Mutations in TFIIIA That Increase Stability of the TFIIIA-5 S rRNA Gene Complex

UNUSUAL EFFECTS ON THE KINETICS OF COMPLEX ASSEMBLY AND DISSOCIATION*

Received for publication, March 10, 2005, and in revised form, May 9, 2005
Published, JBC Papers in Press, May 19, 2005, DOI 10.1074/jbc.M502677200

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We have identified four mutations in Xenopus TFIIIA that increase the stability of TFIIIA-5 S rRNA gene complexes. In each case, the mutation has a relatively modest effect on equilibrium binding affinity. In three cases, these equilibrium binding effects can be ascribed primarily to decreases in the rate constant for protein-DNA complex dissociation. In the fourth case, however, a substitution of phenylalanine for the wild-type leucine at position 148 in TFIIIA results in much larger compensating changes in the kinetics of complex assembly and dissociation. The data support a model in which a relatively unstable population of complexes with multi-component dissociation kinetics forms rapidly; complexes then undergo a slow conformational change that results in very stable, kinetically homogeneous TFIIIA-DNA complexes. The L148F mutant protein acts as a particularly potent transcriptional activator when it is fused to the VP16 activation domain and expressed in yeast cells. Substitution of L148 to tyrosine or tryptophan produces an equally strong transcriptional activator. Substitution to histidine results in genetic and biochemical effects that are more modest than, but similar to, those observed with the L148F mutation. We propose that an amino acid with a planar side chain at position 148 can intercalate between adjacent base pairs in the intermediate element of the 5 S rRNA gene. Intercalation occurs slowly but results in a very stable DNA-protein complex. These results suggest that transcriptional activation by a cis-acting sequence element is largely dependent on the kinetic, rather than the thermodynamic, stability of the complex formed with an activator protein. Thus, transcriptional activation is dependent in large part on the lifetime of the activator-DNA complex rather than on binding site occupancy at steady state. Introduction of intercalating amino acids into zinc finger proteins may be a useful tool for producing artificial transcription factors with particularly high in vivo activity.

TFIIIA† (transcription factor IIIA) is both representative of the large family of zinc finger proteins encoded in eukaryotic genomes (1, 2) and unusually interesting among these proteins in several respects. It has the atypical ability to bind with high affinity and specificity to both DNA (3–6) and RNA (7–9), has an unusually large number of zinc finger domains (nine), and an extended DNA recognition sequence (the ~52-base pair internal control region of 5 S rRNA genes), exhibits rather complex binding energetics to both DNA (10–12) and RNA (13), and is remarkably poorly conserved during the evolution of eukaryotes (14, 15).

We have described previously a method for the genetic analysis of the Xenopus TFIIIA-5 S rRNA gene interaction (16, 17). In this approach, the Xenopus 5 S rRNA gene substitutes for the natural upstream activating sequence in the Saccharomyces cerevisiae CYC1 promoter which, in turn, controls expression of the Escherichia coli $\beta$-galactosidase (lacZ) gene in yeast cells. Transcriptional activity from the CYC1 core promoter is dependent upon binding of an artificial activator Xenopus TFIIIA fused to the transcriptional activation domain of the VP16 protein from the herpes simplex virus (18, 19) (Fig. 1). In this system, the level of $\beta$-galactosidase activity is dependent on the affinity or, as we show in this manuscript, the kinetic stability of the Xenopus TFIIIA-5 S rRNA gene complex (16). Because $\beta$-galactosidase activity in vivo can be assessed readily using X-gal indicator plates, screening large numbers of randomly mutagenized forms of TFIIIA for those mutants with altered DNA binding affinity or specificity is relatively straightforward (16, 17). In fact, we have already described a number of mutant forms of TFIIIA that form complexes with the 5 S rRNA gene that are less stable than is the case with the wild-type protein (16).

