An Accessory DNA Binding Motif in the Zinc Finger Protein Adr1 Assists Stable Binding to DNA and Can Be Replaced by a Third Finger†

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ABSTRACT: The DNA binding domain of Adr1, the protein derived from alcohol dehydrogenase regulatory gene 1, is unusual for zinc finger proteins in that it consists of two classical Cys2His2 zinc fingers and an amino-terminal proximal accessory region termed PAR. PAR is unstructured in the free protein and becomes structured in the DNA-bound form. We investigated the role of PAR in DNA binding using molecular and biochemical approaches, and its importance for activation in vivo, using Adr1-dependent reporter genes. PAR was unimportant for DNA binding when a third finger was added to Adr1, and its importance was diminished but not eliminated by mutations in finger two that increased DNA binding affinity. The kinetic rate constants for three Adr1 proteins containing or lacking PAR were determined by surface plasmon resonance. PAR increased the on rate and decreased the off rate for specific DNA sites for Adr1 containing wild-type fingers one and two. Surprisingly, PAR had no significant effect on the kinetic rate constants when a third finger was present, or when single-stranded DNA was used as the substrate for DNA binding. A mutant form of Adr1-F1F2 in which finger 2 makes three base-specific contacts with DNA had a higher affinity for DNA than Adr1 containing three fingers, yet the mutant protein still depended on PAR for optimal binding affinity. The ability to activate transcription in vivo was correlated with a low dissociation rate, suggesting that stabilizing an activator at the promoter might be rate-limiting for transcription in vivo. PAR may have evolved to lend additional stability to DNA−Adr1 complexes encompassing short binding sites. In addition, PAR may have a role in transcription at a step after DNA binding since deletion of PAR from Adr1 with three fingers decreased activation in vivo but had no effect on DNA binding kinetics.

Zinc fingers of the Cys2-His2 class constitute a ubiquitous DNA binding motif found in all eukaryotes (*1*). The motif is characterized by tetrahedral coordination of a Zn^{2+} ion by Cys and His residues and three conserved aromatic and hydrophobic residues that create a compact domain of 30 amino acids. The structure consists of a reverse β-turn followed by an α-helix (see refs *2* and *3* for reviews). Structural studies of proteins containing up to five fingers and of numerous individual fingers (*4*−*10*) have shown that each finger folds independently and has the same basic tertiary structure.

DNA recognition by many zinc fingers occurs through amino acids at the −1, +2, +3, and +6 positions with respect to the first residue of the α-helix. These amino acids make base-specific contacts with one strand of the DNA through the major groove. The contacts are primarily, if not exclusively, with a single base, usually on the same strand of DNA. Each finger generally recognizes a triplet subsite. Numerous studies have shown that it is possible to change the DNA binding specificity of individual fingers by substituting other amino acids for residues within or just preceding the helix (*11*−*18*). The modularity of zinc fingers has allowed the construction of novel DNA binding proteins capable of being directed to useful targets in vitro and in vivo (*19*−*23*).

ADR1[1] encodes a transcription factor that regulates carbon source utilization in the yeast *Saccharomyces cerevisiae*. *ADR1* was discovered through genetic studies of the regulation of *ADH2* expression (*24*). The binding site for Adr1 in the *ADH2* promoter consists of a palindromic 22 bp sequence, UAS1, which binds two molecules of Adr1 noncooperatively (*25*). Change-of-specificity mutants have been identified within each finger at the the −1, +3, and +6 positions in finger one (F1), and at the −1, +2, +3, and +6 positions of finger 2 (F2) (*12*, *16*). Within each 11 bp binding site Adr1 contacts a G(A/G)GG sequence using residues −1, +3, and +6 in finger 1 and residues −1 and +2 in finger 2. Alanine scanning mutagenesis identified residues that were critical for function within each finger and in the conserved Cys-His linker region (*26*).

Finger 1 is unusual in that it makes three base-specific contacts in its recognition site, using all three positions in the F1 helix. It recognizes the triplets GAG and GGG, using

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[1] Abbreviations: *ADR1*, alcohol dehydrogenase regulatory gene 1; Adr1, protein derived from alcohol dehydrogenase regulatory gene 1; PAR, proximal accesory region of the DNA binding domain of protein Adr1; UAS1, upstream activation sequence 1 in the *ADH2* promoter; EMSA, electrophoretic mobility shift assay; SPR, surface plasmon resonance; SAAB, selection and amplification of binding.

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Table 1: Plasmid Construction[a]

