Designing Zinc-Finger ADR1 Mutants with Altered Specificity of DNA Binding to T in UAS1 Sequences†

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ABSTRACT: Yeast ADR1 contains two Cys_2,His_2 zinc fingers needed for DNA binding to the upstream activation sequence UAS1, with bases $T_5T_6G_7$-$G_8A_9G_{10}$ in the ADH2 promoter. Potential DNA-contacting amino acid residues at −1, +3, and +6 in the α-helical domains of ADR1's fingers one and two include RHR-RLR; however, the latter finger two residues Leu146 and Arg149 had not proved to be crucial for ADR1 binding, even though Leu146-T_6 and Arg149-T_5 interactions with UAS1 DNA were predicted. We altered Leu146 or Arg149 by PCR cassette mutagenesis, to study ADR1 mutant binding to 16 UAS1 variants of thymine bases T_5 and T_6. Mutation of Leu146 to His, making finger two (RLR) like finger one (RHR), decreased binding to wild type UAS1 having T_6, but enhanced its binding strength to sequences having purines G_6 or A_6, similar to binding seen between finger one's His118 and base A_9 of UAS1. Mutating Leu146 to Lys caused this finger two RKR mutant to bind strongly to both G_6 and T_6, possibly by lysine's amine H-bonding to the carbonyl of guanine or thymine. Specificity of ADR1 for UAS1 with T_6 may thus be due to hydrophobic interaction between Leu146 and the T_6 methyl group. ADR1 mutants with either His or Lys in the central +3 residue (146) of zinc finger two, which have Arg149 in the +6 α-helical position, bind with UAS1 mutant sequences having G_5 very strongly, T_5 strongly, A_5 intermediately, and C_5 weakly. Mutation of Arg149 to Gln decreased DNA binding to G_5 and T_5 while increasing its binding to A_5 and C_5 probes, thus showing the expected Arg149 interaction with G_5 or T_5 in UAS1 DNA.

The goal of being able to design a zinc-finger transcription factor which could recognize and bind any given DNA sequence, a critical step in controlling gene expression, has profound consequences. The interaction of a few zinc-finger proteins having only two or three Cys_2,His_2 fingers (i.e., SP1, Zif268 (EGR1), Krox20, or ADR1) with their DNA binding sites is relatively well-understood (Berg, 1992; Desjarlais & Berg, 1993). However, DNA-binding studies of multiple zinc-finger proteins such as GLI or TFIIIA, with five to nine fingers, have revealed complex and unexpected protein−DNA interactions between finger amino acid residues and the bases in bound DNA sequences (Berg, 1992; Pavletich & Pabo, 1993; Clemens et al., 1993).

The prototype zinc-finger protein, TFIIIA, has nine repeats of about 30 amino acids (--Cys-X_{2-4}-Cys-X_3-Phe-X_5-Leu-X_2-His-X_{3-4}-His) including a spacer Thr-Gly-Glu-Lys-Pro (Brown et al., 1985; Miller et al., 1985). A structural model for these Cys_2,His_2 zinc fingers (Berg, 1988; Gibson et al., 1988) was subsequently confirmed by 2D NMR[1] studies of finger peptides, showing two conserved Cys and two His residues coordinate tetrahedrally with a zinc atom at the base of the finger, while conserved Phe and Leu residues nestle in the hydrophobic core of the finger between a β-turn and α-helix (Parraga et al., 1988; Klevit et al., 1990; Lee et al., 1989; Omichinski et al., 1992; Hoffman et al., 1993). X-ray crystallography of the Zif268−DNA complex revealed an elegant architecture for zinc-finger interaction with DNA which is simple, yet attractive (Pavletich & Pabo, 1991). Each finger has three critical amino acids spaced every third residue on the outside of the α-helical domain (at positions −1, +3, and +6), which can potentially contact three consecutive base pairs in inverted order (3′→5′) in the DNA major groove (see Figure 1).

The major protein−DNA interaction, as predicted theoretically by Seeman et al. (1976), was found to be H-bonding between arginine (at α-helix positions −1 and +6) and guanine bases (Figure 6A) observed in the Zif268−DNA structure as well as in most zinc-finger proteins studied to date. Specificity in DNA sequence binding was also determined by the central fingertip (+3) amino acid histidine H-bonding to guanine or adenine (Figure 6C), as seen in zinc fingers of many proteins including mouse Zif268 and related factor Krox20 (Pavletich & Pabo, 1991; Nardelli et

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[1] Abbreviations: PCR, polymerase chain reaction; IPTG, isopropyl thio-β-D-galactoside; βGal, β-galactosidase; NMR, nuclear magnetic resonance; H-bonding, hydrogen bonding; 2D, two dimensional; GEMS, gel electrophoretic mobility shift.

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al., 1991, 1992), human SP1 and Wilm's tumor suppressor WT1 (Desjarlais & Berg, 1992a,b, 1993; Drummond et al., 1994), and yeast ADR1 and MIG1 (Thukral et al., 1992; Lundin et al., 1994), but not Tamtrack (Ttk) of *Drosophila* (Fairall et al., 1992, 1993). These zinc finger (ZF) proteins are shown bound to their cognate DNA elements (3′→5′ of top strand), with presumed H-bonds (|) or "non-contacting" specific interactions (!) between critical (−1, +3, and +6 α-helix) amino acids and bases, indicated below.

reference	Pavletich/ Nardelli	Desjarlais and Berg	Drummond et al.
organism	mouse	human	human
protein	Zif268/Krox20	SP1	Wilms Tumor WT1
Zn finger	ZF1 ZF2 ZF3	ZF1 ZF2 ZF3	ZF1 ZF2 ZF3 ZF4
α-helix #	-136-136-136	-136-136-136	-136-136-136-136
aa residue	RER RHT RER	KHA RER RHK	KHM RQR RHT RER
binding	!| || |!|	|| |!| |||	|| | | || |!|
DNA 3'->5'	GCG-GGG-GCG-5'	GGG-GCG-GGG-5'	GGG-GCG-GGG-GCG-5'
or base(s)	AN	At	t

reference	Thukral	Lundin	Fairall
organism	yeast	yeast	*Dros.*
protein	ADR1	MIG1	Tamtrack
Zn finger	ZF1 ZF2	ZF1 ZF2	ZF1 ZF2
α-helix #	-136-136	-136-136	-1236-136
aa residue	RHR RLR	RHR RER	HSNR RNA
binding	||| |	||| !|	||| ||
DNA 3'->5'	GAG-GTT-5'	GGG-GCG-5'	TAG-GAA-5'
or base(s)	G cG	A	

Alternate DNA bases at ambiguous positions (if known from mutation studies) are shown below the DNA sequence, with weaker binding shown in small letters (N is any base). Fidelity in binding of amino acids Arg at −1 or +6 to G, and His at +3 to A or G, is highly specific. Interaction between Glu at +3 and C could be important in specific recognition of cytosine, in which crucial residues of Arg-Glu-Arg (RER) bind to a GCG base triplet subsite, as shown for Krox20, SP1, WT1, and MIG1. However, this may be indirect (!), since close Glu-C contact was not seen in the Zif268−DNA complex (Pavletich & Pabo, 1991). Furthermore, Asn at the +3 position of both Tamtrack fingers bound specifically with A, like Gln in Figure 6B (Fairall et al., 1993). Thr, Ala, or Met at +6 in various zinc fingers would tolerate proximity to any of the four DNA bases, with a slight preference for G (Nardelli et al., 1992; Kriwacki et al., 1992). Among the known Cys_2,His_2 finger proteins, specific binding of thymine has not been adequately studied. The existence of two T's in the DNA binding site for the yeast ADR1 protein's second zinc finger provides an opportunity to investigate the specific recognition of thymine.

