Nucleic Acids Research, 1998, Vol. 26, No. 3 703–709

The role of zinc finger linkers in p43 and TFIIIA binding to 5S rRNA and DNA

Robert F. Ryan+ and Martyn K. Darby*

Department of Microbiology and Immunology, Kimmel Cancer Institute, Thomas Jefferson University, 233 South 10th Street, Philadelphia, PA 19107, USA

Received November 21, 1997; Accepted December 3, 1997

ABSTRACT

Transcription factor IIIA (TFIIIA) and p43 zinc finger protein form distinct complexes with 5S ribosomal RNA in *Xenopus* oocytes. Additionally, TFIIIA binds the internal promoter of the 5S RNA gene and supports assembly of a transcription initiation complex. Both proteins have nine tandemly repeated zinc fingers with almost identical linker lengths between corresponding fingers, yet p43 has no detectable affinity for the 5S RNA gene. TFIIIA zinc fingers 1–3 are connected by highly conserved linkers, first identified in the *Drosophila* protein Krüppel, that are found in many DNA binding zinc finger proteins. To understand the role of these linkers in RNA and DNA binding we exchanged three TFIIIA linker amino acids with the equivalent amino acids from p43. The major effect of linker substitution is a 50-fold reduction in DNA specificity, concomitant with an 8-fold reduction in affinity. N-Terminal zinc fingers from either TFIIIA or p43 bind to multiple specific sites on 5S RNA that are resistant to competition by tRNA or poly(rA). This mode of RNA binding is unaffected by linker substitution. These data suggest that zinc finger linkers significantly facilitate the specificity of DNA binding.

INTRODUCTION

Zinc finger proteins perform diverse biological functions in regulation of cell growth and differentiation through DNA–, RNA– and protein–protein interactions. Zinc fingers of the C2H2 class coordinate a single zinc atom through two invariant cysteines in an antiparallel β-sheet and two histidines in an α-helix. Molecular determinants for DNA binding have been identified by biochemical experiments and by direct crystallographic observation of zinc finger–DNA complexes (1–3). Zinc finger proteins that mediate binding to RNA have also been identified (4–7). Recently the zinc finger protein WT-1, implicated in Wilm's tumor, has been identified in splicing complexes and is reported to bind RNA *in vitro* (8,9). The molecular determinants for interaction of individual zinc fingers from these proteins with RNA are not fully understood.

The archetypal C2H2 zinc finger protein is the *Xenopus* transcription factor TFIIIA (10,11). TFIIIA has nine tandemly repeated zinc fingers and a C-terminal domain required for transcription (12,13). TFIIIA binds both the 5S RNA gene promoter (internal control region, ICR) and, in developing oocytes, is complexed with 5S RNA in a 7S RNP (14). A related 5S rRNA binding protein, p43 (4), found complexed with 5S RNA in a 42S RNP (15), has identical zinc finger length and similar H/C linker length and appears to bind to the same region of 5S RNA (16). However, binding assays have failed to detect p43 binding to the 5S RNA gene (4). Furthermore, p43 has no sequence homology with TFIIIA in the C-terminal domain, suggesting that it is not a transcription factor for 5S RNA genes. Since p43 binds exclusively to 5S RNA, comparison of the effects of mutation in TFIIIA and p43 provides an excellent model system for investigating the differences in the molecular basis for RNA and DNA recognition.

Within TFIIIA, different groups of zinc fingers have differential binding affinities for RNA and DNA (17–19). The three N-terminal zinc fingers have the highest DNA affinity of any isolated group of three fingers from TFIIIA (19–22). These zinc fingers are uniquely connected by H/C linker sequences similar to a highly conserved linker sequence, TGEKPY, first identified in the *Drosophila* homeotic gene *Krüppel* and present in many DNA binding zinc finger proteins (23). Conservation of the Krüppel-like H/C linker sequence suggests that linkers play a role in enabling adjacent zinc fingers to bind DNA. Mutation of H/C linkers between N-terminal fingers of TFIIIA has been shown to decrease or abolish DNA affinity. Mutation of a single glycine to serine or lysine to glutamic acid between zinc fingers 1 and 2 in full-length TFIIIA results in a weakened TFIIIA footprint on the 5S RNA gene (24). Similar effects for linker mutagenesis, at most a 3-fold reduction in affinity, have been reported recently in extensive scanning mutagenesis of TFIIIA (25). In the context of three N-terminal zinc fingers, mutations in linkers can cause more severe disruption of DNA binding. Substitution of threonine with serine in linker 1 decreases DNA binding affinity 11-fold by increasing the dissociation rate (26). More extensive amino acid changes that substitute both H/C linkers in TFIIIA zinc fingers 1–3 with p43-derived linkers have been reported to completely abolish DNA binding activity (27,28).

The role of H/C linkers in RNA binding is less well understood. The TFIIIA and p43 zinc fingers are generally connected by linkers of similar length. However, TFIIIA zinc fingers 4–7 show the highest discrimination for RNA and these zinc fingers are connected by unusually short H/C linkers (19). In full-length TFIIIA, all zinc fingers contribute to overall 5S RNA binding affinity (17), with mutations in zinc finger 6 that disrupt finger

*To whom correspondence should be addressed. Tel: +1 215 503 4504; Fax: +1 215 923 0249; Email: m_darby@lac.jci.tju.edu

+Present address: The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104, USA

structure having the most profound effects (18,19,29). Deletion analysis of p43 showed that N-terminal zinc fingers 1–4 and full-length p43 have comparable affinity for 5S RNA (17).

To understand the contribution of H/C linkers to specific RNA or DNA binding affinity we have compared the nucleic acid binding properties of N-terminal zinc fingers from TFIIIA and p43 when H/C linkers were exchanged. We find that mutation of H/C linkers has the greatest effect on DNA binding specificity with a smaller effect on affinity. In contrast, 5S RNA binding by three zinc fingers is independent of a unique linker sequence and occurs at multiple specific sites on 5S RNA.