plasmid	symbol	base plasmid	cloned sequences [mutations]
pF1F2	⊂**ΩΩ**	pQE31[b]	ADR1 [(17−160)]
pF1H	⊂**Ω$\underline{\Omega}$**	pQE31	ADR1 [(17−160)], L146H
pF1F1F2	⊂ΩΩ**Ω**	pQE31	ADR1[(17−133) + (106−160)]
pF1F1F1	⊂ΩΩΩ	pQE31	ADR1[(17−133) + 2(106−133)]
pF1F1Q	⊂ΩΩ$\underline{\Omega}$	pQE31	ADR1[(17−133) + 2(106−133)], R115Q
pF1F1F1F1	⊂ΩΩΩΩ	pQE31	ADR1 [(17−133) + 3(106−133)]
pF1QF1Q	⊂Ω$\underline{\Omega}$Ω$\underline{\Omega}$	pQE31	ADR1[(17−133) + 3(106−133)], R115Q
pΔF1F2	**ΩΩ**	pQE31	ADR1 [(94−160)]
pΔF1H	**Ω$\underline{\Omega}$**	pQE31	ADR1 [(94−160)], L146H
pΔF1F1F2	**ΩΩΩ**	pQE31	ADR1 [(94−133) + (106−160)]
pF1F2AD	⊂**ΩΩ**ζ	pRS314	ADR1[(1−160) + (420−462)]
pΔF1F2AD	**ΩΩ**ζ	pGF27	ADR1[(1−20) + (94−160) + (420−462)]
pF1HAD	⊂Ω**$\underline{\Omega}$**ζ	pRS314	ADR1[(1−160) + (420−462)], L146H
pΔF1HAD	Ω**$\underline{\Omega}$**ζ	pGF27	ADR1[(1−20) + (94−160) + (420−462)], L146H
pF1F1F2AD	⊂ΩΩ**Ω**ζ	pRS314	ADR1[(1−133) + (106−160) + (420−462)]
pΔF1F1F2AD	ΩΩ**Ω**ζ	pGF27	ADR1[(1−20) + (94−133) + (106−160) + (420−462)]
pLGC2F		pLG(K)	consensus binding site (GGGGTG)
pLGC3F		pLG(K)	consensus binding site (GGGGGGGTG) + (420−462)]

[a] Construction of the plasmids is described in Materials and Methods. Domains of Adr1 are abbreviated in table and figures as follows: ⊂, PAR (proximal accessory region; ζ, AD (activation domain); Ω, finger 1 of ADR1; $\underline{\Omega}$, finger 1 of ADR1 with R115Q mutation; **Ω**, finger 2 of ADR1; **$\underline{\Omega}$**, finger 2 of ADR1 with L146H mutation. [b] Qiagen Inc.

Arg at the −1 and +6 positions and His at the +3 position. Finger two is unusual in that it appears to make a single base-specific contact in its T-rich subsite, TTG, in which Arg at the −1 position recognizes G. Leu at position +3 and Arg at position +6 do not appear to make important contacts since either can be changed to Ala with little or no effect on DNA binding. The corresponding positions in the binding subsite for F2 are also relatively insensitive to changes.

The minimal DNA binding domain of Adr1 encompasses residues 84−161 (*25*, *27*). Residues 102−161 contain the two zinc fingers. An additional region amino-terminal to the first zinc-finger, called the proximal accessory region or PAR, is also important for DNA binding (*27*−*29*). Single point mutations in PAR can cause either loss of binding or enhanced binding to specific sites. In Swi5 and Tramtrack, two other zinc finger proteins containing just two fingers, the amino acids amino-terminal to the first finger form a third strand of β-sheet in the first finger both in the presence and in the absence of DNA. Unlike Tramtrack, the first finger of Adr1 folds normally in the absence of PAR (*28*, *30*). A similar region amino-terminal to the first finger in proteins containing three fingers has not been observed, suggesting that this region may be unique to proteins containing fewer than three zinc fingers such as Adr1, Swi5, Tramtrack, and GAGA (*10*, *29*, *31*, *32*).

An unexpected feature of PAR is that it is unstructured in the free protein and becomes ordered upon DNA binding (*29*, *33*, *34*). The structure of PAR when bound to DNA consists of a compact domain of three antiparallel strands contacting DNA in the major groove. Related structures may exist in other Cys2-His2 proteins since a search of the database revealed sequences related to PAR proximal to other Cys2-His2 zinc fingers (*29*). Most of the zinc finger proteins containing a sequence related to PAR contain only one or two fingers.

How PAR enhances DNA binding is unknown. The evidence suggests that it is not needed for folding of the fingers themselves (*29*, *33*, *34*). We suggested that it might be important for DNA binding because finger 2 makes a single base-specific contact to DNA (*12*, *26*). The zinc finger proteins with an identified accessory domain contain only one (GAGA; *32*) or two fingers (Adr1, Swi5, Tramtrack; *10*, *29*, *31*). Additional stability is presumably afforded by an accessory domain through additional DNA contacts. The structural data indicate that PAR is in close proximity to both the backbone and the first base 5′ of the subsite for finger 1 (*29*, *33*, *34*).

We tested the hypothesis that PAR is equivalent in function to a third finger by duplicating the first finger of Adr1 to create a three-finger protein, Adr1-F1F1F2, and by mutating finger two so that it makes the same three strong contacts to DNA made by finger one. We used electrophoretic mobility shift assays (EMSA) and surface plasmon resonance (SPR) to determine the binding constants for various forms of Adr1, containing and lacking PAR. Deleting PAR did not reduce the DNA binding affinity of Adr1-F1F1F2, but PAR stabilized binding by both wild-type and mutant Adr1 containing two fingers. PAR seems to act mainly by decreasing the off rate from specific DNA−protein complexes. We conclude that PAR has a unique role in stabilizing the binding of Cys2-His2 proteins containing only two zinc fingers.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions. Yeast strain TYY303 (MATa *adr1*Δ*1*::*LEU2 ade2 can1-100 his3-11,15 trp1-1 ura 3-1*) (*35*) was used for in vivo experiments. After transformation it was grown in synthetic medium (SM) prepared according to standard methods (*36*) containing 5% glucose overnight (repressed conditions) and then switched to SM containing 3% ethanol and 0.05% glucose (derepressed conditions) for 20 h.

Plasmid Construction. Plasmids used in the study are listed in Table 1. Coding regions of the genes were created by PCR and cloned into base plasmids. Mutations were introduced with a Quick Change mutagenesis kit obtained from Stratagene. All mutations were confirmed by sequencing.