The ADR1 transcription factor requires two zinc fingers (Figure 1) (Blumberg et al., 1987), both necessary for activating transcription of mRNA for the glucose-repressible isozyme alcohol dehydrogenase II (ADH2), as well as glycerol kinase (GUT1) and catalase A (CTA1) in the yeast *Saccharomyces cerevisiae* (Denis & Young, 1983; Irani et al., 1987; Pavlik et al., 1993; Simon et al., 1991). Glucose repression of these genes is mediated in part by cAMP-dependent phosphorylation of ADR1, which inhibits activation of transcription (Cherry et al., 1989), while allowing normal DNA binding of ADR1 to the upstream activation site one (UAS1) located at −215 bp in the ADH2 promoter (Taylor & Young, 1990). ADR1 protein binds strongly to UAS1, a 22 bp DNA sequence having dyad symmetry (Eisen et al., 1988). UAS1 has two inverted 11 bp sites (5′-TAAGTTGGAGA-3′) containing four critical purine residues (GGAG) that can bind ADR1 monomers independently (Thukral et al., 1991a; Cheng et al., 1994). Deletion and mutation studies showed the minimal domain of ADR1 required for DNA binding contains the two zinc fingers (amino acids 103−170) plus an additional 20 residues (80−102) located amino-terminal to the finger region, involved in phosphate backbone contacts (Thukral et al., 1989; Camier et al., 1992).

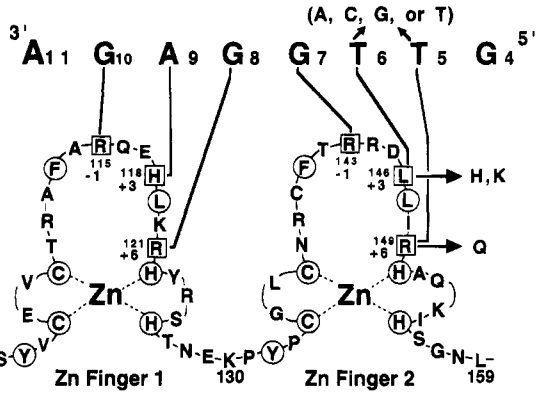

FIGURE 1: UAS1 DNA bases interacting with critical ADR1 zinc finger residues. Top sequence is UAS1 top strand DNA bases 4−11 (in reverse order 3′→5′). Slanted arrows above T_6 and T_5 indicate all (sixteen) possible changes made in UAS1 DNA sequence. Lines beneath DNA connect to putative DNA-contacting residues (boxed). Circled residues indicate amino acids homologous to conserved residues of Cys_2,His_2 zinc finger motif.

Within the two zinc fingers of ADR1, alanine-scanning mutagenesis revealed four DNA-binding residues in the "fingertips" including Arg115, His118, and Arg121 (RHR at −1, +3, and +6 α-helical positions) in finger one, and Arg143 (position −1) in finger two, which were necessary for DNA binding to UAS1 bases (5′-$T_5T_6G_7$-$G_8A_9G_{10}$) (Thukral et al., 1991b, 1993). The conserved Cys, His, and Phe residues were necessary in DNA binding, presumably for zinc coordination and proper folding of each finger (Figure 1, circled residues). Also the two carboxylic amino acids, Glu117 and Asp145, are probably required to coordinate respectively with DNA-binding Arg115 and Arg143 residues located at analogous (−1) positions within the two zinc fingers, similar to the Asp-supported Arg residues that bind to G in the Zif268−DNA structure (see Figure 6A) (Pavletich & Pabo, 1991). Change-of-specificity mutants were made in ADR1 at the four fingertip positions, showing complementation in interaction between mutant ADR1 with mutant UAS sequences for binding of Arg115→Gln (at position −1) to A_{10}, His118→Thr (at +3) to C_9, and Arg121→Asn (at +6) to A_8 UAS mutant DNA (Thukral et al., 1992). Thus, the three crucial amino acid residues Arg115, His118, and Arg121 (RHR) in the first zinc finger are in the correct position to bind respectively to the three bases $G_{10}A_9G_8$ (or a GGG mutant) of UAS1 in inverse order (3′−5′) (Figure 1, and shown above), similar to Zif268's zinc finger two residues RHR binding to the GGG sequence.

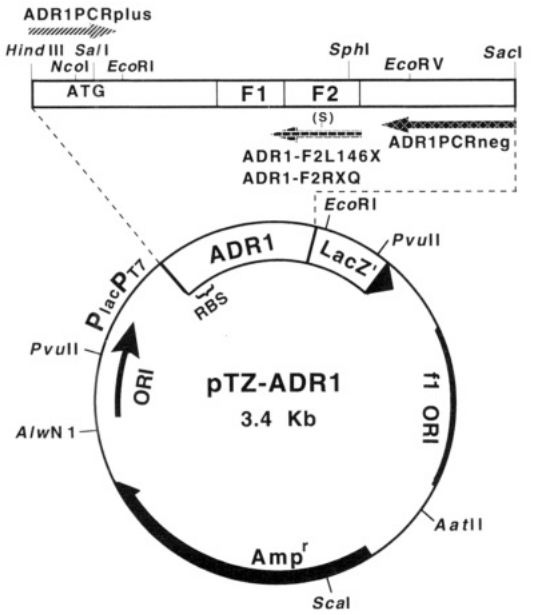

FIGURE 2: Map of pTZ-ADR1 plasmid. The ADR1 gene fragment containing zinc finger one (F1) and finger two (F2) is indicated as a rectangle at the top, with restriction enzyme cut sites used for cloning. It was cloned as a PCR product between *Hin*dIII and *Sac*I sites in the polylinker of vector pTZ19R, in-frame with the open reading frame of lacZ′ (see Materials and Methods). Arrows at the 5′ and 3′ ends of ADR1 show synthetic oligomers ADR1PCRplus and ADR1PCRneg used as PCR primers to amplify the first 510 bp of ADR1 (Figure 3A,B). The arrow under finger two shows synthetic DNA oligomers ADR1-F2L146X and ADR1-F2RXQ (Figure 3C) used for PCR cassette mutagenesis of residues 146 and 149. Shown are ampicillin resistance gene (Amp^r), origin of replication (ORI), and lac and T7 promoters (P_{T7}) for ADR1-βGal expression. Initiation of translation starts at the ATG codon in the *Nco*I site located just downstream from a ribosome binding site (RBS).

