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Identification of Seven Hydrophobic Clusters in GCN4 Making Redundant Contributions to Transcriptional Activation

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GCN4 is a transcriptional activator in the bZIP family that regulates amino acid biosynthetic genes in the yeast *Saccharomyces cerevisiae*. The N-terminal 100 amino acids of GCN4 contains a potent activation function that confers high-level transcription in the absence of the centrally located acidic activation domain (CAAD) delineated in previous studies. To identify specific amino acids important for activation by the N-terminal domain, we mutagenized a *GCN4* allele lacking the CAAD and screened alleles in vivo for reduced expression of the *HIS3* gene. We found four pairs of closely spaced phenylalanines and a leucine residue distributed throughout the N-terminal 100 residues of GCN4 that are required for high-level activation in the absence of the CAAD. Trp, Leu, and Tyr were highly functional substitutions for the Phe residue at position 45. Combined with our previous findings, these results indicate that GCN4 contains seven clusters of aromatic or bulky hydrophobic residues which make important contributions to transcriptional activation at *HIS3*. None of the seven hydrophobic clusters is essential for activation by full-length GCN4, and the critical residues in two or three clusters must be mutated simultaneously to observe a substantial reduction in *GCN4* function. Numerous combinations of four or five intact clusters conferred high-level transcription of *HIS3*. We propose that many of the hydrophobic clusters in GCN4 act independently of one another to provide redundant means of stimulating transcription and that the functional contributions of these different segments are cumulative at the *HIS3* promoter. On the basis of the primacy of bulky hydrophobic residues throughout the activation domain, we suggest that GCN4 contains multiple sites that mediate hydrophobic contacts with one or more components of the transcription initiation machinery.

Transcription initiation by RNA polymerase II requires the assembly of a complex consisting of general transcription factors, RNA polymerase, and other proteins found stably associated with the polymerase (8, 41). Because transcription initiation is a multistep process, activator proteins could function at any of several different steps in the assembly or functional stimulation of the initiation complex. There is evidence that binding of TATA-binding protein (TBP) to the TATA sequence is a rate-limiting step in transcription initiation in yeast cells (11, 40, 69), and certain activators appear to stimulate this step in mammalian cells (1, 10, 19, 37, 38, 42, 72). Several activators bind TBP in vitro in a manner that depends on specific amino acids in the activation domain that are required for transcriptional activation in vivo (6, 10, 19, 24, 34, 36, 43, 50–52). Thus, direct interactions between the activator and TBP may be important in recruiting TBP to the TATA sequence. Other studies suggest that activators interact with the TBP-associated factors in the TFIID complex (13) and that different activators interact with particular TBP-associated factors to stimulate transcription (12, 22, 35, 49, 66). Certain activators also bind TFIIB in vitro (2, 6, 39, 48, 72) and stimulate recruitment of this general factor to preinitiation complexes (13, 39, 47, 48) or interact specifically with the p62 subunit of TFIIH (67, 71).

In addition to binding transcription factors directly, there is evidence that activators interact with mediator proteins, which in turn contact the transcription machinery. For example, the VP16 activation domain binds in vitro to the mammalian coactivator PC4 (18), to the mediator complex found stably associated with yeast RNA polymerase II holoenzyme (26), and to the yeast coactivator ADA2 (4). In addition, activators may enhance the assembly of the initiation complex indirectly by promoting the removal of nucleosomes from the promoter, e.g., by recruiting the SWI-SNF complex (57). Once the initiation complex is formed, an activator might also stimulate the transition from closed to open complexes, the promoter clearance step (54), or even transcription elongation (73).

The structural features of activator proteins responsible for stimulating transcription are not well defined. Activation domains are often rich in acidic residues or glutamines; however, mutational studies of several activators have shown that acidic residues are not the most important for stimulating transcription (6, 14, 59) and that specific aromatic or bulky hydrophobic amino acids are critically required for activation (3, 24, 25, 46, 51). For certain activators, it was shown that multiple subdomains are present and that high-level activation can occur without a full complement of these subdomains. This was first demonstrated for GCN4 (30) and applies to VP16 (59, 68), RelA (25), and the Rta protein of Epstein-Barr virus (6). It appears that the subdomains in these proteins have overlapping functions and that efficient activation requires only a critical number of functionally redundant subdomains. This interpretation is supported by the fact that essentially wild-type levels of activation have been achieved in artificial activators containing reiterated copies of a single subdomain (60, 61, 64).

GCN4 is a transcriptional activator of multiple genes encoding amino acid biosynthetic enzymes in *Saccharomyces cerevisiae*. Expression of *GCN4* is regulated at the translational level such that high levels of the protein are produced only in amino acid-deprived cells (reviewed in reference 28). GCN4 binds to

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DNA as a homodimer (33) through DNA-binding and dimerization (bZIP) domains located at the C terminus (17, 32). The results of deletion analysis suggested that its principal activation function resides in an acidic segment between residues 107 and 144 in the center of the protein (30, 32). We showed that the N-terminal 100 amino acids of GCN4 also contains a potent activation domain that confers high-level transcription when the central acidic activation domain (CAAD) is missing. Similarly, the CAAD is sufficient for high-level activation in the absence of the N-terminal activation domain (NTAD) (16).

The CAAD is critically dependent on two clusters of aromatic and bulky hydrophobic residues, (i) Met-107, Tyr-110, and Leu-113 and (ii) Trp-120, Leu-123, and Phe-124, interspersed among the acidic amino acids (16). NTAD function depends on a pair of Phe residues located near its C-terminal boundary, at positions 97 and 98. Point mutations in the critical hydrophobic residues between positions 107 and 124 in the CAAD impaired *GCN4* function only when combined with a deletion of the NTAD or with substitutions at F-97 and F-98. Likewise, point mutations at F-97 and F-98 substantially impaired *GCN4* function only when the CAAD was missing or contained mutations in the important residues between positions 107 and 124. Combining substitutions at all eight aromatic or hydrophobic residues in full-length GCN4 essentially eliminated activation of *HIS3* and reduced *HIS4* expression to 20% of the wild-type level (16). Thus, GCN4 appeared to contain two functionally redundant segments that can work independently to produce high-level activation, each of which contains hydrophobic and aromatic residues as critical constituents.

A deletion of the entire CAAD and the critical Phe residues at 97 and 98 in the NTAD did not completely abolish the activation of *HIS4* (16), suggesting that GCN4 contains important activation determinants N-terminal to position 97. We set out to map the boundaries of the NTAD more precisely by identifying all of the amino acids that are critically required for activation in the absence of the CAAD. To do so, we randomly mutagenized the NTAD in a construct lacking the CAAD and screened the mutant alleles for reduced activation of the *HIS3* promoter. The results of our analysis show that the NTAD of GCN4 contains three pairs of Phe residues and a leucine residue, in addition to F-97 and F-98, that are required for transcriptional activation in the absence of the CAAD. These results, together with our previous findings, indicate that GCN4 contains a minimum of seven segments bearing critical aromatic or bulky hydrophobic residues located throughout the N-terminal half of the protein. Analysis of a large number of *GCN4* alleles containing different subsets of the hydrophobic clusters suggests that these elements function independently to make redundant contributions to the transcriptional activation process.

MATERIALS AND METHODS

Construction of plasmids and yeast strains. All mutant constructs were derived from pCD35, containing the wild-type *GCN4* allele inserted into YCp50 (56), and its derivative plasmids pCD38 and pCD59, containing *Bgl*II site insertions between *GCN4* codons 17 and 18 (pCD38) or 84 and 85 (pCD59) (16). p1855, all mutant constructs in Table 1 (except for 2-10F, 3-06A, and 4-05H), and constructs pW4, pW48, pW20, pW36, pW46, pW32, pW23, pW4, pW7, pW47, pW42, pW10, pW38, and pW45 were produced by inserting PCR-amplified fragments spanning codons 18 to 100 between the *Bgl*II site at codons 17 and 18 and the *Xba*I site at codon 167 in pCD38 (16). The PCR-generated fragments were produced under conditions that introduce random substitutions at a frequency of ≈0.2% (9). Constructs 2-10F, 3-06A, and 4-05H were generated by inserting synthetic oligonucleotides spanning codons 85 to 100 between the *Bgl*II site at codons 84 and 85 and the *Xba*I site at codon 167 in pCD59 (16). These mutagenic oligonucleotides were synthesized by using precursors contaminated with the other bases at a level expected to yield random substitutions at a frequency of ≈5%. The subcloned fragments and the regions immediately flanking the *Bgl*II and *Xba*I sites were sequenced in their entirety to identify the mutations. The constructs described in Fig. 1, 2, and 6 and Table 2, containing site-directed mutations, were generated by PCR amplification with primers containing the desired mutations and by standard recombinant DNA techniques. A complete description of the primers, templates for PCR amplification, and plasmids into which amplified fragments were inserted is available on request. For all experiments except those in Fig. 7, *GCN4* constructs were introduced by transformation into strain H2384 (*MAT***a** *ura3-52 gcn4-103 leu2-3 leu2-112 trp1-63 ino1* [*HIS3-lacZ*, *LEU2*]), constructed previously (16). The *gcn4-103* allele contains a deletion of 0.645 kb between the *Kpn*I site in the *GCN4* coding region and the *Mlu*I site located 3′ of the gene, and the *HIS3-lacZ* fusion is integrated at *LEU2*.

Plasmid pKN1843i, used in constructing strain KNY27, was formed by inserting the *Sal*I-*Eco*RI fragment bearing *gcn4-1843* from pCD350 between the same sites in YIp5 (56). This strategy was also used to construct pKN2062i from p2062 and YIp5. Strain KNY15 (*MAT***a** *leu2-3 leu2-112 ura3-52 ino1 gcn4*Δ*-103*) was constructed by crossing ascospore clones derived from a cross between strains H2036 (16) and H755 (*MAT*α *leu2-3 leu2-112 ura3-52*). KNY27 was produced by transforming KNY15 to Ura$^+$ with plasmid pKN1843i DNA linearized by *Bam*HI digestion to direct integration to *GCN4*. Recombinants lacking plasmid sequences in which *gcn4-1843* replaced *gcn4-103* were selected on 5-fluoroorotic acid medium and identified by their leaky 3-aminotriazole (3-AT)-sensitive phenotype and the presence of the full-length *GCN4*, with the latter ascertained by PCR amplification of genomic DNA with primers from outside of the segment deleted in *gcn4-103*. KNY29 was constructed by transforming KNY15 to Leu$^+$ with *Bst*EII-digested pKN13, a nonreplicating plasmid containing a *HIS3-GUS* fusion (54a). KNY96 bearing *gcn4-2062* at the *GCN4* locus was derived from KNY29 by the same approach used to generate KNY27, except that pKN2062i DNA linearized by *Pac*I digestion was used to transform KNY29 to Ura$^+$.

