant sequences; the H35N mutation and a HindIII site were introduced using TGGAAAGCTTCAAGGCGAATCTG; the H63N mutation and an EcoRI site were introduced using TTAACCCGGAATCTCAGCTT; the H132N mutation and a DraI site were introduced using GATC- TATATTAAAAAACGTTGACG; the H241N mutation and a HindIII site were introduced using AAGAACCAATATACAAAGGCTTCCAT; the H272N mutation and an EcoRI site were introduced using CTA-GAAAAGGATCAGT; the H327N mutation and an EcoRI site were introduced using CTA-GAAAAGGATCAGT. In the construction of the double mutants, two mutagenic primers were used simultaneously in a standard oligonucleotide-directed mutagenesis experiment (39). Resulting clones were screened for the introduction of both mutations using the restriction site tags associated with the desired changes. The presence of the desired mutations was then verified by sequencing the entire TFIIIA coding sequence from a single positive clone.

**Expression and Purification of Mutant TFIIIA Proteins—**Plasmids encoding each construct were transformed into E. coli strain BL21(DE3), and mutant TFIIIA proteins were expressed and purified as described previously (38). With the following modifications: We found A1–100 to be considerably more soluble than wild-type TFIIIA in E. coli, so recombinant A1–100 was recovered in the initial supernatant after cell lysis by sonication. Further extraction of the insoluble pellet with urea was unnecessary. In contrast, A100–344 is substantially more difficult to solubilize than is wild-type TFIIIA, so initial cell lysis by sonication was performed in the presence of 5 M urea, and the
remaining pellet after centrifugation was extracted in 5 mM urea-containing buffer for several days to recover reasonable yields of solubilized protein. Full-length mutants with two disrupted zinc fingers behaved similarly to wild-type TFIIIA and the single-finger mutants (9). After ammonium sulfate fractionation and binding to Bio-Rex 70 in 5 mM urea as described previously (33), the complexes were dried and eluted in a single step with binding buffer containing 1 mM NaCl and no urea. This modification aided in keeping the proteins soluble without adversely affecting purity of the final product.

In most cases, mutant proteins were further purified on phenyl-Superose or phenyl-Sepharose as described previously (23). In some cases, additional purification was achieved instead by chromatography of the Bio-Rex 70-eluted material on Superose 12 in buffer identical to that used to elute TFIIIA from the Bio-Rex 70 column. Whole recovery was sometimes lower using the Superose 12 column, we often found that a higher percentage of the purified protein was active when assayed for DNA binding. In some cases, further purification beyond the Bio-Rex 70 stage proved technically infeasible, and Bio-Rex 70-eluted material was used directly in the analysis.

To ensure that the double broken finger mutants contained structural disruptions in the proteins only within the mutated fingers and that no unanticipated longer range structural “cooperativity” resulted in more global or extended unfolding of the mutant proteins, we analyzed H33N/H63N, H241N/H272N, H33N/H272N, H33N/H241N, H63N/H241N, and H63N/H272N by partial proteolysis with trypsin or, alternatively, in samples that had been reduced in 8.33M urea but lacking formamide.

**RESULTS**

We have argued previously from the quantitative analysis of equilibrium binding of TFIIIA broken finger mutants to the X. borealis 5 S RNA gene that energetically unfavorable interactions might exist between zinc fingers in the DNA binding reaction (9). Since others had already shown that the first three zinc fingers of TFIIIA bound to DNA fragments from the 5 S RNA gene ICR with high affinity, corresponding to a $\Delta G^o$ for the binding reaction that was greater than 90% of the $\Delta G^o$ for the binding of full-length wild-type TFIIIA (33), it seemed likely that the complementary C-terminal fragment of TFIIIA would not bind the 5 S RNA gene with detectable affinity, unless the two moieties of the protein interacted in an energetically unfavorable fashion when bound to DNA. Consequently, we decided to investigate the binding of such complementary N-terminal and C-terminal TFIIIA fragments to the 5 S RNA gene. Recombinant forms of two polypeptides, A1–100 and A100–344, were prepared and analyzed. A1–100 consists of the first 100 amino acids of wild-type Xenopus laevis TFIIIA, and A100–344 consists of the last 245 amino acids of TFIIIA preceded by a methionine residue. The boundaries of these fragments lie in the linker between histidine 98, the last Zn“+”-binding residue of zinc finger 3 and cysteine 105, the first Zn“+”-binding residue of zinc finger 4. Thus, A1–100 consists of the first three fingers of TFIIIA and A100–344 the last six fingers plus the C-terminal non-zinc finger domain. Between them, A1–100 and A100–344 contain all the components of intact TFIIIA with a duplication of only the single amino acid at position 100.