In finger two of ADR1, Arg143 at position −1 was required for binding to G_7 of UAS1. By analogy with finger one of ADR1, the second zinc finger was predicted to have three crucial residues Arg143, Leu146, and Arg149 (RLR) in positions −1, +3, and +6 which could theoretically interact with the $G_7T_6T_5$ bases 5−7 of UAS1 (Thukral et al., 1992; Klevit, 1991) (Figure 1). However, on the basis of fairly strong DNA binding (about half of normal) measured for Leu146 and Arg149 mutations to alanine, these residues were not implicated as being necessary for DNA binding to the two thymine bases of UAS1 (Thukral et al., 1991b). It may still be possible, as predicted, that Leu146 binds weakly to T_6, and Arg149 binds to T_5 (or even more strongly to G_5), since the alanine mutants may have lacked a negative interaction with DNA near thymine in UAS1. Wild type ADR1 binding to UAS1 mutants showed that only the six bases 5′-$T_5T_6G_7$-$G_8A_9G_{10}$ in each half dyad have a role in base-specific binding to ADR1, where the purines G_7-$G_8A_9G_{10}$ are absolutely required. However, the two thymines are preferred by ADR1 protein over other base substitutions at positions 5 and 6, since UAS1 with T_6 binds 10-fold better than C_6 (purines are not bound), and T_5 binds slightly better than G_5, but about 10-fold more strongly than A_5 (Cheng et al., 1994). Computer modeling of ADR1 zinc fingers bound to UAS1 as in the Zif268−DNA structure, with finger two residues RLR in close proximity to bases $G_7T_6T_5$, showed that the bulky Leu146 residue could clash sterically with the T_6 methyl group to inhibit binding unless the α-helix is moved slightly out away from the DNA major groove (Bernstein et al., 1994). Finding a specific interaction between ADR1's leucine 146 residue and thymine in DNA would add a new dimension to the catalogue of zinc finger interactions with DNA.

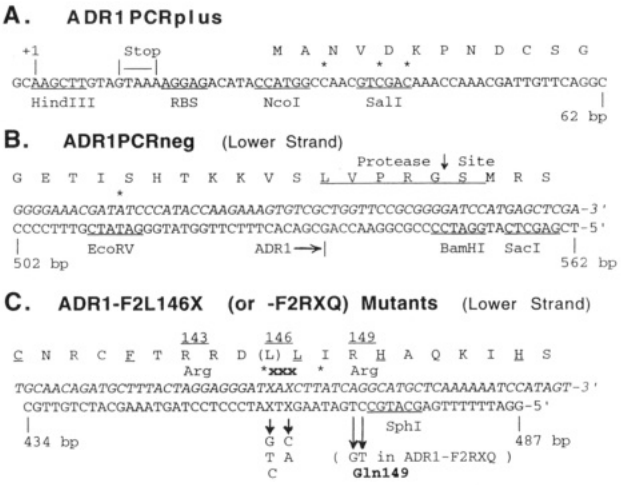

FIGURE 3: DNA sequences of oligomers used as PCR primers. Oligomer is the top strand alone, or the bottom strand of double-stranded DNA (with top in italics). ADR1 amino acid codons (single letter) are shown above, and restriction enzyme cut sites are shown below the sequence, underlined. Base changes within ADR1 open reading frame made to alter restriction sites are marked with asterisks. (A) ADR1PCRplus (64 bases) is the top strand of the 5′ end of ADR1 starting from Met codon "ATG" and including new restriction sites *Nco*I and *Sal*I, fused 9 bp downstream from a ribosome binding site (RBS) and *Hin*dIII site as base 1 (numbered relative to pTZ-ADR1 map, see Figure 2). (B) ADR1PCRneg (61 bases) is the bottom strand of ADR1 downstream from zinc finger two fused with codons for a thrombin site and a *Sac*I restriction site engineered to be in-frame with the *LacZ*′codons in pTZ19R vector. (C) Mutagenic PCR primer ADR1-F2L146X (54 bases), for mutating Leu146, is the lower, noncoding strand of the finger two region. It contains degenerate mutations (equal base fractions) in ADR1 anticodon 146 listed below downward arrows, resulting in codons CAG (Gln), CAT (His), AAG (Lys), AAT (Asn), GAG (Glu), and GAT (Asp) in the coding strand (Figures 2 and 3). Mutagenic PCR primer ADR1-F2RXQ (54 bases) is identical to -F2L146X except it contains two base changes (in parentheses below anticodon 149) for mutation of codon 149 AGG (Arg) to CAG (Gln), in addition to the degenerate mutations in ADR1 codon 146 listed previously.

Our goal is to learn how the amino acid residues in ADR1 zinc finger two recognize the thymine T_5T_6 bases of UAS1, since this is one of the unsolved problems in recognition and binding of DNA sequences (Berg, 1992; Nardelli et al., 1992). By site-directed mutagenesis of the second zinc finger, we have systematically modified the two crucial residues (Leu146 and Arg149) that could contact the two T DNA bases, and then tested if a given mutant abolishes or changes the specificity of DNA binding to the core of UAS1 (TTGGAG) or thymine base variant sequences.

MATERIALS AND METHODS

Construction of pTZ-ADR1 and Zinc-Finger Mutants. For expression of yeast ADR1 protein in *Escherichia coli* bacteria, we constructed plasmid pTZ-ADR1, containing the ADR1 gene fragment encoding two zinc fingers. It was inserted downstream from the lac promoter and fused in-frame with the *lacZ*′ gene in vector pTZ19R (Figure 2). Plasmid pTZ-ADR1 (3.38 kb) was constructed by cloning a 546 bp PCR fragment including from 1 to 510 bp in the yeast ADR1 open reading frame into the 2.9 kb pTZ19R

Table 1: DNA Sequences of pTZADR1-RXX Mutants of Zn Finger Two

pTZADR1 -F2RXX Name[a]	aa Mutations[b] aa146	aa149	Sequence of Zn Finger 2 α-helix Codons
			(-1) (+3) (+6) α-helix #
RLR	*Leu146*	*Arg149*	5'AGG AGG GAC CTC CTG ATC AGG CAT GCT CAA
	Wild Type		*Arg* Arg Asp *Leu* Leu Ile *Arg* His Ala Gln
			143 146 149 aa#
RLQ	*Leu146*	Gln149	5'AGG AGG GAT CTC CTT ATC CAG CAT GCT CAA
			Arg Arg Asp *Leu* Leu Ile Gln His Ala Gln
RHR	His146	*Arg149*	5'AGG AGG GAT CAT CTT ATC AGG CAT GCT CAA
			Arg Arg Asp His Leu Ile *Arg* His Ala Gln
RHQ	His146	Gln149	5'AGG AGG GAT CAT CTT ATC CAG CAT GCT CAA
			Arg Arg Asp His Leu Ile Gln His Ala Gln
RKR	Lys146	*Arg149*	5'AGG AGG GAT AAG CTT ATC AGG CAT GCT CAA
			Arg Arg Asp Lys Leu Ile *Arg* His Ala Gln
RKQ	Lys146	Gln149	5'AGG AGG GAT AGG CTT ATC CAG CAT GCT CAA
			Arg Arg Asp Lys Leu Ile Gln His Ala Gln