In this paper, the description of the isolation, using this genetic system, of several mutant forms of TFIIIA that interact with the 5 S rRNA gene to form complexes that are more stable than is the case with wild-type TFIIIA. One of these variant proteins is particularly interesting in that it exhibits only a small increase in equilibrium binding affinity, but the rates of both DNA binding and dissociation are reduced greatly in comparison to the wild-type or other gain-of-function mutants. A more detailed analysis of various amino acid substitutions at the site mutated in this kinetically altered form of TFIIIA suggests that the kinetic effects result from intercalation of a planar amino acid side chain between adjacent base pairs in the DNA helix. The fact that the ability of these variant proteins to activate transcription by RNA polymerase II correlates with kinetic and not equilibrium binding properties also suggests an unorthodox mechanism whereby transcriptional activators stimulate transcription by RNA polymerase II.

**EXPERIMENTAL PROCEDURES**

*Genetic Screen—The genetic screen used to isolate the mutant forms of TFIIIA discussed in this paper has been described previously (16, 17)
and is illustrated in Fig. 1. Error-prone PCR using primers GP5 and VP3 along with pG1-803 as a template was used to generate pools of mutant TFIIIA sequences. These were introduced via homologous recombination into pG1-803 that had been doubly cut with SacI and SalI. The recombination event occurred after co-transformation with both gapped pG1-803 and the error-prone PCR product into strain BJ2168 containing the plasmid pY25SR-Z. After selection of transformants on C-Ura-Trp plates, β-galactosidase activity was assessed by replica plating onto SSX-Ura-Trp plates. Four colonies that were darker blue than was the case with pG1-803 (wild-type TFIIIA) were chosen for further analysis, and viable cells were recovered from the original transformation plate. The TRP1 plasmids were recovered from these strains and used to transform E. coli. After further recovery from E. coli, these pG1-803 variants were used to transform BJ2168 containing pY25SR-Z, and the β-galactosidase phenotype was again assessed on X-gal plates to confirm that the elevated β-galactosidase activity was plasmid-dependent. We used Western blots with a polyclonal rabbit anti-TFIIIA antibody to measure the level of variant TFIIIA-VP16 expression in the TRP1 ‘URA3’ yeast strains to ensure that the expression level was similar to that observed with wild-type TFIIIA-VP16. Finally, the TFIIIA moiety of the pG1-803 variants was sequenced to determine the amino acid changes responsible for the elevated transcriptional activity of the reporter gene.

**Measurement of β-Galactosidase Activity**—We used a variation of the method of Kippert (20) to measure β-galactosidase activity in yeast cells. Cells were grown in C-Ura-Trp to an A500 of 0.5–1.0 (≈7–21 × 10^6 cells/ml) and pelleted from 4 ml of this culture. After washing one time in 4 ml of Z buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgCl2, and 40 mM β-mercaptoethanol, pH 7.0) and resuspending the pellet in 4 ml of the same buffer, the A500 of a 1-ml aliquot was determined in a 200-µl aliquot of the resuspended cells was then added to 400 µl of Z buffer containing 0.2% (wt/vol) sodium Sarcosyl. After 30 min at 30 °C, 150 µl of a 4 mg/ml solution of o-nitrophenol-β-d-galactoside in Z buffer was added, and the incubation continued at 30 °C until a yellow color developed. The reaction was stopped by the addition of 400 µl of Na2CO3 (1 m), and insoluble material was pelleted by a brief spin in a microcentrifuge. Absorbance of the supernatant at 420 nm was measured, to that from a control reaction mixture containing no yeast cells. Units of β-galactosidase activity were determined according to Equation 1,

\[
\text{Units} = \frac{\text{A}_{420} \times 1000}{\text{A}_{500} \times \text{V}}
\]  

(1)

where V is the volume (in milliliters) of washed and resuspended yeast cells added to the reaction mixture, t is the time (in minutes) of the incubation after addition of o-nitrophenol-β-d-galactoside, A500 is the absorbance at 420 nm after stopping the reaction with Na2CO3, and A500 is apparent absorbance of the yeast cell suspension after resuspending in Z buffer. All assays were performed in triplicate using independent yeast cultures, and triplicate assays were repeated on different days as a total of three times. Therefore, nine independent determinations were used for each reported β-galactosidase activity measurement. Relative activities were determined by normalizing to the level of activity measured for BJ2168 containing pY5SR-Z and pG1-803 (wild-type TFIIIA-VP16).

