Is There a Dynamic DNA-Protein Interface in the Transcription Factor IIIA-5 S rRNA Gene Complex?*

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Others (Foster, M. P., Wuttke, D. S., Radhakrishnan, I., Case, D. A., Gottesfeld, J. M., and Wright, P. E. (1997) Nat. Struct. Biol. 4, 605–608; Wuttke, D. S., Foster, M. P., Case, D. A., Gottesfeld, J. M., and Wright, P. E. (1997) J. Mol. Biol. 273, 183–206) have proposed that several amino acid side chains exhibit considerable conformational mobility at the DNA-protein interface in the transcription factor IIIA-5 S rRNA gene complex and that the rapid movements of these side chains permit them to make fluctuating contacts with adjacent bp in the DNA target site. This “dynamic interface” model makes biochemical predictions concerning the consequences of truncating specific amino acid side chains and the effects of these truncations on sequence selectivity in DNA binding. The model also makes predictions concerning the effects of DNA sequence context on the apparent energetic contributions to binding made by individual bp. We have tested these predictions, and our results are inconsistent with any significant energetic role being played by the contact of multiple bp by conformationally mobile amino acid side chains. They do, however, show that some individual amino acids affect the recognition of multiple bp through mechanisms other than direct interaction.

Transcription factor IIIA (TFIIIA)1 from Xenopus laevis exhibits a number of unusual features that make it of special interest in the study of nucleic acid-protein interactions. These include its ability to recognize both DNA (the internal control region of the 5 S rRNA gene) and RNA (5 S rRNA) with high affinity and specificity (1–4), its unusually large number of zinc fingers (nine), and correspondingly large DNA binding site (5–8), the complex thermodynamics with which it recognizes both DNA (9, 10) and RNA (11, 12), and its status as the archetypal zinc finger protein (13). Although it has been the subject of numerous biochemical studies, direct determination of the structure of the TFIIIA-5 S rRNA gene complex has been limited to two TFIIIA fragments bound to portions of the 5 S rRNA gene internal control region. Wright and colleagues (14, 15) have used nuclear magnetic resonance methods to determine the structure of the first three zinc fingers of TFIIIA bound to a 13-bp fragment of the 5 S rRNA gene, and Nolte et al. (16) have described a structure for the first six zinc fingers of TFIIIA bound to a 32-bp fragment of the same gene in a DNA-protein co-crystal. The structural models resulting from these studies confirmed the existence of some DNA-protein interactions in the TFIIIA-5 S rRNA gene complex that were predicted from the previously determined structures of other zinc finger proteins of the TFIIIA class (17–20) and also revealed previously undescribed features of DNA recognition by a zinc finger protein.

Among the most interesting novel features of recognition proposed by Wright and colleagues was the existence of a dynamic DNA-protein interface in the complex of the three N-terminal zinc fingers of TFIIIA bound to bp 80–92 in the 5 S rRNA gene. In particular, Foster et al. (15) and Wuttke et al. (14) proposed that several amino acid side chains involved in direct contact with DNA bp in the complex undergo rapid conformational fluctuations (in the μs–ms time range). Furthermore, the authors proposed that these fluctuations were of a spatial magnitude that permitted the amino acid side chains in question to make nearly isoenergetic contacts with multiple DNA bp through rapid, sequential conformational interconversions. This mechanism would thereby permit individual functional groups to contribute to the specific recognition of multiple bp within the target DNA molecule. Furthermore, the mobility of these amino acid side chains at the DNA-protein interface would contribute directly to the thermodynamics of binding by reducing the entropic penalty associated with immobilizing the side chain at a specific, static position in the DNA-protein complex. The existence of a dynamic, rather than a more conventional static, DNA-protein interface is of considerable interest and could have a profound effect on our understanding of DNA-protein recognition in general if such a mechanism were widespread in DNA-protein complexes.

The dynamic interface model for the TFIIIA-5 S rRNA gene complex was based in part on the observation that the spatial locations of the several amino acid side chains in question were poorly constrained by the distance constraints provided by the NOE data (14, 15). Of course, it is possible that the apparent flexibility of the amino acid side chains in question is an artifact of an inadequate number of distance constraints that, if obtained, would have eliminated models in which amino acid side chains assume conformations that are not permitted in reality. Foster et al. (15) nonetheless offer several arguments, based on the broadening of particular lysine side chain resonances and rapid relaxation times for 13C nuclei in heteronuclear HCCH-TOCSY spectra of the same lysine side chains, that make such an interpretation less likely. Furthermore, one of these residues, Lys-92, exhibits NOEs to bases on opposite DNA strands, strongly suggesting that this amino acid side chain populates, at some level, multiple conformational states. As a further complication, however, a model for the structure of

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* The abbreviations used are: TFIIIA, transcription factor IIIA; NOE, nuclear Overhauser effect.

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the same region of the TFIIIA-5 S rRNA gene interface derived from x-ray crystallography gave no indication of conformational flexibility involving the several amino acid side chains in question (16). Of course, it is possible that the more distributed electron density associated with mobile amino acid side chains would be difficult to detect crystallographically, particularly if some conformations were underpopulated relative to others. It is also worth noting that the reported resolution of the crystallographic data was only ~3.1 Å, making assignment of specific protein-DNA contacts somewhat difficult and the prospects of detecting multiple conformational states rather poor.