[a] ADR1 Zinc finger two DNA-binding residues (RXX) at critical a-helical positions −1 (aa# 143), +3 (146), and +6 (149). [b] Critical wild type (italicized) or mutant residues 146 and/or 149 (underlined) are shown by plasmid DNA sequence (see Methods).

vector (USBC) between the *Hin*dIII and *Sac*I sites. The ADR1 gene was amplified by 25 PCR cycles using 1 ng of pBR-ADR1 digested with *Eco*RV as the template and 100 pmol of synthetic DNA oligomer primers, ADR1PCRplus and ADR1PCRneg (Figure 3A,3B), synthesized by Microchemical Core Facility at San Diego State University. PCR was done as described previously (Thukral et al., 1991b), except we used Vent DNA polymerase and buffer (New England Biolabs). The DNA fragment was purified and ligated with vector, and *JM109* or DH5α *E. coli* cells were transformed and grown on LB ampicillin plates containing X-Gal and IPTG for selection of blue colonies as described. Mutant plasmids encoding amino acid substitutions in ADR1 zinc finger two (positions +3 and +6 in the α-helix) were constructed by oligonucleotide-directed, PCR cassette mutagenesis of pTZ-ADR1 using ADR1PCRplus and ADR1-F2L146X or ADR1-F2RXQ as the primers. The oligonucleotides used for site-directed mutagenesis and PCR amplification of ADR1 contained degenerate changes in codons for Leu146 and/or Arg149 (see Figures 2 and 3C). The 484 bp PCR ADR1 fragments were digested with *Sal*I and *Sph*I which are located respectively 5′ and 3′ of the finger region. This 436 bp *Sal*I−*Sph*I ADR1 fragment was used for transplacement of the mutant ADR1 cassette in the wild type vector by cloning as described above. The identity of ADR1 mutations in purified plasmid DNA was verified by both restriction mapping and Sanger DNA sequencing (Sequinase 2.0 kit with the −40 primer, USBC). ADR1 finger two mutations in these plasmids, designated pTZADR1-F2RXX, included the following: wild type (wt) Leu146 and Arg149 (RLR), single mutations His146 (RHR), Lys146 (RKR), and Gln149 (RLQ), and double mutants His146 + Gln149 (RHQ) and Lys146 + Gln149 (RKQ). Mutants in zinc finger two are named by their three critical amino acid residues (RXX) at α-helix positions: −1 (143), +3 (146), and +6 (149). The DNA sequence within the second finger region of each mutant ADR1 is shown in Table 1 (P. J. Naraghi-Arani, Master's Thesis, 1993; and Hubert Suruki, Master's Thesis, 1994, Department of Chemistry and Biochemistry, California State University, Fullerton (CSUF)).

ADR1 Protein Extract Preparation. ADR1 protein extracts were made essentially as described (Thukral et al., 1989). Bacterial cultures containing pTZ-ADR1 (wild type Leu146, Arg149) or mutant pTZADR1-F2RXX plasmids were grown to early log phase and then induced with IPTG to express the ADR1-βGal mutant fusion proteins. Cultures were grown an additional 3.5 h, and then cells were harvested, sonicated in A50 buffer, and clarified to produce cell lysates. The protein extract was then aliquoted and stored at −70 °C. Total protein concentration was determined by the Folin protein assay with a BSA standard. β-Galactosidase activity (A_{420}/(min·mg of protein)) was determined as described previously, in order to measure the level of ADR1-βGal fusion protein expressed in *E. coli* strain JM109 or DH5α, with α-complementation.

UAS1 DNA Probes. The DNA probes for DNA binding assays were made by San Diego State University's Microchemical Core Facility (MCF) and then purified by HPLC. UAS1 WT (wild type binding site for ADR1) which contains a 22 bp dyad (symmetrical at colon) has the following sequence: 5′-GATCTGTCTCCAACGTC·GACGTTGGAGACA-3′. Note that the dyad's central 6 bases (5′-GTCGAC-3′) in UAS1-WT were changed from the ADH2 promoter's UAS1 sequence (5′-TTATAA-3′) without affecting the ADR1 binding site (Cheng et al., 1994), and bases 12 and 13 (numbered from dyad center) were changed to make a 5′ overhang *Bgl*II site in self-annealed, double-stranded DNA. Mutations in the UAS1 sequence were introduced at either base position 5 and/or 6 along with their complementary dyad positions (underlined) to make wild type (T_5T_6) and all 15 possible mutant sequences (named N_5N_6, where N is any base). The UAS1 oligomers were self-annealed and then labeled by filling in the 5′ overhang sites using [α-^{32}P]dATP with Klenow DNA polymerase. Labeled DNA was purified from free [^{32}P]dATP by centrifugation through Chroma Spin-10 columns (Clontech).

Gel Electrophoretic Mobility Shift Assay. Gel electrophoretic mobility shift assays were done essentially as described (Eisen et al., 1988; Thukral et al., 1989). Assays were done in a time course with 16 parallel reactions each

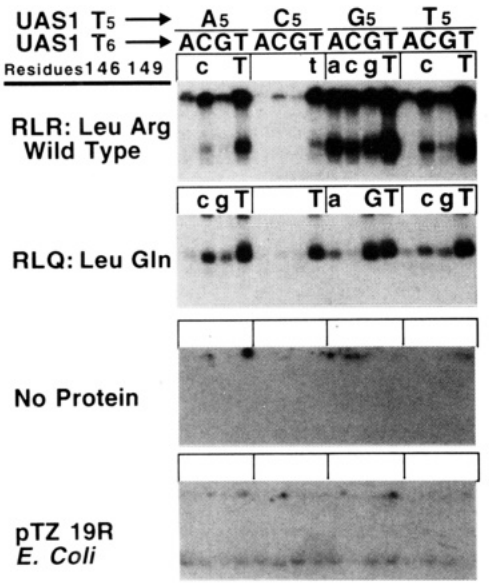

FIGURE 4: DNA binding of UAS1 mutant DNA probes with protein extracts. Top portions of autoradiograms are compared for DNA binding of the 16 different UAS1 probes with wild type ADR1-βGal (RLR), the Gln149 mutant (RLQ), no protein, and protein extracts from *E. coli* with vector pTZ19R (no ADR1). A different UAS1 probe was used in each lane, as shown above autoradiogram. The top row indicates the four possible DNA bases at position five, and for each of these the second row lists four DNA bases at position six of UAS1 mutants (see Figure 1). Relative DNA-binding affinities of UAS1 variants are indicated above gel lanes, as strong (capital letter), intermediate (lower case letter), and weak or none (no letter). GEMS assays were done as described in Materials and Methods.

