Zinc fingers in transcription factor IIA (TFIIIA) contribute differentially to RNA and DNA binding affinity. We investigated whether the same putative α-helix amino acids in TFIIIA zinc fingers are essential for both RNA and DNA binding. In published structures, zinc fingers make DNA base contacts through amino acids −1, +2, +3, and +6 of the recognition helix. Alanine substitution at these four positions were made in TFIIIA RNA binding zinc fingers, tz4–7 and DNA binding zinc fingers, tz1–3. Substitution in zinc fingers 4 or 6 of tz4–7 reduced RNA affinity 77- and 38-fold, respectively, whereas substitution in zinc fingers 5 or 7 had little effect. DNA binding affinity of tz1–3 was eliminated by alanine substitution in any one zinc finger. We determined which amino acids supported RNA binding by phage display of a library of zinc finger 4 mutants. Lysine at helix position −1 of zinc finger 4 was conserved in all selected tz4–7 fusions. Point mutation of Lys−1 to alanine in zinc finger 4 reduced tz4–7 RNA affinity 30-fold. We propose that RNA binding by TFIIIA shows similarity to DNA binding in the use of the recognition helix. Helix positions −1 and +2 may have particular significance for RNA binding.

In Xenopus oocytes, transcription factor IIA (TFIIIA) has dual functions as a positive transcription factor that binds to the 5 S rRNA gene internal control region and as a storage protein in a 7 S ribonucleoprotein complex with 5 S rRNA (1, 2). Nine tandemly repeated C2H2 zinc fingers at the amino terminus function in binding both 5 S rRNA and the internal control region of the 5 S rRNA gene with high affinity and specificity. Site-specific zinc fingers 4–7, and zinc fingers 1–3 bind independently and specifically to 5 S rRNA and DNA, respectively (3–6). With the exception of the Wilim’s tumor protein WTI (7), other RNA binding C2H2 zinc fingers do not yet have well defined RNA binding sites (8, 9). The other major class of zinc fingers that bind to RNA, the C2HC retroviral nucleocapsid protein, are structurally distinct from TFIIIA (10).

Three structures of C2H2 zinc fingers, from Zif268 (11), Tramtrack (12), and GLI (13), complexed with their cognate DNA have been solved. In each structure, α-helices lie in the major groove and make base-specific contacts. Zinc fingers 1–3 from TFIIIA bind the 5 S rRNA gene promoter with high affinity and produce a compact footprint similar to Zif268 (14–16). A recent mutagenesis study of TFIIIA has shown that some of the potential amino acid contacts with DNA are at positions predicted to be α-helical by comparison with the Zif268 crystal structure (17). The significance of these putative α-helical amino acids for RNA binding is less clear.

We have investigated whether amino acids in the putative α-helices of zinc fingers are important for RNA and DNA binding affinity.

**EXPERIMENTAL PROCEDURES**

**Ala**nine Substitute Mutant Construction—Site-directed mutagenesis was done using a PCR based procedure. cDNA fragments encoding zinc fingers were amplified from pSPTF15 (18) using specific primers and subcloned into pET28b (+) (Novagen). Mutations were introduced by overlapping PCR using primer pairs encoding the desired amino acid changes.