MATERIALS AND METHODS

Construction of TFIIIA and p43 mutants

TFIIIA cDNA from plasmid pTA102, kindly provided by Dr David Setzer, was tailed with a double-stranded DNA adapter oligonucleotide, 5′-d(AATTCCCGGGCTAGCTGACTAG) and 5′-d(GATCCTAGTCAGCTAGCCCGGG), and inserted via a *Bam*HI linker into pGEM®5Zf(–) (Promega) to create pRR0. The cDNA was then altered by site-directed mutagenesis to introduce nucleotides encoding amino acids found at the equivalent position in p43 (30). The mutagenic oligonucleotides used were 5′-d(GCAGGCGCATCTGTGCAAACA**CTCAG**AACAGAAACCATTTCCATG), to change TGE to SEQ in the first H/C linker, and 5′-d(CCCGCCACTCACTCACTCATCTTGCC**TTTAAA**ACTTCACATGTG), to change TGE to LAL in the second H/C linker (nucleotides that differ from wild-type are underlined and the restriction sites introduced are in bold). These oligonucleotides were used separately or in combination with single-stranded pRR0 DNA as a template to introduce linker mutations. The complementary strand was synthesized with T7 DNA polymerase (US Biochemical) (30). Plasmids containing mutated sequence were identified by the presence of either *Dde*I or *Dra*I restriction sites, introduced by mutagenic oligonucleotides, or the absence of a *Xmn*I site and confirmed by dideoxy sequencing. The full-length wild-type and mutated cDNAs were subcloned into the expression vector pET-15b (Novagen), which introduces an N-terminal polyhistidine tag and thrombin recognition site.

To express the N-terminal zinc fingers from TFIIIA, a DNA fragment encoding zinc fingers 1–3 was generated by PCR amplification of the full-length TFIIIA cDNA using the T7 primer [5′-d(TAATACGACTCACTATAGGG)], 5′ of the methionine start in pET-15b, and a 3′ primer corresponding to the end of zinc finger 3 [5′-d(GGCGCTAGCTCAGATCTTG-ATGTTATGGAA)]. Fragments were inserted into pET-15b using the 5′ *Nde*I and 3′ *Bam*HI sites and sequenced. Similarly, DNA encoding the first three zinc fingers from p43 were amplified from the full-length p43 cDNA (4) by PCR using primers PZN1 [5′-d(CGGGATCCCATGAAAAA-CGTTGGT)] and PZC3 [5′-d(AGTGGATCCTATACGGCCTCC-CCGT)]. The product was digested with *Bam*HI, gel purified and subcloned into the *Bam*HI site of pET15b. The H/C linker between p43 zinc fingers 1 and 2 was introduced into the p43 cDNA using the mutagenic oligonucleotide 5′-d(GGCTGGGCACACCGGTG-AGAAG-CCGTGG) to generate pHPZ1. The mutation in the second H/C linker was generated by overlapping PCR. In one PCR a forward primer corresponding to the N-terminal end of finger 1, PZN1, was used with a reverse primer complementary to the H/C linker mutation, 5′-d(GAGTTTCTTCTCGCCGGTGTGTCGCT-TC), with pHPZ1 as template. In a second PCR, also with pHPZ1 as template, a forward primer corresponding to the H/C linker mutation, 5′-d(GAAGCGACACACCGGCGAGAAGAAACTC), was used with a reverse primer to the C-terminal end of finger 3, PZC3. The products of these reactions were purified by agarose gel electrophoresis and used as templates in a third PCR with primers corresponding to the N-terminus of zinc finger 1 (PZN1) and C-terminus of zinc finger 3 (PZC3). Final PCR products were subcloned into the pET-15b *Bam*HI site and sequenced.

Purification of zinc finger proteins

Xenopus laevis rTFIIIA was expressed in *Escherichia coli* strain BL21(DE3) from plasmid pTA102 and purified by ammonium sulfate precipitation and Biorex70 ion exchange chromatography as described by Del Rio and Setzer (31). Three zinc finger polypeptides derived from TFIIIA or p43 were expressed from pET vectors as polyhistidine tagged molecules in *E.coli* strain BL21(DE3), which harbors an IPTG-inducible T7 RNA polymerase gene (32). Cells harboring the appropriate plasmid were grown to an OD_{600} of 0.4–0.6 in Rich broth supplemented with 100 µM zinc sulfate, 40 mM glucose and 200 µg/ml ampicillin. Protein expression was induced by addition of 0.5 mM IPTG for 3–4 h. Cells were harvested, lysed with lysozyme (150 µg/ml) and deoxycholate (0.05%) and disrupted by sonication. Inclusion bodies containing the fusion protein were precipitated by centrifugation and extracted overnight in 15 ml binding buffer (5 mM imidazole, 500 mM NaCl, 50 µM $ZnSO_4$, 100 µM PMSF, 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 binding buffer containing 6 M urea. The column was washed with 10 column vol binding buffer containing 6 M urea and proteins eluted with 400 mM imidazole, 200 mM NaCl, 6 M urea, 50 µM $ZnSO_4$, 8 mM Tris, pH 8.0 (elution buffer). Protein-containing fractions were analyzed by Tris–tricine gel electrophoresis (33) and peak fractions were pooled. Zinc fingers were renatured by step-wise dialysis from elution buffer containing 6 M urea to 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 150 mM NaCl, 50 µM $ZnSO_4$, 10 mM DTT, 10% glycerol, 50 mM Tris, pH 8.0, prior to digestion. Proteins were digested for 2 h with 1–2 U thrombin (Calbiochem)/100 µg protein at 25°C and dialyzed at 4°C prior to storage in buffer containing 1 mM benzamidine at –80°C. Protein concentration was determined by BioRad protein assay. BSA was used as the standard and final protein concentrations were determined by multiplying the apparent concentration by 0.62 to correct for dye binding differences between BSA and TFIIIA (34). Amino acid compositions and concentrations of TFIIIA-derived protein fragments were confirmed by amino acid analysis.

DNA and RNA substrates

The DNA substrate used was a 66 bp *Ava*I fragment containing the *Xenopus borealis* somatic 5S RNA gene internal control region (ICR) from bp 39 to 99 (17). DNA concentration was determined as OD_{260}. Gel-purified DNA (2 µg) was end-labeled with [α-^{32}P]dCTP using the Klenow fragment of DNA polymerase I and purified by spin column chromatography over Sephadex G-25 (Boehringer Mannheim).