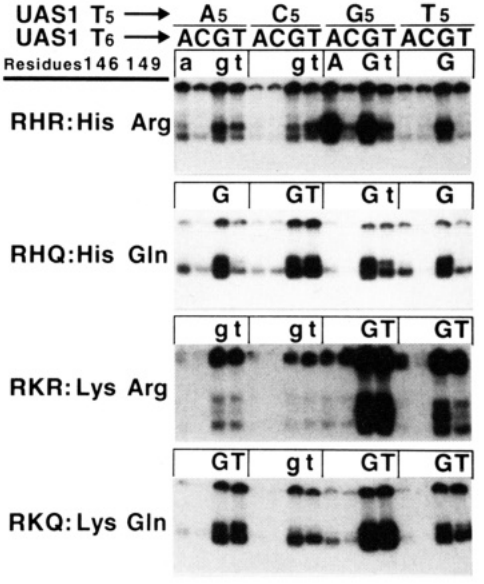

FIGURE 5: DNA binding of ADR1-βGal mutants with UAS1 variant DNA probes. Top portions of the autoradiograms are compared for DNA binding of the 16 different UAS1 probes with ADR1-βGal zinc finger two mutants RHR (His146), RHQ (His146 and Gln149), RKR (Lys146), and RKQ (Lys146 and Gln149). GEMS assays are labeled as in Figure 4.

in a total volume of 20 μL of buffer A50, and containing one of 16 different UAS1 DNA probes (200 000 cpm/(1−3 ng)) plus 2 μg of poly(dI-dC) (Sigma), to which was added 5 μg of ADR1-βGal protein extract. After incubation at room temperature for 5 min, reaction mixtures were immediately run on a 6% polyacrylamide gel and subjected to autoradiography as described. A positive control of wild type ADR1-βGal with the wild type UAS1 probe was included with each experiment, to calibrate relative DNA binding between ADR1 mutant proteins. Experiments were repeated at least five times with all 16 probes (two different preparations) binding every ADR1-βGal mutant protein, and a representative autoradiogram is shown (Figures 4and 5). Each ADR1 protein extract was also titrated from 0 to 20 μg of total protein (i.e., 0, 2.5, 5.0, 7.5, 10, 15, and 20 μg) for three representative UAS1 mutant probes, showing that the 5 μg amounts of protein used in these assays was nonsaturating and in the linear range of increasing amount of DNA complex formation for each probe tested (data not shown).

RESULTS

ADR1 DNA Binding by Gel Mobility Shift. ADR1 mutants made in plasmid pTZ-ADR1 by PCR cassette cloning were expressed in *E. coli* as ADR1-βGal fusion proteins, then extracted from the cell culture, measured, and tested *in vitro* by the gel electrophoretic mobility shift (GEMS) assay for DNA binding to synthetic, radiolabeled mutant UAS1 sequences. We tested the DNA binding of all 16 possible UAS1 sequences (N_5N_6) having mutations at the two T's (but all other bases normal) with different ADR1 mutations having hydrophobic Leu146 replaced by the basic amino acids histidine or lysine, and with Arg149 replaced by glutamine. Equal amounts (in counts per minute) of each of the 16 UAS1 mutant sequences were mixed and incubated with equal amounts of a given ADR1-βGal protein extract (Materials and Methods). The use of the ADR1-βGal fusion protein, which had activity for both ADR1 and β-galactosidase, allowed measurement of βGal activity for quantification of the relative amounts of ADR1 protein; only extracts with over 6 units of βGal activity were used in assays. Reactions were analyzed by GEMS assay to separate the smaller free DNA from the large protein−DNA complexes, seen by autoradiography.

Three size classes of ADR1-dependent DNA complexes were seen for each ADR1-βGal protein extract, including networks of ADR1-βGal linked with DNA (stuck at gel top), large ADR1-βGal complexes (high in gel), and degraded ADR1-βGal zinc-finger fragments (low in gel), located above free UAS1 DNA at the bottom (data not shown for full gel; see Eisen et al., 1988). In order to compare DNA binding affinities of the 16 mutant UAS1 DNA probes for a given protein, we will analyze intensities of the main ADR1-βGal bands located high in the gel since these DNA complexes probably have similar intact forms of ADR1. However, we cannot compare the affinities of different mutant proteins for a given DNA sequence due to variations in the degree of ADR1-βGal degradation. Top portions of autoradiograms are shown from representative experiments with the five ADR1-βGal mutants along with wild type protein (Figures 4 and 5). ADR1 dependence of the DNA complexes was shown by the fact that control extracts, lacking ADR1 fusion protein but containing only the vector pTZ19R, do not form these complexes, as seen in previous work (Eisen et al., 1988). In the controls, all sixteen UAS1 DNA probes formed only one very weak band just below the position of ADR1-βGal (Figure 4, bottom).

Wild Type ADR1 (F2RLR) DNA Binding. Wild type ADR1-βGal protein having zinc finger two residues RLR bound strongly to the wild type UAS1 DNA sequence, T_5T_6,

as expected (Figure 4, top right). It also bound well to all other UAS1 probes having thymine six, including G_5T_6, T_5T_6, A_5T_6, and C_5T_6 (listed from strong to weaker), but bound more weakly to the T_5C_6 and A_5C_6 probes having cytosine six. Binding to T_6 may be due to a favorable hydrophobic interaction between the isopropyl group of Leu146 and the methyl group of T_6 in the DNA major groove. The wild type protein bound strongly to G_5T_6, but it formed complexes with the entire group of four probes having G_5 quite well, while binding less with A_5, and poorly with the C_5 UAS1 variant sequences.

Leu146,Gln149 Mutant F2RLQ. The alteration of Arg149 to Gln149 in the RLQ mutant caused only a few subtle changes in specificities of DNA binding to UAS1 sequences. As in wild type ADR1, every DNA probe that has T_6 formed complexes with this mutant protein (Figure 4). The RLQ mutant also bound well to G_5G_6 and bound weakly to UAS1 probes having C_6 including T_5C_6 and A_5C_6. However, unlike the wild type protein, the Gln149 mutant bound equally well with all four DNA probes having T_6, but with variable bases in the fifth base position.