**Saturation Mutagenesis of Residue 148—Overlap extension PCR was used to generate a population of Xenopus TFIIIA-encoding DNA fragments in which the nucleotide sequence for codon 148 was randomized. Specifically, two initial amplifications were performed using pG1-803 as a template. One made use of primers GP5 (16) and an antisense oligonucleotide (L148-AS) containing wild-type sequence flanking codon 148, but with the three nucleotides of codon 148 being synthesized as a random mixture. The second amplification used primer VP3 (16) and the reverse complement of L148-AS, that is, the corresponding TFIIIA sense sequence with a randomized codon 148 (L148-S). The products of these two PCRs were gel-purified and used as templates in a single amplification reaction containing primers GP5 and VP3. The product of this reaction was also gel-purified and used in combination with the large SacI/SalI fragment of pG1-803 to transform yeast strain BJ2168 containing either plasmid pY5SR-Z or pY25SR-Z (16). URA4 colonies were selected on C-Ura-Trp plates and replicates plated onto 5-SSX-Ura-Trp. Colonies were selected based on their color in comparison to control strains containing pG1-803 (expressing wild-type TFIIIA-VP16) and pG1 (expressing no TFIIIA-VP16) in addition to the relevant reporter plasmid (pY5SR-Z or pY25SR-Z). The TRP1-containing plasmid was recovered, digested through E. coli, and used to transform BJ2168 containing pY25SR-Z. The β-galactosidase phenotypes of these strains were confirmed on 5-SSX-Ura-Trp plates. The

**RESULTS**

**Identification of Mutant TFIIIA**—We used the approach outlined in Fig. 1 and described in detail elsewhere (16, 17) to screen for mutant forms of TFIIIA with reduced or altered
binding affinity for the *X. borealis* somatic type 5 S rRNA gene. As expected, this screen identified many yeast colonies that were white or light blue on X-gal indicator plates; those chosen for further detailed analysis were shown to express mutant forms of TFIIA-VP16, the TFIIIA moiety of which exhibited reduced affinity for the 5 S rRNA gene (16). Interestingly, however, we also recovered four colonies that stained a darker blue than was observed with wild-type TFIIA-VP16. Quantitative measurements of β-galactosidase activity were then made for each of these strains, along with control strains expressing wild-type TFIIA-VP16 or containing the parent expression vector lacking TFIIA-VP16 sequences. Enzyme activities were normalized to that obtained with wild-type TFIIA-VP16. Error bars represent S.E.