**Protein Expression and Purification**—Zinc finger polypeptides derived from TFIIIA were expressed from pET28b as polyhistidine-tagged molecules in Escherichia coli strain BL21 (DE3). Cells were grown to anopt 0.6 in 100 ml of LB, supplemented with 100 μM ZnSO4, 40 mM glucose, and 30 μM kanamycin and induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3–4 h. Inclusion bodies containing the fusion protein were collected by centrifugation and extracted by sonication in 5 ml of His-binding buffer (5 mM imidazole, 500 mM NaCl, 50 mM ZnSO4, 100 mM phenylmethylsulfon fluoride, 1 mM benzamidine, 20 mM Tris, pH 8.0) containing 6 M urea. The extract was clarified by centrifugation and passed over a 1–2 ml nickel-agarose column (HIS-bind resin, Novagen) equilibrated with His-binding buffer containing 6 M urea. The column was washed with 10 column volumes of His-binding buffer containing 6 M urea and 6 volumes of column buffer (6 M urea, 500 mM NaCl, 20 mM Tris, pH 8.0) containing 40 mM imidazole. Proteins were eluted with 1 M imidazole in column buffer. Protein-containing fractions were pooled, dialyzed twice against 500 ml of column buffer over a 12-h period and once against 500 ml of 2 M urea, 150 mM NaCl, 1 mM DTT, 2 mM CaCl2, 20 mM Tris, pH 7.9, for 4 h. Derivatives of tz4–7 were digested for 2–4 h with 1–2 units of thrombin (Novagen)100 μg of protein at 25 °C. All proteins were denatured by dialysis against 6 M urea in 500 mM NaCl, 50 mM ZnCl2, 1 mM DTT, 20 mM MES, pH 6.5, and renatured by stepwise dialysis in the same buffer containing progressively lower concentrations of urea. The final dialysis buffer was 100 mM NaCl, 50 mM ZnCl2, 1 mM DTT, 20 mM MES, pH 6.5, 20% glycerol.

**RNA and DNA Binding Assays**—A 66-base pair AsoI fragment of the Xenopus borealis oocyte 5 S rRNA gene internal control region was end-labeled with α-32PdCTP using the Klenow fragment of DNA polymerase I (5). *Xenopus laevis* oocyte-type 5 S rRNA was made by in vitro runoff transcription in the presence of α-32PdCTP (T7 Megascript kit, Ambion). Protein binding to 32P-labeled RNA and DNA was measured by electrophoretic mobility shift (5). For Scatchard analysis, fixed concentrations of protein that produce between 20 and 80% shifted nucleic acid were incubated with DNA (0.4–50 nm) or RNA (0.4–20 nm) in 10 μl of binding buffer (0.05% Nonidet P-40, 90 mM KCl, 10 μM ZnCl2, 5 mM DTT, 8% glycerol, 1 mM MgCl2, 20 mM Tris, pH 7.5) for 30 min at room temperature (21 °C). Nonspecific competitors were also included at 20 μg/ml poly[d(I-C)] for RNA reactions and 20 μg/ml poly(I-C) for DNA binding reactions. Kd values (Table I) are the mean of at least three independent determinations.

**Expression and Selection**—Four codons (−1, +2, +3, and +6 of the α-helix) in zinc finger 4 were randomized by constructing zinc finger 4 from two oligonucleotides, which were annealed and filled in with the Klenow fragment of DNA polymerase I. Randomized zinc finger 4 DNA was digested with BsiWI, ligated to DNA coding for fingers 5–7 synthesized...
Phage Display of RNA Binding Zinc Fingers

RESULTS AND DISCUSSION

The positions of zinc-liganding amino acids in TFIIIA zinc fingers are similar to those of Zif268, as are the positions of conserved hydrophobic residues required for zinc finger structure (20). By analogy to the Zif268 structure, we assumed position 1 of the zinc finger α-helix to be six amino acids amino-terminal to the zinc-ligated histidine. Nucleic acid binding was determined by Scatchard plot. The assay conditions we used (20–80% shifted RNA), provide an accurate measurement of the fraction of shifted RNA, but absolute values for KD could have significant errors if the data are not truly linear in a bound/free versus bound plot, despite correlation coefficients greater than 0.8 (Figs. 1 and 2). To estimate potential errors, we also determined the KD by an alternative method using protein titration and curve-fitting (3). The values differ by 60% (0.1 nM by Scatchard versus 0.16 nM), but the error is probably less, since the determination by protein titration assumes fully active protein. We used scatchard analysis since the fraction of active protein need not be known and we place greater emphasis on relative affinities rather than absolute values for KD.