Equilibrium binding constants ($K_d$ values) and $\Delta G^o$ values for the binding reactions were determined for each of the two truncated forms of TFIIIA using exactly the same methodology we used previously in the analysis of the broken finger series of TFIIIA mutants (9). The results are shown in Table I. A1–100 binds with 90% the binding energy of wild-type TFIIIA, consistent with the result of Liao et al. (39) in which an A1–101 fragment had 95% the binding energy of wild type. Although qualitative studies had not detected binding of C-terminal fragments of TFIIIA to the 5 S RNA gene (26, 33–35, 45), we found that A100–344 binds to the ICR with considerable affinity, the $\Delta G^o$ of the binding reaction between A100–344 and the 5 S RNA gene is 83% that exhibited by wild-type TFIIIA. This result suggests that the two moieties of TFIIIA contained in A1–100 and A100–344 are not interacting with the 5 S RNA DNA complex.
independently in the context of full-length, wild-type TFIIIA. In fact, the affinity of the intact protein for the 5 S rRNA gene is severely compromised relative to that which could be achieved by the two halves of the protein binding independently in a single polypeptide. It thus appears that the N- and C-terminal moieties of TFIIIA interfere with one another energetically when binding to the 5 S rRNA gene.

This conclusion depends upon the assumption that the portions of TFIIIA contained in A1–100 and A100–344 occupy the same sites on the 5 S rRNA gene whether present in intact TFIIIA or in the truncated polypeptides. We have addressed this issue through DNase I protection analysis of complexes containing the 5 S rRNA gene and TFIIIA, A1–100, or A100–344. The results of such an analysis are shown in Fig. 1A. Consistent with several earlier reports (28, 32, 33), the N-terminal three-finger construct protects about 20 base pairs at the 3’ end of the ICR. The 3’ end of the A1–100 footprint is identical to that of full-length TFIIIA, both with respect to the boundary of protection and to the presence of a hypersensitive site between nucleotides 92 and 93. A100–344 protects about 20 base pairs at the 5’ end of the ICR, essentially the entire region not protected by A1–100. There is strong protection of about 15 base pairs from nucleotides 45 to 61 and weaker protection of another 15 base pairs extending to nucleotide 76. The 5’ boundary of the A100–344 footprint is identical to that of intact TFIIIA. Template strand protection by the TFIIIA fragments was less complete but consistent with the nontemplate strand footprints (data not shown). The existence and location of these footprints show that A1–100 and A100–344 bind to the 5 S rRNA gene not only with high affinity but also with sequence specificity, a conclusion that we have confirmed by analysis of covariance using data sets from the independent Kd determinations. The relative Kd is normalized to that of wild-type TFIIIA. The data for wild-type TFIIIA are from Table II of Del Rio et al. (9).

| Fragment     | Fingers deleted | N   | n  | Kd          | Relative Kd | ΔG°   | Percent of wild-type ΔG° |
|--------------|----------------|-----|----|-------------|-------------|-------|-------------------------|
| Wild-type    |                | 6   | 48 | 0.416 ± 0.03| 1.0         | −12.9 |                         |
| A1–100      | 4–9            | 6   | 52 | 4.00 ± 0.66 | 9.6         | −11.47| 89.7                    |
| A100–344    | 1–3            | 3   | 29 | 15.5 ± 2.3  | 37.2        | −10.66| 83.3                    |