Surprising Effects of L148F on Equilibrium Binding Affinity—We expressed the four mutant forms of TFIIA initially identified in the genetic screen (K92R, D209G/K309E, and E270K) in *E. coli* and determined the equilibrium binding constants for the interaction of these proteins with the *X. borealis* somatic type 5 S rRNA gene (10, 22) (Fig. 4). As anticipated, the relatively modest increases in β-galactosidase activity associated with the K92R, D209G/K309E, and E270K mutants were reflected in relatively modest increases in equilibrium binding affinity (1.7–5-fold decreases in the *Kd*). Surprisingly, however, L148F also exhibited a relatively modest increase in affinity for the 5 S rRNA gene (3.4-fold), despite the much larger effect on β-galactosidase activity. Thus, it appears that the strong correlation noted previously (16) between transcriptional activity as measured by expression of the β-galactosidase reporter gene and the equilibrium binding constant of the TFIIA-5 S rRNA gene interaction does not hold in the case of the L148F mutant. Effects of Mutations on Kinetic Stability of the TFIIA-5 S rRNA Gene Complex—To investigate this discrepancy further, we measured the *in vitro* dissociation rates of TFIIA-5 S rRNA gene complexes for wild-type TFIIA and for each of the four mutants under analysis (Fig. 5). For K92R, D209G/K309E, and E270K, the dissociation rate data could be fit readily to simple first-order decay curves, and the measured first-order rate constants for the dissociation reaction were roughly consistent with the equilibrium constants for the binding reaction, indicating that the effects of the mutations were mediated primarily through effects on complex stability and not on complex formation. In contrast, the rate of L148F dissociation from the 5 S rRNA gene was both much slower and kinetically complex, making it impossible to extract a single first-order rate constant that described the dissociation process accurately. The initial equilibrium binding and kinetic experiments were carried out under our standard conditions for analyzing
TFIIA-5 S rRNA gene interactions (10), which include an incubation time of 30 min to allow the binding reaction to come to equilibrium. This is much longer than is necessary for wild-type TFIIA-DNA complexes (data not shown). Because the kinetically complex dissociation curve obtained with L148F suggested that multiple forms of TFIIA-DNA complexes existed after such a 30-min incubation, however, we measured dissociation rates for this particular complex following a wide range of preincubation times ranging from 5 min to 48 h. A comparison of the dissociation curves obtained with wild-type TFIIA and L148F after a 5-min preincubation is shown in Fig. 6, and a comparison of the curves obtained with L148F after 5-min and 30-hour preincubations is shown in Fig. 7. These results demonstrate that at least two forms of DNA-protein complexes with different kinetic stabilities form initially in the case of the L148F mutant but that the complicated dissociation rate data resolve into a single, first-order process after prolonged preincubation times. Remarkably, however, the first-order dissociation rate constant for L148F is very low, corresponding to a half-life of 3 h in comparison with a \( t_{1/2} \) of 6–9 min for the wild-type TFIIA-DNA complex. In contrast to the multi-component kinetics observed after short preincubations with L148F, simple first-order dissociation kinetics are observed after preincubation times of only 5 min in the case of wild-type TFIIA, as well as when the K92R, D209G/E309K, and E270K mutants are analyzed (Fig. 5). Furthermore, the measured half-life of the wild-type TFIIA-DNA complex is independent of the preincubation time (Fig. 8).

Despite the fact that preincubations of many hours are required for L148F-5 S rRNA gene complexes to form a kinetic-
cally homogeneous population, those DNA-protein complexes that are formed rapidly (less than a few minutes) are indistinguishable in quantity and electrophoretic mobility from those present after much longer incubations, as assessed by electrophoretic mobility shift assays (data not shown). This result suggests that formation of the L148F-DNA complex occurs rapidly, but the initial complex that forms is less kinetically stable than the complex that predominates after long incubations. Thus, the data described here are consistent with a model in which L148F binds to DNA rapidly to form a relatively unstable complex, but this initial complex then undergoes a slow conformational change to assume a kinetically very stable form.

Our original measurements of apparent equilibrium binding constants for complexes of the 5 S rRNA gene with the various mutant forms of TFIIIA had been made using 30-min incubation times. Because of the very long time required to reach a true equilibrium state for the L148F mutant, it is clear that our original measurements of $K_d$ for L148F were not made at equilibrium. Thus, the discrepancy between $K_d$ and $k_{off}$ remains, consistent with the model in which the overall rate of formation of the stable L148F-DNA complex is quite low.

No Major Structural Changes in the L148F-5 S rRNA Gene Complex Were Detected in Footprinting Experiments—It seemed possible that the conformational change associated with stable DNA-binding by L148F could be detected in footprinting experiments. Consequently, we analyzed the DNase I protection pattern produced on both strands of the 5 S rRNA gene by wild-type TFIIIA and by the K92R, L148F, D209G/K309E, and E270K mutants. In comparison to the protection pattern conferred by wild-type TFIIIA, it is probable that the L148F mutant (and possibly the D209G/K309E mutant as well) results in very slight alterations in the pattern and extent of protection of the relatively unprotected bands around position +75 on the non-template strand of the gene (data not shown). These alterations are quite subtle, however, and are not dependent upon incubation time. Thus, it is unlikely that they reflect the conformational alteration in the DNA-protein complex that results in kinetic stabilization.