His146,Arg149 Mutant F2RHR. Substitution of His for Leu146 in ADR1 caused significant changes in binding specificity, eliminating all binding with the wild type UAS1 sequence T_5T_6 (Figure 5, top). The His146 mutant RHR bound most strongly with UAS1 mutant probes G_5G_6 and G_5A_6, containing guanine or adenine at base six, and also having G_5. It bound to all other UAS1 mutant probes having G_6, including G_5G_6, T_5G_6, A_5G_6, and C_5G_6 (listed from strong to weak), and also bound weakly to the G_5T_6 and C_5T_6 probes having thymine at base six. All four DNA sequences having G_5 were bound by the RHR mutant, as in wild type ADR1 protein.

His146,Gln149 Mutant F2RHQ. The ADR1 RHQ mutant with His146 plus Gln149 was similar to the RHR mutant, in binding to all UAS1 mutant probes having guanine six (Figure 5). However, the band intensities were about equal for G_5G_6, T_5G_6, A_5G_6, and C_5G_6. This ADR1 RHQ mutant also bound specifically to the G_5T_6 and C_5T_6 probes having thymine at base six. The most notable difference between the two His146 mutants is that the change from Arg149 to Gln149 caused a remarkable loss of binding to G_5A_6 as well as a general decrease in binding to all probes having G_5, and an increase in binding to the sequences with A_5 and C_5.

Lys146,Arg149 Mutant F2RKR. The zinc finger two mutant RKR, which has Leu146 changed to positively-charged Lys146, binds most strongly to G_5G_6 or G_5T_6 having G or T at UAS1 base six, and also having G_5 (Figure 5). ADR1-F2RKR bound to all other UAS1 mutant probes having either G_6 or T_6, including G_5G_6 and G_5T_6, T_5G_6 and T_5T_6, A_5G_6 and A_5T_6, and C_5G_6 and C_5T_6 (listed from strong to weak, in the same order as in RLR (wt) and RHR mutant). Zinc-finger RKR protein binding was very strong with DNA sequences having G_5, but weak for sequences having C_5.

Lys146,Gln149 Mutant F2RKQ. In a pattern very similar to that of the Lys146 mutant RKR, the RKQ mutant with Lys146 plus Gln149 bound to all UAS1 mutant probes having either G_6 or T_6 at base six, including G_5G_6 and G_5T_6, A_5G_6 and A_5T_6, T_5G_6 and T_5T_6, and C_5G_6 and C_5T_6 (listed from strong to weaker). The main difference between these two Lys146 mutants is that the change of Arg149 to Gln149 caused a subtle decrease in binding to the UAS1 probes having G_5, and a substantial increase in DNA binding to sequences having A_5 and C_5 (Figure 5, bottom).

DISCUSSION

Changes in DNA binding specificity of mutant ADR1 zinc fingers were found to confirm previously-observed interactions between amino acids and DNA bases, including binding of basic residues Arg, His, or Lys with guanine by ionic or hydrogen bonds. In addition, we demonstrated an interaction between hydrophobic Leu and thymine which had not been shown previously. ADR1-βGal zinc finger two residues at α-helix position +3 were mutated from Leu to basic His or Lys, while Arg at +6 was mutated to the amide Gln. DNA binding was measured using GEMS assays of ADR1-βGal protein extracts with UAS1 DNA sequences having all combinations of bases five and six (wild type T's). A summary of the ADR1 zinc finger two binding data are shown below, where weaker interactions are given in lower case letters, and N is any base.

protein	WT ADR1	F2RLQ	F2RHR	F2RHQ	F2RKR	F2RKQ
finger #	Zn-F2	Zn-F2	Zn-F2	Zn-F2	Zn-F2	Zn-F2
αhelix #	-1 3 6	-1 3 6	-1 3 6	-1 3 6	-1 3 6	-1 3 6
ADR1 aa	R L R	R L Q	R H R	R H Q	R K R	R K Q
binding	| | |	| |	| | |	| |	| | |	| |
UAS1 bp	G T T-5'	G T N	G G G	G G N	G G G	G G N
(3'->5')	c G	c	A t	t	T t	T
base #	7 6 5	7 6 5	7 6 5	7 6 5	7 6 5	7 6 5

ADR1 proteins with Arg149 bind very strongly with G_5, strongly with T_5, moderately with A_5, and weakly with C_5 at UAS1 base five. Mutating Arg149 to Gln eliminates preferential DNA binding to G_5 and limits binding of His146 (RHQ) only to guanine G_6 (not A_6). Mutants with basic residues His146 and Lys146 in the +3 position both display strong binding to guanine at base six, but Lys146 also binds thymine T_6. Proteins having wild type Leu146 bind strongly to T_6 at base six, and weakly to C_6. Thus, specificity of interaction between finger two's +3 position and UAS1's sixth base is maintained when Leu146 interacts with T_6, His146 binds G_6, and Lys146 binds G_6 or T_6, even though the +6 position varies between Arg149 or Gln149.

Arg149 Binding to G_5 or T_5. ADR1-βGal binding to G_5 DNA sequences is favored only if ADR1 finger two has wild type Arg149 instead of Gln at the +6 position. Arg149 binding to guanine at UAS1 base five could account for moderately stronger binding as well as nonspecific toleration of all four bases seen for UAS1 base six, especially evident when Leu146 at the +3 helix position is mutated to His or Lys. Strongest Arg binding to G_5 or T_5 sequences relative to weaker binding to A_5 or C_5 is consistent with the arginine interaction with guanine seen in the −1 or +6 helical position of ADR1 finger one (Thukral et al., 1992) and all other Cys_2, His_2 zinc-finger proteins discussed previously. X-ray crystal structures of zinc-finger proteins Zif268, GL1, and Tamtrack bound to DNA (Pavletich & Pabo, 1991, 1993; Fairall et al., 1993) showed H-bonding between the two protonated guanidinium amines of Arg and the electronegative N7 and carbonyl O6 atoms of guanine (see Figure 6A) as predicted by Seeman et al. (1976).

In ADR1 zinc finger two, Arg149 is located to interact with wild type UAS1 base T_5. Our data showed about equal