Our interest in the general problem of DNA-protein recognition and our particular focus on the TFIIIA-DNA complex, as well as the intellectual attractiveness of the dynamic interface model, led us to design and carry out a series of biochemical studies designed to provide an independent set of tests of this model. We report the results of these studies here and conclude that any mobility in amino acid side chains that does exist at the TFIIIA-5 S rRNA gene interface is unlikely to contribute significantly to the energetics of multiple bp recognition by a single amino acid side chain. Instead, our data are consistent with a more conventional static interface, albeit one in which there is substantial thermodynamic coupling in the recognition of at least some adjacent bp. We also believe that the experimental approach we describe could prove useful in biochemically defining DNA-protein contacts in other complexes and in testing for thermodynamic coupling in the recognition of multiple bp.

**EXPERIMENTAL PROCEDURES**

**Preparation of Labeled DNA Fragments**—A collection of mutant Xenopus borealis somatic type 5 S rRNA genes in the plasmid pST5HD (21) was prepared in our laboratory a number of years ago. This collection includes all possible single point mutations between and including bp 45 and 96 in the 5 S rRNA gene; a number of the mutants have been analyzed previously with respect to TFIIIA binding affinity (22). In the current study, labeled DNA fragments containing the wild-type or mutant gene were prepared from these plasmids using the PCR primer 759 (5’-CCGGGGAGAGAAAATGAGATU-3’) and either primer 190 (5’-GCTTGCATGCCACGCGTCTC-3’) or primer 290 (5’-GGTGCGATGCCACGCGTCTC-3’) or primer 290 (5’-GGTGCGATGCCACGCGTCTC-3’). Primer 759 was labeled with [γ-32P]ATP and polynucleotide kinase prior to its use in the PCR. The fragments were generated with primers 190 and 290 were 279 and 184 bp in length, respectively. Similar fragments containing double mutant 5 S rRNA genes were produced by overlap extension PCR. Complementary primers flanking the desired bp changes were synthesized and used individually in combination with either oligonucleotide 290/190 or oligonucleotide 759 to prepare, by PCR, short DNA fragments each consisting of a 5’- or 3’-moiety of the 5 S rRNA gene with flanking sequences. The products of the two reactions were gel purified, combined with 32P-labeled primer 759 and either oligonucleotide 290 or 190, and used to synthesize labeled PCR products of 279 (primer 190) or 184 (primer 290) bp, as described above for the single mutant 5 S rRNA gene fragments. All labeled fragments were purified by PAGE before being used in equilibrium binding reactions.

**TFIIIA Mutagenesis and Purification**—We used oligonucleotide-directed mutagenesis (21) to generate variants of phageencoded pTA105 (10) encoding various mutant forms of TFIIIA. Wild-type and mutant TFIIIA were expressed in *Escherichia coli* and purified through the Bio-Rex 70 column step as described previously (10, 23).

**Equilibrium Binding Reactions and Analysis**—Equilibrium binding reactions were set up in our standard binding buffer: 20 mM Tris-Cl, pH 7.5, 70 mM KCl, 7 mM MgCl2, 10 μM ZnCl2, 1 mM dithiothreitol, 10% (v/v) glycerol, 10 μg/ml poly(dI-dC), 100 μg/ml bovine serum albumin (8). Each reaction mixture also contained two 32P-labeled 5 S rRNA gene-containing DNA fragments of 279 and 184 bp, prepared as described above. Generally, DNA fragments were present at a concentration of 0.05–0.5 nM. Wild-type or mutant TFIIIA was added at a range of empirically determined concentrations, with the goal of obtaining between 15 and 80% of the DNA bound for each of the two DNA fragments. Reaction mixtures were incubated at 25 °C for 30 min before being loaded onto a running nondenaturing polyacrylamide gel prepared and run as described previously (8). After electrophoresis, the gel was dried and scanned using an Ambis Radioanalytic Imaging System. Radioactivity in the four bands obtained (two free and two bound) was quantified, and the relative *Kd* for binding of the form TFIIIA being analyzed to the two DNA fragments was determined using Equation 4 applied to each binding reaction mixture. If the fraction of DNA bound for either fragment was less than 0.15 or greater than 0.80, the data were discarded. All other results were used to prepare the data summary presented in Table I.

**RESULTS**

**Rationale and Experimental Design**—The dynamic interface model predicts that particular amino acid side chains contribute to the recognition of multiple bp in the internal control region of the 5 S rRNA gene. In particular, Wuttke et al. (14) predicted that at least five residues, Lys-26, Lys-29, His-58, His-59, and Lys-92, are involved in such fluctuating base contacts. Lys-26 was proposed to interact with the T residue of an AT bp at position 90, the G of a CG bp at position 91, and the G in a CG bp at position 92. Lys-29 makes putative contacts with the A of an AT bp at position 88, the G of a GC bp at position 89, and the intervening phosphate. His-58 and His-59 in finger 2 were also proposed to make fluctuating contacts to multiple bases, although multiple, specific contacts were suggested only for His-59, which could interact with G residues in GC bp at positions 86 and 87. As originally noted by the authors, however, the NMR data did not permit determination of the precise nature of the interactions of His-58 or His-59 with the DNA. Lys-92 in finger 3 may also contact multiple bp as a result of conformational fluctuations. Specifically, it was proposed to interact with adjacent G residues in GC bp at positions 81 and 82 as well as with a T residue on the opposite strand at position 83.