RNA Binding by Zinc Fingers 4–7—Sequence-specific RNA binding by TFIIIA is mediated through central zinc fingers (3, 6). Mobility shift assays with tz4–7 show a single shifted species at protein-RNA ratios that generate multiple shifted bands for tz1–3 (compare Figs. 1A and 2A). When individual zinc fingers in tz4–7 were substituted at four positions predicted to be within the α-helix, substitution in zinc fingers 4 and 6 showed the most profound reduction in RNA affinity. The estimated dissociation constant for zinc finger 4 substitution (tz4–7A4) was 7.7 nM, a 77-fold reduction from the native tz4–7 KD of 0.1 nM. Alanine substitution in zinc finger 6 (tz4–7A6) reduced RNA affinity 30-fold (Table I). In contrast, alanine substitution in zinc fingers 5 or 7 (tz4–7A5 and tz4–7A7) produced no significant difference in RNA affinity compared with native tz4–7. The absence of large effects for alanine substitution in zinc fingers 5 and 7 suggests that alanine substitutions in tz4–7 do not grossly disrupt the structure of the finger 4–7 fragment.

Clemens et al. (3) previously showed that tz5–7 RNA affinity was 20-fold lower than tz4–7 (using our nomenclature), suggesting an important contribution for zinc finger 4. Our data suggest that substitution of one or more of amino acids Lys2, Asn3, and Thr4 in the context of tz1–7, a Glu3 → Arg substitution in finger 4 results in a 4-fold reduction in affinity, which indicates that one or more of the other alanine-substituted positions, -1, +2, +6, make more significant contributions to RNA binding affinity. Alanine substitutions in zinc finger 6 reduce 5 S RNA affinity to approximately the same degree as a previously reported Thr2 → Ile substitution (3), suggesting that Thr1 may be the most important residue within zinc finger 6 for 5 S RNA affinity. Individual zinc finger "knockout" mutations in TFIIIA that disrupt the zinc liganding capacity of zinc fingers are in agreement with our findings. Substitution of histidine with asparagine in central zinc fingers 4, 5, or 6 reduced RNA affinity 2.6–4.1-fold (21). Alanine substitution in zinc fingers 5 and 7 produced no change in RNA affinity. Yet qualitative assays showing reduced 5 S RNA affinity of fingers 5–9 compared with 6–9 and quantitative assays showing a 60-fold reduction in affinity for fingers 1–6 compared with 1–7 suggest that specific RNA contacts are made by both finger 5 and finger 7 (3, 5). Such contacts presumably lie outside of the zinc finger amino acid positions that we mutated.

RNA and DNA Binding by Zinc Fingers 1–3—Alanine substitution at four putative α-helical amino acids in zinc fingers 1 or 2, within tz1–3, abolished DNA binding (Fig. 2). A discrete band shift was detectable only at a 1500:1 molar ratio of protein to DNA, and DNA titration with a fixed protein concentration was not attempted. Alanine substitution in zinc finger 3 appeared to be less severe, since a discrete band shift appeared at a high molar ratio of protein to DNA (300:1). In contrast, alanine substitutions in zinc finger 1 or 2 (tz1–3A1 and tz1–3A2) reduced RNA binding only 3–4-fold. Substitution in finger 3 (tz1–3A3) has no significant effect on RNA binding by these zinc fingers. These amino-terminal zinc fingers contribute to overall RNA affinity of TFIIIA with lower sequence specificity.