Xenopus laevis oocyte-type 5S RNA was made by *in vitro* run-off transcription (T7 Megascript kit; Ambion) from plasmid pT7OD, containing a T7 promoter 5′ of the RNA coding region and a *Dra*I site at the 3′-end. The resultant RNA is 121 nt long and has one additional uridine at the 3′-end not found in native 5S RNA. Helix IV/V RNA was similarly generated by run-off

transcription of plasmid pMC2, encoding 5S RNA nt 64–112, linearized with *Bsr*GI. RNA was radiolabeled by inclusion of 100 µCi [α-^{32}P]CTP (456 µM) in a 20 µl transcription reaction. 5S RNA was purified by denaturing electrophoresis through 12% polyacrylamide, 8 M urea, 40 mM Tris–acetate, pH 7.2. After elution from polyacrylamide gel slices using 40 mM Tris–acetate, 1 mM EDTA, pH 7.4, in DEPC-treated H_2O, RNA was renatured by heating to 65°C for 15 min in 10 mM $MgCl_2$, followed by slow cooling over 30 min to 25°C. RNA was equilibrated with DEPC H_2O by spin column chromatography on Sephadex G-50 (Boehringer Mannheim).

Determination of dissociation constants

Apparent equilibrium dissociation constants were determined by Scatchard analysis of bound and free nucleic acid (35). A fixed protein concentration (10–200 nM) was incubated with a range of nucleic acid concentrations (1–200 nM DNA, 0.6–20 nM RNA) in binding buffer (70 mM KCl, 1 mM $MgCl_2$, 10 µM $ZnSO_4$, 0.1% NP-40, 5% glycerol, 10 mM DTT, 10 µg/ml poly(dI·dC), 2 µg/ml poly(rA), 20 mM Tris, pH 8.0) in a total volume of 10 µl. Free and protein-bound DNA or 5S RNA were separated by non-denaturing gel electrophoresis through 5% polyacrylamide, 1% glycerol, 20 mM HEPES, pH 8.3, at 4°C, with the upper buffer chamber containing 2 mM DTT. Gels were pre-electrophoresed for 1 h at 23 V/cm and allowed to re-equilibrate to 4°C prior to loading. Samples were loaded with the gel running at 4.5 V/cm and switched to 25 V/cm for the duration of the run (45–90 s). Appropriate protein concentration ranges were determined empirically in preliminary experiments to give a broad range of data points in the Scatchard plot of bound/free nucleic acid against amount of nucleic acid bound. The quantity of radiolabeled substrate in free and bound fractions was determined by laser densitometric scanning of autoradiograms and/or by phosphorimage analysis of dried gels. Apparent K_d values for each protein were calculated by fitting data to a modified form of the Scatchard equation

$$[\text{nucleic acid}_{\text{bound}}]/[\text{nucleic acid}_{\text{free}}] = \{-(1/K_d) \times [\text{nucleic acid}_{\text{bound}}]\} + \{[\text{protein}_{\text{total}}]/K_d\}$$

by linear regression using Cricket Graph (Computer Associates) software (22,31,36). Standard errors of the mean were determined by combining values for individual experiments with correlation coefficients >0.7 derived from eight or more data points using SYSTAT (Systat Inc.) software.

Approximate ratios of affinity constants for the first (K_1) and second (K_2) shifted species were derived from the equation

$$I_0 I_2/(I_1)^2 \approx K_2/K_1, \text{ when } K_1 >> K_2 \text{ (37)}.$$

I_0, I_1 and I_2 are the intensities of radioactive bands representing free nucleic acid, nucleic acid bound in the first complex and nucleic acid bound in the second complex respectively. The error inherent in this equation is $(1 + K_2/K_1)^2$. The error is small when K_1/K_2 is large (e.g. when $K_1/K_2 = 20$ the error is 1%), but becomes much larger when the ratio of affinity constants is small (when $K_1/K_2 = 2$ the error = 56%).

RESULTS

Construction of H/C linker mutants

To compare the influence of linker amino acids on RNA and DNA affinities of zinc fingers we substituted linker amino acids between zinc fingers from *Xenopus* TFIIIA, which bind DNA and RNA, with the corresponding amino acids from a related zinc finger protein, *Xenopus* p43, which binds exclusively to 5S RNA. Ideally, the influence of an individual linker is best studied in a two zinc finger fragment that is connected by a single linker. However, while two N-terminal zinc fingers are sufficient for 5S RNA binding, three are required for DNA binding (17). Therefore, to determine the effects of linker changes on RNA and DNA affinity of zinc fingers we substituted amino acids linking zinc fingers in the minimal three finger N-terminal fragment of TFIIIA that binds both DNA and RNA.

Three substitution mutants of TFIIIA zinc fingers 1–3 (*tz1–3*) were made by oligonucleotide-mediated site-directed mutagenesis of the cDNA. Proteins *tz1–3.pl1*, *tz1–3.pl2* and *tz1–3.pl1/2* have three linker amino acids substituted between zinc fingers 1 and 2, zinc fingers 2 and 3 and zinc fingers 1, 2 and 3 respectively with corresponding amino acids from p43. Similarly, H/C linkers between the three N-terminal zinc fingers of p43 were substituted with the TGE sequence from TFIIIA. Only three amino acids in the linker were changed, because the other amino acids in the KrYppel-like linker are either the same in TFIIIA and p43 (KP in linker 1 or K in linker 2) or else they are predicted to be part of the zinc finger β-sheet from the Zif268 crystal structure and not part of the linker (2). Amino acid substitutions and protein nomenclature are described in Figure 1A.

Zinc finger proteins were expressed in *E.coli* BL21(DE3) cells with an N-terminal polyhistidine tag and purified to homogeneity by affinity chromatography over nickel–agarose. The N-terminal fusion was proteolytically cleaved from the zinc fingers with thrombin prior to use (Fig. 1B). All proteins contain three vector-derived amino acids at the N-terminus (Gly-Ser-His) after removal of the polyhistidine tag. The mobility of p43 fingers after cleavage with thrombin is significantly slower than TFIIIA fingers in this gel system (33). However, prior to cleavage all recombinant protein molecular weights were as predicted from conceptual translation.