Sum of fragment ΔG° values: −22.13 173.0

(1B), we have also analyzed the simultaneous binding of A1–100 and A100–344 to the 5 S rRNA gene using DNase I protection. When these TFIIIA fragments are bound to the 5 S rRNA gene at the same time, they result in a DNase I protection pattern that clearly demonstrates binding at both ends of the internal control region. In fact, the footprint conferred by the simultaneous binding of A1–100 and A100–344 is exactly that predicted by combining the individual A1–100 and A100–344 footprints seen in Fig. 1A. These results therefore demonstrate that A1–100 and A100–344 can bind simultaneously to the 5 S rRNA gene, strengthening the conclusion that the two truncation mutants bind to the same sites occupied by their corresponding moieties in intact, wild-type TFIIIA. It is interesting to note that the low mobility complex generated by the simultaneous binding of A1–100 and A100–344 migrates more rapidly than the complex containing full-length TFIIIA and the 5 S rRNA gene (Fig. 2). We consider the implications of this observation more fully below.

The preceding results clearly demonstrate the existence of an unfavorable thermodynamic interaction between one or more elements contained in the two moieties of TFIIIA represented in A1–100 and A100–344. To localize this energetically unfavorable interaction to smaller structural elements and to quantify it in the context of an intact TFIIIA molecule, a series of full-length TFIIIA variants was constructed in which two zinc fingers at a time were disrupted by the same histidine to asparagine mutations used in our previous study of broken finger mutants (9). To explore the possibility of both long range and short range effects, the two outermost fingers at each end of TFIIIA were disrupted in all six pairwise combinations. In addition, a finger-6/finger-8 double mutant was constructed since these two single finger disruptions resulted in footprint alterations that overlapped, suggesting that structural interactions between fingers 6 and 8 might exist. In the absence of energetically significant interactions between a particular pair of zinc fingers and subject to the caveat noted below, the equilibrium binding constant (and ΔG°) of the double mutant should be predicted by the Kd values of the corresponding single mutants. If a thermodynamically unfavorable interaction takes place between a pair of fingers, the double mutant should bind more weakly than predicted by the binding properties of the relevant single mutants. Jencks (46) has treated the additivity of binding energies formally when considering interactions between different moieties of a protein and its substrate. In general, such binding energies are not simply additive. The free energy change upon binding of a protein to a substrate containing moieties A and B can be represented as ΔG AB = ΔG A + ΔG B + ΔG conn where ΔG conn describes the thermodynamic interaction between domains or recognition subsites involving the A and B moieties. In the simplest case, ΔG conn can be considered a favorable and largely entropic term that derives from the presence of two substrate-binding moieties in the same protein and the two subsites for binding in the
same substrate molecule; in the current instance of multivalent DNA binding by TFIIIA, a favorable entropic contribution would result from the presence of multiple DNA-binding moieties in TFIIIA and the presence of the subsites for binding in the same DNA molecule. This means that any thermodynamically unfavorable interaction detected by comparing the binding of a double broken finger mutant to the binding predicted from the properties of the single finger mutants would represent a minimum estimate of the energetic interference. Small, energetically unfavorable effects would be masked by the favorable entropic effect that must exist as a consequence of multivalent binding.

Table II shows the equilibrium dissociation constants of all seven TFIIIA double mutants. In Table III, we have tabulated the arithmetic differences in the $\Delta G^0$ values for the binding reactions involving each double mutant and wild-type TFIIIA ($\Delta \Delta G^0$) and have further compared them to the differences that would be predicted if the energetic effects of the individual finger disruptions were merely additive in each case. The actual $\Delta \Delta G^0$ is greater than would be expected in the case of simple additivity for four double mutants, those with disruptions in fingers 1 and 8, fingers 1 and 9, fingers 2 and 8, or fingers 2 and 9. We have treated the discrepancy between predicted and actual $\Delta \Delta G^0$ values as "strain" existing in the TFIIIA-DNA complex. The amount of strain energy for each pairwise interaction is indicated in the last column of Table III; because of the energetically favorable entropic effect noted above, this is a minimum estimate. For double mutants containing mutations in fingers 1 and 2, 8 and 9, or 6 and 8, the effects of finger disruptions are simply additive, within experimental error, meaning that any unfavorable interaction which might exist between fingers 1 and 2, fingers 8 and 9, or fingers 6 and 8 is smaller than the favorable entropic interaction. The magnitude of this entropic effect is difficult to predict, although it is likely to be small in this particular analysis because of the retention of seven or eight of the nine zinc fingers in the mutants analyzed.