Immunoblot analysis of GCN4 proteins expressed in *S. cerevisiae*. GCN4 protein levels in transformants of *S. cerevisiae* H2384 were measured in whole-cell extracts by immunoblot analysis, as described previously (16). Briefly, cultures were grown in SC medium (63) lacking uracil and histidine to an optical density at 546 nm of 1.0 and 3-AT was added to 40 mM. Cells were harvested 12 to 14 h later, and whole-cell extracts were prepared and analyzed for protein concentration by the Bradford method (7). Aliquots of total protein were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (8 to 16% polyacrylamide), blotted to nitrocellulose filters, and probed with GCN4-specific antiserum raised against the C-terminal 60 amino acids of the protein (16). Immune complexes were visualized with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (heavy plus light chains) (Bio-Rad).

Assays of β-galactosidase expressed from the *HIS3-lacZ* fusion in H2384. Transformants were grown for 2 days to saturation in SD medium (63) supplemented with 0.2 mM inositol, 2.0 mM leucine, 0.5 mM isoleucine, 0.5 mM valine, 0.4 mM tryptophan, and 0.25 mM arginine and diluted 1:50 into the same medium. For starvation conditions, 3-AT was added to 10 mM after 2 h of growth and cultures were harvested 6 h later. Whole-cell extracts were prepared, and β-galactosidase specific activities were determined as previously described (53). The values obtained for each construct are the means of replicate determinations on at least three independent transformants, with standard errors of 10% or less. The β-galactosidase activities measured for a large number of transformants of H2384 bearing vector YCp50 alone were averaged and equated with the GCN4-independent component of expression from this fusion. This value was subtracted from the activities measured in transformants bearing the *GCN4* constructs to yield the GCN4-dependent component of fusion enzyme expression for each construct. The GCN4-dependent expression conferred by each construct was expressed as the percentage of the GCN4-dependent expression measured for the wild-type allele on pCD35.

RESULTS

Random mutagenesis identifies multiple Phe residues as critical determinants of the NTAD. To identify residues located N-terminal to F-97 required for activation by GCN4, we mutagenized the NTAD in a *GCN4* construct lacking the CAAD and screened the mutant alleles for reduced *GCN4* function in vivo. In one strategy, we replaced the wild-type sequence from codons 18 to 167 with PCR-generated fragments containing codons 18 to 100 (and thus lacking the CAAD) synthesized under conditions that produce one or two substitutions per fragment. In a second approach, the sequence containing codons 85 to 166 was replaced with oligonucleotides containing codons 85 to 100 synthesized under conditions expected to produce one substitution in the 16 codons of the NTAD contained in each oligonucleotide. A construct lacking the CAAD and containing a *Bgl*II site between codons 17 and

18 that was used for inserting the PCR-generated fragments (p1855) served as the parental construct for these mutant alleles. This construct was phenotypically indistinguishable from one described previously (pCD162) which lacks the CAAD but is devoid of *Bgl*II insertions in the NTAD (16). Alleles with reduced function relative to the parental construct were identified by their 3-AT-sensitive phenotype in a *gcn4*Δ strain. Because 3-AT inhibits the *HIS3* product (a histidine biosynthetic enzyme), growth on 3-AT medium requires GCN4-mediated derepression of *HIS3* transcription. Plasmids were recovered from 3-AT-sensitive transformants and sequenced in the mutagenized region to identify the amino acid changes responsible for their Gcn4$^-$ phenotypes.

We isolated 33 plasmids from the PCR mutagenesis and 3 plasmids from the oligonucleotide mutagenesis that conferred a marked reduction in 3-AT resistance relative to p1855 when introduced into the *gcn4*Δ strain (Table 1). Most of these alleles contained two or more amino acid substitutions; however, it was noteworthy that the first 29 listed in Table 1 contained a mutation at one or more of the six Phe residues in the NTAD between positions 18 and 100: F-45, F-48, F-67, F-69, F-97, and F-98. The mutant phenotypes of two alleles containing a single serine substitution at F-97 (construct 24) or F-98 (construct 27) and that containing a deletion of F-98 (construct 29) were similar to those described previously for alleles containing an Ala substitution at position 97 or 98 (16). To determine whether changing the Phe residues at position 45, 48, 67, or 69 could account for the phenotypes of the other 23 alleles containing Phe substitutions, we used site-directed mutagenesis to make single- and double-Ala substitutions of the four Phe residues in the parental construct p1855. Each single-Ala substitution at position 45, 48, 67, or 69 led to a substantial reduction in *GCN4* function (Fig. 1B and C), comparable or greater in degree to that observed for the corresponding randomly selected mutant alleles in Table 1. In addition, the A-67/A-69 double mutation conferred a greater defect than did the corresponding single-Ala substitutions, whereas the A-45 single substitution produced the same severe Gcn4$^-$ phenotype shown by the A-45/A-48 double mutation (Fig. 1B and C). From these results, we concluded that the Gcn4$^-$ phenotypes of the first 29 alleles listed in Table 1 were derived, in large part, from substitution of Phe residues at positions 45, 48, 67, 69, 97, and 98. Consequently, we did not attempt to determine whether any substitutions of non-Phe residues in this group contributed to their mutant phenotypes.

We then asked whether the two Phe residues at positions 9 and 16, N-terminal to the region mutagenized randomly, are also required for NTAD function. Substitution of F-16 with Ala in the construct lacking the CAAD had little effect; however, the corresponding Ala substitution at F-9 reduced *GCN4* function roughly to the same extent as did single Ala substitutions at the other six Phe residues in the NTAD (Fig. 1A). The A-9/A-16 double substitution was more impaired than the A-9 single substitution, suggesting that F-16 also contributes to *GCN4* function; however, it appears to be less important than are the other seven Phe residues in the N-terminal domain.

The last seven alleles in Table 1 did not involve substitutions of Phe residues. Because construct 9.01 displayed only a minor defect and construct 60.01 contained five separate mutations (constructs 30 and 36), neither was analyzed further. In construct 4-05H, E-88 was changed to Lys and S-99 was changed to Ala (construct 33). We showed previously that an A-99 substitution alone has little effect on *GCN4* function (16), suggesting that the mutant phenotype of 4-05H resulted from the K-88 substitution. To verify this deduction, we used site-directed mutagenesis to introduce a single K-88 mutation and

TABLE 1. *GCN4* alleles lacking the CAAD with point mutations in the NTAD that confer reduced derepression of *HIS3* expression under histidine starvation conditions

Construct no.	Construct name[a]	*HIS3* expression (3-AT^r)[b]	Amino acid substitution(s)[c]
1	4.06	±	F45S
2	43.01	2+	G41R F45L
3	57.01	2+	F45Y D90E
4	53.01	2+	S24P F45I A80T
5	49.01	2+	F48I
6	36.01	1+	F48S
7	47.01	3+	F48L Q59R
8	52.01	3+	E27K F48I E53V
9	18.12	±	V40D F48I L84S
10	29.01	1+	F48L V94G E95N
11	58.01	3+	E27K V29A F48I
12	7.13	±	T33A F48S L65H
13	39.02	3+	K37I Q42K F67L A80T
14	11.03	−	T33I K37R E52G S63P F67S D68G
15	41.01	3+	F69L L84P D91G
16	6.01	2+	T25A M39V P55T F69S
17	34.02	±	V40A D66V F69S A80V
18	1.03	±	M39K F69Y D79N
19	17.06	−	P38S F69L P78S E88K
20	42.01	1+	E27V K37E I45T F69S T76A L84S
21	59.01	1+	K23R N28T M39V D46N D54G F69S A80G I86T
22	28.01	−	F67A F69A
23	37.02	3+	G41S F67L F69L D79G
24	27a.01	1+	F97S
25	38.02	1+	T25A F97S
26	40.01	2+	N28I F97S
27	14.02	±	F98S
28	2-10F	4+	F98V
29	3-06A	1+	F98Δ
30	9.01	4+	V94K
31	30.01	2+	V40A I86T
32	15.06	−	D46G T74K
33	4-05H	2+	E88K S99A
34	16.01	±	D46G D60G N64S
35	24.02	3+	T51P E53G D54V
36	60.01	2+	N28Y S34P Q42H D54V Q73R
37	p1855	5+	None (parental construct)

[a] All constructs are single-copy *URA3* plasmids bearing *GCN4* alleles that lack the CAAD and contain the indicated substitution mutations in the NTAD. Mutations affecting one of the six Phe residues present in the mutagenized portion of the NTAD (codons 18 to 100) are underlined. Constructs 2-10F, 3-06A, and 4-05H were obtained from oligonucleotide mutagenesis of codons 85 to 100. They contain the sequence AGA-TTC (a *Bgl*II site, encoding Arg-Ser) inserted between codons 84 and 85, lack codons 101 to 166 comprising the CAAD, and contain the sequence AGA-TCC (encoding Arg-Ser) at the deletion junction between codons 100 and 167. All the remaining constructs were obtained from PCR mutagenesis of codons 18 to 100. These, as well as the parental construct p1855, contain a *Bgl*II site inserted between codons 17 and 18, lack codons 101 to 167, and contain the sequence AGA-TCC-AGT (encoding Arg-Ser-Ser) at the deletion junction between codons 100 and 168.

[b] Constructs were introduced into the *gcn4*Δ *ura3-52* strain H2384, and Ura$^+$ transformants were replica plated to SD medium containing all 20 amino acids except histidine, 3-AT (30 mM), and excess leucine (40 mM) (29) and incubated at 30°C. Growth was scored after 3 days relative to the parental wild-type construct p1855 (assigned a score of 5+) and the empty vector (assigned a score of −). The *HIS3* product is inhibited by 3-AT, and the level of resistance to 3-AT (3-AT^r) is an accurate indicator of the GCN4-mediated derepression of *HIS3* transcription under conditions of histidine starvation (16).

[c] The mutations present in each allele are indicated by the amino acid present in wild-type *GCN4* followed by its location relative to the N terminus and the amino acid found in the mutant allele, using the single-letter code for amino acids.