Mutation of the conserved H/C linker sequence reduces DNA affinity

Estimates for K_d were obtained from linear regression of Scatchard plots, since the determination of K_d from the slope of the line is independent of any accurate knowledge of protein concentration (35). This is particularly important for these experiments, because we know the fraction of active protein to be variable between mutant proteins and between preparations of the same protein. A linear Scatchard plot assumes equal binding affinity of all sites on a protein for ligand. Since this is unlikely in the cases where we detect multiple shifted species, we used high RNA concentrations that produce a low fraction of bound material to estimate K_d for the first stoichiometric binding constant (38).

Wild-type TFIIIA zinc fingers 1–3, *tz1–3*, bind DNA with a dissociation constant of ~3 nM (Fig. 2 and Table 1). Substitution of amino acids TGE (Thr-Gly-Glu) in the conserved Krüppel-like H/C linker, TGEKPF between zinc fingers 1 and 2, with amino acids SEQ (Ser-Glu-Gln) from p43 (*tz1–3.pl1*), reduced DNA affinity 5-fold. A similar reduction in affinity (3-fold) was observed when the second H/C link, TGE, was substituted with LAL (*tz1–3.pl2*). The DNA binding affinity of *tz1–3.pl1/2*, in which both linkers were substituted, was reduced 8-fold ($K_d = 24 \pm 4$ nM) compared with the native TFIIIA fragment (*tz1–3*).

Substitution of the linker sequences caused a striking reduction in DNA binding specificity that is readily apparent from protein binding isotherms (Fig. 3). The DNA retardation assay using a wild-type TFIIIA fragment (*tz1–3*) generated a single discrete band representing the binary DNA–protein complex when as much as 90% of the RNA was bound (Fig. 3A). In contrast, the TFIIIA mutants with either one or both H/C linker substitutions generated multiple bands when 50% of the RNA was bound, suggestive of protein binding to multiple sites on the DNA fragment (Fig. 3B). To determine the relative affinity of the multiple species formed with the double linker mutant, complexes were titrated with a non-specific competitor DNA, poly(dI·dC) (Fig. 4). The difference in affinity of the higher order complexes is readily apparent from their titration at low, 10-fold, excess of non-specific competitor. The binary *tz1–3*–DNA complex is reduced by 50% with a 2000-fold base pair excess of poly(dI·dC), whereas the binary complex formed with the double linker substitution mutant is eliminated with a 500-fold base pair excess. The affinity of the binary complex is estimated to be 200-fold greater than the ternary complex for the wild-type zinc fingers, but only 4-fold for the mutant in which both H/C linkers had been changed to p43 sequences (Table 2; 37).

Mutation of the H/C linker does not affect RNA binding

The three N-terminal zinc fingers from TFIIIA have a comparable affinity (within 5-fold) for both 5S RNA and the ICR DNA fragment under our assay conditions, in contrast to an earlier report of a 20-fold difference (19), but in agreement with Setzer *et al.* (39). One striking feature of RNA binding for all TFIIIA three zinc finger fragments is formation of multiple shifted species in an RNA mobility shift assay, suggesting that RNA binding by these zinc fingers is inherently less specific than DNA binding (Fig. 3). We measured dissociation constants at low protein excess (10–40% shifted substrate) to give an approximate affinity for the first 5S RNA binding site. 5S RNA affinity of wild-type TFIIIA and the three linker substitution mutants was comparable (Fig. 2 and Table 1), demonstrating that the p43 linker amino acids support RNA binding in the context of TFIIIA zinc fingers. Rates for association and dissociation of the TFIIIA fragments from 5S RNA, like DNA, were also faster than we could measure by gel retardation (data not shown).

Native p43 zinc fingers 1–3 (*pz1–3*) bind 5S RNA with a 2- to 3-fold higher affinity than the corresponding TFIIIA zinc fingers. Substitution of the p43 H/C linkers between fingers 1, 2 and 3 with TGE to generate Krüppel-like H/C linkers did not confer DNA binding to the resulting p43/TFIIIA hybrid (*pz1–3.tl1/2*) nor was the affinity for 5S RNA decreased (Figs 2 and 3 and Table 1).

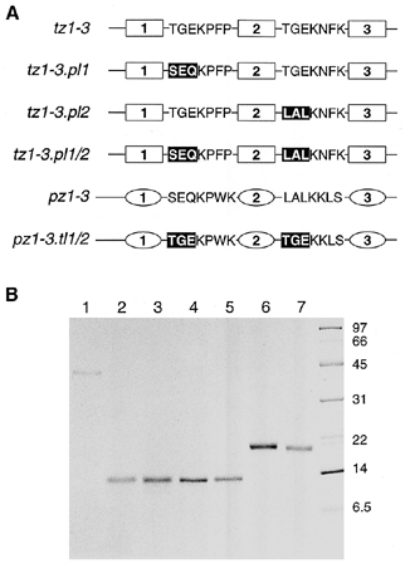

Figure 1. TFIIIA and p43 wild-type and mutant three zinc finger proteins. (**A**) Schematic drawing of the proteins used in this study. The first three zinc fingers of TFIIIA (rectangles) or p43 (ovals) were used to construct proteins containing either wild-type or mutant H/C links. Protein nomenclature is *tz* or *pz* to denote TFIIIA or p43 zinc fingers respectively. The linker origin is denoted by *tl* for the TFIIIA linker or *pl* for the p43 linker and the position of the linker substitution is denoted by the number which follows. (**B**) Purified, recombinant proteins (~0.5 µg) were electrophoresed on a 15% Tris–tricine polyacrylamide gel in the presence of SDS and visualized by Coomassie blue staining. Lane 1, TFIIIA; lane 2, *tz1–3*; lane 3, *tz1–3.pl1*; lane 4; *tz1–3.pl2*; lane 5, *tz1–3.pl1/2*; lane 6, *pz1–3*; lane 7, *pz1–3.tl1/2*. Molecular weight markers (kDa) are shown on the right side.