FIG. 1. 5 S rRNA binding activity of alanine substitution mutants of tz4–7. A, complexes between tz4–7 alanine substitution mutants (black boxes are zinc fingers with alanine substitutions at -1, +2, +3, and +6 of the α-helix) were formed with 5 nM 32P-labeled 5 S RNA and increasing concentrations of each protein and resolved by non-denaturing gel electrophoresis. Concentration of each protein is as follows: 6 nM (lane 1), 12 nM (lane 2), 25 nM (lane 3), 50 nM (lane 4), 100 nM (lane 5), 200 nM (lane 6). Lanes marked F indicate control reactions without added protein. Open triangles indicate the position of unbound 5 S RNA. B, representative Scatchard plots for each substitution mutant were derived from titrations of constant protein with variable 5 S RNA, not from the protein titrations above which are included to illustrate the number of shifted bands. KD values derived from at least three plots of each mutant are summarized in Table I. ■, tz4–7; □, tz4–7A4; ▲, tz4–7A5; ●, tz4–7A6; ○, tz4–7A7.
than tz4–7 and can likely achieve this through two zinc fingers, which is the minimal RNA binding fragment from the amino terminus of TFIIIA (5). Consistent with this notion, when any two zinc fingers of tz1–3 are substituted, a clear band shift was observed, suggesting that some of the two zinc fingers of tz1–3 are substituted, a clear band shift was generated by a single-shift. tz1–3 (Fig. 2C), tz1–3A1 (C), tz1–3A2 (C), and tz1–3A3 (C). C, complexes formed with double α-helix mutants of tz1–3 and 5 S rRNA. 32P-Labeled 5 S rRNA (2 nM) was incubated with the following protein concentrations: 1.5 nM (lane 1), 6 nM (lane 2), 24 nM (lane 3), 95 nM (lane 4), 396 nM (lane 5). The position of free 5 S rRNA is indicated with an open triangle.

These experiments suggest amino acids that make significant contributions to TFIIIA DNA affinity are in part the same as that of a-helical amino acids substituted in the RNA contacts made by tz1–3 (Fig. 2C).

Phage Display of Zinc Fingers tz4–7—To determine which amino acids in zinc finger 4 were able to support RNA binding, we used phage display to screen a large number of mutant tz4–7 sequences (23). Native codons for amino acids 1, +2, +3, and +6 in zinc finger 4 were replaced with a degenerate sequence, NNN(G/C), that encodes all 20 amino acids and the amber stop (Fig. 3A). Enrichment of 5 S rRNA binding phage from the zinc finger 4 library over 4 rounds of selection is shown in Fig. 3B. The final round included competitor tRNA at 10-, 100-, or 1000-fold molar excess over 5 S rRNA to reduce nonspecific RNA binding. DNA sequencing of 24 clones from the selection round using a 1000-fold excess tRNA revealed complete conservation of lysine at the −1 position of the zinc finger 4 α-helix. The sequence of 24 clones at the selected positions is shown in Table II. Identity with wild type at positions other than −1 were found in 5 sequences, at Glu 13 and Val 14. However, the majority of clones had only Lys −1 in common with wild type, suggesting an important zinc finger structural or RNA contact role for this amino acid. Serine was present in 14 of 24 clones at +2, suggesting a role for a polar amino acid at this position. Serine is present in many zinc finger proteins at +2, and within the known zinc finger-DNA structures, serine makes contacts with G and T (11, 13, 24). Serine can be represented by three codons in the degenerate sequence, NN(G/C), that encodes all 20 amino acids and the amber stop (Fig. 3A).
codon NNS and therefore may be selected on the basis of its frequency in the library. However serine was not predominant at any other position.

RNA binding activity of selected zinc fingers was confirmed by expression and purification of His-tagged zinc finger mutants from *E. coli*. In protein titration assays, the binding of selected zinc fingers is comparable to wild-type tz4–7 (3). Zinc fingers 5 and 7 may make fewer contacts with 5S rRNA than other zinc fingers. We believe this report is the first demonstration that RNA binding can be successfully assayed by phage display of zinc fingers. Phage display of RNA binding zinc fingers with more extensive degeneracy in individual zinc fingers should reveal the primary structural requirements for RNA binding by the extensive C$_{H2}$ class of zinc finger proteins.

Acknowledgments—We thank E. Rebar and C. Pabo for providing fd.tet.7000 and *E. coli* strains MC1061 and K91.