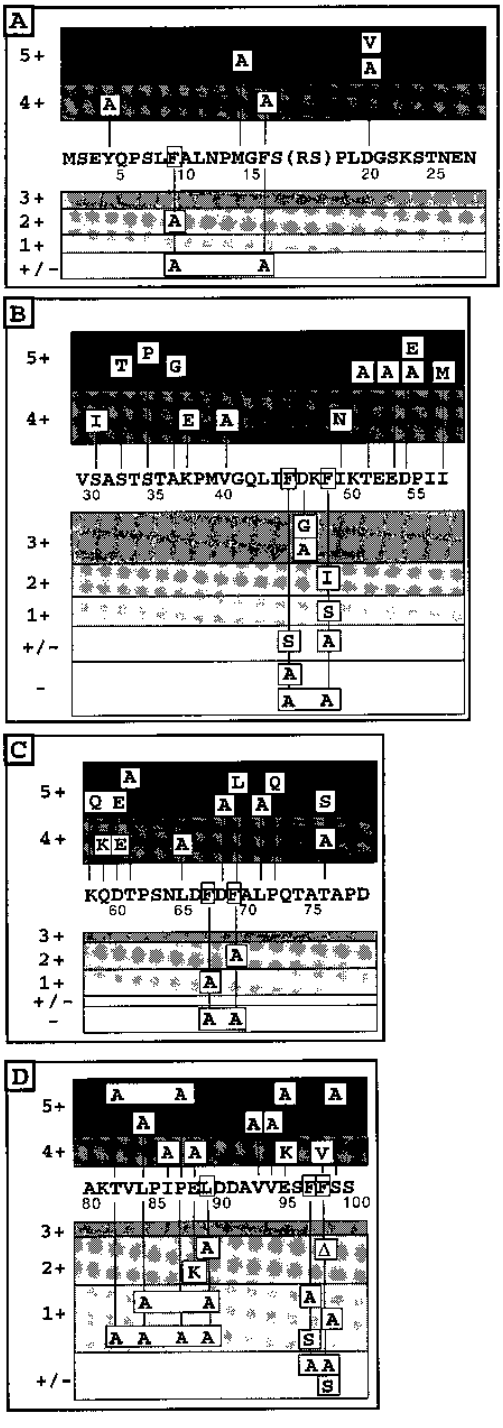

FIG. 1. Effects of point mutations in the NTAD in *GCN4* alleles lacking the CAAD on derepression of *HIS3* expression under histidine starvation conditions. The effects of point mutations in successive intervals of the NTAD (numbered relative to the N terminus of GCN4) in plasmid-borne constructs lacking the CAAD on resistance to 3-AT conferred in *gcn4*Δ strain H2384 are shown. Alleles containing single, double, or quadruple amino acid substitutions in the NTAD are depicted by boxes containing, respectively, one, two, or four letters indicating the substituted residues drawn directly above or below the corresponding wild-type residues. Resistance to 3-AT was measured as described in Table 1, footnote *b*. The height of the box showing the mutations above or below the wild-type sequence is indicative of the 3-AT^r phenotype of the allele, as shown on the left side of each panel. The following list contains the names of plasmids bearing the alleles depicted in the figure that were constructed in the present study. (A) p2001 (Y4A), p1884 (F9A), p2002 (M14A), p2003 (F16A), p1885 (F9A, F16A), p2004 (D20A), pW4 (D20V); (B) pW48 (S30I), pW20 (S32T), pW45 (S34P), pW36 (A36G), pW46 (K37E), p2005 (V40A), p1888 (F45A), construct 4.06 (F45S [Table 1]), p2006 (D46A), p2007 (D46G), p1889 (F48A), construct 49.01 (F48I [Table 1]), construct 36.01 (F48S [Table 1]), p1890 (F45A, F48A), pW32 (I49N), p2010 (T51A), p2011 (E53A), p2012 (D54A), pW23 (D54E), pW4 (I57M); (C) pW7 (K58Q), pW47 (Q59K), pW7 (D60E), p2013 (L65A), p1856 (F67A), p2014 (D68A), p1857 (F69A), pW45 (F69L), p1858 (F67A, F69A), p2015 (L71A), pW42 (P72Q), pW10 (T76S), pW38 (T76A); (D) p2016 (L84A), p2017 (I86A), p2019 (T82A, P87A), p1887 (E88K), p2018 (L89A), p2020 (L84A, L89A), p2021 (T82A, L84A, P87A, L89A), p1886 (E88A), p2022 (V94A), p2023 (E95A), pW38 (E95K), construct 27a.01 (F97S), construct 3-06A (F98Δ [Table 1]), construct 14.02 (F98S [Table 1]), p2027 (F98V [Table 1]). With one exception, the parental wild-type construct for the alleles just listed is p1855, which lacks residues 101 to 167 (encompassing the CAAD) and contains the sequence AGA-TCT (a *Bgl*II site) inserted between residues 17 and 18. The allele on p2027 differs from these others by two codons at the junctions of the CAAD deletion, as described in Table 1. Note that three of the plasmids listed above, pW4 (D20V, I57M), pW45 (S34P, F69L), and pW38 (T76A, E95K), each contain two mutations but are depicted as singly mutated in a given panel because they contain mutations widely separated in the protein. Plasmids bearing the V93A (pCD298), F97A (pCD294), F98A (pCD295), F97A and F98A (pCD297), or S99A (pCD296) substitution were described previously (16) and differ from those constructed here in lacking both the *Bgl*II site between residues 17 and 18 and residues 101 to 169 (instead of 101 to 167) and in containing the sequence AGA-TCT instead of AGA-TCC-AGT at the junction between residues 101 and 169.

found that it reduced NTAD function (Fig. 1D) to the same extent seen for the double mutation in construct 4-05H (Table 1, construct 33). An Ala substitution at position 88 had only a small effect, however, suggesting that Glu is not uniquely required at this position (Fig. 1D). Interestingly, E-88 falls within the only cluster of amino acids in the N-terminal domain (A-80–X–T–X–L–X–X–P–X–L-89–X–D-91) that appears to be conserved between GCN4 and *Neurospora crassa* cpc-1 (55). By making Ala substitutions of the central four conserved residues in this cluster, we found that L-89 is required for activation by the NTAD whereas T-82, L-84, and P-87 are not (Fig. 1D). From these findings, we suggest that substitution of nonconserved E-88 with Lys reduces activation primarily by interfering with the function of conserved L-89.

Two other members of the group of seven alleles in Table 1 which lack Phe substitutions had Gly replacing Asp at position 46 (Table 1, constructs 32 and 34). By site-directed mutagenesis, we found that the G-46 substitution alone could partially account for the defect in *GCN4* function associated with these two multiply mutant alleles. Presumably, substitutions at the other positions augmented the defect associated with altering D-46 to Gly. For constructs 31 and 35 in Table 1, we observed little or no effect of making single Ala substitutions at any of the positions altered in these two alleles (V-40, I-86, T-51, E-53, or D-54). Perhaps only particular amino acids (excluding Ala) at these positions reduce *GCN4* function, as suggested above for position 88. Alternatively, none of the positions may be critically required, such that several must be changed simultaneously for a detectable reduction in *GCN4* function.

In addition to analyzing the various positions altered in our pool of mutant alleles, we made site-directed mutations in the parental construct (p1855) to investigate the effects of changing other bulky hydrophobic or aromatic residues or of substituting acidic residues located near one of the seven critical Phe residues in the NTAD. We also sequenced 14 randomly mutagenized plasmids with a wild-type phenotype to identify noncritical positions in the NTAD. The results of these experiments (Fig. 1), combined with those summarized above, indicate that alterations of various aromatic or bulky hydrophobic residues near one of the seven critical residues in the NTAD (including Y-4, M-14, V-40, I-49, L-65, L-71, L-84, I-86, V-93, and V-94) did not significantly reduce *GCN4* function, demonstrating a requirement for hydrophobic or aromatic residues

TABLE 2. GCN4-mediated activation of *HIS3* expression with different amino acids at position 45 in the NTAD in alleles lacking a functional CAAD[a]

Amino acid at residue 45	With CAAD deleted		With CAAD mutations M107A, Y110A, L113A, W120A, L123A, F124A	
	Construct	3-AT^r	Construct	3-AT^r
Phe (WT)[b]	p1855	5+	p2060	5+
Trp	p2042	5+	p2185	4+
Leu	p2041	4+	p2183	4+
Tyr	p2040	3+	p2184	3+
Ile	p2039	2+	p2186	2+
Val	p2038	1+	p2182	±
Ser	p2043	±	ND[c]	ND
Cys	p2037	−	ND	ND
Gln	p2036	−	p2181	−
Ala	p1888	−	p2062	−
Thr	p2035	−	ND	ND
Gly	p2031	−	ND	ND
Pro	p2033	−	ND	ND
Glu	p2034	−	p2179	−
Lys	p2030	−	p2180	−
Stop	p2032	−	ND	ND

[a] All constructs are single-copy *URA3* plasmids bearing *GCN4* alleles. The amino acids encoded at codon 45 in the NTAD of these alleles are listed in the left-hand column. The constructs listed in column 2 lack codons 101 to 167 and contain the sequence AGA-TCC-AGT (encoding Arg-Ser-Ser) at the deletion junction between codons 100 and 168. The constructs listed in column 4 contain the listed Ala substitutions in the CAAD and AGA-TCT insertions between codons 17 and 18. Constructs were introduced into the *gcn4*Δ *ura3-52* strain H2384, and Ura$^+$ transformants were tested for 3-AT resistance (3-AT^r) as described in Table 1, footnote *b*.

[b] WT, wild type.

[c] ND, not determined.

only at particular sites in the NTAD. In addition, among the seven acidic residues in the NTAD that were altered (D-20, E-53, D-46, D-54, D-68, E-88, and E-95), only D-46 appeared to make a substantial contribution to *GCN4* function.

Further evidence indicating the functional primacy of bulky hydrophobic residues is that substitution of F-69 with Leu (Fig. 1C) or F-98 with Val (Fig. 1D) produced little (V-98) or no (L-69) reduction in *GCN4* function whereas an Ala substitution at each of these sites was very detrimental. On the other hand, substitution of F-45 with Ile partially impaired *GCN4* function, suggesting that not all bulky hydrophobic side chains are equally functional at position 45. To investigate more thoroughly the side chain requirements at position 45, we randomized this codon in the parental construct lacking the CAAD and screened the resulting pool of mutant alleles for different levels of *GCN4* function. Sequence analysis of the collection of mutant plasmids revealed a functional hierarchy for different residues at position 45 (Table 2). These results support the idea that activation by the NTAD is dependent on the presence of an aromatic or bulky hydrophobic residue at position 45. It is interesting that Trp and Leu are the best substitutes for Phe at position 45, considering that the only critical non-Phe residue uncovered in the NTAD is L-89 and that W-120 appears to be the most important residue among the six hydrophobic amino acids analyzed previously in the CAAD (16).