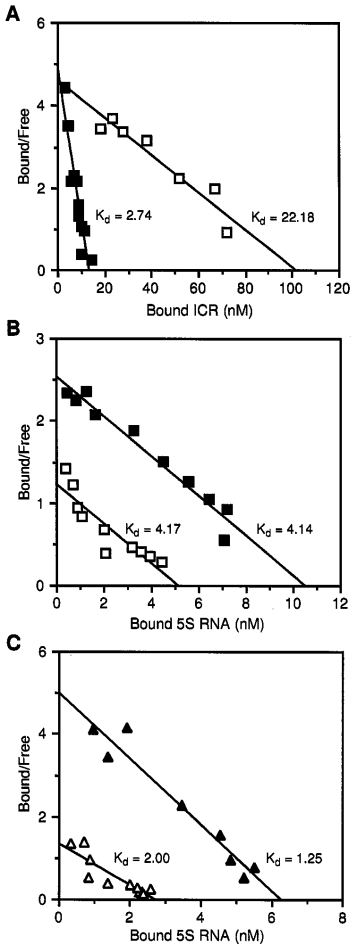

Figure 2. Representative Scatchard plots for DNA and RNA affinity of H/C linker mutants. Gel mobility shift assays were done using constant protein concentrations and varying the concentration of a 5S RNA gene ICR fragment (0.6–200 nM) (**A**) or oocyte-type 5S RNA (0.6–20 nM) (**B** and **C**). Representative examples are shown of Scatchard plots used to derive equilibrium binding constants presented in Table 1. For the analyses shown protein concentrations used were: in (A) 20 nM *tz1–3* (■) and 200 nM *tz1–3.pl1/2* (❑); (B) 20 nM *tz1–3* (■) and 20 nM *tz1–3.pl1/2* (❑); (C) 30 nM *pz1–3* (▲) and 15 nM *pz1–3.tl1/2* (Δ).

Table 1. Apparent dissociation constants for H/C linker substitution mutants

Protein	5S RNA		Helix IV/V RNA		ICR DNA	
	K_d ± SEM (nM)[a]	n[a]	K_d ± SEM (nM)	n	K_d ± SEM (nM)	n
rTFIIIA	0.4 ± 0.1	2			0.9 ± 0.1	3
tz1–3	6 ± 1	4	6 ± 1	5	3 ± 0.1	4
tz1–3.pl1	7 ± 1	4			15 ± 3	4
tz1–3.pl2	7 ± 1	5			9 ± 1	3
tz1–3.pl1/2	9 ± 2	5	15 ± 2	4	24 ± 4	4
pz1–3	2 ± 0.2	3	5 ± 1	4		
pz1–3.tl1/2	2 ± 0.1	2	4 ± 1	4		

[a]Standard error of the mean of *n* experiments.

Multiple RNA–protein complexes were formed by both wild-type and mutant fragments of TFIIIA and p43 (Fig. 3). To determine the specificity of binding we titrated complexes formed between 5S RNA and subsaturating amounts of wild-type and double linker mutants with non-specific and specific RNA substrates (Fig. 5). Unlabeled RNA that contained the predicted protein binding site, i.e. 5S RNA and a helix IV/V RNA fragment from 5S RNA, competed with radiolabeled 5S RNA for protein binding. Non-specific RNA, i.e. tRNA or poly(rA), were unable to compete for binding at the same base pair concentration. These results suggest that the multiple shifted species are sequence- or structure-specific complexes. We estimate the affinity of the binary complex to be only 2- to 3-fold greater than the ternary complex for wild-type TFIIIA fingers (*tz1–3*) or the double linker substitution mutant (*tz1–3.pl1/2*) (Table 2). There appears to be no difference in RNA specificity or affinity between wild-type and linker substitution mutants. A 5S RNA gene fragment containing the TFIIIA binding site competed for *tz1–3* binding better than 5S RNA, but competed poorly for the double substitution mutant *tz1–3.pl1/2*, as predicted by our K_d determinations (Fig. 5A and B). The same DNA fragment did not compete for binding to p43-derived zinc fingers (Fig. 5C and D).

DISCUSSION

Our findings demonstrate a difference in the contribution of H/C linkers to RNA and DNA binding affinity by two related zinc finger proteins. Although a reduction in DNA binding affinity is observed, qualitatively similar to previous reports, the major effect of linker mutation is a reduction in binding site specificity. RNA binding by the same N-terminal zinc fingers occurs at multiple specific sites. The affinity for these sites is unchanged by linker mutation.

Crystallographic and NMR studies of DNA–zinc finger protein complexes show that linkers are an extended and mobile connection between adjacent zinc fingers (2,40,41). It has been suggested that since no direct contacts with the DNA are made by the linker, the relative orientation of zinc fingers is determined by DNA contacts made with the α-helix (40). However, a potentially crucial role for linkers may exist in the light of the strong evolutionary conservation of H/C linkers in zinc fingers that bind DNA. N-Terminal TFIIIA fingers have a rod-like structure with extended linkers when free in solution (42). If TFIIIA binds DNA along the major groove, bending around the DNA axis, the necessary change in relative finger orientation may be permitted only by particular linker amino acids. The linker glycine residue has been shown to make an important contribution to TFIIIA DNA binding affinity, perhaps by providing sufficient flexibility to wrap in the DNA major groove, regardless of its position within the linker (24). TFIIIA zinc fingers 1–3, which provide much of the DNA binding affinity, are the only zinc fingers connected with a Krüppel-like linker. If N-terminal p43 zinc fingers interact with

