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Altered DNA-binding Specificity Mutants of GCN4 and TFIID

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Mutant DNA-binding proteins with altered sequence recognition properties have been extremely useful for defining specific protein-DNA contacts mediated by the helix-turn-helix structural motif (*1–8*). Previously characterized specificity mutants contain single amino acid substitutions and alter DNA sequence recognition at a particular base pair, thus providing strong functional evidence for direct contacts between individual amino acids and base pairs. In general, direct interactions inferred from genetic and biochemical studies have been confirmed by high resolution structures of the protein-DNA complexes (*9–11*). This paper will review our studies (*12, 13*) concerned with the isolation and characterization of altered specificity mutants of two transcriptional regulatory proteins from the yeast *Saccharomyces cerevisiae*, GCN4 and TFIID.

I. GCN4

GCN4 protein binds to the promoters of many amino acid biosynthetic genes and coordinately activates their transcription (*14, 15*). Optimal

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binding is observed with a 9-bp dyad symmetric sequence, ATGA(C/G)TCAT, with the central 7-bp being most important (*16*, *17*). GCN4 binds as a dimer to overlapping and non-equivalent half-sites (*18*), and the optimal half-site is ATGAC (*19*). The 56 C-terminal amino acids of GCN4 are sufficient both for dimerization and for specific DNA-binding (*18*, *20*, *21*), and this DNA-binding domain folds independently of the remainder of the protein (*22*).

The GCN4 DNA-binding domain contains the bZIP structural motif found in a class of eukaryotic transcription factors that includes C/EBP and the Jun and Fos oncoproteins (*23*). The bZIP domain is largely α-helical (*21*, *24–26*) and consists of a dimerization element, the leucine zipper, and an adjacent basic region that directly contacts DNA (*24*, *27–31*). As predicted by models of the protein-DNA complex (*25*, *32*), the leucine zipper symmetrically positions a diverging pair of α-helical basic regions to make sequence-specific contacts with the DNA target (*33*). The basic region contains a quartet of uncharged residues including two alanines, a serine/cysteine, and an invariant asparagine (corresponding to asn235, ala238, ala239, and ser242 in GCN4) that have been proposed to lie on the face of the α-helix that contacts the DNA (*25*). In support of this idea, DNA-binding activity is retained in a derivative in which 5 non-conserved residues on the putative solvent-exposed surface are changed to alanine or glutamine (*25*). Surprisingly, however, these highly conserved residues are not essential for DNA-binding by GCN4 (*34*).

As a general approach for identifying GCN4 derivatives with altered DNA-binding specificity, we utilized a set of yeast strains that differ only in the GCN4 recognition sequence upstream of the TATA element in the *his3* promoter. GCN4 binding to a functional target sequence activates *his3* transcription, which permits cells to grow in the presence of aminotriazole (AT), a competitive inhibitor of the *his3* gene product. Symmetrical double mutants representing equivalent changes in each of the adjacent half-sites (TTGACTCAA, ACGACTCGT, ATTACTAAT, ATGTCAC-AT) bind GCN4 poorly and are unable to support GCN4-dependent activation in yeast cells (*19*). To isolate potential altered specificity mutants, collections of GCN4 proteins, generated by mutagenesis *in vitro* with degenerate oligonucleotides, are screened for their ability to activate *his3* transcription from the symmetrically mutated target sequences.

The first example of an altered specificity mutant of GCN4 came from an analysis of the invariant asparagine (asn235). Although most substitutions of asn235 abolish DNA-binding, the trp235 protein displays nearly wild-type function, and the gln235 and ala235 proteins show

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detectable activity. The ability of an amino acid to functionally substitute for asn235 does not correlate with its preference for assuming the N-cap position of an α helix (*35*), thus arguing against a prediction (*32*) that the invariant asparagine forms an N-cap structure that permits the α-helical basic region to bend sharply around the DNA. The most interesting result was that the trp235 protein activated transcription from TTGACTCAA, a sequence refractory to activation by wild-type GCN4. In contrast, when the target sequence was GTGACTCAC, the site that naturally occurs in the *his4* promoter, the asn235 protein activates strongly, whereas the trp235 protein appears inactive. Both proteins activate transcription from a promoter containing the optimal binding site, ATGACTCAT, although wild-type GCN4 is more efficient because it permits strains to grow at higher concentrations of aminotriazole.