Substitutions at critical Phe residues must be combined with point mutations in the CAAD to reduce activation by full-length GCN4. It was important to establish that the newly identified Phe residues at positions 9, 45, 48, 67, and 69 and L-89 are important for the function of full-length GCN4. For constructs containing the wild-type CAAD and Ala substitutions at F-9 and F-16 (p2047 [Fig. 2A]), F-45 (p2048 [Fig. 2B]), F-67 and F-69 (p2052 [Fig. 2C]), L-89 (p2187 [Fig. 2D]), or F-97 and F-98 (p2067 [Fig. 2E]), we saw no reduction in the 3-AT^r phenotype relative to the parental wild-type construct (p2044 [Fig. 2A]). This was true even at an elevated 3-AT concentration (150 mM), fivefold greater than that used previously, which significantly retards the growth of wild-type *GCN4* strains. These mutations also had little or no effect on the *GCN4*-dependent expression of a *HIS3-lacZ* reporter assayed in the same strains following growth in the presence of 30 mM 3-AT (Fig. 2).

Combining Ala substitutions at W-120, L-123, and F-124 in the CAAD with Ala substitutions in the NTAD at positions 9 and 16 (p2054), 45 (p2055), 67 and 69 (p2059), or 89 (p2188) led to a modest reduction in 3-AT^r (at 150 mM) and *HIS3-lacZ* expression (Fig. 2A to D), with the greatest effects being observed for p2055 (Fig. 2B) and p2059 (Fig. 2C). By comparison, combining the substitutions at F-97 and F-98 with those at W-120, L-123, and F-124 (pCD355) had a significantly greater effect on 3-AT^r and *HIS3-lacZ* expression (Fig. 2E).

As expected, activation was severely impaired when each of the NTAD substitutions was combined with Ala substitutions at all six critical residues in the CAAD (M-107, Y-110, L-113, W-120, L-123, and F-124). The constructs in this group containing the F-9 and F-16 (p2061 [Fig. 2A]) and L-89 (p2189 [Fig. 2D]) substitutions conferred low-level activation, whereas those substituted at F-45 (p2062 [Fig. 2B]), F-67 and F-69 (p2066 [Fig. 2C]), and F-97 and F-98 (pCD350 [Fig. 2E]) were essentially nonfunctional. Results similar to those just described were obtained when the NTAD substitutions were combined with a deletion of the CAAD (Fig. 2, constructs p1885, p1888, p1858, p2018, and pCD297). As shown in Table 2, the relative effects of various substitutions at position 45 were also very similar whether they were combined with the six Ala substitutions in the CAAD or with a deletion of the entire CAAD. Together, these results indicate that the newly identified residues in the NTAD contribute to the activation function of this domain in full-length GCN4 protein, not just in the truncated constructs lacking the CAAD.

Effects of substituting critical residues in the NTAD on steady-state GCN4 protein levels. To examine possible effects on GCN4 protein levels of mutations in the newly identified residues in the NTAD, we carried out immunoblot analysis of GCN4 proteins in whole-cell extracts after growth in the presence of 3-AT to derepress GCN4 synthesis. In accordance with previous findings (16), the parental construct for mutants in which the CAAD was deleted (p1855) produced GCN4 at levels ca. fivefold higher than did the wild-type *GCN4* allele (Fig. 3A, lanes 7 and 8), presumably because of removal of protein instability determinants (16). Most of the highly functional alleles derived from p1855 produced proteins at levels indistinguishable from that of the parental construct (E95A, F16A, T51A, D68A, L71A, and F45L [Fig. 3]), whereas most of the reduced-function alleles produced GCN4 at somewhat lower levels. By analyzing serial dilutions of these extracts, we estimated that single Ala substitutions at positions 9, 45, 48, 67, and 69 plus the A-9/A-16 double substitution led to reductions of only ca. 30% whereas the double Ala substitutions at positions 45 and 48 and positions 67 and 69 reduced protein levels by 50 to 70% relative to that in the parental construct p1855 (data not shown).

Even though all of these mutant proteins are produced at higher levels than is full-length GCN4, the reductions in their steady-state levels relative to those in the parental construct p1855 could be partly responsible for their reduced ability to

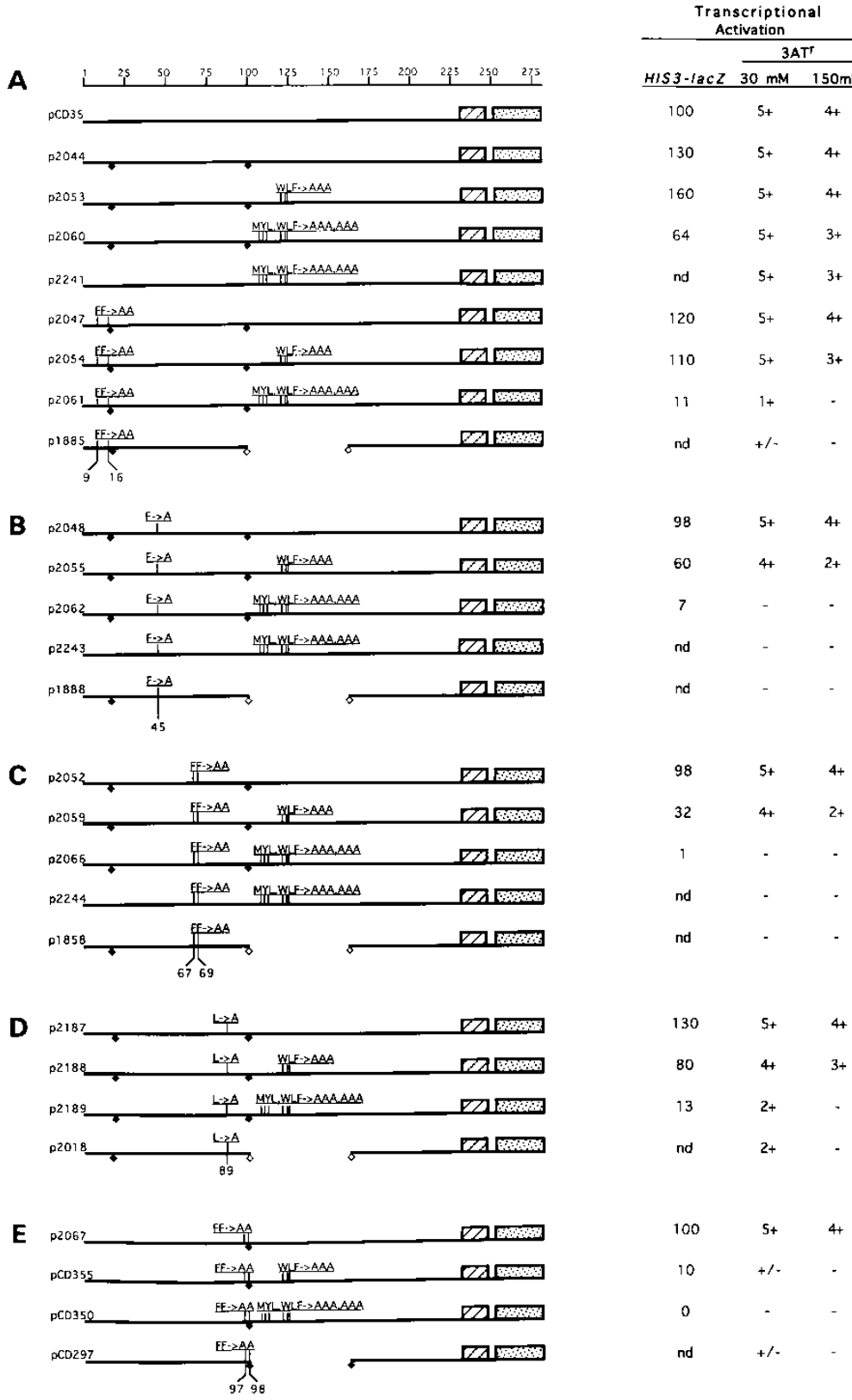

FIG. 2. Effects of point mutations in the NTAD in *GCN4* alleles bearing point mutations in the CAAD on GCN4-mediated derepression of *HIS3* expression. The linear amino acid sequence of GCN4 is shown for each construct as a solid line, with the DNA-binding and leucine zipper segments of the bZIP domain shown as hatched and speckled rectangles, respectively, at the C terminus. Amino acid positions relative to the N terminus are shown on the ruler line at the top. The locations of in-frame Arg-Ser codon insertions (AGA-TCT) that introduce *Bgl*II sites into the coding sequence between codons 17 and 18, 100 and 101, and 169 and 170 (for construct pCD297 only) are shown as solid diamonds below the lines depicting the GCN4 sequences. For constructs p1885, p1888, p1858, and p2018, the corresponding open diamonds signify the sequence AGA-TCC-AGT inserted at the junction between codons 100 and 168. The locations of Ala substitutions are shown for F-9 and F-16 (A), F-45 (B), F-67 and F-69 (C), L-89 (D), and F-97 and F-98 (E), as are the positions of Ala substitutions at M-107, Y-110, and L-113 (MYL → AAA) and at W-120, L-123, and F-124 (WLF → AAA). The names of the constructs bearing the depicted *GCN4* alleles are listed to the left of each schematic. All constructs were introduced into *gcn4*Δ *ura3-52* strain H2384, and the resulting transformants were tested for 3-AT resistance (3-AT^r) as described for Fig. 1, except that medium containing 150 mM 3-AT was used in addition to medium containing 30 mM 3-AT. The wild-type *GCN4* strain grows more slowly on medium containing 150 mM 3-AT (4+) than on medium containing 30 mM 3-AT (5+). Transcriptional activation of *HIS3* was quantitated by assaying the *HIS3-lacZ* fusion in H2384 under conditions of histidine starvation induced with 3-AT. Expression of *HIS3-lacZ* under histidine starvation conditions was 20 to 25% of wild-type levels when no *GCN4* allele was present in H2384 (data not shown). This GCN4-independent expression was subtracted from the observed results, and only the GCN4-dependent component of *HIS3* expression is presented, given as a percentage of that conferred by the wild-type construct (pCD35) in the same strain. nd, not determined.

activate transcription. One observation at odds with this interpretation is that the L65A (Fig. 3C) and F45W (Fig. 3D) mutant proteins were produced at 30 to 50% of the level of the parental construct but conferred wild-type (F45W) or nearly wild-type (L65A) activation. Thus, it appears that much of the excess protein produced by constructs lacking the CAAD is not required for high-level activation of *HIS3*. Consequently, the levels of protein produced by mutant alleles containing substitutions at the critical Phe residues are probably sufficient for wild-type activation. It is also noteworthy that Lys-45 confers much lower activation than does Trp, Tyr, Ile, or Val at this position, even though the abundance of the Lys-45-substituted protein is greater than or equal to those of the last four mutant proteins (Fig. 3D).