For illustrative purposes, the structure of the TFIIIA-5 S rRNA gene complex in the vicinity of the Lys-26, Lys-29, His-59, and Lys-92 side chains is provided in Fig. 1. The structures shown are based on the crystallographically determined spatial coordinates (16) and do not depict the amino acid side chain mobility predicted by the dynamic interface model. Alternative representations illustrating conformational fluctuations of these same residues can be found in Ref. 14.

The presence of specific contacts between amino acid side chains and particular DNA bp makes several biochemical predictions. Assuming a specific DNA-protein contact is energetically significant, mutation of the relevant bp should result in a decrease in equilibrium binding affinity (an increase in the *Kd*) of wild-type TFIIIA for the mutant 5 S rRNA gene relative to that observed with the wild-type gene. Furthermore, preferential binding to the wild-type gene should be dependent on the presence of the interacting amino acid side chain. Stated differently, removal of the relevant amino acid side chain should reduce the ability of the protein to distinguish between wild-type and mutant versions of the 5 S rRNA gene, if and only if the amino acid side chain in question is at least partially responsible for recognition of the wild-type bp. An experimental test of this prediction requires two things. First, one must be able to remove or truncate the putatively interacting amino acid, which can be accomplished readily by alanine substitution. Second, one must be able to measure with some precision the relative equilibrium binding constants for the interaction of a particular protein with either the wild-type or mutant 5 S rRNA gene.

To accomplish this second goal, we have used an assay in which the wild-type 5 S rRNA gene is contained on one radioactively labeled DNA fragment, and the mutant 5 S rRNA gene is present on a second labeled DNA fragment of a distinguishable size. The protein in question is incubated with a mixture of the two labeled DNA fragments, and the binding reaction is allowed to reach equilibrium. Under these conditions, two com-

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**Equation 4**

\[ K = \frac{[DNA][Protein]}{[DNA][Protein]} \]

where 

- \( [DNA] \) is the concentration of DNA
- \( [Protein] \) is the concentration of protein
- \( K \) is the equilibrium constant

**Table I**

| Protein | Wild-type | Mutant | Ratio |
|---------|-----------|--------|-------|
| Lys-26  | 1.0       | 0.5    | 0.5   |
| Lys-29  | 1.0       | 0.5    | 0.5   |
| His-58  | 1.0       | 0.5    | 0.5   |
| His-59  | 1.0       | 0.5    | 0.5   |
| Lys-92  | 1.0       | 0.5    | 0.5   |

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**Figure 1**

This figure illustrates the crystallographically determined spatial coordinates of the TFIIIA-5 S rRNA gene complex in the vicinity of the Lys-26, Lys-29, His-59, and Lys-92 side chains. The structures shown are based on the crystallographically determined spatial coordinates and do not depict the amino acid side chain mobility predicted by the dynamic interface model. Alternative representations illustrating conformational fluctuations of these same residues can be found in Ref. 14.
peting equilibria are established, represented by the equilibrium binding equations

$$K_{d1} = \frac{[P_f][D_f]}{[PD_1]}$$  \hspace{1cm} (Eq. 1)  \\
and  \\
$$K_{d2} = \frac{[P_f][D_f]}{[PD_2]}$$  \hspace{1cm} (Eq. 2)

where $P_f$ is free protein, $D_f$ is free DNA, $PD$ is the DNA-protein complex, and the superscripts denote the two different DNA fragments.

These two binding equilibria are coupled through the free (unbound) protein term, such that

$$\frac{[P_f]}{K_{d1}} = \frac{[PD_1]}{[D_f]}$$  \hspace{1cm} (Eq. 3)

Rearranging terms,

$$K_{d1}/K_{d2} = \frac{[PD_2]}{[PD_1]}$$  \hspace{1cm} (Eq. 4)

Thus, the ratio of equilibrium binding constants for the interaction of the binding protein with the two different DNA fragments can be determined from a single equilibrium binding reaction, so long as one can measure the four relevant experimental parameters: the concentrations of the two free (unbound) DNA fragments as well as the concentrations of the two DNA-protein complexes. In fact, it is necessary only that the relative concentrations be determined. By adjusting the DNA fragment lengths appropriately, we can readily resolve not only the two different free DNAs on nondenaturing polyacrylamide gels, but also the TFIIIA-bound forms of the two DNA fragments from each other and from both of the free fragments, as shown in the example of Fig. 2. Fragments of appropriate sizes are prepared by PCR using a common end-labeled primer for both fragments and a second primer that results in the production of fragments of differing length. The use of a common end-labeled primer ensures that the two DNA fragments are of identical specific activity; this simplifies some of the experimental analysis, even though it is not formally necessary. An advantage of this method of measuring relative equilibrium binding constants is that it requires no independent knowledge of protein or DNA concentration and instead requires only the empirical measurement of four experimental quantities in a single lane of a gel. Most systematic errors cancel out in the data analysis, and the relative binding affinities can be measured in a single binding reaction. Of course, multiple measurements at a variety of protein concentrations are carried out for
each comparison, and determination of the relative $K_d$ is most precise and exhibits the lowest variance when binding is carried out under certain conditions, including the use of DNA concentrations that are at or below the $K_d$ and when the fraction of DNA bound is neither very high nor very low (we limit our data analysis to those binding reactions in which the fraction of DNA bound is greater than 0.15 but less than 0.80). We have carried out control binding reactions using fragments of differing length but with the same 5 S rRNA gene sequence to ensure that there is no difference in binding affinity that results from variation in fragment length (Fig. 2, for example), and we have also analyzed pairs of 5 S sequences in which each member of the pair is present in the shorter and longer DNA fragments in separate experiments (data not shown). These controls demonstrated that there is no dependence of $K_d$ on DNA fragment length, at least over the size range we have used. We have used a variation of this approach previously to analyze binding of TFIIIA to 5 S rRNAs with backbone breaks in loop A (12). It is also conceptually similar to methods described by Gartenberg et al. (24) some number of years ago.