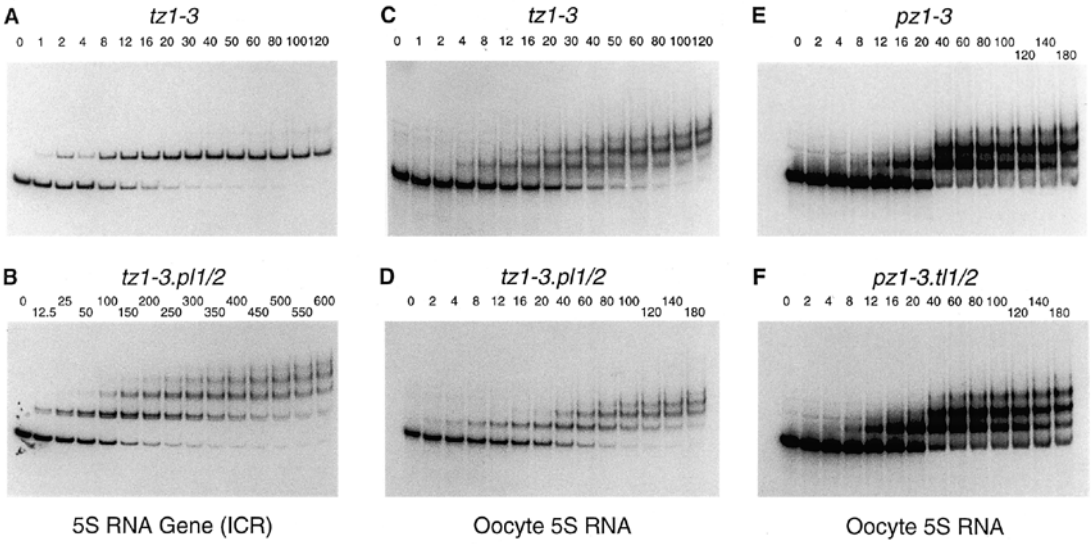

Figure 3. DNA and RNA binding by zinc finger H/C linker mutants. The indicated concentration of protein (nM) was incubated with radiolabeled internal control region DNA (9 nM) (**A** and **B**) or radiolabeled oocyte-type 5S RNA (7.5 nM) (**C–F**) and electrophoresed through a non-denaturing polyacrylamide gel.

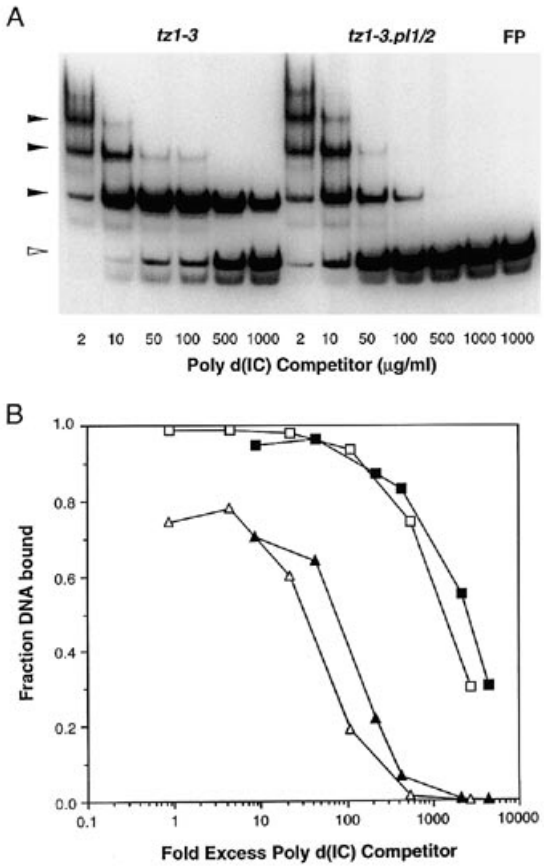

Figure 4. Mutation of the H/C linkers reduces DNA specificity. (**A**) Wild-type TFIIIA zinc fingers (*tz1–3*, 200 nM) or a substitution mutant with both linkers changed (*tz1–3.pl1/2*, 200 nM) were incubated with a radiolabeled 5S gene fragment (5 nM) in the presence of increasing concentrations of the non-specific DNA competitor poly(dI·dC). Protein-bound DNA was separated from free by non-denaturing gel electrophoresis. Closed arrows indicate the major binding species and the open arrow the free probe position. FP denotes the free probe lane not containing protein. (**B**) Gels from two separate experiments were quantified by phosphorimage analysis: *tz1–3* [100 nM (❑) and 200 nM (■)] and *tz1–3.pl1/2* [100 nM (Δ) and 200 nM (▲)]. The fraction of DNA in all bound species is plotted against the fold base pair excess of poly(dI·dC) competitor over 5S DNA fragment.

helical regions of 5S RNA they may not require the same degree of linker flexibility, since the Krüppel-like linker is absent.

Substitution of amino acids in both H/C linkers between TFIIIA zinc fingers 1–3 with linker amino acids that connect equivalent fingers in p43 reduces DNA affinity 8- to 10-fold. This decrease in DNA binding affinity is substantially lower than previously reported by Choo and Klug (27), who were unable to detect DNA binding by a similar hybrid protein. The difference may arise from our use of recombinant protein compared with *in vitro* translated proteins assayed in translation extracts, which contain endogenous competitor 5S RNA. Similarly, Clemens *et al.* (26) reported formation of aggregates with a mutant similar to *tz1–3pl1*. We have not observed aggregation and instead resolve specific complexes and estimate a K_d of 15 nM. The absence of changes in RNA affinity provides an internal control for the integrity and activity of our recombinant proteins. Exchange of amino acids between full-length TFIIIA and p43 to change single H/C linker sequences has recently been done by Zang *et al.* (25). Single linker changes decrease DNA affinity by at most 3-fold in full-length TFIIIA, with no detectable change in RNA affinity.

Table 2. Estimated ratio of association constants for the first and second protein binding sites

Protein	K_1/K_2[a]	
	5S RNA	ICR DNA
tz1–3	1.8	203.2
tz1–3.pl1	2.3	21.3
tz1–3.pl2	2.2	19.2
tz1–3.pl1/2	2.4	4.0
pz1–3	2.8	
pz1–3.tl1/2	2.4	

[a]K_1 and K_2 are the apparent association constants for formation of the binary and ternary complexes respectively. The ratio was derived from gel mobility shifts as described in Materials and Methods.