We also analyzed the levels of full-length GCN4 proteins bearing Ala substitutions in the critical residues of the NTAD. This experiment was complicated initially by the *Bgl*II insertions in the parental construct (p2044) from which the mutant alleles were derived. Although these insertions had no effect on *GCN4* function (Fig. 2), they conferred heterogeneity in electrophoretic mobility. In agreement with previous findings (27, 31), wild-type GCN4 expressed from pCD35 migrated more slowly than expected from its molecular weight of 31,920, running as a major band with an apparent M_r of 44,000 and a

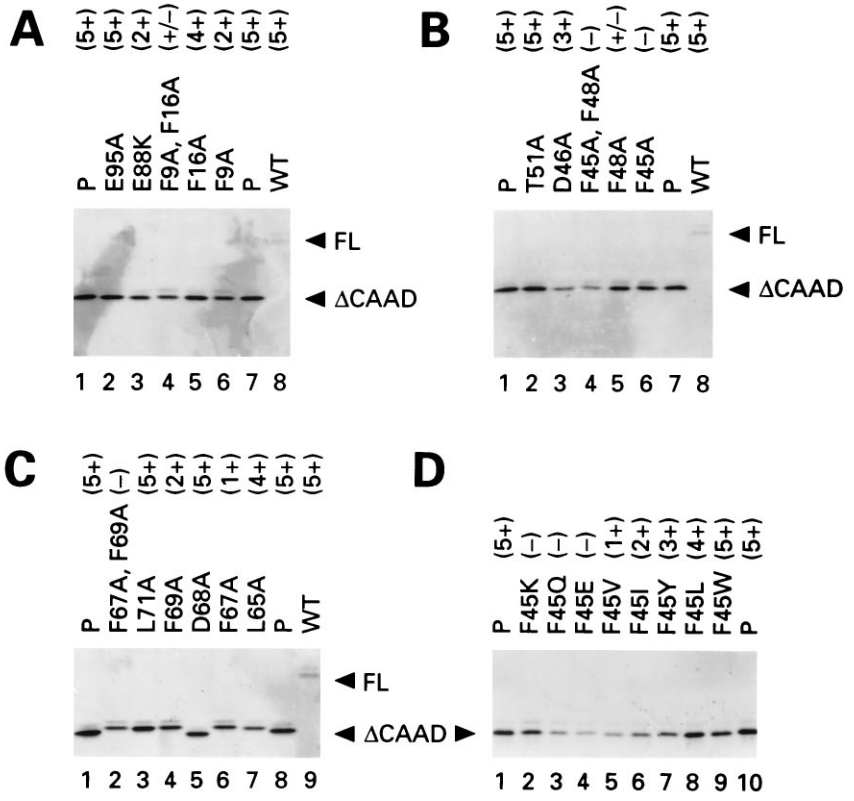

FIG. 3. Immunoblot analysis of mutant GCN4 proteins lacking the CAAD and containing NTAD point mutations following expression in yeast cells. Aliquots of whole-cell extracts containing equal amounts of protein from transformants of strain H2384 containing the wild-type allele on pCD35 (WT), the parental construct p1855 (P) lacking the CAAD, and mutant constructs derived from p1855 were separated by SDS-PAGE (8 to 16% polyacrylamide), electroblotted to nitrocellulose filters, and probed with GCN4-specific antiserum specific for the C terminus of the protein. Immune complexes were visualized with alkaline phosphatase-conjugated goat anti-rabbit serum. The amino acid substitutions and 3-AT^r phenotypes (−, ±, or 1+ to 5+) are indicated above the lanes for each construct. FL indicates the position of full-length GCN4 encoded by pCD35; ΔCAAD indicates the position of the truncated proteins lacking the CAAD encoded by p1855 (P) or one of the indicated mutant alleles.

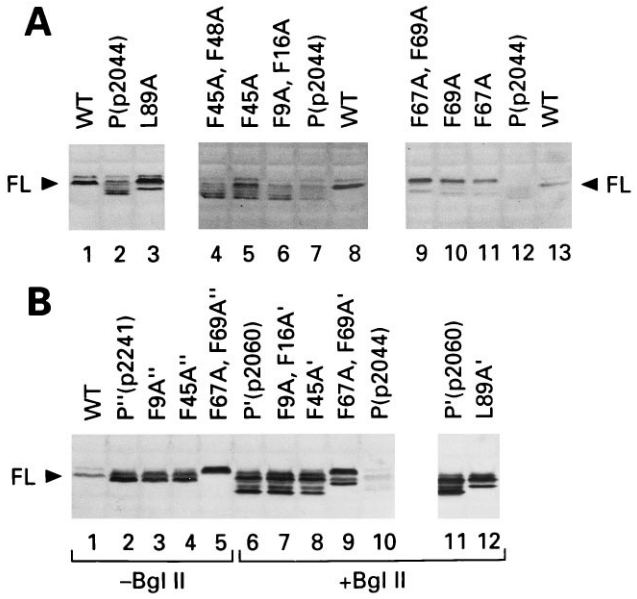

FIG. 4. Immunoblot analysis of full-length mutant GCN4 proteins bearing NTAD point mutations following expression in yeast cells. Aliquots of whole-cell extracts containing equal amounts of protein from transformants of strain H2384 containing wild-type or mutant *GCN4* alleles were separated by SDS-PAGE (8 to 16% polyacrylamide), electroblotted to nitrocellulose filters, and probed with GCN4-specific antiserum specific for the C terminus of the protein. (A) Analysis of extracts from transformants containing the wild-type allele pCD35 (WT), the parental construct p2044 (P) bearing *Bgl*II insertions between codons 17 and 18 and codons 100 and 101 (with wild-type CAAD sequences), and mutant derivatives of p2044 containing Ala substitutions at the indicated Phe residues (lanes 3, 4 to 6, and 9 to 11, respectively). (B) Analysis of extracts from transformants containing the wild-type allele pCD35 (WT), the parental construct p2044 (P) analyzed in panel A, the parental construct p2060 (P′) bearing *Bgl*II insertions between codons 17 and 18 and codons 100 and 101 and Ala substitutions at M-107, Y-110, L-113, W-120, L-123, and F-124 in the CAAD, mutants derived from parental construct P′ (lanes 7 to 9 and 12), the parental construct p2241 (P″) containing mutations in the six critical residues in the CAAD but lacking the *Bgl*II insertions, and mutants derived from p2241 (lanes 2 to 4). FL indicates the mobility of the major electrophoretic species observed for wild-type GCN4 protein.

minor band with an M_r of 47,000 (Fig. 4A, lane 1). The otherwise wild-type construct containing the two *Bgl*II insertions produced six electrophoretic species with M_r ranging from 36,000 to 47,000 (Fig. 4A, lane 2). The faster-migrating species could be degradation products, conformational isomers, or modified forms of the protein. Introducing Ala at position 9, 16, 45, 48, 67, 69, or 89 into the p2044 construct had little or no effect on levels of GCN4 protein (Fig. 4A), suggesting that the substitutions do not destabilize full-length GCN4. The Ala substitutions at L-89, F-67, and F-69 (Fig. 4A, lanes 3, 9, and 10) additionally altered the relative amounts of the different electrophoretic isoforms. The effects of these mutations in the full-length construct bearing Ala substitutions at the six critical positions in the CAAD and the *Bgl*II insertions in the NTAD (construct p2060 [Fig. 2B]) were also examined. The six Ala substitutions in the CAAD of construct p2060 led to a substantial increase in GCN4 protein levels, estimated from serial dilutions to be ≈fourfold (Fig. 4B, compare construct p2060 [P′] in lane 6 with p2044 [P] in lane 10). Subsequently introducing the Ala substitutions at the critical residues in the NTAD had variable effects on the distribution of electrophoretic species but little or no effect on total GCN4 protein levels (Fig. 4B, compare lanes 7 to 9 with lane 6 and lanes 11 and 12).

In an effort to simplify the analysis of full-length GCN4 proteins, we examined a group of NTAD substitutions in a construct containing the six Ala substitutions in the CAAD but lacking the *Bgl*II insertions in the NTAD. This construct (p2241 [Fig. 2A]) produced higher steady-state levels of GCN4 protein than did the wild-type allele; however, most of the protein migrated as a single electrophoretic species (Fig. 4B, compare lanes 1 and 2). Introducing the Ala substitutions at F-9, F-45, or F-67 and F-69 altered the mobility of the principal electrophoretic isoform but had little effect on the overall steady-state levels of the protein (Fig. 4B, lanes 2 to 5). Combining the results shown in Fig. 3 and 4, we conclude that the Ala substitutions at positions 9, 45, 48, 67, 69, and 89 impair transcriptional activation when combined with mutations in the CAAD by decreasing the activation function of GCN4 rather than reducing its steady-state abundance. At present, we cannot explain the idiosyncratic effects of point mutations in the activation domain on the electrophoretic mobility of GCN4.

We found that introducing the NTAD mutations into a *GCN4* allele containing substutitions at the six critical residues in the CAAD led to a dominant-negative phenotype. Strain H1486 containing wild-type chromosomal *GCN4* was transformed with plasmid p2060 (Fig. 2A) bearing the six Ala substitutions in the CAAD or with constructs additionally containing the mutations F9A and F16A, F45A, F67A and F69A, L89A, or F97A and F98A (all described in Fig. 2). The constructs containing mutations in both the NTAD and CAAD conferred 3-AT^s relative to the p2060 construct, which contains mutations in the CAAD alone (Fig. 5). If the mutations in the NTAD impaired *GCN4* function only by decreasing the abundance of GCN4 protein, they should not cause a dominant-negative phenotype relative to p2060. The fact that they do so strongly suggests that the mutant proteins are defective for activation and are produced at levels sufficient to interfere with the function of wild-type GCN4, forming defective het-

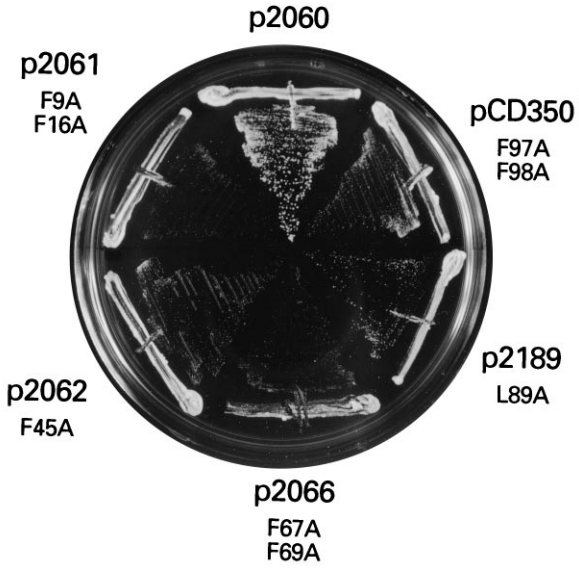

FIG. 5. Point mutations in the NTAD cause dominant-negative phenotypes. The indicated plasmid constructs bearing *GCN4* alleles were introduced into wild-type *GCN4* strain H1486, and transformants were streaked for single colonies on 3-AT medium. All six *GCN4* constructs contain the six Ala substitutions at M-107, Y-110, L-113, W-120, L-123, and F-124 in the CAAD and the additional substitution(s) listed under the plasmid name.