Oligonucleotides and Probes. Double-stranded probes were prepared by annealing synthetic oligonucleotides.

Probes for electrophoretic mobility shift assays (EMSA) were end-labeled by using T4 polynucleotide kinase with [γ-^{32}P]-ATP under standard conditions. The double-stranded 2F consensus probe is composed of 2FCONB, CACCGGG-CATCCCCACTTACCTAGTC, and 2FCONA, BioGAC-TAGGTAAGTGGGGATGCCCGGTG. The double-stranded 3F consensus probe is composed of 3FCONB, CAC-CGGGCATCCCCCCCACTTACCTGAGTC, and 3FCO-NA, BioGACTCAGGTAAGTGGGGGGGATGCCCGGTG. Double-stranded probes were purified on a 15% polyacrylamide gel.

Proteins. All pQE31(Qiagen Inc.)-based constructs were expressed in the *Escherichia coli* strain MC1061 (pREP) and grown in 2× LB medium with 20 μM $ZnCl_2$ for 4 h after IPTG induction. Cells were harvested by centrifugation and resuspended in A150 buffer (50 mM Hepes, pH 7.6, 150 mM KCl, 0.1 mM EGTA, 2 mM DTT, 10 μM $ZnCl_2$, and 0.05 Tween 20) with protease inhibitors and lysozyme. The cells were passed through a French press and the extract was spun at 15000*g* for 30 min. Proteins were purified on a cation-exchange (HS/M) column (Perspective BioSystem) with an elution gradient of 0.26−1.20 M NaCl in 40 mM Hepes, pH 7.6. DTT (10 μM) was added to the purified fractions. Mass spectrometry indicated pure proteins of the correct molecular weight. Protein concentration was determined by absorbance readings at 280 nm. Molar extinction coefficients were calculated from the amino acid composition.

SAAB (Selection and Amplification of Binding Sites). SAAB was performed essentially as described in ref *37* except binding conditions used were specific for Adr1 (*25*) and advantage was taken of the fact that all the proteins used in the study had an attached His_6 tag. The 65 bp oligonucleotide used for binding had a centered 25 bp degeneracy. After incubation of protein and DNA, 20 μL of Ni^{2+} resin saturated with A150 buffer was carefully added to the reaction and incubated for 30 min on ice, washed twice with 100 μL of A150 buffer, and eluted with an equal volume of 0.5 M imidazole. One or two microliters of the supernatant was used directly for amplification in the PCR reaction. Three rounds of such SAAB proved to be sufficient for consensus binding site determination.

Electrophoretic Mobility Shift Assays. DNA binding and mobility shift assays were performed as described in ref 25 and gels were quantified with a Molecular Dynamics phosphorimager. λ DNA was used as a nonspecific competitor. K_A and K_{NS} (nonspecific) of binding were determined from the equation $K_{app} = K_A/(1 + C_{NS}K_{NS})$ (*38*).

Surface Plasmon Resonance. SPR experiments were carried out with a Biacore 2000 (Biacore). Biotinylated oligonucleotides were injected over a streptavidin-coated sensor chip (SA5, Biacore) to achieve ∼100−300 RU. Unused cavities of streptavidin were filled in with ImmunoPure D-biotin from Pierce to decrease nonspecific binding. Buffer A150 was used as the flow buffer. For each protein the experiments were conducted at five different concentrations. The amount of DNA bound to the chip and the flow rates were adjusted to minimize artifacts arising due to bulk effect and mass transfer. Bound proteins were eluted by two pulses of 0.1% SDS (20 μL, at a flow rate of 100 μL/min). Association and dissociation of the protein−DNA complex was followed for 10 min at a flow rate of 20 μL/min. Each protein was injected at five different concentrations. A control sensorgram obtained over the surface modified only with biotin was subtracted for each experiment. Kinetic constants k_{on} and k_{off} were calculated with the BIA evaluation software version 2.1 (Biacore).

β-Galactosidase Assays. β-Galactosidase activity was measured in permeabilized cells (*39*). Three transformants were assayed for each plasmid construct. Standard deviations were less than 25%.

RESULTS

Modular Nature of Adr1 Zinc Fingers. Before attempting to manipulate PAR in Adr1, we needed to demonstrate that Adr1 zinc fingers behave in a modular fashion, as do those based on proteins containing three fingers. Table 1 shows schematically the zinc finger proteins we created that contain up to four tandem fingers derived from Adr1. These proteins all start with Adr1 amino acid 17 preceded by a His6 amino acid tag and a start codon derived from the expression vector. Arg115 in finger 1 was mutated to Gln in some cases to test the dependence of binding specificity on finger position in a tandem array of Adr1 fingers. Finger 1 with this mutation is designated Q. In wild-type Adr1 (Adr1-F1F2), replacing Arg115 with Gln (R115Q) changes the binding specificity at a single position in the finger one subsite, from G to A (*12*).

A binding site selection and amplification protocol (SAAB) was used to determine the binding site preferences of the new zinc finger proteins. Table 2 shows the frequency of the most common nucleotide found at each position within the binding site. Position 1 corresponds to the first nucleotide in the binding site for finger 1. Most of the frequencies are near unity for finger 1, indicating that there is a strong preference for each nucleotide, as was found in the two-finger protein. However, F1 at the C-terminus of a protein containing four F1 fingers showed less base discrimination, suggesting that its ability to influence DNA binding was compromised by its C-terminal position. Changing Arg 115 to Gln in F1 at different positions within a tandem array of three or four F1 fingers produced the same change of specificity as the analogous change in the two-finger protein. Thus, a tandem array of identical, wild-type Adr1 F1 zinc fingers shows the same type of modularity as found for a consensus finger or zinc finger proteins that naturally contain three fingers.