DNA-binding experiments *in vitro* indicate that the asn235 protein binds more efficiently to the optimal (ATGACTCAT) site than the trp235 protein. GCN4 binds the native *his4* site (GTGACTCAC) almost as strongly as the optimal site, while the trp235 protein does not bind detectably. In contrast, the two proteins bind TTGACTCAA with comparable affinity. In terms of relative affinities, the order for GCN4, from highest to lowest binding, is ATGACTCAT > GTGACTCAC > ATGACTCTT > TTGACTCAA. For the trp235 protein, the order is TTGACTCAA = ATGACTCAT > ATGACTCTT > GTGACTCAC. Analysis of the gln235 and ala235 proteins for their sequence preferences at the $\pm$ 4 position surprisingly reveal that both proteins favor the optimal site ATGACTCAT, bind weakly to TTGACTCAA and CTGACTCAG and strongly discriminate against GTGACTCAC. As expected from the *in vivo* results, the gln235 and ala235 proteins bind with lower affinity than the trp235 protein.

The trp235, gln235, and ala235 proteins can be interpreted as having lost some of the high affinity interactions at position $\pm$4 because all of these strongly discriminate against GTGACTCAC, a site efficiently bound by GCN4. This strongly suggests that asparagine 235 contributes to high-affinity GCN4 binding by recognizing, directly or indirectly, the $\pm$4 position. However, asn235 is clearly not responsible for all specificity at position $\pm$4, because the trp235, gln235, and ala235 substitutions retain some of the normal sequence preferences. On the other hand, the trp235 protein is not simply a "loss of specificity" mutant because it has the novel property of binding with comparable affinity to TTGACTCAA and the optimal site. Moreover, as tryptophan is the only substitution at position 235 that permits activation from TTGACTCAA,

the altered specificity is not easily explained simply by the absence of asn235. Although indirect, conformationally transmitted effects cannot be excluded, we favor the idea of a direct contact between amino acid 235 and position ±4, especially because tryptophan, glutamine, and alanine are structurally very different yet all result in a strong discrimination against GTGACTCAC. Moreover, in a chimeric protein containing the Jun basic region in place of the GCN4 basic region, the only position 235 derivative that appears to activate transcription from TTGACTCAA is the equivalent change from asparagine to tryptophan.

Other protein-DNA contacts involved in high affinity binding have come from recently identified derivatives that display altered DNA-binding specificity at different positions of the binding site (J. Kim, D. Tzamarias, and K. Struhl, unpublished). At the +3 position, a protein containing tyr238 instead of the conserved ala238 has the novel property of activating transcription from ACGACTCGT. DNA-binding experiments indicate that the tyr238 protein can recognize this sequence, whereas the wild-type protein cannot. The tyr238 protein does not bind to any symmetrical double mutants at the ±2 or ±4 position indicating specificity at ±3. Interestingly, however, this protein retains the ability to bind the optimal GCN4 site. At the ±2 position, changing the moderately conserved ser242 to leu242 results in a protein that has near wild-type affinity for ATTACTAAT, a site not recognized by GCN4. Again, the ser242 protein is unable to recognize any symmetrical double mutants at ±3 or ±4, but retains the ability to bind the optimal GCN4 target.

Taken together, these observations strongly support a model in which the α-helical surface defined by amino acid positions 235, 238, and 242 is aligned along the DNA with direct contacts to ±4, ±3, and ±2, respectively. However, we do not understand the structural basis for why all of our altered specificity mutants retain the ability to recognize the optimal binding site. Hopefully, conclusive proof of the above hypothesis as well as more detailed knowledge of the chemical nature of the protein-DNA interactions will be addressed by a forthcoming high resolution structure of the protein-DNA complex (Tom Ellenberger and Steve Harrison, personal communication).

II. TFIID

TFIID is the highly conserved component of the RNA polymerase II transcription machinery that binds specifically to the TATA element (consensus TATAAA). The C-terminal 180 residues of the various

TFIIDs are at least 80% identical in amino acid sequence, and this core domain is necessary and sufficient for TATA-element binding and basal transcription *in vitro* (*36–39*), and for the essential functions of TFIID in yeast cells (*40–43*).

TFIID appears to be an unusual sequence-specific DNA-binding protein. It is extremely slow at binding to and dissociating from TATA elements (*44*), and it has a surprisingly high affinity for single-stranded DNA (*45*). Unlike most specific DNA-binding proteins, TFIID binds as a monomer (*37*) and it undergoes a conformational change upon binding to the TATA element (*39*). Optimal binding is not constrained to a simple target DNA site that conforms to the consensus of naturally occurring TATA elements, and a variety of non-consensus sequences interact efficiently with TFIID (*45*, *46*). Deletion analysis (*37*) and proteolytic cleavage experiments (*39*) indicate that the DNA-binding domain is not localized to a short region but instead requires the structural integrity of the entire core domain. Another unusual feature of the TFIID conserved core is the presence of two direct 67 amino acid repeats that are separated by a highly basic region. Dominant negative mutations in either repeat of yeast TFIID have been isolated that eliminate DNA binding while maintaining at least some aspects of normal structure and function (*42*). These mutations suggest that the TFIID monomer contains a bipartite DNA-binding domain in which each repeat contributes to sequence recognition, but they do not address the issue of binding specificity.