**Analysis of Lys-29**—A prediction of the dynamic interface model is that a single amino acid side chain is responsible in part for recognition of multiple bp. For example, recognition of the AT bp at position 88 and the GC bp at position 89 should depend, at least in part, on the presence of the wild-type lysine residue at position 29 in TFIIIA. Thus, using the assay described above, we measured the ability of wild-type TFIIIA to distinguish between the wild-type 5 S rRNA gene and mutant forms of the gene with substitutions at bp 88 or 89 compared with the ability of a K29A mutant of TFIIIA to do the same. The results are summarized in Table I and shown graphically in Fig. 3. Clearly, Lys-29 is important for the ability of TFIIIA to distinguish wild-type from mutant bp at both positions 88 and 89 in the 5 S rRNA gene. Although these data are consistent with the dynamic interface model, they do not exclude an alternative possibility involving cooperativity between static interactions. For example, it is possible that another amino acid contacts the GC bp at position 89, but does so in an energetically optimal way only when Lys-29 also contacts the AT bp at position 88. Similarly, optimal binding of Lys-29 to AT88 would be reciprocally dependent upon the interaction of the other amino acid residue with GC89. This would be a case of positive cooperativity in the binding of different amino acids to adjacent bp and could result from a mutually interdependent network of amino acid-bp interactions.

Fortunately, the dynamic interface and positive cooperativity models can be distinguished experimentally. Assuming the proposed multiple conformational states of the dynamic interface model are reasonably populated, the fluctuating side chain must make roughly isoenergetic contacts to multiple bp. Thus, mutation of one of these bp would have a relatively modest effect on binding affinity because the bp acting as the alternative binding partner for the amino acid side chain in question would remain available for interaction. As a consequence, the energetic cost associated with this mutation would be roughly equal to the entropic penalty of restricting the conformational freedom of the amino acid side chain. *A priori*, it would be difficult to define just what this energetic penalty might be in absolute terms, but it can be measured relative to the effect of the same bp mutation in the context of a 5 S rRNA gene containing a second mutation in the bp that constitutes the alternative binding partner. A clear prediction of the dynamic interface model is that the apparent energetic cost of mutating an interacting bp will be higher when the alternative binding partner is also mutated. In contrast, the positive cooperativity model makes the opposite prediction: the energetic cost of a bp mutation in this case will be greater when the 5 S rRNA gene is otherwise wild-type in sequence rather than when the cooperatively interacting bp is also mutated. This follows from the fact that a single point mutation exacts a thermodynamic penalty both because of the loss of direct contacts with TFIIIA and because of the loss of cooperative binding energy. Another way of describing the experimental test is to ask whether wild-type TFIIIA exhibits a decrease or an increase in its ability to distinguish a wild-type from a mutant bp when a second bp is also mutated. The difference between predicted results with the dynamic interface and positive cooperativity models can be formalized as

$$K_{d}^{wt}/K_{d}^{mut} < K_{d}^{mut1}/K_{d}^{mut2}$$  \(^{(Eq. 5)}\)

for the dynamic interface model and

$$K_{d}^{mut1}/K_{d}^{mut2} > K_{d}^{mut1}/K_{d}^{mut2}$$  \(^{(Eq. 6)}\)

for the positive cooperativity model, where $K_{d}^{mut1}/K_{d}^{wt}$ is the ratio of equilibrium binding constants for the binding of wild-type TFIIIA to either a 5 S rRNA gene containing a single bp mutation in position 1 or to a wild-type 5 S rRNA gene. Similarly, $K_{d}^{mut1}/K_{d}^{mut2}$ is the ratio of equilibrium binding constants for binding of wild-type TFIIIA either to a 5 S rRNA gene containing two mutations (bp 1 and 2) or to a 5 S gene containing only a single mutation in bp 2. A graphical representation of the predicted results of the two models is shown in Fig. 4. The results of an actual analysis of the role of Lys-29 in recognizing bp 88 and 89 are shown in Fig. 5, with the details of the quantitative analysis tabulated in Table I. We find that the apparent energetic importance of GC89 is greatest when AT88 is wild-type; that is, the apparent energetic contribution of GC89 to wild-type TFIIIA binding is reduced when AT88 is mutated, and this is true regardless of the identity of the mutation at position 89. This finding is clearly at odds with the prediction of the dynamic interface model but is fully consistent with the existence of a static interface with strong cooperativity in the recognition of adjacent bp by distinct functional groups. Surprisingly, the apparent energetic contribution of AT88 is largely insensitive to the identity of the bp at position 89. It is perhaps relevant that TFIIIA is only marginally capable of distinguishing between the wild-type (AT) and mutant (TA) bp at position 88 (relative $K_r \sim 1.85$) and that this discrimination is not entirely Lys-29-dependent in the GC89 mutant background. Nonetheless, we conclude that a dynamic interface involving fluctuating contacts of Lys-29 with both AT88 and GC89 is unlikely to exist unless the energetic consequences of such contacts are negligible.