While the "strain energies" we calculate for interactions between pairs of zinc fingers are generally small relative to the actual $\Delta G^0$ values of the binding reactions, we believe that there is little doubt that they are genuine relative to the precision of the $K_d$ measurement. This is illustrated for three of the double mutants in Fig. 3, where aggregate data from multiple $K_d$ determinations for a single protein are rescaled to permit display on a single Scatchard plot. In each case, the
actual data and a best-fit line that describes the $K_d$ values are compared with the line corresponding to the predicted $K_d$ if finger disruptions were merely additive with respect to $\Delta G^0$. The finger 1 + 9 and 2 + 8 double mutants, the latter of which exhibits the smallest finger interaction energy that we consider significant, clearly bind with higher $K_d$ values than would be predicted. In contrast, the finger 1 + 2 double mutant binds to the 5 S rRNA gene with a $K_d$ that is indistinguishable from that predicted by the binding affinities of the single finger mutants.

In Fig. 4, we have plotted the discrepancies in the measured versus predicted $K_d$ values of the double finger mutants as a function of the distance between the mutated fingers. The striking result is that the greatest thermodynamically unfavorable interactions occur between zinc fingers that are distant from one another in the protein and that the effect decreases monotonically as the inter-finger distance decreases. Thus, adjacent fingers or those separated by a single intervening zinc finger exhibit no apparent energetic interaction. We consider this pattern to be most remarkable and discuss its implications further below (see “Discussion”).

While the thermodynamic data just presented demonstrate unequivocally the existence of energetic strain in the TFIIIA-5 S rRNA gene complex, they say little about the structural basis for the strain. It is noteworthy, however, that others have described previously the existence of TFIIIA-induced distortions in the 5 S rRNA gene (40–42, 47). One experimental manifestation of this distortion is the existence of systematic variations in the electrophoretic mobility of TFIIIA-DNA complexes depending upon the position of the 5 S rRNA gene ICR within the DNA fragment being studied. In fact, these “circular permutation assays” have been used to define an apparent bend angle in the DNA of about 60° in TFIIIA-DNA complexes (40–42). Because it seemed possible that DNA bending could account for some or all of the energetic strain detected in TFIIIA-DNA complexes, we decided to measure the apparent bend angles produced by binding of wild-type TFIIIA and by A1–100 and A100–344, the N- and C-terminal fragments of TFIIIA that both exhibited high affinity binding to the 5 S rRNA gene. Using a circular permutation assay and methods of analysis very similar to those described previously (41, 42), we obtained the results summarized in Table IV.

Our estimate of the bend angle induced by wild-type TFIIIA binding, 61°, is in excellent agreement with values reported previously by others (40–42). Interestingly, however, both A1–100 and A100–344 individually produce only very small bends (about 25°) in the 5 S rRNA gene. Furthermore, simultaneous binding by A1–100 and A100–344 results in a similar small bend (about 30°) in the DNA. Thus, the major bend produced by wild-type TFIIIA is dependent upon linkage of the N- and C-terminal zinc fingers of the protein in a single polypeptide. Separation of the N- and C-terminal fingers results in high affinity binding of the two protein fragments to their normal sites without the necessity of bending the 5 S rRNA gene. These results are consistent with but do not prove that at least some of the energetic strain we have demonstrated in the TFIIIA-5 S rRNA gene complex results from bending of the DNA necessary to permit simultaneous binding by the N- and C-terminal zinc fingers of TFIIIA.