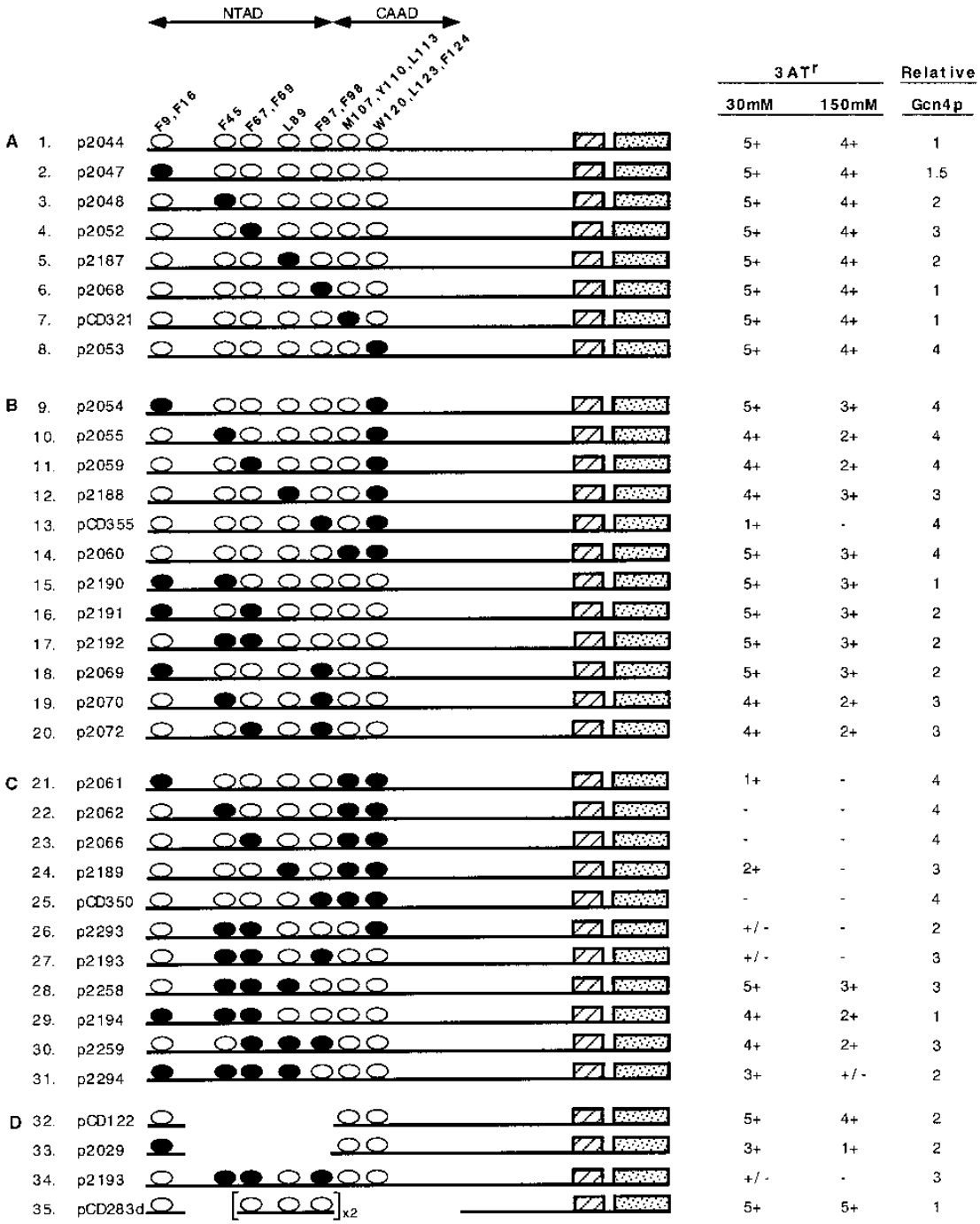

FIG. 6. Effects of different combinations of point mutations in the NTAD and CAAD on GCN4-mediated derepression of *HIS3* expression. The seven segments containing critical hydrophobic residues in the NTAD and CAAD are depicted schematically as ovals that are open when they contain the wild-type sequence and solid when they bear mutations at the residues listed above each oval. Constructs pCD122 (no. 32) and p2029 (no. 33) lack residues 18 to 100 and contain the sequence AGA-TCT at the junction between codons 17 and 101. Constructs were introduced into the *gcn4*Δ *ura3-52* strain H2384, and Ura$^+$ transformants were tested for 3-AT resistance (3-AT^r) on medium containing 30 or 150 mM 3-AT, as described in the legends to Fig. 1 and 2. Relative GCN4 protein levels were determined by immunoblot analysis as described in the legend to Fig. 4 and are listed in the right-hand column.

erodimers or mutant homodimers that compete with wild-type protein for promoter binding.

Evidence for functional redundancy among the hydrophobic clusters in GCN4. Combining Ala substitutions at F-97 and F-98, F-45, or F-67 and F-69 with a deletion of the CAAD (Fig. 1) or Ala substitutions at six residues in the CAAD (Fig. 2) led essentially to complete inactivation of *GCN4* function. These data are consistent with the idea that the Phe residues at positions 45, 67, 69, 97, and 98 are components of a single activation domain, the NTAD, and that substitutions at any of these residues are sufficient to inactivate the NTAD. However, combining substitutions at F-97 and F-98 with those at W-120,

L-123, and F-124 reduced *GCN4* function much more than did combining the last three mutations with substitutions in any other NTAD residues (Fig. 6B, compare constructs 9 to 13). This was the first of several observations indicating that F-97 and F-98 and the other critical hydrophobic residues in the NTAD are not functionally equivalent.

If the clusters containing the residues at positions 9 and 16, 45 and 48, 67 and 69, 89, and 97 and 98 all belonged to the same activation domain, then combining mutations at two of these clusters should reduce *GCN4* function less than combining each with mutations in the CAAD, because two activation domains would be impaired in the latter case. At odds with this prediction, we found that combining mutations at position 45 or positions 67 and 69 with mutations at either F-97 and F-98 or W-120, L-123, and F-124 was equally deleterious (Fig. 6B, compare constructs 10, 11, 19, and 20 for 150 mM 3-AT). Even more significant, the p2193 construct containing mutations in the 97 and 98, 45 and 48, and 67 and 69 clusters is essentially nonfunctional. This was the same result obtained for the two constructs combining mutations at positions 97 and 98 or at 45, 67, and 69 with substitutions at positions 120 to 124 in the CAAD (Fig. 6B and C, compare construct 27 with constructs 13 and 26). All three of these constructs produce similar steady-state levels of GCN4 protein (Fig. 6B and C). The large additive effect of combining mutations at F-97 and F-98 with those at F-45, F-67, and F-69 seems inconsistent with the idea that these residues belong to a single functional domain, the NTAD, that would be dispensable in the presence of the CAAD. Instead, it could be proposed that the two hydrophobic clusters at positions 45 and 48 and 67 and 69 and the one at positions 97 and 98 belong to two different domains, one of which must be present with the W-120, L-123, and F-124 cluster in the CAAD to achieve high-level activation by GCN4.

Combining the mutation at L-89 or F-9 with substitutions at positions 45, 67, and 69 led to significant reduction in *GCN4* function compared with that produced by the F45A, F67A, and F69A group of mutations alone (Fig. 6B and C, compare constructs 28, 29, and 31 with construct 17). As above, these functional differences cannot be attributed to alterations in GCN4 protein levels (Fig. 6B and C). It is noteworthy, however, that combining mutations in these N-terminal clusters generally impaired *GCN4* function much less than did combining them with mutations at F-97 and F-98 or W-120, L-123, and F-124 (Fig. 6C). This may indicate that F-9 and F-16, F-45, F-67 and F-69, and L-89 belong to one functional unit that differs from the NTAD as originally defined (16) only in lacking the F-97 and F-98 cluster and in being required for wild-type transcription even when the CAAD is intact (Fig. 6C, construct 31) (see Discussion).

The fact that combining mutations at positions 45, 48, 67, 69, 97, and 98 essentially eliminates *GCN4* function is ostensibly at odds with our previous finding (16) that a large deletion removing all of these critical residues left a high level of *GCN4* function intact (Fig. 6D, compare constructs 32 and 34). These previous results with construct pCD122 led to the conclusion that the CAAD is sufficient for wild-type activation in the absence of the NTAD (16). The deletion in pCD122 (Fig. 6D, construct 32) does not remove F-9 and F-16, however, raising the possibility that these residues near the N terminus of GCN4 can complement the functions of the M-107, Y-110, and L-113 and the W-120, L-123, and F-124 clusters when the two segments are juxtaposed by deletion of the intervening residues. In support of this idea, we found that making Ala substitutions at F-9 and F-16 in pCD122 significantly reduced *GCN4* function (Fig. 6D, construct 33) without decreasing the steady-state level of the GCN4 protein. The fact that the F-9 and F-16 cluster can complement the CAAD when these residues are abutting (as in pCD122) but not when they are separated by defective subdomains (as in p2193) could be explained by proposing that the defective subdomains in the p2193 construct interfere with activation by additional copies of the CAAD borne by the companion subunit in a GCN4 dimer or by other GCN4 molecules bound at the same promoter. This explanation is consistent with the fact that the CAAD functions more effectively when present alone in the p2029 construct than it does in the full-length protein bearing a combination of functional and nonfunctional N-terminal subdomains encoded by p2193 (Fig. 6D, compare constructs 33 and 34). The latter implies that multiple copies of the CAAD bound at the promoter can partially compensate for the absence of all N-terminal activation determinants, imparting measurable, albeit reduced, activation of *HIS3* transcription.

The idea that multiple copies of a portion of the activation domain can compensate for a loss of other activation determinants in GCN4 is supported by the final results presented in Fig. 6D for construct pCD283d. In this construct, both the CAAD and the segment spanning residues 18 to 54 (encompassing the critical residues F-45 and F-48) are deleted and residues 55 to 100 (containing the F-67 and F-69, L-89, and F-97 and F-98 clusters) are tandemly duplicated. Judging from other constructs lacking both the CAAD and the F-45 and F-48 clusters (Fig. 6C, construct 22; Fig. 1B, F45A and F48A alleles), the pCD283d allele should be nonfunctional; however, it actually conferred wild-type activation of *HIS3* even though it was expressed at lower steady-state levels than were these other nonfunctional constructs (Fig. 6D). This finding suggests that the presence of two tandem copies of residues 55 to 100 functionally compensates for the absence of both the CAAD and the hydrophobic cluster containing F-45 and F-48.