**Analysis of Lys-26, His-59, and Lys-92**—A similar analysis has been carried out with the other residues proposed to be involved in a dynamic DNA-protein interface involving specific bp: Lys-26, His-59, and Lys-92. Data are summarized in Table I, and the dependence of specific bp recognition on these amino acid residues is depicted graphically in Fig. 6. In this representation, we have expressed the fraction of the $\Delta \Delta G^0$ that can be measured for each bp substitution (relative to the wild-type 5 S rRNA gene sequence) that is dependent on the amino acid side chain in question. The important role played by Lys-29 in recognition of bp AT88 and GC89 (Fig. 3) is also obvious in this analysis; 75–95% of the $\Delta \Delta G^0$ associated with bp substitutions at either of these positions is dependent on Lys-29. In contrast, we find that greater than 70% of the $\Delta \Delta G^0$ for the substitution of GC for CG at position 91 is dependent upon Lys-26 in TFIIIA, whereas less than 20% of the $\Delta \Delta G^0$ resulting from substitution of GC for AT at position 90 is dependent on Lys-26. Thus, it would appear that Lys-26 is primarily involved in
recognizing CG91 and only marginally significant in the recognition of AT90. Similarly, Lys-92 appears to play an important role in recognizing GC82, with a lesser role in recognition of GC81. Notably, Lys-92 actually appears to be inhibitory in the recognition of AT83, one of the bp proposed by Wright and colleagues (14, 15) to be involved in fluctuating contacts with Lys-92. Mutating His-59 to alanine has approximately equivalent proportional effects on recognition of GC86 and GC87, although the effect is modest in both cases. Thus, at least for Lys-26 and Lys-92, the results are more consistent with a dominant role for the amino acid side chain in the recognition of a particular bp rather than the multiple bp predicted by the dynamic interface model.

In each case, an analysis similar to that described above for Lys-29 has been used to assess whether the dynamic interface model or the static interface/positive cooperativity model provides a better explanation for even small effects of particular TFIIA mutations on multiple bp recognition. With the exception of