If DNA bending is the source of the thermodynamic strain we detect in the TFIIIA-DNA complex, then there should be a correlation between pairs of zinc fingers exhibiting the greatest unfavorable thermodynamic interaction and those most important for DNA bending. We have shown above that the thermodynamic interaction between individual zinc fingers in TFIIIA is greatest when the fingers are most distant from one another. Thus, assuming that DNA bending is the source of the thermodynamic strain we have identified, one would predict that the largest effects of individual zinc finger disruption on DNA bending would be observed when terminal zinc fingers were mutated. We therefore tested the hypothesis that the thermodynamic strain in the TFIIIA-DNA complex derives primarily or exclusively from DNA bending by measuring the apparent bend angle induced by binding of each of the broken finger mutants to the 5 S rRNA gene.

In fact (Table V), the results we obtained are not consistent with this simple hypothesis. When the nine single finger mutants are analyzed, we find that all but three bend the 5 S rRNA gene equivalently to wild-type TFIIIA. The three exceptions are the finger-1, finger-2, and finger-3 mutants; among these, the finger-3 mutant results in the greatest reduction in
apparent bend angle (to 46°), and the finger-1 mutant produces a bend of 53°, a marginal reduction from the 61° produced by wild type. In particular, the finger-8 and finger-9 mutants produce apparent bend angles that are not significantly different from that exhibited by wild-type TFIIIA, and the same is true of the finger-8/finger-9 double mutant. In fact, the double finger mutants produce bend angles that are consistent with combining the effects of the single finger mutants. Thus, it is unlikely that bending the DNA to accommodate simultaneous binding of all nine zinc fingers of TFIIIA can account fully for the thermodynamic interactions we have observed between zinc fingers in the DNA binding reaction. Instead, the structural basis for the energetic effects we observe is likely to be more complex and involve elements of TFIIIA structure and its distortion as well.

**DISCUSSION**

Our results with complementary N- and C-terminal truncation mutants as well as double finger disruption mutants demonstrate the existence of an unfavorable thermodynamic interaction between zinc fingers of TFIIIA in the DNA binding reaction. Although the mechanistic source of this interaction has not been determined directly, we have considered three possibilities. First, it is possible that the high binding affinities observed for the N- and C-terminal truncation mutants are artifacts resulting from an altered mode of interaction of the TFIIIA fragments with the 5 S rRNA gene. This seems unlikely, however, since the footprints obtained with the truncation mutants are non-overlapping and, when taken together, correspond to the full-length TFIIIA footprint. Furthermore, A1–100 and A100–344 are capable of simultaneous binding to

**TABLE IV**

| Fragment | n  | Apparent bend angle |
|----------|----|---------------------|
| Wild-type| 28 | 61.4 ± 3.1          |
| A1–100  | 7  | 23.6 ± 3.1          |
| A100–344| 4  | 24.3 ± 2.1          |
| A1–100 + A100–344 | 3 | 30.7 ± 2.6 |