It could be imagined that different hydrophobic clusters are devoted to distinct functions in transcriptional activation and that only a subset of these functions must be carried out to achieve high-level transcription. If so, it might be possible to observe functional complementation between *gcn4* alleles bearing different subsets of the seven hydrophobic clusters that are individually incapable of significant activation. This could occur through formation of heterodimers, because only the bZIP region of GCN4 is required for dimerization in vitro (17, 33) and in vivo (16, 32); alternatively, two different homodimers bound to adjacent sites in the promoter could provide complementary functions needed for activation. If, instead, the hydrophobic clusters perform the same molecular function(s) and the critical determinants of activation are simply the number and potency of hydrophobic clusters present at the promoter, we would not expect to observe functional complementation between defective *gcn4* alleles. In the latter case, roughly the same number of functionally interchangeable hydrophobic clusters would be brought to the promoter when the two mutant proteins were coexpressed or when each was expressed individually from two copies of the respective allele.

To investigate these possibilities, we transformed strain KNY27 containing the defective *gcn4-1843* chromosomal allele with plasmids bearing three other impaired *gcn4* alleles (Fig. 7A, constructs 3 to 5), a second copy of *gcn4-1843* (construct 2), or vector alone (construct 1) and measured the 3-AT resistance of the resulting transformants. In a control experiment, the same five plasmids were analyzed in an isogenic *gcn4*Δ strain (Fig. 7A). Each of the plasmid-borne *gcn4* alleles exhibited strong 3-AT sensitivity in the *gcn4*Δ strain (Fig. 7A, constructs 2 to 5) similar to that shown for these constructs in Fig. 6. Introducing a plasmid-borne copy of *gcn4-1843* into the *gcn4-1843* strain did not increase the level of 3-AT resistance

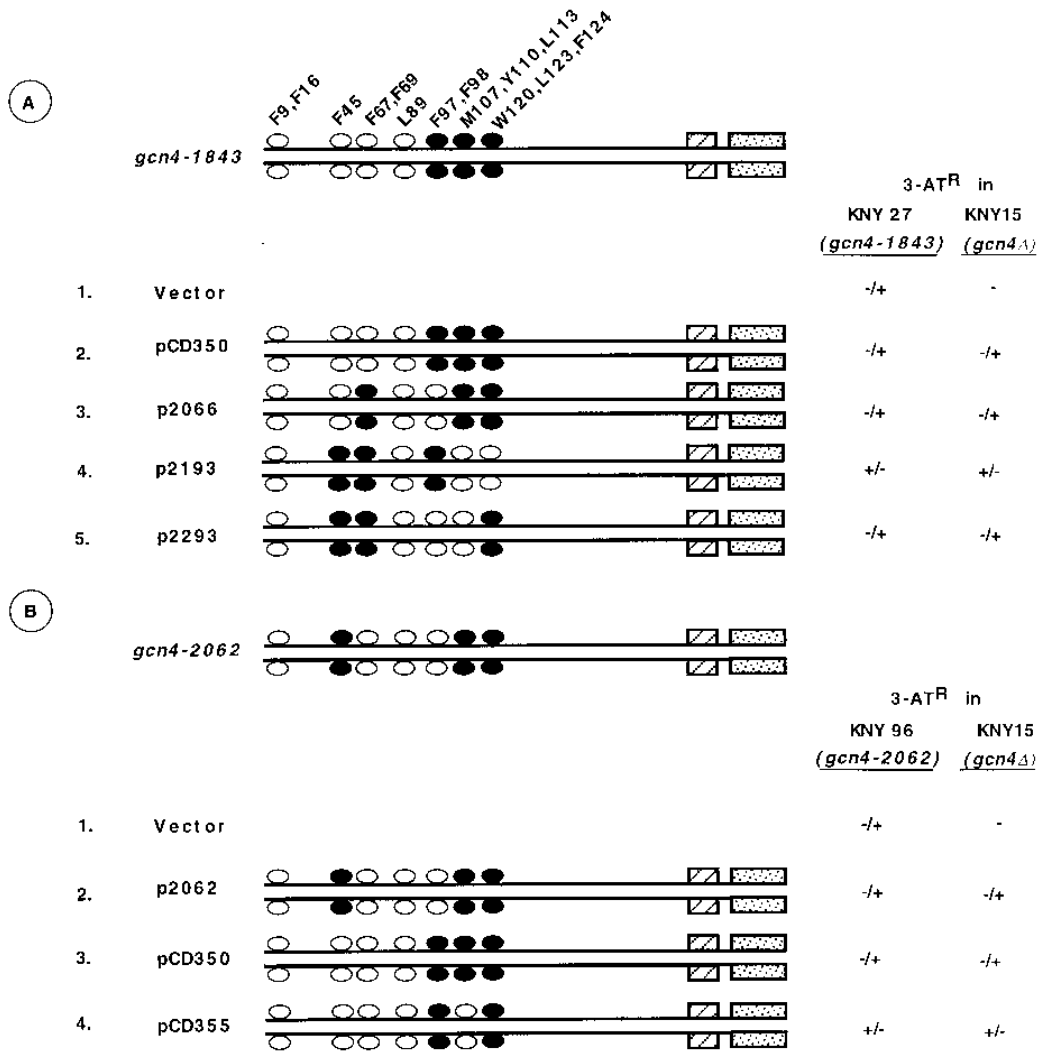

FIG. 7. Analysis of functional complementation between defective *gcn4* alleles containing different subsets of the seven hydrophobic clusters in the activation domain. The schematics depict the various GCN4 homodimers that would be produced from each allele under consideration, with the seven hydrophobic clusters labeled above the schematic shown at the top of panel A. As in Fig. 6, solid ovals designate Ala substitutions in the critical residues of the cluster. (A) Strain KNY27 containing *gcn4-1843* (the same allele carried on pCD350 [construct 2]) integrated at the chromosomal locus or strain KNY15 containing *gcn4*Δ was transformed with YCp50 (vector), pCD350, p2066, p2193, or p2293, and growth on medium containing 20 mM 3-AT was measured after 2 days, as described in Table 1, footnote *b*. (B) Isogenic strain KNY96 containing *gcn4-2062* (the same allele carried on p2062 [construct 2]) at the chromosomal locus and KNY15 were transformed with YCp50, p2062, pCD350, and pCD355 and analyzed for growth on medium containing 20 mM 3-AT. KNY15 transformed with wild-type *GCN4* on plasmid pCD35 conferred 3-AT resistance at a level scored as 5+.

(construct 2). In addition, each of the other plasmid-borne *gcn4* alleles conferred the same or nearly identical levels of 3-AT sensitivity whether coexpressed with *gcn4-1843* in KNY27 or expressed alone in the *gcn4*Δ strain. Similar results were obtained when three different plasmid-borne *gcn4* alleles were introduced into a strain bearing chromosomal *gcn4-2062* (Fig. 7B). The inability of constructs p2066 and p2193 to complement *gcn4-1843* (Fig. 7A) and of pCD350 and pCD355 to complement *gcn4-2062* (Fig. 7B) cannot be attributed to unequal protein levels, because these mutant alleles are expressed at nearly the same levels (Fig. 6); therefore, we can expect that 50% of the GCN4 proteins in strains coexpressing these alleles exist as heterodimers (17). These results suggest that heterodimers in which the two subunits contain different arrays of hydrophobic clusters do not activate transcription significantly better than do the corresponding defective homodimers.

DISCUSSION

GCN4 contains multiple hydrophobic clusters that make redundant contributions to transcriptional activation. In this study, we identified hydrophobic residues at five sites in the N-terminal 100 residues of GCN4 that are required for transcriptional activation in the absence of the CAAD. At three sites, two closely spaced Phe residues contribute to the activation function of that segment (F-45 and F-48, F-67 and F-69, and F-97 and F-98), while at the other two, a single Phe (position 9) or Leu (position 89) appears to be predominant. In our previous study (16), two clusters of hydrophobic residues, which were required for activation in the absence of residues 18 to 100 or when only F-97 and F-98 were substituted with alanines, were identified in the CAAD (M-107, Y-110, and L-113, and W-120, L-123, and F-124). Combining the results of both studies, we conclude that seven clusters of bulky hydro-

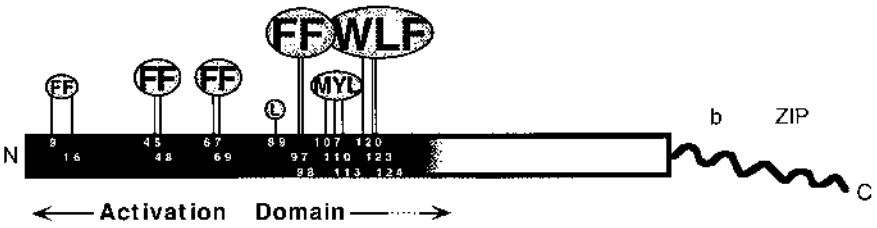

FIG. 8. GCN4 contains seven hydrophobic clusters that make redundant contributions to transcriptional activation. The GCN4 protein is depicted with the DNA-binding (b) and dimerization (ZIP) domains at the extreme C terminus shown in α-helical conformation as predicted from X-ray crystallography (17, 32) and the rest of the protein shown as a rectangular box. The critical hydrophobic residues identified here and in our previous study (16) are shown above the sequence at the appropriate positions in the protein in the single-letter code. The critical residues are grouped in seven hydrophobic clusters, symbolized by shaded ovals. The size and height of the lettering above the protein for each cluster is proportional to the reduction in *GCN4* function seen in response to Ala substitutions at that site, based on the following results shown in Fig. 6. Mutating the F-97 and F-98 and the W-120, L-123, and F-124 clusters together severely impaired activation (Fig. 6B, construct 13), whereas all other alleles lacking only two of the seven clusters retained relatively high-level *GCN4* function (Fig. 6B). Therefore, we designated the F-97 and F-98 and the W-120, L-123, and F-124 clusters to be the two most potent activation units in GCN4. A comparison of pCD355 (Fig. 6B), p2293 (Fig. 6C), and p2193 (Fig. 6C) suggested that the F-45 and F-48 and the F-67 and F-69 clusters are each about one-half as potent as the F-97 and F-98 or W-120, L-123, and F-124 cluster, and comparing constructs 21 to 25 in Fig. 6C suggested that the three clusters containing F-9 and F-16, L-89, and M-107, Y-110, and L-113 are roughly one-half as potent as either the F-45 and F-48 or the F-67 and F-69 cluster. In this analysis, we assumed that the Ala substitutions completely inactivated each cluster, which is not necessarily the case. The seven clusters may function independently of one another, making cumulative contributions to transcriptional activation in proportion to their strengths as activation units. Alternatively, there is evidence that the four clusters at positions 9 and 16, 45 and 48, 67 and 69, and 89 are interdependent components of a single N-terminal activation domain and that the two clusters at 107 to 113 and 120 to 124 belong to the CAAD as defined previously (16). It appears that the F-97 and F-98 cluster makes a strong contribution to activation that is independent of the other six hydrophobic clusters. The functions of these hydrophobic clusters are redundant; therefore, several must be inactivated simultaneously to destroy *GCN4* function. Mutating positions 97 and 98 and positions 120 to 124 is the only situation when *GCN4* function is greatly impaired by substitutions in only two of the seven clusters. At the other extreme, substituting all four clusters at positions 9 and 16, 45 and 48, 67 and 69, and 89 was the only situation when a substantial amount of *GCN4* function occurred with only three clusters left intact (see the text).