### Table 1

| Protein  | DNA 1  | DNA 2  | \(K_{d1}^{2}/K_{d1}^{1}\) | st err | % st err | \(\Delta G^0\) | n   |
|----------|--------|--------|---------------------------|--------|----------|----------------|-----|
| Wild-type | Wild-type | A88T   | 1.85  | 0.14 | 7.6   | 0.365 | 39 |
| K29A     | Wild-type | A88T   | 5.69  | 0.57 | 10.0  | 1.031 | 9  |
| K29A     | Wild-type | G89A   | 1.23  | 0.07 | 6.0   | 0.123 | 12 |
| Wild-type | Wild-type | G89A   | 8.56  | 0.53 | 6.2   | 1.274 | 14 |
| K29A     | Wild-type | G89C   | 1.62  | 0.07 | 4.3   | 0.287 | 23 |
| Wild-type | Wild-type | G90T   | 7.22  | 0.33 | 4.6   | 1.172 | 10 |
| K29A     | Wild-type | G90T   | 1.15  | 0.06 | 5.0   | 0.038 | 4  |
| Wild-type | Wild-type | Wild-type | 1.16  | 0.05 | 4.2   | 0.087 | 7  |
| K29A     | Wild-type | Wild-type | 1.01  | 0.08 | 7.9   | 0.004 | 6  |
| Wild-type | Wild-type | A88T/G89T | 1.43  | 0.24 | 17.0  | 0.213 | 2  |
| K29A     | Wild-type | A88T/G89T | 1.69  | 0.24 | 14.4  | 0.312 | 3  |
| Wild-type | Wild-type | A88G   | 6.09  | 0.09 | 7.9   | 0.004 | 6  |
| K26A     | Wild-type | A88G   | 1.11  | 0.03 | 2.3   | 0.105 | 15 |
| Wild-type | Wild-type | G89A   | 1.30  | 0.06 | 4.9   | 0.156 | 8  |
| K26A     | Wild-type | G89A   | 2.50  | 0.06 | 2.4   | 0.544 | 7  |
| Wild-type | Wild-type | A88T   | 1.65  | 0.03 | 2.0   | 0.267 | 2  |
| K26A     | Wild-type | A88T   | 2.60  | 0.11 | 4.4   | 0.567 | 8  |
| Wild-type | Wild-type | A90G   | 1.82  | 0.14 | 7.5   | 0.355 | 6  |
| K26A     | Wild-type | A90G   | 2.96  | 0.31 | 10.4  | 0.644 | 15 |
| Wild-type | Wild-type | G86C   | 3.47  | 0.39 | 11.1  | 0.737 | 7  |
| H59A     | Wild-type | G86C   | 1.55  | 0.06 | 4.1   | 0.260 | 6  |
| Wild-type | Wild-type | G86C/G87A | 1.93  | 0.08 | 4.2   | 0.389 | 10 |
| H59A     | Wild-type | G86C/G87A | 1.55  | 0.04 | 2.3   | 0.290 | 11 |
| Wild-type | Wild-type | G87A   | 1.92  | 0.09 | 4.5   | 0.388 | 8  |
| H59A     | Wild-type | G87A   | 1.39  | 0.07 | 4.9   | 0.196 | 14 |
| Wild-type | Wild-type | G86C   | 1.41  | 0.11 | 7.9   | 0.204 | 16 |
| H59A     | Wild-type | G86C   | 2.17  | 0.10 | 4.8   | 0.458 | 11 |
| Wild-type | Wild-type | G81T   | 2.48  | 0.47 | 18.8  | 0.540 | 9  |
| K29A     | Wild-type | G81T   | 2.06  | 0.20 | 9.7   | 0.429 | 10 |
| Wild-type | Wild-type | G82A   | 1.86  | 0.09 | 4.6   | 0.367 | 10 |
| K29A     | Wild-type | G82A   | 1.46  | 0.04 | 2.6   | 0.226 | 13 |
| Wild-type | Wild-type | G82A   | 2.21  | 0.07 | 3.2   | 0.470 | 11 |
| K29A     | Wild-type | G82A   | 1.22  | 0.06 | 4.5   | 0.118 | 8  |
| Wild-type | Wild-type | G81T   | 1.38  | 0.09 | 6.8   | 0.192 | 11 |
| K29A     | Wild-type | G81T   | 1.08  | 0.07 | 6.6   | 0.046 | 9  |
| Wild-type | Wild-type | A83T   | 1.74  | 0.20 | 11.5  | 0.329 | 11 |
| K29A     | Wild-type | A83T   | 3.00  | 0.12 | 3.9   | 0.651 | 3  |
| Wild-type | Wild-type | G82A   | 4.21  | 0.31 | 7.3   | 0.852 | 11 |
| K29A     | Wild-type | G82A   | 3.14  | 0.28 | 9.0   | 0.679 | 17 |
| Wild-type | Wild-type | A83T   | 4.09  | 0.25 | 6.1   | 0.535 | 7  |
| K29A     | Wild-type | A83T   | 1.56  | 0.06 | 3.7   | 0.264 | 8  |
| Wild-type | Wild-type | G81T   | 2.32  | 0.08 | 3.5   | 0.499 | 10 |
| K29A     | Wild-type | G81T   | 2.65  | 0.15 | 5.8   | 0.577 | 11 |
| Wild-type | Wild-type | A83T   | 3.07  | 0.31 | 10.2  | 0.604 | 11 |
| K29A     | Wild-type | A83T   | 1.45  | 0.07 | 4.7   | 0.221 | 8  |
tion of results involving the AT83 bp (see below), the data (Table I) are analogous to those we observed for Lys-29 and suggest that the DNA-protein interface involving these amino acid residues is a static one, with modest degrees of positive cooperativity in the recognition of adjacent bp. This cooperative effect is most pronounced with AT88 and GC89, making Lys-29 almost equally important in the recognition of both bp, although it is unlikely that it does so by the kinds of direct, fluctuating contacts suggested by the dynamic interface model (10, 14).

Thermodynamic interactions between the AT83 bp and GC bp at positions 81 and 82 are invariably unfavorable, in contrast to the cooperativity that we have observed elsewhere; that is, the effects of mutations at positions 81 or 82 are greater when the bp at 83 is mutant (TA) rather than wild-type (AT). Reciprocally, the effect of mutating AT83 to TA is also greater when bp 81 or 82 is mutant. This would be consistent with the predictions of the dynamic interface model except for the fact that Lys-92 does not appear to be involved in recognition of AT83 at all (Fig. 6). Thus, the dynamic interface model is not supported by these data either. Instead, it appears that there are unfavorable functional interactions between the AT83 bp and the wild-type bp at positions 81 and 82 and that the energetic importance of AT83 in binding to TFIIIA is actually enhanced when Lys-92 is mutated to alanine.

DISCUSSION

The model originally proposed by Foster et al. (15) and Wuttke et al. (14) for the complex of the three N-terminal zinc fingers of TFIIIA bound to bp 80–92 of the 5 S rRNA gene is noteworthy in several respects. Perhaps the most novel and striking aspect of this model is the proposal that certain amino acid side chains at the TFIIIA-DNA interface are conformationally mobile and participate in the recognition of multiple bp through rapidly fluctuating, nearly isoenergetic contacts with the same conformationally mobile amino acid side chain. Specifically, this apparent energetic contribution will be greater when the adjacent bp is mutated, eliminating this adjacent bp as a potential site for making nearly isoenergetic contacts. In contrast, the static interface/positive cooperativity model makes the opposite prediction: the apparent energetic contribution of an individual bp will be dependent upon the identity of adjacent bp involved in putative contacts with the same conformationally mobile amino acid side chain. Specifically, this apparent energetic contribution will be greater when the adjacent bp is mutated, eliminating this adjacent bp as a potential site for making nearly isoenergetic contacts. In contrast, the static interface/positive cooperativity model makes the opposite prediction: the apparent energetic contribution of an individual bp to the overall binding energy will be greatest in an otherwise wild-type DNA sequence background.