Genetic Screen of Other Mutations at Position 148—It was not immediately obvious why the substitution of phenylalanine for the normal leucine at position 148 in TFIIIA resulted in such a major change in the kinetics of DNA binding and dissociation. To investigate this matter further, we subjected a pool of TFIIIA-encoding DNA sequences containing a randomized codon 148 to the same genetic screen (depicted in Fig. 1) that was used in the initial identification of the mutant proteins described above. Four classes of resulting yeast colonies were identified based on colony color on X-gal-containing plates. Class 1 colonies were no different in color from those expressing the wild-type TFIIIA-VP16 fusion protein. Those in class 2 were white or a lighter blue than with the wild-type control. Class 3 colonies were very dark blue, comparable in color to that observed with the original L148F mutant. Members of class 4 were intermediate in color between that associated with wild-type TFIIIA and the L148F mutant.

After confirming that the colony color phenotypes were plasmid-dependent, the TFIIIA-encoding DNA sequences in several plasmids of each class were determined, with a particular emphasis on plasmids from classes 3 and 4. In every case of a

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**FIG. 7.** Dissociation kinetics of L148F-5 S rRNA gene complexes after varying preincubation times. Complexes between the 5 S rRNA gene and the L148F variant of TFIIIA were allowed to form for either 5 min or 30 h. Dissociation of these complexes was then monitored as a function of time (10, 22). A logarithmic plot of the fraction of complexes remaining at various times is shown.

**FIG. 8.** Half-lives of TFIIIA-DNA complexes after variable preincubations. Dissociation rate constants and half-lives were determined for complexes of either wild-type or L148F TFIIIA with the 5 S rRNA gene after 5 min or 2, 30, or 48 h of preincubation. In the case of L148F, multi-component dissociation curves prevented the determination of a single first-order rate constant that accurately described the process. Half-lives of the complexes are plotted here. Error bars represent the S.E.
class 3 colony (very dark blue), the amino acid at position 148 was phenylalanine, tyrosine, or tryptophan. Every class 4 mutant contained a histidine at position 148. A variety of other mutations were identified for the relatively small number of clones analyzed from classes 1 and 2, with no particular pattern being apparent. Quantitative β-galactosidase assays confirmed that the L148F, L148Y, and L148W mutations greatly increased transcriptional activation (~20-fold above wild-type), whereas the L148H mutant resulted in a more modest increase above wild-type (~2.7-fold) for the L148H mutant (Fig. 9).

Analysis of DNA Binding by the L148H Mutant—We determined the equilibrium binding constant and the rate constant for dissociation of the L148H mutant protein-DNA complex (Figs. 4 and 10). Again, there is a very modest increase in equilibrium binding affinity compared with wild-type TFIIIA, and complex kinetics of dissociation that resolve into a single first-order process upon prolonged preincubation. After long preincubation, the half-life of the L148H-5 S rRNA gene complex is ~5-fold greater than that of wild-type (Fig. 11). Thus, a histidine substitution at position 148 mirrors what we observed previously for the phenylalanine substitution (and what we presume to be the case for tryptophan and tyrosine substitutions), but with less stabilization of the equilibrium complex.

Specificity of the Effects at Position 148—To ensure that the enhanced transcriptional activity associated with the L148F mutant was still dependent on the presence of the VP16 activation domain, we generated a strain expressing TFIIIA with the L148F mutation but with no VP16 moiety. There was no detectable β-galactosidase activity in this strain above that observed with the pG1 expression vector control. Thus, the mutation does not create a novel activation domain that makes the VP16 domain dispensable.