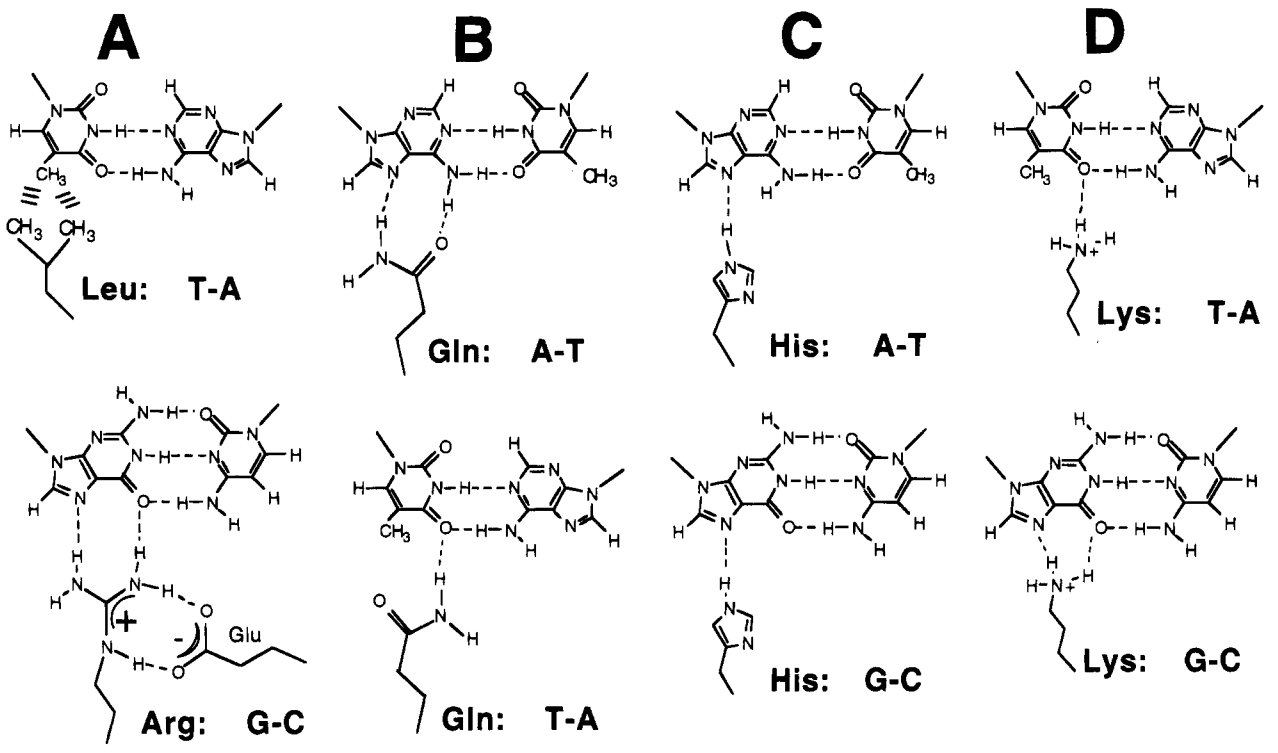

FIGURE 6: Molecular models of amino acid residues contacting various DNA nucleotides. DNA base pairs in the major groove of the top strand are shown interacting with Leu or Arg (A), Gln (B), His (C), or Lys (D). Hydrogen bonds are shown as dashed lines connecting hydrogen to an electronegative atom such as O or N. Hydrophobic interaction is shown as short parallel lines.

ADR1-βGal binding to either wild type UAS1 T_5T_6 or the mutant G_5T_6, with which stronger specific binding to G_5 was expected. However, in another study, ADR1 interaction with T_5 was 3-fold stronger than with G_5 and 10-fold stronger than with A_5 UAS1 mutants (Cheng et al., 1994). Arg149 binding with G_5 may be weakened by the proposed hydrophobic interaction of the bulky Leu146 residue with T_6, which prevents Arg149 from making close contact with DNA (Bernstein et al., 1994). The structural basis for strong binding of Arg149 with T_5 may be an H-bond between the Arg amines and the carbonyl O4 atom of thymine. However, Arg149 does not bind to A_5 or C_5 due to repulsive interaction with their amines (Figure 6A).

Gln149 Binding to N_5. Gln149 showed equal preference for binding A_5, C_5, G_5, and T_5 bases at UAS1 position five. This lack of specificity indicates either similar binding or a loss of DNA contact by Gln149. Compared to the wild type Arg side chain, the shorter Gln149 propylamide may not reach the DNA. Seeman et al. (1976) proposed Gln interaction with adenine via two hydrogen bonds (Figure 6B). Indeed, an ADR1 mutation of Arg115 to Gln resulted in the strongest binding to A_{10} (Thukral et al., 1992). Similar DNA binding to adenine was seen for the Asn amides in both Tramtrack zinc fingers at the +3 position (Fairall et al., 1993). Theoretically, amide residues can H-bond to any base pair (Figure 6B), where DNA base bonding with either the amine or carbonyl group is made possible by rotating the Gln amide side chain. This is consistent with Gln149 binding equally to all bases.

His146 Binding to G_6 (or A_6 with G_5). Mutating Leu146 to His made zinc finger two (RHR) essentially similar to zinc finger one. Our data showed strong His146 binding to G_6 (or A_6 with G_5). Thus, DNA-contacting residues RHR in both ADR1 zinc fingers one and two specifically bind to GGG or GAG subsites (Thukral et al., 1992; Cheng et al., 1994). It is remarkable that the DNA-binding specificity of these zinc fingers is determined primarily by the three critical RHR residues, with little contribution from other residues of the α-helical domain which are different between ADR1 fingers one and two. Histidine binding to guanine or adenine has been observed with a similar ADR1 finger two mutant (Cheng and Young, personal communication) in addition to many other zinc finger proteins (Nardelli et al., 1992; Desjarlais & Berg, 1992a,b; Lundin et al., 1994; Drummond et al., 1994). As in the Zif268–DNA structure (Pavletich & Pabo, 1991), ADR1 His146 probably binds to the purine ring N7 of either G or A bases. Stronger His146 interaction with guanine versus adenine may be due to alternative H-bonding between the His ϵ-N hydrogen and the guanine carbonyl O6, as opposed to blocking by the N6 amine of adenine (Figure 6C).

This preference of His146 interaction with G versus A could explain the apparent change in DNA-binding specificity at His in the +3 position, seen as a result of mutation of Arg at the +6 position. The His146 mutant bound G_6 well, but His bound A_6 only if G_5 was also present in UAS1, which was presumably bound by Arg149. When this Arg149 residue was mutated to Gln in the RHQ mutant, His146 still bound all G_6 sequences, but did not bind to G_5A_6, probably due to loss of strong Arg149 (at +6) binding of G_5 that had stabilized the complex. The Gln149 mutant, as mentioned before, has a shorter side chain and weaker interaction with UAS1 base five, so His146 binding specificity in RHQ was limited to G_6, with weak binding to T_6, possibly via the thymine carbonyl O4.

Lys146 Binding to G_6 or T_6. Lys146 mutants bind to G_6 or T_6 sequences, probably by lysine's protonated amine H-bonding to carbonyl oxygen atoms O6 on guanine or O4 on thymine (Figure 6D). Although either guanine's or adenine's N7 could accept an H-bond from lysine's amine,

adenine's N6 amine may sterically hinder or repel this bond as discussed above for His. Lys146 bonding to G_6 through its N7 or O6 is about equal in strength to Lys interaction with T_6 by two possible ways: hydrophobic interaction of its alkyl chain with thymine's methyl, in addition to H-bonding to O4.