H/C linkers appear to facilitate specific DNA binding. When p43 linker amino acids are substituted into a three zinc finger fragment from TFIIIA multiple DNA complexes are detected at protein to DNA ratios that generate a single DNA complex for native zinc fingers. This observation suggests that zinc fingers have an inherent non-specific DNA binding activity that is revealed when specific protein–DNA contacts cannot be made. The K_d for the double substitution mutant (24 nM) is close to the K_d for non-specific DNA binding by TFIIIA (30 nM; 43), suggesting that specific DNA binding affinity is close to a minimum. Wild-type TFIIIA zinc fingers also bind to multiple DNA sites at high protein concentrations. However, competition with poly(dI·dC) shows that wild-type zinc fingers have a 60-fold greater resistance to non-specific competitor DNA when compared with the double substitution mutant at the titration midpoint. Comparison of relative affinities for binary and ternary DNA complexes shows that wild-type fingers form a DNA binary complex with 50-fold greater discrimination than the double linker substitution mutant. These two different methods reveal a substantial change in specificity as a consequence of the linker substitutions. Reduced DNA affinity *per se* does not result in multiple shifted DNA species, since mutation of four α-helical amino acids in zinc finger 3 essentially abolishes DNA binding, yet the residual band shift is a single species (36).

Xenopus zinc finger proteins p43 and TFIIIA both have nine tandemly repeated zinc fingers and bind the same target RNA, yet they show only 15% identity at amino acid positions not directly involved in forming the zinc finger motif (4). Despite these differences in amino acid composition, the basic skeleton of the proteins is almost identical. For example, changes in linker length between equivalent zinc fingers are highly conserved, suggesting that the sequence or length of all H/C linkers may be important for 5S RNA recognition. However, RNA binding affinity of zinc fingers 1–3 is unaffected by the linker substitutions in either protein. Estimated changes in K_d for the first 5S RNA complex are at most 1.4-fold between wild-type *tz1–3* or *pz1–3* and the corresponding double linker substitution mutants (*tz1–3.pl1/2* and *pz1–3.tl1/2*). In addition, p43 N-terminal zinc fingers have a 3-fold greater affinity for 5S RNA than equivalent TFIIIA zinc fingers. Since the affinity of full-length p43 is similar to TFIIIA (44), the distribution of RNA binding affinity over individual zinc fingers of p43 may be different from TFIIIA, which makes RNA contacts primarily through zinc fingers 4–7 (19,28).

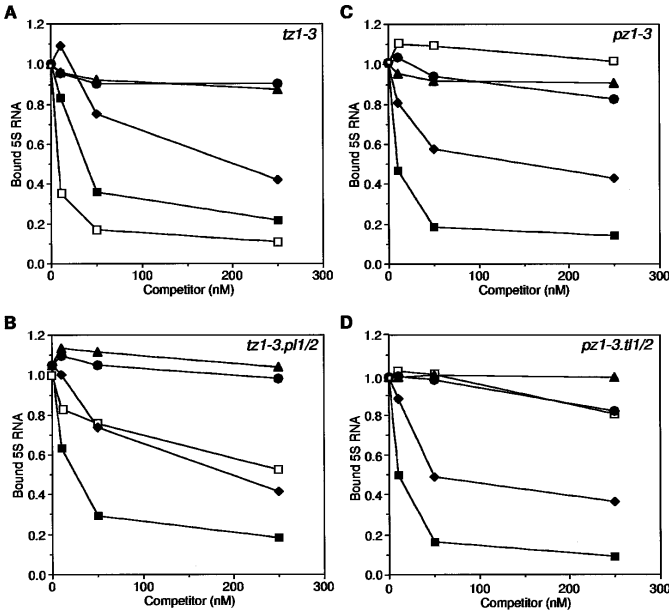

Figure 5. Specificity of RNA binding by H/C linker mutants. TFIIIA zinc fingers 1–3 (12 nM) or p43 zinc fingers 1–3 and linker-substituted proteins (10 nM) were incubated with radiolabeled 5S RNA (1 nM) in the presence of unlabeled competitor nucleic acid. The fraction of protein-bound 5S RNA was determined by non-denaturing gel electrophoresis. The plots shown are the average of at least three experiments normalized to 1.0 for the fraction of 5S RNA bound in the absence of competitor. This represented ~60% bound RNA. Competitors are 5S RNA (■); 5S RNA helix IV/V (◆); yeast tRNA (●), poly(rA) (▲); ICR DNA (❑).

In contrast to the marked reduction in DNA specificity, linker substitution does not change the pattern of multiple RNA complexes formed with *tz1–3* or *pz1–3*. Competition binding assays show these complexes to be specific, either for sequence, structure or both. 5S RNA binding by three finger polypeptides from TFIIIA and p43 are competed by specific competitors, 5S RNA and helix IV/V RNA, but not by similar concentrations of non-specific competitors, tRNA and poly(rA). In addition, the primary RNA–protein complex formed by wild-type and mutant proteins is resistant to a 25 000-fold mass excess of poly(rA) (data not shown).

N-Terminal zinc fingers of p43 may have evolved as a structure optimal for RNA binding, whereas the equivalent TFIIIA zinc fingers represent an evolutionary compromise for binding RNA and DNA. However, absence of Krüppel-like H/C linkers alone cannot account for the inability of p43 to bind DNA. This is demonstrated by the p43-derived mutant *pz1–3.tl1/2*, which has Krüppel-like linkers but fails to bind DNA. RNA binding affinity is also not reduced by these substitutions, suggesting that affinity of p43 zinc fingers for 5S RNA derives entirely from contacts made by amino acids within the bounds of the zinc ligands. Clearly, N-terminal zinc fingers of p43 and TFIIIA are sufficiently divergent that RNA binding fingers in p43 are no longer able to make specific DNA contacts even if TFIIIA linkers are present.

ACKNOWLEDGEMENTS

We would like to thank Keith Joho for providing us with plasmid pMC2, David Setzer for plasmid pTA102 and Ken Lanclos and Qin Ryan for constructive criticism of the manuscript. This material is based upon work supported by the National Science Foundation under grant no. MCB-9206873.