We have developed a new approach for addressing aspects of TFIID function that involves the isolation of derivatives that are transcriptionally active on mutated TATA elements (*13*). Such altered specificity mutants, which have been obtained for prokaryotic σ factors (*47–49*) provide strong genetic evidence for a direct protein-DNA contact. Moreover, by "genetically marking" TFIID with the property of altered TATA-element specificity, the activities of heterologous or mutated TFIIDs can be specifically assayed *in vivo* even in the presence of wild-type TFIID, which might be required for viability of the organism. Saturation mutagenesis of the yeast *his3* T_R TATA element revealed that almost all single base pair substitutions in the core sequence (TATAAA) severely compromised promoter function *in vivo* and *in vitro* (*50*, *51*). Consequently, cells carrying *his3* alleles with such defective TATA elements grow poorly on medium lacking histidine and not at all in the presence of aminotriazole (AT), a competitive inhibitor of the *his3* gene product. Thus, TFIID mutants with altered specificity for TATA ele-

ments would be isolated by virtue of their ability to increase transcription from a defective *his3* TATA element and hence to confer AT resistance.

To search for altered specificity mutants, the region of TFIID between amino acids 190 and 205 was heavily mutagenized by replacing it with a degenerate oligonucleotide containing 8% non-wild-type residues per base pair. This small region was targeted because it displays a weak sequence similarity (*52*) with a region of prokaryotic σ factors thought to interact with DNA (*53*). A library (10^6 independent clones) containing the collection of TFIID mutant proteins was introduced into a set of yeast strains with defective *his3* TATA elements (TGTAAA, GATAAA, TAGAAA, and TATAGA) whose transcriptional activities *in vitro* are at least 10-fold reduced compared to the wild-type TATAAA (*51*). All of these strains contain the wild-type TFIID gene on the chromosome to carry out the essential functions for cell growth.

From this genetic selection, four TFIID derivatives that permitted growth of the yeast strain containing the TGTAAA element were obtained; one of these, mutant 3, grew at higher drug concentrations suggestive of higher transcriptional activity. Indeed, a yeast strain carrying the TGTAAA element shows substantially increased *his3* RNA level in the presence of TFIID mutant 3 only. Moreover, this basal transcription is stimulated by GCN4, with the fold-induction being comparable to that expected for a wild-type promoter. The absolute level of *his3* transcription mediated by TFIID mutant 3 on the TGTAAA promoter is about 20% of that mediated by wild-type TFIID on an equivalent promoter containing TATAAA (*54*). Thus, the altered TFIID protein not only allows constitutive transcription to occur from a defective TATA element, but is also responsive to an acidic activator protein. Most importantly, the mutated TFIID derivatives do not function at defective TATA elements (GATAAA, TGTAAA, TCTAAA, TAGAAA, TATAGA) other than the TGTAAA sequence used in the original screening. Thus, as expected for an altered specificity mutant, these TFIID derivatives suppress mutations of the *his3* TATA element in an allele specific manner.

By analogy with altered specificity mutants of several DNA-binding proteins, TFIID derivatives functioning at mutated TATA sequences might be expected to be defective in activating transcription from promoters with natural TATA elements. Because the wild-type TFIID gene was present in these cells, the ability of the different mutants to carry out this and other essential functions of wild-type TFIID could not be tested. However, as determined by the plasmid shuffle complementation assay

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(*55*), yeast cells containing mutants 1, 2, or 4 as the sole source of TATA-binding protein are viable, but they grow significantly less well than wild-type. For yeast cells containing mutant 3, very slow growing colonies are observed only after prolonged incubation. Thus, TFIID proteins bearing mutations that allow them to induce transcription from a defective TATA element are less efficient in promoting transcription from wild-type promoters. Moreover, the derivative allowing the highest *his3* expression from altered TATA element is also the least effective in carrying out the essential functions.