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**DISCUSSION**

Our results with complementary N- and C-terminal truncation mutants as well as double finger disruption mutants demonstrate the existence of an unfavorable thermodynamic interaction between zinc fingers of TFIIIA in the DNA binding reaction. Although the mechanistic source of this interaction has not been determined directly, we have considered three possibilities. First, it is possible that the high binding affinities observed for the N- and C-terminal truncation mutants are artifacts resulting from an altered mode of interaction of the TFIIIA fragments with the 5 S rRNA gene. This seems unlikely, however, since the footprints obtained with the truncation mutants are non-overlapping and, when taken together, correspond to the full-length TFIIIA footprint. Furthermore, A1–100 and A100–344 are capable of simultaneous binding to
the 5 S rRNA gene, which would not be expected if either of the polypeptides assumed a substantially different position relative to that occupied by the same zinc fingers in the context of wild-type TFIIIA. Nonetheless, it is difficult to exclude completely the possibility that local interactions with the 5 S rRNA gene are altered in the truncation mutants. It is therefore prudent to interpret results obtained with protein fragments with some caution, at least if one is concerned primarily with the mechanism of binding of the intact protein. Second, it is possible that there is a direct structural or steric interference between zinc fingers when they bind to their respective sites in the 5 S rRNA gene. This seems unlikely, however, since the greatest energetic interference we observe is between zinc fingers at opposite ends of the protein, and adjacent fingers show little or no thermodynamic interaction. It is difficult to imagine that direct steric clashes are the cause of the functional interaction between fingers 1 and 9, whereas no effect is observed between fingers 1 and 2 or fingers 8 and 9. The third possibility, which we favor, is that simultaneous binding of zinc fingers at opposite ends of TFIIIA requires energetically unfavorable distortions in the DNA, the protein, or both relative to the preferred conformations of the free DNA and protein. This model accounts for the largest interaction occurring between well separated zinc fingers with a monotonic decrease in the magnitude of the unfavorable energetic interaction as the inter-finger separation is decreased.

Interestingly, previous data have shown that the 5 S rRNA gene is distorted structurally as a consequence of binding to TFIIIA (41, 42, 47), and we have confirmed these earlier studies in circular permutation assays of DNA bending induced by TFIIIA. It is certainly possible that DNA bending is required for simultaneous occupancy of the 5 S ICR by all nine zinc fingers of TFIIIA and that this bending is responsible for a portion of the unfavorable thermodynamic interaction we have described. Our results demonstrating that the A1–100 and A100–344 fragments of TFIIIA bind the 5 S rRNA gene with high affinity while producing only a small bend in the DNA, even when bound simultaneously, are consistent with a contribution of DNA bending to the energetic strain present in the TFIIIA-DNA complex. On the other hand, analysis of the DNA bending angles induced by the various single and double broken finger mutants indicates that DNA bending alone is unlikely to account for all of the thermodynamic interference we observe between zinc fingers. In a recent paper, Brown et al. (40) have estimated the energetic cost of bending the 5 S rRNA gene to accommodate TFIIIA binding at only about 1 kcal/mol. While the method used to make this estimate might well under-estimate the actual cost of TFIIIA-induced distortion in the DNA, a value of 1 kcal/mol is nonetheless much less than even a minimum estimate of the degree of thermodynamic interference we have noted (at least 9.2 kcal/mol for the interaction between the A1–100 and A100–344 moieties of TFIIIA, for example). Thus, these results also suggest the existence of an alternative structural source of energetic strain. It is possible that there are additional TFIIIA-induced distortions in the 5 S rRNA gene that are not detected in circular permutation assays (as suggested in the electron spectroscopic imaging experiments of Bazett-Jones and colleagues (47), for example). It is perhaps even more likely that TFIIIA must also undergo energetically unfavorable conformational changes to permit simultaneous binding to the 5 S rRNA gene by all nine zinc fingers. Analysis of DNA binding by TFIIIA using fluorescence spectroscopy suggests that the protein does undergo some conformational changes upon binding to the 5 S rRNA gene (48), but the energetic costs of such changes have not been estimated. Ultimately, it will be important to link quantitatively the results of structural and thermodynamic assays to provide a rigorous structural explanation for the thermodynamic interactions we have observed. Unfortunately, this is will be technically difficult and is unlikely to be achieved in the near future.