Finger 2 showed less dramatic base preferences in Adr1 containing three and four fingers, as was found in the two-finger protein (*40*). In wild-type Adr1-F1F2, guanine is the only base that was detected at position 1 in the subsite for finger 2 (*40*; position 4 or 7 in Table 2), and this preference is maintained in Adr1 containing three fingers. The ability to discriminate at this position appeared to be significantly reduced when PAR was deleted from Adr1-F1F2. However, Adr1-F1F2 lacking PAR had a low binding affinity and it was consequently difficult to assign a consensus sequence for this protein. Finger 2 in all proteins had a slight preference for G at the third position in its subsite (*40*).

The requirement for G at positions 1, 4, and 7 in the binding site was tested by EMSA using Adr1-F1F1F2 and specific oligonucleotide probes. Mutating G1 or G7 to C reduced the binding affinity about 10-fold. Mutating G4 to

Table 2: Proteins Composed of Different Arrays of Adr1 Zn Fingers Bind in a Modular Manner[a]

	binding site (position no.)													
	IV				III			II			I (N-terminal)			
protein I	+1	12	11	10	9	8	7	6	5	4	3	2	1	−1
⊂ΩΩΩ	T/A				G	G	G	G	G	G	G	G	G	T/A
F1F1F1	0.7				1.0	0.8	1.0	1.0	0.9	0.9	0.9	0.9	0.9	0.7
⊂ΩΩΩ	T				G	G	A	G	G	G	G	G	G	T
F1F1Q	0.7				1.0	1.0	0.9	1.0	1.0	1.0	1.0	0.9	1.0	1.0
⊂ΩΩΩΩ	N	G	G	G	G	G	G	G	G	G	G	G	G	A
F1F1F1F1		1.0	0.3	0.7	1.0	1.0	1.0	1.0	0.7	1.0	1.0	0.7	1.0	0.7
⊂ΩΩΩΩ	N	G	G	A	G	G	G	G	G	A	G	G	G	T/A
F1QF1Q		0.8	0.9	0.4	1.0	1.0	1.0	1.0	1.0	0.8	1.0	0.7	0.8	0.8

	finger 2				finger 1 (N-terminal)			
protein II	+1	6	5	4	3	2	1	−1
⊂ΩΩ F1F2*	T/A	G	T	G	G	G	G	T/A
ΩΩ ΔF1F2	N	N	T 0.5	G 0.6	G 1.0	G 0.7	G 1.0	N

	finger 2				finger 1			finger 1 (N-terminal)			
protein II	+1	9	8	7	6	5	4	3	2	1	−1
⊂ΩΩΩ	N	G	T	G	G	G	G	G	G	G	T/A
F1F1F2		0.7	0.4	0.9	1.0	1.0	1.0	1.0	1.0	1.0	0.6
ΩΩΩ	N	N	G/T	G	G	G	G	G	G	G	N
ΔF1F1F2			0.9	0.9	1.0	1.0	1.0	1.0	0.8	1.0	

[a] The nucleotide sequences are given in the customary 5′-to-3′ orientation, corresponding to a C-to-N-terminal orientation of the fingers. For the two-finger proteins the nucleotides corresponding to the second finger are shown in the left panel as indicated. The Adr1−F1F2 data are from Cheng et al. (40). Relative frequencies of binding were determined from binding site selection and amplification experiments as described in Materials and Methods. Generally 15−20 sequences were determined for each construct and a consensus sequence was determined by inspection. The relative frequency is the fraction of sequences that conformed to the consensus nucleotide, rounded to the nearest tenth of a unit. N indicates that no nucleotide occurred at a frequency higher than 0.4 at that particular position.

C resulted in a 100-fold decrease in binding affinity (data not shown). Thus, the central finger contributes more significantly to stability of the complex than the N- or C-terminal fingers, but all three F1 fingers contribute to binding affinity.

In summary, Adr1 proteins containing two, three, or four finger 1 domains had the binding specificity that was predicted on the basis of the binding specificity of finger 1 in the wild-type protein. Thus, altering the position of finger 1 within a tandem array of fingers does not alter its binding specificity. This result demonstrates that Adr1 F1 is modular in the same way that consensus and Zif268- and SP1-derived zinc fingers are modular.

PAR Is Not Required for DNA Binding by Adr1-F1F1F2. Previous studies of the effect of deleting or mutating PAR indicated that it had an important influence on DNA binding by Adr1-F1F2. To determine whether PAR acts independently of the fingers in DNA binding, we tested several constructs that we suspected would have enhanced DNA binding affinity. If PAR contributes to DNA binding in a manner that is independent of the association of the fingers with DNA, we predicted that increasing the number of contacts made by the fingers would compensate for the loss of PAR.

We used EMSA to determine specific and nonspecific equilibrium constants (*38*) for Adr1-F1F2, Adr1-F1F1F2, and Adr1-F1F2 (L146H) proteins with and without PAR. Adr1-F1F2 (L146H) (abbreviated Adr1-F1H) has a single amino acid change, Leu146-to-His, in the recognition helix of finger 2 that changes the binding site specificity from TTG to GGG, the same specificity as F1 (*40*). This protein recognizes 6 bp as compared to 4 bp by wild-type Adr1-F1F2, and we suspected that it would have a higher binding affinity than wild-type Adr1-F1F2. An oligonucleotide containing the appropriate consensus sequence that was determined from the binding site selection experiments was used as a probe. An example of an EMSA for Adr1-F1F1F2 is shown in Figure 1, and the calculated equilibrium association constants are presented in Table 3.