Surprisingly, all four TFIID altered specificity derivatives bear the same double amino acid substitution, ile_{194} to phe_{194} and leu_{205} to val_{205}. Mutant 3, which supports the highest *his3* expression from the TGTAAA containing promoter, also contains a third amino acid change, val_{203} to thr_{203}, that is not present in the other molecules. Substitutions of either glutamine (molecule 2) or asparagine (molecule 4) for the lysine at position 199 appear to be neutral because these molecules are phenotypically indistinguishable from molecule 1, which contains the wild-type residue. Given that all four altered specificity mutants share two particular base pair substitutions, it is very likely that any other pair of nucleotide changes within the mutagenized region could not yield TFIID derivatives that would pass the genetic selection employed here. Thus, increased activity from TGTAAA promoters cannot be accomplished with at least 80% of the possible single amino acid substitutions. The presence of the same double mutation in four independent mutants strongly suggests that both changes are required to produce a TFIID protein with altered specificity. Indeed, all other possible combinations of the mutations found in molecule 3 were completely unable to induce transcription from the TGTAAA containing promoter.

The DNA-binding specificities of wild-type TFIID and mutant 3 were directly examined on the complete set of TATA sequences that differ at position 2. As expected from *in vivo* (*50*) and *in vitro* (*51*) transcriptional analyses of the identical TATA sequences, wild-type TFIID binds efficiently to TATAAA, weakly to TTTAAA, and extremely poorly to both TGTAAA and TCTAAA. In striking contrast, the mutant protein binds much more efficiently than wild-type TFIID to the TGTAAA sequence. Otherwise, TFIID mutant 3 behaves very similarly to the wild-type protein on the four TATA sequences tested. Consistent with the observation that the mutant 3 stimulates transcription *in vivo* from TGTAAA with less than full wild-type efficiency, the mutant protein binds TGTAAA with somewhat lower affinity than TATAAA. These

biochemical experiments directly demonstrate that TFIID mutant 3 displays altered DNA-binding specificity in that it gains the ability to efficiently bind TGTAAA while retaining other recognition properties of wild-type TFIID.

By analogy with altered specificity mutants of a variety of DNA-binding proteins, our results provide strong genetic evidence that the region of TFIID between residues 190 and 205 directly interacts with the TATA element. This very region had previously been implicated as being important for DNA binding because single substitutions at positions 196, 203, and 207 abolish TATA-element interaction while retaining at least some aspects of normal TFIID structure and function (*42*). In addition, a different substitution at position 205 is observed in the *spt15*-122 allele of TFIID, which alters the transcription pattern at the *his4*-917δ locus possibly by affecting TATA element utilization (*56*) (F. Winston, personal communication). Given that mutant 3 specifically increases binding to TGTAAA, the most likely explanation for altered specificity is the existence of a new contact between protein and position 2 of the TATA element that does not interfere with the normal TATA element interactions mediated by TFIID. However, the standard interpretation cannot be easily applied because altered TATA-element specificity requires two amino acid substitutions spaced 11 residues apart and is increased by a third substitution within the region. It is possible that residues 194, 203, and 205 are in close proximity in the folded structure of TFIID, with one or more of them directly interacting with position 2 of the TATA element. Alternatively, the mutations might subtly disrupt the conformation of a critical surface of TFIID and affect specificity more indirectly through another amino acid residue.

SUMMARY

Mutant DNA-binding proteins with altered sequence recognition properties are useful for defining specific protein-DNA contacts. This paper describes altered specificity mutants of two transcriptional regulatory proteins from the yeast *S. cerevisiae*, GCN4 and TFIID. TFIID is the highly conserved, but species-specific, component of the RNA polymerase II transcription machinery that binds specifically to the TATA element (consensus TATAAA). Using a genetic selection, we isolate an altered specificity derivative of yeast TFIID that permits transcription from promoters containing a mutated TATA element (TGTAAA). Biochemical analysis indicates that this TFIID derivative has specifically

gained the ability to efficiently bind TGTAAA. The mutant protein contains three substitutions within a 12 amino acid region; two of these are necessary and primarily responsible for the altered specificity. These results define a surface of TFIID that directly interacts with the TATA element. By genetically selecting for derivatives of yeast GCN4 that activate transcription from promoters containing mutant binding sites, we isolate an altered-specificity mutant in which the invariant asparagine in the basic region of bZIP proteins (asn235) has been changed to tryptophan. Wild-type GCN4 binds the optimal site (ATGACTCAT) with much higher affinity than the mutant site (TTGACTCAA), whereas the trp235 protein binds these sites with similar affinity. Moreover, the trp235, ala235, and gln235 derivatives differ from GCN4 in their strong discrimination against GTGACTCAC. These results suggest a direct interaction between asn235 and the ±4 position of the DNA target site. This and other specificity mutants will be very useful in interpreting a X-ray structure of the GCN4/AP-1 complex.

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