The fact that TFIIIA, either alone or in complexes with DNA or RNA, has been refractory to production of highly ordered crystals (49) and the fact that it is too large to be studied by multi-dimensional NMR methods has made it necessary to approach high resolution structural questions through the analysis of TFIIIA fragments. We have noted above that, although the possibility cannot be excluded, nothing in our data suggests that the local interactions between DNA and TFIIIA are altered as a result of the protein truncation. Nonetheless, our data do clearly make the point that the properties of TFIIIA bound to DNA, and particularly the thermodynamic parameters governing the binding equilibrium between TFIIIA and the 5 S rRNA gene, cannot be deduced from the DNA binding properties of TFIIIA fragments. In fact, the thermodynamics of the binding reaction for TFIIIA is dominated by a large energetically unfavorable term that is lost when the two ends of the protein are separated from one another. It is therefore clear that factors governing the binding equilibrium must be assessed in the context of the full-length protein and that a complete description of all the local contacts made between TFIIIA and the 5 S rRNA gene would not provide an adequate accounting of the forces governing the binding reaction. Previous studies on TFIIIA fragments have given rise to the attractive and powerful notion that the remarkable ability of TFIIIA to bind specifically to both 5 S rRNA and the 5 S rRNA gene can be explained by the use of different subsets of zinc fingers for specific recognition of DNA and RNA (29, 30, 35). Thus, DNA binding would be conferred primarily by the three N-terminal zinc fingers while RNA binding would be the responsibility of the middle 3–4 fingers of TFIIIA. Our current and previous data (9, 50) make it clear that this simple model is inadequate and, in some ways, misleading. Clearly, all the zinc fingers of TFIIIA are involved in sequence-specific DNA binding, and the relative importance of various fingers or subsets of fingers cannot be assessed in a context-independent fashion. For example, do the three N-terminal fingers of TFIIIA really provide most of the binding energy for 5 S rRNA gene recognition because A1–100 binds with a $\Delta G^0$ that is 90% the $\Delta G^0$ for wild-type TFIIIA binding? If so, then one must paradoxically conclude that fingers 4–9 also provide most of the binding energy, since the $\Delta G^0$ for A100–344 binding is 83% that for wild-type TFIIIA. Similarly, it is impossible to define

| Mutant        | Fingers mutated | $n$ | Apparent bend angle |
|---------------|-----------------|-----|---------------------|
| Wild-type     | None            | 28  | 61.4 ± 3.1          |
| H3N           | 1               | 3   | 53.0 ± 0.5          |
| H6N           | 2               | 3   | 48.0 ± 0.6          |
| H9N           | 3               | 3   | 46.1 ± 1.8          |
| H12SN         | 4               | 3   | 58.6 ± 2.1          |
| H155N         | 5               | 5   | 63.2 ± 4.9          |
| H183N         | 6               | 3   | 68.7 ± 1.0          |
| H210N         | 7               | 3   | 63.2 ± 3.1          |
| H241N         | 8               | 3   | 58.4 ± 1.4          |
| H272N         | 9               | 5   | 64.6 ± 7.6          |
| H33N/H272N    | 1 + 9           | 4   | 57.0 ± 6.5          |
| H241N/H272N   | 8 + 9           | 3   | 62.0 ± 6.0          |
| H33N/H63N     | 1 + 2           | 4   | 46.6 ± 3.7          |
the energetic contribution made by any particular zinc finger to DNA binding without specifying the status of the rest of the protein. Does finger 1 really contribute 0.96 kcal/mol greater binding energy in the absence of finger 9 than in its presence, as a simple-minded interpretation of the data of Table III suggests? The resolution to these apparent paradoxes is to realize that the thermodynamics of DNA binding by TFIIIA is affected to a large extent by functional interactions between zinc fingers, probably as a result of energetically unfavorable distortions that are necessary to accommodate simultaneous binding by all nine zinc fingers. As a consequence, it is simply impossible to define the absolute energetic importance of individual fingers in a way that is truly meaningful.

It is interesting to consider the possible functional implications of this mode of DNA binding. Previously, we referred to it as compensatory binding (9), because the unfavorable interactions between zinc fingers at the opposite ends of the protein provide a kind of energetic buffering in the TFIIIA-5 S rRNA gene complex. Thus, loss of binding by one or more zinc fingers results in a complex that has only marginally reduced stability. One of the puzzling features of 5 S rRNA gene transcription is that direct disruption of a subset of zinc fingers, probably as a result of energetically unfavorable distortions that are necessary to accommodate simultaneous binding by all nine zinc fingers. As a consequence, it is simply impossible to define the absolute energetic importance of individual fingers in a way that is truly meaningful.

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