An additional F1 finger increased the apparent association constant 29-fold for specific DNA and 7-fold for nonspecific DNA, compared to Adr1-F1F2. Surprisingly, changing the binding specificity of finger 2 to that of finger 1 (Adr1-F1H) had an even greater effect on the apparent association constants, increasing the specific and nonspecific association constants 46-fold.

The addition of a third finger domain counteracted the negative effect that deleting PAR had on DNA binding affinity. However, this effect is not due simply to the increased binding affinity by Adr1-F1F1F2 since Adr1-F1H has a higher binding affinity than Adr1-F1F1F2, but deleting PAR still reduced its specific DNA binding affinity by about a factor of 10. Deleting PAR had an effect of similar magnitude on the apparent nonspecific binding constants.

PAR Affects both the On and Off Rates of Specific DNA Binding. EMSA provides important information about the stoichiometry and nature of the components in a protein−DNA complex, but it is more difficult to extract quantitative data from the assay. Surface plasmon resonance (SPR) is a valuable adjunct to EMSA since it allows the investigator to monitor the course of the reaction in real time, thus providing kinetic constants of the interaction.

We used SPR to measure on and off rates during DNA binding to determine which component(s) of the equilibrium constant was affected by PAR. The sensorgrams of a representative experiment with Adr1-F1F2 is shown in Figure 2A. Figure 2 panels B and C show single sensorgrams of Adr1-F1F1F2 and Adr1-F1H plus and minus PAR at the same protein concentrations. One of the flow cells contained single-stranded DNA to measure binding to nonstructured DNA as well. Rate and equilibrium binding constants were determined as described in Materials and Methods.

The results from the surface plasmon resonance experiments confirm and extend the conclusions reached on the basis of the EMSA data. Table 3 compares the equilibrium association constants measured by the two techniques for Adr1 proteins containing and lacking PAR. The two techniques consistently showed the same effect when comparing two different proteins or when comparing the effects of deleting PAR from one of the proteins. An additional F1 finger increased the apparent association constant 18-fold for specific DNA and 1.5-fold for single-stranded DNA, compared to Adr1-F1F2. As in the EMSA, changing the binding specificity of finger 2 to that of finger 1 (Adr1-F1H) had a greater effect on the apparent association constants than did adding a third finger, increasing the specific and nonspecific association constants 72- and 20-fold, respectively.

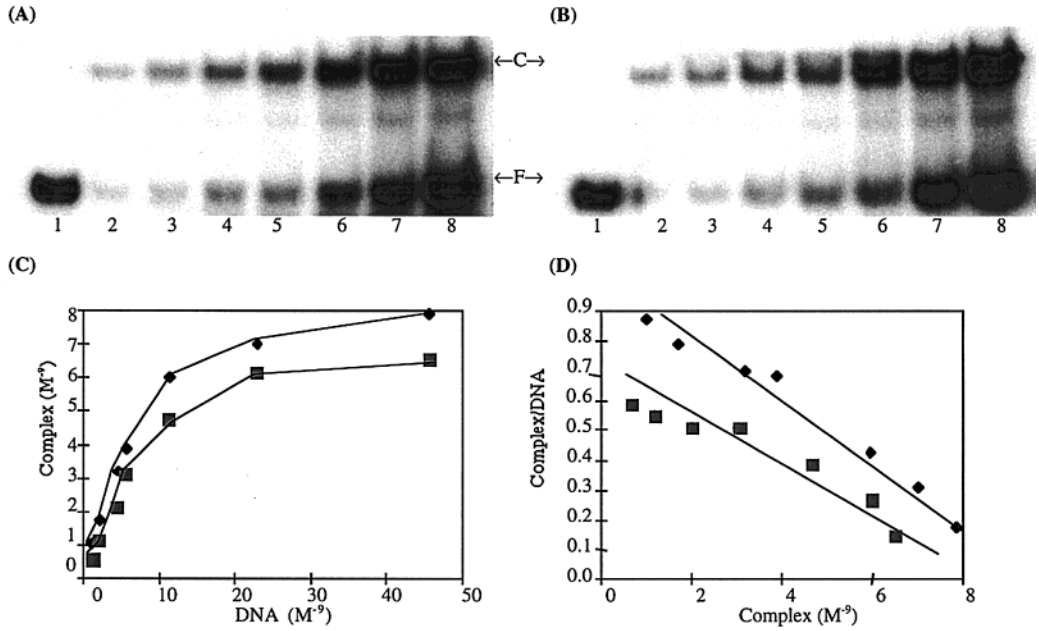

FIGURE 1: Determination of association constants of specific and nonspecific binding (K_A and K_{NS}) for ⊂**ΩΩΩ** (F1F1F2) protein by EMSA. (A, B) Two different concentrations of nonspecific competitor (200 and 400 ng/reaction λ DNA, respectively) were used in the binding reactions, where protein concentration was kept constant and increasing amount of radioactive probe was titrated in (lanes 2−8). F, free probe; C, DNA−protein complex. (C, D) Results of quantification by phosphorimager. (◆) 200 ng of λ DNA/reaction; (■) 400 ng of λ DNA/reaction.