In most zinc fingers, the position occupied by TFIIIA L148 corresponds to position −1 relative to the start of the α-helix. In TFIIIA finger 5, however, there is a proline at the normal +1 helical position, resulting in a delayed start to the helix, as seen in the crystallographically determined structure of the first six zinc fingers of TFIIIA (6). We considered the possibility that placing a phenylalanine (Phe) residue, or a phenylalanine-proline (Phe-Pro) dipeptide in a corresponding position in other zinc fingers of TFIIIA would lead to effects similar to those we observed in the case of the L148F mutant. We therefore generated Phe and Phe-Pro substitutions at corresponding positions in fingers 1, 7, and 9 of TFIIIA and tested the effects of these substitutions in our genetic assay with a β-galactosidase reporter. In no case did we observe an increase in transcriptional activity (data not shown).

DISCUSSION

Of the four gain-of-function TFIIA mutations we identified in our genetic screen for mutants with altered DNA binding properties, three can be rationalized on the basis of what is known structurally about the TFIIIA-5 S rRNA gene interaction (5, 6, 25) or on the basis of general features of zinc finger-DNA interactions that have been described previously (24). In the K92R mutant, a lysine residue at position +6 in the α-helix of finger 3 is replaced by an arginine. The bidentate H-bonding properties of the arginine side chain could make possible an additional contact beyond that afforded by the wild-type lysine, particularly considering that the contacted base is a guanine. The wild-type aspartic acid residue at position 209 in wild-type TFIIA also occurs at the +6 helical position, one that is often involved in direct DNA contacts (24–26). It is possible that replacement of the negatively charged aspartic acid with an uncharged glycine residue eliminates an unfavorable electrostatic repulsion between the protein and the sugar-phosphate backbone of the DNA, or that the greater conformational flexibility afforded by the glycine residue enhances binding. The glutamic acid at position 270 in finger 9 is predicted to occur at helical position +5, again likely to be in close proximity to the negatively charged DNA backbone. The E270K mutation re-
results in a charge reversal that could be more favorable electrostatically. Of course, direct structural evidence in support of these speculations is not available, and the actual mechanisms whereby these mutations result in the observed increases in binding affinity could be much more complex.

In contrast to the relatively small genetic and biochemical effects mediated by these three mutations, however, L148F results in a large increase in the transcriptional activity conferred by the artificial TFIIIA-VP16 activator, as well as striking changes in the kinetics of protein-DNA complex formation and dissociation. The leucine at position 148 in wild-type TFIIIA probably makes a hydrophobic contact with the 5-methyl group of a thymine base in the AT base pair at position 74 in the 5 S rRNA gene (6); but why a substitution to phenylalanine should result in such major alterations in the DNA-binding properties of the protein was not immediately clear. Randomization of codon 148 and screening of the randomized mutant pool in our genetic assay provided a strong indication of the likely mechanism, however. A planar, aromatic side chain (phenylalanine, tyrosine, or tryptophan) at this position appears to be necessary and sufficient to produce the high β-galactosidase phenotype initially identified in the case of the L148F mutation. This phenotype is associated with altered kinetics of DNA binding and dissociation, consistent with either of two mechanistic models. In the first model, initial rapid binding of the mutant protein is followed by a slow conformational change to yield a protein-DNA complex that dissociates very slowly. Alternatively, the two kinetically distinct complexes could form directly and independently from the free protein and DNA pools, without a relatively unstable intermediate in the pathway leading to the kinetically stable complex. It seems likely that the molecular event resulting in formation of the stable complex is intercalation of the planar amino acid side chain between adjacent base pairs in the DNA helix. If this is indeed the case, the mechanism involving rapid formation of a comparatively unstable complex followed by conformational conversion to the stable form seems more plausible. We propose that this intercalation occurs relatively slowly under our standard binding conditions, explaining the multi-component dissociation kinetics observed with short preincubation times, but yields a very stable protein-DNA complex once formed, explaining the simple, slow ($t_{1/2} \approx 3$ h) kinetics of complex dissociation after long preincubations. This rationale is strengthened further by the fact that a histidine substitution at the same position results in a protein with genetic and biochemical phenotypes that are intermediate between those observed with wild-type TFIIIA and L148F. Specifically, β-galactosidase levels are elevated relative to wild-type but not to the extent observed with the aromatic substitutions at the same position. Furthermore, L148H exhibits multi-component dissociation kinetics after short preincubations but simple first-order kinetics after long preincubations, just as was observed for L148F. In comparison to L148F, however, the stability of the resulting protein-DNA complex is considerably lower, although greater than with wild-type TFIIIA. The simplest interpretation, again, is that binding occurs rapidly to form an initial complex or set of complexes that isomerize slowly, presumably through intercalation of the planar imidazole ring of the histidine side chain, to form a protein-DNA complex whose stability is intermediate between that afforded by intercalation of the larger, aromatic rings of phenylalanine, tyrosine, and tryptophan and the simple hydrophobic interaction of the wild-type aliphatic leucine side chain and the 5-methyl group of a thymine base exposed in the major groove. It should be noted, however, that we have no direct physical evidence for an intercalation event and that stabilization of the complex by a planar side chain at position 148 in TFIIIA could occur by other mechanisms.