REFERENCES

1 Fairall,L., Schwabe,J.W., Chapman,L., Finch,J.T. and Rhodes,D. (1993) *Nature* (*Lond.*), **366**, 483–487.
2 Pavletich,N.P. and Pabo,C.O. (1991) *Science*, **252**, 809–817.
3 Pavletich,N.P. and Pabo,C.O. (1993) *Science*, **261**, 1701–1707.
4 Joho,K.E., Darby,M.K., Crawford,E.T. and Brown,D.D. (1990) *Cell*, **61**, 293–300.
5 Koster,M., Kuhn,U., Bouwmeester,T., Nietfeld,W., el-Baradi,T., Knochel,W. and Pieler,T. (1991) *EMBO J.*, **10**, 3087–3093.
6 Andreazzoli,M., De Lucchini,S., Costa,M. and Barsacchi,G. (1993) *Nucleic Acids Res.*, **21**, 4218–4225.
7 Klocke,B., Koster,M., Hille,S., Bouwmeester,T., Bohm,S., Pieler,T. and Knochel,W. (1994) *Biochim. Biophys. Acta*, **1217**, 81–89.
8 Caricasole,A., Duarte,A., Larsson,S.H., Hastie,N.D., Little,M., Holmes,G., Todorov,I. and Ward,A. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 7562–7566.
9 Larsson,S.H., Charlieu,J.P., Miyagawa,K., Engelkamp,D., Rassoulzadegan,M., Ross,A., Cuzin,F., van Heyningen,V. and Hastie,N.D. (1995) *Cell*, **81**, 391–401.
10 Brown,R., Sander,C. and Argos,P. (1985) *FEBS Lett.*, **186**, 271–274.
11 Miller,J., McLachlan,A.D. and Klug,A. (1985) *EMBO J.*, **4**, 1609–1614.
12 Vrana,K.E., Churchill,M.E., Tullius,T.D. and Brown,D.D. (1988) *Mol. Cell. Biol.*, **8**, 1684–1696.
13 Mao,X. and Darby,M. (1993) *Mol. Cell. Biol.*, **13**, 7496–7506.
14 Pelham,H. and Brown,D. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 4170–4174.
15 Picard,B., le Maire,M., Wegnez,M. and Denis,H. (1980) *Eur. J. Biochem.*, **109**, 665–669.
16 Sands,M.S. and Bogenhagen,D.F. (1991) *Nucleic Acids Res.*, **19**, 1797–1803.
17 Darby,M.K. and Joho,K.E. (1992) *Mol. Cell. Biol.*, **12**, 3155–3164.
18 Theunissen,O., Rudt,F., Guddat,U., Mentzel,H. and Pieler,T. (1992) *Cell*, **71**, 679–690.
19 Clemens,K.R., Wolf,V., McBryant,S.J., Zhang,P., Liao,X., Wright,P.E. and Gottesfeld,J.M. (1993) *Science*, **260**, 530–533.
20 Del Rio,S., Menezes,S.R. and Setzer,D.R. (1993) *J. Mol. Biol.*, **233**, 567–579.
21 Hansen,P.K., Christensen,J.H., Nyborg,J., Lillelund,O. and Thøgersen,H.C. (1993) *J. Mol. Biol.*, **233**, 191–202.
22 Liao,X.B., Clemens,K.R., Tennant,L., Wright,P.E. and Gottesfeld,J.M. (1992) *J. Mol. Biol.*, **223**, 857–871.
23 Schuh,R., Aicher,W., Gaul,U., Côté,S., Preiss,A., Maier,D., Seifert,E., Nauber,U., Schröder,C., Kemler,R. and Jäckle,H. (1986) *Cell*, **47**, 1025–1032.
24 Smith,J.F., Hawkins,J., Leonard,R.E. and Hanas,J.S. (1991) *Nucleic Acids Res.*, **19**, 6871–6876.
25 Zang,W.-Q., Veldhoen,N. and Romaniuk,P. (1995) *Biochemistry*, **34**, 15545–15552.
26 Clemens,K.R., Zhang,P., Liao,X., McBryant,S.J., Wright,P.E. and Gottesfeld,J.M. (1994) *J. Mol. Biol.*, **244**, 23–35.
27 Choo,Y. and Klug,A. (1993) *Nucleic Acids Res.*, **21**, 3341–3346.
28 McBryant,S., Veldhoven,N., Gedulin,B., Leresche,A., Foster,M., Wright,P., Romaniuk,P. and Gottesfeld,J. (1995) *J. Mol. Biol.*, **248**, 44–57.
29 Rollins,M.B., Del Rio,S., Galey,A.L., Setzer,D.R. and Andrews,M.T. (1993) *Mol. Cell. Biol.*, **13**, 4776–4783.
30 Vandeyar,M., Weiner,M., Hutton,C. and Batt,C. (1988) *Gene*, **65**, 129–133.
31 Del R'o,S. and Setzer,D.R. (1991) *Nucleic Acids Res.*, **19**, 6197–6203.
32 Studier,F.W., Rosenberg,A.H., Dunn,J.J. and Dubendorff,J.W. (1990) *Methods Enzymol.*, **185**, 60–89.
33 Schägger,H. and von Jagow,G. (1987) *Anal. Biochem.*, **166**, 368–379.
34 Zwieb,C. and Brown,R.S. (1990) *Nucleic Acids Res.*, **18**, 583–587.
35 Scatchard,G. (1949) *Annls NY Acad. Sci.*, **51**, 660–672.
36 Friesen,W.J. and Darby,M.K. (1997) *J. Biol. Chem.*, **272**, 10994–10997.
37 Fried,M. and Crothers,D.M. (1981) *Nucleic Acids Res.*, **9**, 6505–6525.
38 Bailey,J.M. (1979) *Anal. Biochem.*, **93**, 204–206.
39 Setzer,D.R., Menezes,S.R., Delrio,S., Hung,V.S. and Subramanyan,G. (1996) *RNA*, **2**, 1254–1269.
40 Nakaseko,Y., Neuhaus,D., Klug,A. and Rhodes,D. (1992) *J. Mol. Biol.*, **228**, 619–636.
41 Liao,L., Clemens,K., Cavanagh,J., Tennant,L. and Wright,P. (1994) *J. Biomol. NMR*, **4**, 433–454.
42 Bruschweiler,R., Liao,X. and Wright,P. (1995) *Science*, **268**, 886–889.
43 Romaniuk,P.J. (1990) *J. Biol. Chem.*, **265**, 17593–17600.
44 Zang,W.Q. and Romaniuk,P.J. (1995) *J. Mol. Biol.*, **245**, 549–558.