We have tested these predictions for four of the five amino acid side chains predicted by Wuttke et al. (14) to be involved in fluctuating isoenergetic contacts to multiple bp. For Lys-26 and Lys-92, the data are most consistent with primary contacts being made to bp CG91 and GC82, respectively, although each amino acid side chain may also make very modest energetic contributions to the recognition of another bp, AT90 for Lys-26 and GC81 for Lys-92. It is worth noting that the primary contacts that we identify biochemically for these two amino acids are identical to those proposed by Nolte et al. (16) in their crystallographic analysis. His-59 makes modest, but more or less equivalent proportional contributions to the recognition of bp GC86 and GC87. Most interestingly, Lys-29 is essential for the specific recognition of both AT88 and GC89 bp in the 5 S rRNA gene. Strikingly, however, measurements designed to test the third prediction of the dynamic interface model, as described above, were not consistent with the model. Instead, they were entirely consistent with a model involving strong positive cooperativity in the recognition of adjacent bp, such as might result from a thermodynamic network of static interactions at the DNA-protein interface.
Although the hypotheses we have tested in the studies described here were based on the original structural papers from Wright’s laboratory (14, 15), it is important to note that subsequent work from the same research group has provided a refinement in the proposed structure of the TFIIIA/H18528 DNA complex (25). This refined structure was made possible by analysis of the positions of interfacial water molecules using NOEs and molecular dynamics simulations (25). Most importantly, Lys-29 in the refined structure contacts only a backbone phosphate in the 5 S rRNA gene; fluctuating contacts to AT88 and GC89 are no longer proposed to exist. Presumably, however, the original NMR data showing peak broadening in the Lys-29 (as well as in the Lys-26, His-58, and His-59) side chain resonances are not in question and continue to suggest the existence of multiple, interconverting conformational states, even though these conformations do not include contacts of Lys-92 with bp AT88 and GC89. Thus, the higher resolution structural model is consistent with our conclusion that conformational dynamics in the Lys-29 side chain do not play a role in the energetics of recognition of AT88 and GC89. We also note that the higher resolution structural model eliminates GC81 as a contact point for the mobile Lys-92 side chain (25).
FIG. 5. Distinguishing models for Lys-29. An analysis similar to the theoretical treatment outlined in Fig. 4 was used to distinguish the dynamic interface and static interface/positive cooperativity models for the proposed interactions of Lys-29 with bp AT88 and GC89 in the 5 S rRNA gene. Mutant 5 S rRNA genes are designated as described in the legend to Fig. 3. Error bars are S.E. of the mean.
Although it may appear that the biochemical results described here contradict the NMR data that gave rise to the dynamic interface model, we believe that there is no inherent conflict in the data themselves. The results of Foster et al. (15) and Wuttke et al. (14) demonstrate clearly that there is heterogeneity in the chemical environments experienced by the amino acid side chains in question, consistent with the idea that there is some mobility of these side chains in the DNA-protein complex. Nonetheless, these data do not, by themselves, demonstrate that the same amino acid side chains make the proposed contacts with multiple bp or even that they undergo conformational movements on a scale that would be necessary to permit these proposed multiple contacts. Alternatively, it is possible that the proposed conformational fluctuations occur but have no significant thermodynamic consequences. This would be the case, for example, if all but one of the conformers were accessed relatively infrequently, resulting in significant population of only a single conformational state in the equilibrium mixture of complexes. This scenario, however, approximates the conventional view of a static DNA-protein interface and would be incompatible with the idea of multiple, roughly isoenergetic interactions available to a single amino acid side chain (15). It is also possible that the predicted thermodynamic effects resulting from rapid, alternating contacts of a single amino side chain with multiple bp are small relative to those resulting from static contacts by other amino acid side chains that are in contact with the same bp. For example, the AT90 bp appears to make a water-bridged contact with Trp-28 in TFIIIA in addition to its proposed fluctuating contact with Lys-26. Similarly, AT83 makes static contacts with Asn-89 in addition to its proposed contact with the dynamic Lys-92 side chain. If these static contacts dominate the energetics of bp recognition and if they further exhibit a high degree of positive cooperativity, then it is possible that our experiments would not detect the thermodynamic signature associated with a dynamic interface of the kind originally proposed by Wright and colleagues. If this explanation applies, however, then dynamic base contacts at the DNA-protein interface would contribute relatively little to the energetics of bp recognition. The fact that we usually find significant dependence of specific bp recognition on the presence of the lysine/histidine side chains in question argues against this scenario. On balance, we believe the simplest interpretation of the entire body of evidence is that there is some conformational mobility of select amino acid side chains at the DNA-protein interface in the TFIIIA-5 S rRNA gene complex but that specific bp recognition is carried out by conventional, essentially static interactions, albeit interactions that can exhibit a high degree of cooperativity involving recognition of adjacent bp by different functional groups in the protein.