It is surprising that the L148F, L148Y, and L148W (and, to a lesser extent, L148H) mutations produce such large effects on transcriptional activation. The effects of the L148F and L148H mutations on equilibrium binding affinity are quite modest; although the L148Y and L148W mutants have not been analyzed biochemically, we have no reason to think they will behave differently from L148F. Conventionally, one thinks about site occupancy being the critical feature determining the activity of a cis-acting transcriptional regulatory element. Occupancy is, in turn, a thermodynamically determined parameter; that is, occupancy is determined by the binding constant ($K_o$ or $K_d$) describing the interaction of a DNA sequence element and a DNA binding transcription factor at equilibrium. Interestingly, however, the Leu-148 mutations that we describe in this paper have relatively minor effects on the equilibrium binding constant, and these small changes seem inadequate to account for the large increases in transcriptional activity associated with the mutant proteins. Instead, transcriptional activity correlates much more closely with a kinetic parameter, i.e. the rate constant for dissociation of TFIIIA-DNA complexes. This observation suggests that site occupancy, per se, is not the most important parameter affecting activity of a cis-acting regulatory element. Thus, it is the lifetime of the complex formed between a regulatory element and a transcription factor that is most relevant and not the fractional occupancy of the site at equilibrium. In other words, two regulatory elements could be

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**FIG. 11.** Half-lives of L148F-DNA and L148H-DNA complexes after long preincubations. Dissociation rate constants and half-lives of complexes of wild-type TFIIIA, L148F, or L148H with the 5 S rRNA gene were determined after 30 h of preincubation. Half-lives of the complexes are plotted here. Error bars represent the S.E.
occupied to the same extent at any given moment in time but still exhibit widely different activities; our results suggest that the site with the slower kinetics of binding and dissociation would be much more active. Ukiyama et al. have proposed previously a similar idea based on analysis of variant SFY proteins with altered kinetic parameters (27). Remarkably, a stabilizing and activating mutation in that case also involves substitution to a DNA-intercalating phenylalanine residue.

The use of the zinc finger motif as a structural scaffold to assemble artificial DNA-binding proteins of pre-determined sequence binding specificity has attracted considerable interest, and impressive successes in the construction of such proteins have been reported (28–33). The striking effects of a single amino acid substitution on the kinetics of DNA binding and dissociation that we report here could provide a new tool in the rational design of DNA binding transcription factors. Although our initial studies suggest that the observed effects on kinetic stabilization and enhanced transcriptional activation cannot be reproduced simply in the context of all zinc fingers, it is possible that additional analysis will identify the peculiar structural features of TFIIIA finger 5 that make planar side chain intercalation and the concomitant biochemical and genetic effects possible in a variety of sequence and structural contexts. Certainly, the ability to produce artificial transcription factors with greatly enhanced transcriptional activation potential could be a valuable tool in a variety of rational protein design projects, including some involving targeted gene therapy approaches.

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J. Biol. Chem. 2005, 280:26743-26750.
doi: 10.1074/jbc.M502677200 originally published online May 11, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M502677200

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