Table 3: Equilibrium Association Constants of Miniproteins Binding to DNA Measured by SPR and EMSA[a]

	equilibrium association constants				
	specific		nonspecific		relative specific K_A SPR
protein	EMSA	SPR	EMSA	SPR	
⊂**ΩΩ** F1F2	3.5×10^7	7.2×10^7	9.5×10^5	1.3×10^7	1.0
ΩΩ ΔF1F2	$>2 \times 10^6$	5.3×10^6	nd	2.3×10^7	0.07
⊂**Ω**$\underline{\mathbf{Ω}}$ F1H	1.6×10^9	5.2×10^9	4.4×10^7	2.6×10^8	72
Ω$\underline{\mathbf{Ω}}$ ΔF1H	1.4×10^8	1.1×10^9	5.1×10^6	2.1×10^8	15
⊂**ΩΩΩ** F1F1F2	1.0×10^9	1.3×10^9	6.5×10^6	1.9×10^7	18
ΩΩΩ ΔF1F1F2	6.0×10^8	1.4×10^9	2.5×10^6	1.7×10^7	19

[a] Association constants were determined as described in Materials and Methods. The specific association constants were determined by use of the consensus oligonucleotides determined by SAAB. The nonspecific constants for EMSA were determined by use of λ DNA. For SPR experiments the nonspecific constants were determined by use of single-stranded oligonucleotides.

Deletion of PAR did not reduce the equilibrium association constant for Adr1-F1F1F2. Deleting PAR had the greatest effect on binding of Adr1-F1F2 to specific double-stranded DNA, reducing the association constant about 14-fold, and had no effect on binding to single-stranded DNA for any of the proteins. The change-of-specificity mutation in finger 2 increased the binding affinity 72-fold relative to Adr1-F1F2 and deletion of PAR reduced the association constant about 5-fold.

The on and off rates measured for the six protein samples studied by EMSA are shown in Table 4. Deleting PAR did not affect either the on or the off rate for binding to DNA by Adr1-F1F1F2. Deleting PAR increased the off rate on specific DNA for both two-finger proteins and decreased the on rate for Adr1-F1F2. Thus, the PAR can affect both the on and off rates for Adr1, but whether it does so depends on the number of fingers and on their composition. Deleting PAR had no effect on the kinetic rate constants for binding to single-stranded DNA.

PAR May Also Act after DNA Binding during Transcription Activation in Vivo. In addition to a role in DNA binding, PAR could have a role in transcription acting at a step after DNA binding. The ability of Adr1-F1F1F2 and Adr1-F1H to bind DNA with high affinity in the absence of PAR allowed us to test this hypothesis. The six proteins described above were expressed in yeast after fusion to TADIII, a strong Adr1 activation domain representing amino acids 420−462 (*35*), and activation of a *UAS1/CYC1/lacZ* reporter gene was measured (*25*, *39*).

The data for the strains grown in derepressing growth conditions (active Adr1) and containing reporter genes with a single binding site are shown in Table 5. In repressing growth conditions, none of the constructs had significant levels of activity (data not shown), indicating that increasing the DNA binding affinity by up to 70-fold did not overcome glucose repression. Addition of a third finger to Adr1-F1F2, or mutating Leu146 to His in F2, had two significant effects (Table 5). Germane to this work, both alterations reduced the dependence of activation on PAR. In addition, both alterations allowed Adr1 to activate transcription at a high level from a single binding site. In this experiment as in previous experiments, deletion of PAR abolished activation by Adr1-F1F2 (*25*, *27*). The decrease in activation in the absence of PAR was even more dramatic when an inverted repeat was present in the promoter of the *CYC1/lacZ* reporter gene (data not shown).

PAR had another unexpected effect. It appeared to enhance the stability of Adr1 since proteins lacking PAR were present at a lower level than those containing PAR. Thus, we expressed mutants lacking PAR from a high-copy plasmid and wild-type proteins from a low-copy plasmid. With this expression system, proteins containing and lacking PAR were present at equivalent levels as determined by Western blotting (data not shown). In conclusion, these results suggest that in addition to a role in DNA binding, PAR might act a