Similar to Lys146, lysine residues in other Cys_2,His_2 zinc fingers including ADR1 finger one mutants also can bind to guanine. When G-contacting Arg residues at −1 or +6 helical positions in ADR1 were replaced by lysine, Lys115 bound strongly to G_{10} in UAS1, while Lys121 and Lys143 bound G_7 and G_8 guanines weakly relative to Arg (Thukral et al., 1992). Other zinc-finger proteins including SP1 and GLI (finger five) have Lys at −1 or +6 positions which bind to guanine bases in the cognate DNA element (Berg, 1992; Pavletich & Pabo, 1993). The lysine residue most similar to our Lys146 mutant in the +3 α-helix position is the central His118 to Lys mutation in ADR1 finger one. Like Lys146, Lys118 did not bind to adenine (A_9) in UAS1, but its binding to guanine and thymine was not tested (Thukral et al., 1992).

Leu146 Discriminates T_6. The wild type Leu146 residue at the +3 helical position of zinc finger two specifically recognizes the central T_6 base of the $T_5T_6G_7$ subsite (Figure 1). Leu146 binds more weakly with C_6 sequences, consistent with previous studies (Thukral et al., 1992; Cheng et al., 1994). Changes in DNA-binding specificity at UAS1 base six (T_6) were seen when Leu146 was mutated to either His or Lys. This confirms that ADR1 zinc fingers interact with UAS1 bases in the pattern revealed by Zif268−DNA interaction (Pavletich & Pabo, 1991). Leu at α-helix position +3 discriminates among the four bases either by positive binding to T_6 (or weakly to C_6) or by neutral tolerance of T_6 and hydrophobic repulsion of purines A_6 and G_6. Leu146 interaction with T_6 could form a hydrophobic pocket between Leu146's isopropyl group and thymine's methyl group in the DNA major groove (Figure 6A). Molecular computer modeling of ADR1 zinc fingers docked with the UAS1 sequence has shown that the hydrophobic methyl groups on Leu146 and T_6 are probably located close together within the protein−DNA complex, at a proper distance to allow van der Waals attraction (Igarashi, unpublished), instead of hindrance due to steric clash between methyl groups (Bernstein et al., 1994).

ADR1 is the only protein shown to have leucine as a critical residue for specific recognition of T, but several zinc fingers have Leu at the central +3 position. SP1 zinc finger two, with critical residues RER binding bases GCG, was changed to RLR by a Glu mutation to Leu at α-helix position +3 (Desjarlais & Berg, 1992b). This SP1 RLR mutant, unlike ADR1 with Leu146, bound equally strongly to GCG, GAG, and GTG subsites, indicating that this +3 Leu residue interacted positively with C, A, or T. The ability of ADR1 to distinguish T_6 may involve Leu146 interaction with other residues within the context of zinc finger two.

Conclusions. DNA-binding studies of ADR1 mutants at the +3 position of zinc finger two demonstrated strong interaction of Leu with T_6, His with G_6, and Lys with G_6 or T_6, while Arg at the +6 position binds G_5 best. These results not only add to the growing catalog of Cys_2,His_2 zinc finger−DNA binding specificities, but also give the first report of leucine engaging in base-specific interaction with thymine in these DNA-binding proteins. Alanine is another hydrophobic amino acid residue which is involved in zinc finger binding with thymine in DNA sequences by hydrophobic interaction, as seen in the GLI−DNA complex (Pavletich & Pabo, 1993). Ala discriminates T bases moderately well in SP1 zinc finger one (Berg, 1992), especially when its zinc atom is replaced by nickel (Nagaoka et al., 1993). When ADR1 Leu146 was mutated to the shorter Ala residue, it still bound UAS1 with T_6 about half as strongly as wild type ADR1 (Thukral et al., 1991b). Future work will show whether base-specific interaction between thymine and the hydrophobic amino acid residues such as leucine, isoleucine, or valine is a general rule in zinc finger binding to DNA.

By testing all possible DNA base combinations at relevant UAS1 positions, we observed subtle modulations of DNA-binding specificity caused by interactions between neighboring critical residues Leu146 and Arg149 within a zinc finger. DNA-binding specificity for a central +3 residue (for instance, His binding to A) can be modulated by the parallel interaction of an adjacent DNA-binding residue at the +6 α-helical position with its cognate DNA base (i.e., Arg with G or T), and vice versa. Apparently, stable DNA complex formation with ADR1 depends upon a balance between binding of the +3 residue with UAS1 base six, and binding of the +6 residue with base five. The strongly-interacting bases T_6 and G_5 in these two positions of the ADR1 binding site can compensate for each other in the overall energy balance of ADR1 binding to DNA, allowing at least weak binding of the protein if one strong interaction is missing. That is, all G_5- or T_6-containing sequences including G_5N_6 and N_5T_6 bind ADR1 at least weakly. The ADR1 DNA consensus sequence $(T/G/A)_5(T/C)_6G_7$-$G_8(A/G)_9G_{10}$ from Cheng et al. (1994) agrees well with our data, except mutants C_5T_6 and $G_5(G/A)_6$ also bind ADR1 weakly. Low-affinity ADR1 binding sites GAG-GAG (G_5A_6) were found near UAS1 in the yeast ADH2 promoter (at −158 and −255) by DNaseI footprinting (Eisen et al., 1988), but may have no role in ADH2 regulation. However, strong binding of ADR1 with variant UAS1 sequences including GTG-G(A/G)G is important for regulation of several genes in *S. cerevisiae* including GUT1 and CTA1 (Cheng et al., 1994; Pavlik et al., 1993; Simon et al., 1991). Yeast gene expression for many genes can thus be regulated by differential binding of ADR1 to variant UAS sequences, due to inter-residue interactions of DNA-binding amino acids within zinc finger two.

The DNA-binding specificity of a given residue can also be altered by inter-finger interactions due to the order of a zinc finger within a group of several finger domains. For instance, both fingers of ADR1 have four Arg residues that can bind to guanine, but G is very specifically required by two internal "fixed" Arg residues at +6 in finger one and at −1 in finger two (Thukral et al., 1992). In contrast, two critical external Arg residues at −1 in finger one and especially at +6 in finger two (Arg149) bind less specifically to G (and can be substituted in function by other residues), perhaps because of being located at more flexible end positions on both zinc fingers. Like ADR1, the Tamtrack protein's two zinc fingers have two internal Arg residues which bind to guanine, while external "flexible" residues His and Ala do not contact DNA; instead, unique Ser or Asp residues at the +2 positions bind to pyrimidine bases (Fairall et al., 1993).

Simple models used to represent zinc-finger interaction with DNA sequences must be fleshed out by computerized

molecular 3D modeling, to define steric constraints on molecular interactions. Affinity selection of ambiguous DNA sequences by binding to purified zinc-finger proteins, using either physical or biological methods, will continue to be important in generating an unbiased data bank of strong protein−DNA interactions (Cheng et al., 1994; Rebar & Pabo, 1994). However, our systematic approach of testing all DNA sequence combinations is also needed for clearly detecting subtle binding interactions. Making an accurate catalog of empirical rules for protein−DNA binding is vital to the goal of correctly designing zinc fingers to recognize and bind predetermined DNA sequence elements.

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