REFERENCES

1. Engelke, D. R., Ng, S.-Y., Shastry, B. S., and Roeder, R. G. (1989) *Cell* **19**, 717–728
2. Patel, H., and Brown, D. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77**, 4170–4174
3. Clemens, K. R., Wolf, V., McBeary, S. J., Zhang, P., Liao, X., Wright, P. E., and Gottesfeld, J. M. (1993) *Science* **260**, 530–533
4. Clemens, K. R., Zhang, P., Liao, X., McBeary, S. J., Wright, P. E., and Gottesfeld, J. M. (1994) *J. Mol. Biol.* **244**, 23–35
5. Darby, M. K., and Joho, K. E. (1992) *Biochim. Biophys. Acta* **1197–1202
6. Theunissen, O., Rutz, F., Gudhat, U., Mentzel, H., and Pieder, T. (1992) *Cell* **71**, 879–890
7. Caricasole, A., Duarte, A., Larsson, S. H., Haste, N. D., Little, M., Holmes, G., Todorov, I., and Ward, A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 7562–7566
8. Kotter, M., Kuhn, U., Bouwmeester, T., Nijssel, W., el-Baradi, T., Knoehl, W., and Piefer, T. (1991) *EMBO J.* **10**, 3087–3093
9. Gründl, B., Buzinet, M., and Aubry, M. (1996) *J. Biol. Chem.* **271**, 15453–15457
10. Darlix, J. L., Lapadatapolsky, M., Deroquigny, H., and Roques, B. P. (1995) *J. Mol. Biol.* **254**, 523–537
11. Pavleitish, N. P., and Pabo, C. O. (1991) *Science* **252**, 806–817
12. Fairall, L., Schwabe, J. W., Chapman, L., Finch, J. T., and Rhodes, D. (1993) *Nature* **366**, 483–487
13. Pavleitish, N. P., and Pabo, C. O. (1993) *Science* **261**, 1701–1707
14. Hanssen, P. K., Christensen, J. H., Nyborg, J., Lieblund, O., and Thegersen, H. C. (1993) *J. Mol. Biol.* **233**, 191–202
15. Hayes, J. J., and Clemens, K. R. (1992) *Biochemistry* **31**, 11600–11605
16. Liao, X. B., Clemens, K. R., Tennant, L., Wright, P. E., and Gottesfeld, J. M. (1992) *J. Mol. Biol.* **223**, 857–871
17. Zang, W.-Q., Veldhoen, N., and Rumanlou, P. (1995) *Biochemistry* **34**, 15455–15552
18. Vrana, K. E., Churchill, M. E., Tullius, T. D., and Brown, D. D. (1988) *Mol. Cell. Biol.* **8**, 1684–1696
19. Rebar, E. J., and Pabo, C. O. (1994) *Science* **263**, 671–673
20. Kuchyan, M., Keutmann, H. T., and Weiss, M. A. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 8455–8459
21. Setzer, D. R., Menezes, S. B., Delrio, S., Hung, V. S., and Subramanyan, G. (1996) *RNA* **2**, 1254–1269
22. Del Rio, S., Menezes, S. B., and Setzer, D. R. (1993) *J. Mol. Biol.* **233**, 567–579
23. Smith, G. P., and Scott, J. K. (1993) *J. Mol. Biol.* **217**, 228–257
24. Kim, C. A., and Berg, J. M. (1996) *Nat. Struct. Biol.* **3**, 940–945
25. Ginsberg, A. M., King, B. O., and Roeder, R. G. (1984) *Cell* **33**, 479–489
26. Arakawa, H., Nagase, H., Hayashi, N., Ogawa, M., Nagata, M., Fujitani, T., Takahashi, E., Shin, S., and Nakamura, Y. (1996) *Cytogenet. Cell. Genet.* **70**, 205–208
27. Joho, K. E., Darby, M. K., Crawford, E. T., and Brown, D. D. (1990) *Cell* **61**, 293–300
28. Huber, P., and Wool, I. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 1593–1597
29. Wimmerly, B., Varani, G., and Timco, I., Jr. (1993) *Biochemistry* **32**, 1078–1087
30. Christiansen, J., Brown, B., Spratt, B., and Garrett, R. (1987) *EMBO J.* **6**, 453–460
31. McBeary, S. J., Veldhoen, N., Gedulin, B., Leresche, A., Foster, M. P., Wright, P. E., Rumanlou, P. J., and Gottesfeld, J. M. (1995) *J. Mol. Biol.* **248**, 44–57