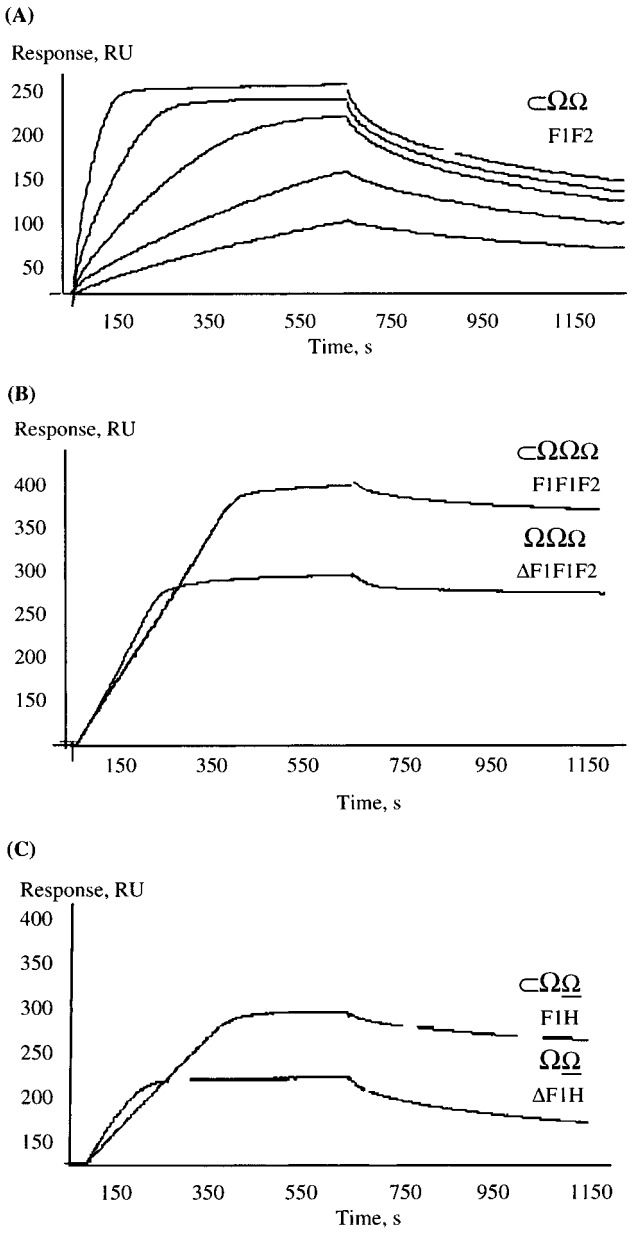

FIGURE 2: Superimposed sensorgrams obtained at (A) five concentrations (25, 50, 100, 200, and 400 μM) of F1F2 protein interacting with double-stranded 2F consensus oligo immobilized on the sensor chip surface. (B) F1F1F2 (200 μM) and ΔF1F1F2 (200 μM) interacting with 3F consensus oligo. (C) F1H (20 μM) and ΔF1H (20 μM) interacting with 3F consensus oligo.

subsequent step in transcription activation.

An important question in gene activation by transcription factors is the role the rates of DNA association and dissociation have on the effectiveness of an activator in vivo. Table 6 compares the relative activity in vivo of the different activators with the relative DNA binding constants derived from the SPR analysis using specific DNA binding oligonucleotides. The ratios of the binding constants were calculated to make them comparable to the in vivo activity. Thus, the relative dissociation rate constants were calculated so that values greater than 1 reflect slower dissociation than Adr1-F1F2, and the relative association constants were calculated so that values greater than 1 reflect more rapid association than Adr1-F1F2.

The relative in vivo activity of the Adr1 activators is positively correlated most closely with the dissociation rate constants, rather than with either the equilibrium or association rate constants. This is observed for Adr1 containing and lacking PAR, for both Adr1-F1F1F2 and Adr1-F1H. This correlation suggests that, for activation by Adr1, accessibility to its binding site in vivo is not the rate-limiting step in transcription activation. Instead, the rate of dissociation appears to determine its ability to stimulate transcription.

DISCUSSION

Formation of an Adr1−DNA complex induces a conformational change in PAR (*29*, *34*). This conformational change is likely to be important for formation of a stable protein−DNA complex since deletion of PAR reduces DNA binding affinity. Single amino acid changes within PAR also affect DNA binding, either in a negative manner as with Leu-to-Pro changes at amino acids 87 and 97 or positively as with Arg91 to Lys (*25*, *30*, *41*). We addressed the role of PAR by altering the zinc fingers either qualitatively or quantitatively to determine if these changes altered the importance of PAR for either specific or nonspecific DNA binding.

Addition of a third zinc finger to Adr1 negated the effect of deleting PAR. This does not appear to be simply a consequence of enhancing the DNA binding affinity, since mutating finger 2 in Adr1-F1F2 so that the association constant was increased 72-fold did not negate the effect of deleting PAR. Thus, PAR seems to play an important role that appears to be unique to Adr1 containing just two fingers.

It is surprising that a single amino acid change, Leu146 to His in finger 2 of Adr1-F1F2, increased the equilibrium association constant more than adding an additional finger 1. The F1 fingers in Adr1-F1F1F2 make the same six contacts to a string of guanine residues that are made by the two fingers in Adr1-F1F2 (L146H). In addition, Arg 143 in F2 in the three-finger proteins makes at least one additional contact to a guanine residue. Since Adr1-F1F1F2 binds a larger site than Adr1-F1F2 (L146H), enthalpic considerations would suggest that the three-finger protein would bind tighter than the two-finger protein. Since this is not the case, it seems likely that entropic considerations favor the binding of the mutant two-finger protein relative to the three-finger protein. For example, more extensive ordering of the three-finger protein might be required to allow all three fingers to interact with their DNA subsites. Having fewer fingers could allow Adr1-F1F2 (L146H) to achieve a highly stable complex without as large a positive change in entropy as might occur when the three-finger protein goes from the free to the bound state.

The exceptionally high binding affinity of Adr1-F1F2 (L146H) suggests that it might be possible to design two-finger proteins that have nanomolar binding constants. This might be useful when it is desirable to minimize the binding site of the protein and still achieve a high binding affinity. Inclusion of PAR might be necessary to achieve additional stability without requiring additional base-specific interactions.