**FIG. 6. Dependence of specific bp recognition on Lys-26, Lys-29, His-59, and Lys-92.** The ability of wild-type TFIIIA to distinguish between the wild-type and various mutant 5 S rRNA genes was determined (relative $K_d$ for wild-type TFIIIA). The same parameter was measured for the relevant mutant form of TFIIIA (K26A, K29A, H59A, or K92A). In each case the relative $K_d$ was converted to the $\Delta G^\circ$ (reduction, attributable to the 5 S rRNA gene mutation, in the change in binding free energy), and the fraction of the wild-type TFIIIA $\Delta G^\circ$ that is dependent on a particular amino acid side chain (as deduced from analysis of the relevant alanine-substituted TFIIIA mutant) was then calculated. This value is plotted on the ordinate in each case and provides a measurement of the dependence of specific bp recognition on a particular amino acid. Mutant 5 S rRNA genes are designated as described in the legend to Fig. 3.
Although the conceptual and technical framework for the approach we describe here has been anticipated in earlier studies (12, 24), we believe that the specific methodology we describe should be generally useful in defining thermodynamically significant nucleic acid-protein contacts. In the case of the TFIIIA-5 S rRNA gene complex, the large size of the interaction surface, coupled with what we have called previously a “compensatory” mode of binding (10), makes the effects of individual bp or amino acid substitutions on binding of the full-length protein relatively small. Thus, it was imperative that we make use of an assay that could reproducibly measure relative equilibrium binding constants with high precision. We believe the assay we have used to be well suited to this problem for several reasons. First, it permits estimation of relative $K_d$ values from single binding reaction mixtures, making it possible to accumulate a large number of multiple determinations with only moderate effort. Second, because the two binding equilibria under analysis are analyzed in the same tube, systematic errors in measurement tend to cancel out. Third, the relative $K_d$ values are determined with no requirement for an independent determination of protein or DNA concentration, eliminating other sources of measurement error. It is also worth noting that analogous approaches could be developed using methods other than electrophoretic mobility shift to resolve protein-bound and free DNAs, so long as means exist to distinguish between the two populations of DNA molecules. For example, filter binding assays could be used if the DNA fragments under analysis were labeled with distinguishable isotopes.

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REFERENCES
1. Pelham, H. R., and Brown, D. D. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 4170–4174
2. You, Q., Veldhoen, N., Baudin, F., and Romaniuk, P. J. (1991) Biochemistry 30, 2495–2500
3. Clemens, K. R., Wolf, V., McBayant, S. J., Zhang, P., Liao, X., Wright, P. E., and Gottesfeld, J. M. (1990) Science 260, 530–533
4. Rawlings, S. L., Matt, G. D., and Huber, P. W. (1996) J. Biol. Chem. 271, 868–877
5. Engelke, D. R., Ng, S. Y., Shastry, B. S., and Roeder, R. G. (1980) Cell 19, 717–729
6. Hayes, J. J., and Tullius, T. D. (1992) J. Mol. Biol. 227, 407–417
7. Hayes, J. J., and Clemens, K. R. (1992) Biochemistry 31, 11600–11605
8. Del Rio, S., Menezes, S. R., and Setzer, D. R. (1993) J. Mol. Biol. 233, 567–579
9. Romaniuk, P. J. (1990) J. Biol. Chem. 265, 17593–17600
10. Kehres, D. G., Subramanyan, G. S., Hung, V. S., Rogers, G. W., Jr., and Setzer, D. R. (1997) J. Biol. Chem. 272, 20152–20161
11. Romaniuk, P. J. (1985) Nucletic Acids Res. 13, 5369–5387
12. Setzer, D. R., Menezes, S. R., Del Rio, S., Hung, V. S., and Subramanyan, G. (1996) RNA (N. Y.) 2, 1254–1269
13. Miller, J., McLachlan, A. D., and Klug, A. (1985) EMBO J. 4, 1609–1614
14. Wuttke, D. S., Foster, M. P., Case, D. A., Gottesfeld, J. M., and Wright, P. E. (1997) J. Mol. Biol. 273, 183–206
15. Foster, M. P., Wuttke, D. S., Radhakrishnan, I., Case, D. A., Gottesfeld, J. M., and Wright, P. E. (1997) Nat. Struct. Biol. 4, 605–608
16. Nolte, R. T., Conlin, R. M., Harrison, S. C., and Brown, R. S. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 2938–2943
17. Pavletich, N. P., and Pabo, C. O. (1991) Science 252, 809–817
18. Elrod-Erickson, M., Rouid, M. A., Nekludova, L., and Pabo, C. O. (1996) Structure 4, 1171–1180
19. Pavletich, N. P., and Pabo, C. O. (1993) Science 261, 1701–1707
20. Fairall, L., Schwabe, J. W., Chapma, L., Finch, J. T., and Rhodes, D. (1993) Nature 366, 483–487
21. Setzer, D. R., Hnuel, R. M., and Liao, S. Y. (1990) Nucleic Acids Res. 18, 4175–4178
22. Veldhoen, N., You, Q., Setzer, D. R., and Romaniuk, P. J. (1994) Biochemistry 33, 7568–7575
23. Del Rio, S., and Setzer, D. R. (1991) Nucleic Acids Res. 19, 6197–6203
24. Gartenberg, M. R., Ampe, C., Stitz, T. A., and Crothers, D. M. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6034–6038
25. Tsui, V., Radhakrishnan, I., Wright, P. E., and Case, D. A. (2000) J. Mol. Biol. 302, 1101–1117