phobic residues located throughout the N-terminal 124 amino acids make important contributions to transcriptional activation by GCN4. Only two additional phenylalanines are present in GCN4, at positions 108 and 162. F-108 is located within the M-107, Y-110, and L-113 cluster and may contribute to the activation function of that segment. An Ala substitution of F-162 did not reduce activation in a construct lacking residues 18 to 100 (unpublished observations), suggesting that it is not a critical component of the activation domain. At F-45, no amino acid replacements that functioned better than Phe were found; however, Trp, Leu, and Tyr each conferred high-level activation (Table 2). In addition, Leu functioned indistinguishably from Phe at position 69 and Val functioned nearly as well as Phe at position 98 (Fig. 1). Thus, residues that function at critical sites in the GCN4 activation domain include aromatic and the most hydrophobic amino acids.

In the random mutagenesis of residues 18 to 100, we identified mutations that impaired activation at only two acidic residues. These residues (D-46 and E-88) are located immediately adjacent to critical hydrophobic residues, and Ala substitutions at these positions had modest effects on *GCN4* function (Fig. 1). Thus, it appears that individual acidic residues are not critically required for activation. On the other hand, the region encompassing L-89, F-97 and F-98, and the important residues at positions 107 to 113 and 120 to 124 (residues 88 to 147 in total) has a net negative charge of −16, and we showed previously that simultaneously substituting four acidic residues between positions 109 and 115 significantly impaired activation by the CAAD (16). In addition, F-45, F-67, F-69, L-89, Y-110, W-120, and F-124 are flanked by acidic residues (Fig. 1), a feature characteristic of critical hydrophobic residues in the activation domains of several acidic activators (45, 60). One interesting possibility is that the acidic residues in GCN4 increase the solubility of the hydrophobic clusters; alternatively, charge repulsion among the acidic residues may inhibit the formation of stable structures by the isolated activation domain, and these charges would be neutralized by interaction with basic residues on the surface of one or more transcription factors (61).

In our earlier study, we suggested that GCN4 contains two largely redundant activation domains, the NTAD and CAAD; this conclusion was based partly on the fact that residues 18 to 100 (containing most of the NTAD) or residues 101 to 169 (containing the CAAD) could be deleted without significantly reducing transcription of *HIS3* or *HIS4*. At odds with this previous conclusion, the strong additive effect of combining the mutations at F-97 and F-98 with those at F-45, F-67, and F-69 (p2193 [Fig. 6C]) suggests that these residues do not belong to a single activation domain that is functionally redundant with the CAAD. Instead, it could be proposed that the Phe residues at positions 45, 48, 67, and 69 and at positions 97 and 98 belong to two distinct domains, either of which must be present with the hydrophobic cluster at positions 120 to 124 for high-level activation to occur. The fact that combining substitutions in all four clusters at positions 9 and 16, 45 and 48, 67 and 69, and 89 left a significant amount of *GCN4* function intact (p2294 [Fig. 6C]) is consistent with the idea that these residues are interdependent components of a single large N-terminal activation domain. The significant reduction in *GCN4* function seen when all four of these clusters are mutated simultaneously (Fig. 6C, construct 31) indicates that this putative N-terminal domain would be required for wild-type activation even when the F-97 and F-98 cluster and the CAAD are both intact.

While the data in Fig. 6 are consistent with the idea that GCN4 contains three domains with overlapping functions in activation (the CAAD, F-97 and F-98, and the remainder of the NTAD), we can also account for most of our results by proposing that all seven hydrophobic clusters act independently to provide redundant means of activating transcription and that their functions are cumulative. The exact number of clusters required for wild-type activation would depend on the particular set present in each allele because of differences in their strengths as activation units. On the basis of the results shown in Fig. 6, we would designate the F-97 and F-98 and the W-120, L-123, and F-124 clusters as the two strongest activation units in GCN4, the F-45 and F-48 and the F-67 and F-69 clusters as being ca. one-half as potent, and the three clusters containing F-9 and F-16, L-89, and M-107, Y-110, and L-113 as being ca. one-fourth as potent as the two strongest clusters (Fig. 8 legend). Using these estimates of activation potency, we

can account for the functional ranking of most of the alleles in Fig. 6 by summing up the relative strengths of the clusters present in each allele. This model can explain why construct pCD355 (Fig. 6B), which contains mutations in the two clusters judged to be the strongest, is more impaired than construct p2294 (Fig. 6C), which contains mutations in four of the weaker clusters. It can also explain how the F-9 and F-16 cluster can functionally complement the CAAD in construct pCD122 (Fig. 6D), even when the rest of the hydrophobic clusters in the N-terminal region of the protein are deleted. Our estimates of the relative activation strengths of the hydrophobic clusters (Fig. 8) may not be precise if, for example, the hydrophobic clusters are not fully impaired by the Ala substitutions we made in them. Nevertheless, our ability to account for the results in Fig. 6 by assuming that the mutations reduce activation by an amount unaffected by the presence or absence of adjacent clusters supports the idea that these segments make independent contributions to activation by GCN4.

Possible functions for the hydrophobic clusters in the GCN4 activation domain. Mutational analysis of the VP16 protein suggests that it contains two subdomains, each containing phenylalanines as critical residues, that function additively in transcriptional activation (14, 59, 68). Physical studies indicate that these Phe residues exist in solvent-exposed unstructured protein segments (61) that adopt a more constrained conformation in the presence of purified TBP (62). These results suggest that the VP16 subdomains provide duplicate or complementary means of activating transcription by recruitment of TBP to the promoter. In addition to binding TBP (34), VP16 has been implicated in direct interactions with TFIIB (48), TFIIH (71), *Drosophila* $TAF_{II}40$ (23), the mediator complex of yeast holoenzyme (26), and the coactivators PC4 (18) and ADA2 (4). One way to explain these findings would be to propose that many different factors in the initiation complex contain similar surfaces that can interact with the same residues in a given activation domain. Alternatively, the structure of an activation domain may be induced upon interaction with a target protein, allowing it to interact with different factors or with different segments of the same factor (15, 44, 45). In either case, the same activation domain could function sequentially, exchanging one interaction for another as the preinitiation complex is assembled and activated (70).

We interpreted our results to indicate that GCN4 contains multiple autonomously functioning units that perform redundant functions in stimulating transcription. The functional segmentation of the GCN4 activation domain and the fact that most amino acids surrounding the critical hydrophobic residues contribute little to activation (Fig. 1) are both consistent with the idea that the activation domain has a relatively simple structure in which the critical hydrophobic residues are exposed on the surface to mediate hydrophobic interactions with one or more targets in the transcription machinery. One way to explain the functional redundancy in the activation domain would be to propose that each hydrophobic cluster (or group of adjacent clusters) is dedicated to making a unique contact with a particular transcription factor. No single interaction would be absolutely required for activation as long as a minimum number of factors were contacted in the course of assembling the initiation complex. A simpler explanation would be that the hydrophobic clusters are functionally interchangeable, each being capable of interacting with the same surface(s) on one or more transcription factors with an efficiency that varies from one cluster to the next. To use the latter model to explain the observed requirement for multiple hydrophobic clusters, we could propose that a high concentration of hydrophobic surfaces is required to attract and sequester initiation factors effectively. Alternatively, as in the first model, GCN4 might contact several surfaces on the same factor or interact with several different factors simultaneously to stimulate high-level transcription.

The idea that all seven hydrophobic clusters can mediate the same contact(s) with the transcription machinery is in accord with the results obtained for pCD283d (Fig. 6D, construct 35), in which a second copy of the segment containing F-67, F-69, L-89, F-97, and F-98 compensated for the absence of the F-45 and F-48, the M-107, Y-110, and L-113, and the W-120, L-123, and F-124 clusters. It is also more consistent with the findings in Fig. 7, where the presence of various pairs of defective *gcn4* alleles containing different arrays of hydrophobic clusters in the activation domain did not confer greater *HIS3* transcription than did two copies of the same allele. If various clusters carried out complementary functions in activation, we might find that *gcn4* alleles lacking one of the N-terminal clusters but retaining either the F-97 and F-98 or the M-107, Y-110, and L-113 and the W-120, L-123, and F-124 clusters would at least partially complement a *gcn4* allele containing only the N-terminal clusters intact. The failure to observe any significant complementation between such alleles is more easily explained if the hydrophobic clusters carry out the same function(s) in activation and the principal determinants of transcription efficiency are the number and potency of hydrophobic clusters.

It has been reported that the CAAD of GCN4 interacts with TBP, although this interaction is weak compared with that observed between GAL4 and TBP and was not shown to be dependent on specific residues or segments of the CAAD (50). Genetic analysis has implicated the ADA2, ADA3, and GCN5 proteins as important mediators of GCN4 transcriptional activation (5, 16, 20, 21, 58). ADA2 interacted with the GCN4 activation domain in cell extracts, although it was not determined whether this interaction was direct or required any of the hydrophobic residues involved in activation by GCN4 (4). We reported previously that two *GCN4* alleles containing only the N-terminal or centrally located hydrophobic clusters showed similar dependencies on *ADA2* for activation of the *HIS3* and *HIS4* promoters. This observation makes it unlikely that ADA2 discriminates between GCN4 proteins containing different sets of activation units (16). A significant component of GCN4 activation in vivo is independent of the ADA2-ADA3-GCN5 complex (16) and may involve recruitment of TFIIB (65). It is now important to examine these and other possible interactions between GCN4 and general factors and coactivators for their dependence on the seven hydrophobic clusters in GCN4 that mediate the activation of the *HIS3* promoter in vivo.

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