Although Adr1-F1F2 (L146H) binds DNA with higher affinity than does Adr1-F1F1F2, the three-finger protein activates transcription in vivo about 3-fold better than the two-finger protein. We attribute the higher activity in vivo to two effects. The first is the greater specificity of Adr1-

Table 4: Association and Dissociation Rate Constants Calculated with Biacore Evaluation Software[a]

	DS consensus DNA		SS DNA	
protein	k_a (M^{-1} s^{-1})	k_d (M^{-1})	k_a (M^{-1} s^{-1})	k_d (M^{-1})
⊂**ΩΩ** F1F2	$(1.3 \pm 0.2) \times 10^5$	$(1.8 \pm 0.6) \times 10^{-3}$	$(4.4 \pm 1.1) \times 10^4$	$(3.3 \pm 0.2) \times 10^{-3}$
ΩΩ ΔF1F2	$(3.2 \pm 1.4) \times 10^4$	$(6.1 \pm 1.5) \times 10^{-3}$	$(4.7 \pm 0.5) \times 10^4$	$(2.1 \pm 1.5) \times 10^{-3}$
⊂**ΩΩ** F1H	$(1.3 \pm 0.1) \times 10^6$	$(2.5 \pm 0.1) \times 10^{-4}$	$(6.2 \pm 0.8) \times 10^5$	$(2.4 \pm 0.2) \times 10^{-3}$
⊂**ΩΩ** ΔF1H	$(1.3 \pm 0.2) \times 10^6$	$(1.2 \pm 0.1) \times 10^{-3}$	$(5.3 \pm 2.1) \times 10^5$	$(2.5 \pm 0.4) \times 10^{-3}$
⊂**ΩΩΩ** F1F1F2	$(1.4 \pm 0.2) \times 10^5$	$(1.1 \pm 0.5) \times 10^{-4}$	$(2.4 \pm 0.8) \times 10^4$	$(1.3 \pm 1.3) \times 10^{-3}$
ΩΩΩ ΔF1F1F2	$(1.8 \pm 0.5) \times 10^5$	$(1.2 \pm 0.1) \times 10^{-4}$	$(2.5 \pm 0.5) \times 10^4$	$(1.5 \pm 0.2) \times 10^{-3}$

[a] Rate constants were determined as described in Materials and Methods with Biacore evaluation software. For each protein five different concentrations were used.

Table 5: Activation of Transcription by Miniproteins[a]

protein	symbol	β-galactosidase activity
	GGG GTG	
none		5
F1F2AD	⊂**ΩΩ**ζ	20
ΔF1F2AD	**ΩΩ**ζ	5
	GGG GGG GTG	
none		20
F1HAD	⊂**ΩΩ**ζ	140
ΔF1HAD	**ΩΩ**ζ	40
F1F1F2AD	⊂ΩΩ**Ω**ζ	500
ΔF1F1F2AD	ΩΩ**Ω**ζ	100

[a] Yeast strain TYY303 was transformed with plasmids encoding one of the miniproteins and a reporter (pLGC2F or pLGC3F) containing a single consensus binding site upstream of a *CYC1/lacZ* fusion. β-Galactosidase activities are in Miller units, and the standard deviations were less than 25%.

Table 6: Comparison of in Vitro DNA Binding Activities and Transcription Efficiency in Vivo[a]

			relative DNA binding (specific, SPR)		
protein	symbol	relative in vivo activity	K_A	k_d	k_a
F1F2AD	⊂**ΩΩ**ζ	1	1.0	1.0	1.0
ΔF1F2AD	**ΩΩ**ζ	0	0.07	0.30	0.25
F1HAD	⊂**ΩΩ**ζ	8	72	7.2	10
ΔF1HAD	**ΩΩ**ζ	1	15	1.5	10
F1F1F2AD	⊂ΩΩ**Ω**ζ	30	18	16	1.1
ΔF1F1F2AD	ΩΩ**Ω**ζ	7	19	15	1.4

[a] Activities are calculated relative to wild-type Adr1−F1F2 for each of the miniproteins. The data for in vitro DNA binding are derived from Tables 3 and 4, and the in vivo efficiences are derived from Table 5.

F1F1F2. Specificity, which is the ratio of the specific to the nonspecific equilibrium constants, is higher for the three-finger than for the two-finger proteins. Adr1-F1F1F2 has a specificity of 150 compared to a specificity of 37 for both two-finger proteins (based on the EMSA measurements). The higher specificity of Adr1-F1F1F2 could be due to the larger binding site of the three-finger protein. The higher specificity of the three-finger protein allows it to spend less time on nonproductive binding sites in vivo, thus enhancing its ability to activate transcription from binding sites in promoters. Deletion of PAR had little effect of the specificity of either protein, an observation that is consistent with PAR primarily contributing to DNA binding affinity by making nonspecific contacts with the DNA backbone (*29*).

A second reason for the higher activity of Adr1-F1F1F2 in vivo could be its slower rate of dissociation compared to Adr1-F1F2 (L146H). As noted in Table 6, the activity of the Adr1 proteins in vivo showed the best correlation with the dissociation rates: the slower the rate of dissociation, the more activity was observed in vivo. This correlation suggests that, in vivo, in derepressing growth conditions, the rate of dissociation influences the ability of Adr1 to activate transcription more than its rate of association.

The Adr1 binding site in the *ADH2* promoter is apparently free of nucleosomes in vivo (*42*), and Adr1 is necessary for the chromatin remodeling that occurs over the TATA box and RNA initiation sites (*43*). Thus, it appears that DNA binding by Adr1 may be unperturbed by nucleosomes, allowing DNA dissociation to play a more important role in transcription activation.

If the role of PAR in DNA binding can be played by a third zinc finger, it is surprising that PAR was not replaced during evolution by a third zinc finger. One reason PAR may have been retained is that it appears to play a role in transcription activation independent of its role in DNA binding. Even though DNA binding by Adr1-F1F1F2 is unaffected by deletion of PAR, activation of transcription in vivo is reduced about 5-fold. It could be that PAR acquired a role in transcription independent of its role in DNA binding and that this role cannot be replaced by an additional finger.

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