Association of distinct yeast Not2 functional domains with components of Gcn5 histone acetylase and Ccr4 transcriptional regulatory complexes

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The NOT genes were originally identified in a yeast genetic screen that selected mutations resulting in increased utilization of a non-consensus Tc TATA element of the HIS3 promoter. Here, we present evidence that the N-terminus of Not2 interacts with components of the Ada/Gcn5 histone acetyltransferase complex. Loss of this interaction either through abrogation of Not2 N-terminal function or deletion of ada2 or gcn5 results in derepression of the HIS3 Tc element. This suggests that association of Not2 with the Ada/Gcn5 histone acetyltransferase complex is involved in regulation of the HIS3 promoter. Association between the Not and CCR4 transcriptional regulatory complexes has also been observed recently. Our phenotypic analyses suggest that these CCR4-related Not2 functions are mediated by a functionally independent domain of Not2 that includes the highly conserved C-terminus. Chimeric proteins containing the yeast Not2 N-terminus fused to the human C-terminus function in yeast, suggesting that the Not2 C-terminus represents a distinct modular domain whose function is conserved between higher and lower eukaryotes.

Keywords: Ccr4/histone acetylase/Not2/transcriptional regulation

Introduction

The NOT genes were first identified in Saccharomyces cerevisiae through a genetic screen designed to identify mutations that enhanced the transcriptional activation of a crippled Gcn4 protein (Collart and Struhl, 1993, 1994), and were discovered to act as global negative transcriptional regulators of several genes, including HIS3. The HIS3 promoter contains two proximal elements, Tg and Tc, a conventional TATA sequence, is required for efficient transcriptional activation by Gcn4, and supports transcript initiation at nucleotide +13 (Struhl, 1986; Iyer and Struhl, 1995). The HIS3 Tc promoter element consists of a cluster of weak non-conventional TATA elements that function constitutively and respond only weakly to Gcn4. The Tc element supports transcription initiation at nucleotide +1 of HIS3 (Mahadevan and Struhl, 1990; Iyer and Struhl, 1995). NOT mutations result in increased utilization of the +1 HIS3 transcription start site, presumably through increased utilization of the Tc TATA element, thus increasing the ratio of +1 to +13 HIS3 transcripts. This Gcn4-independent augmentation of HIS3 expression results in the increased 3'-AT resistance phenotype for which the not mutants were originally selected.

The four described Not proteins (Not1, Not2, Not3 and Not4) exist in a large multiprotein complex (Collart and Struhl, 1994), but little is known about the manner in which these proteins regulate transcription. Action of the Not repressor complex is not limited to regulation of HIS3. not mutant yeast also display increased transcription of a diverse set of unrelated genes, including HIS3, STE4, HIS4, TBP and BIK1 (Collart and Struhl, 1993, 1994). Cloning and sequencing of NOT1 and NOT2 revealed that these genes had been identified previously as CDC39 and CDC36, respectively. In their original characterization, cdc39 and cdc36 mutants growth arrested in G1 at restrictive temperature (37°C), at which time they bore a morphological resemblance to pheromone-arrested cells (Reed, 1980; Breiter et al., 1983; Ferguson et al., 1986; deBarros Lopes et al., 1990). With the subsequent discovery that NOT genes mediate transcriptional repression, cdc36 and cdc39 growth arrest was attributed to the inability of these and the not mutants to suppress expression of pheromone response pathway genes (Collart and Struhl, 1993, 1994).

Like the Not proteins, the multiprotein Ccr4 transcriptional regulatory complex controls expression of a diverse but distinct variety of yeast genes. Physical association between the Not proteins and the Ccr4 complex was discovered recently (Liu et al., 1998). The importance of the physical interaction between Ccr4 and the Not proteins was supported by the observation that not mutants share many ccr4 phenotypes, including defects in cell wall integrity and growth arrest at 37°C. not and ccr4 mutations also resulted in loss of FUS1–lacZ negative regulation, as well as decreased inducibility of the ADH2 promoter under ethanol growth conditions. These observations suggested that the previously defined Not and Ccr4 complexes are physically and functionally associated, and that these proteins collaborate to regulate transcription positively or negatively (Liu et al., 1998).

Many transcriptional regulatory proteins interact directly with components of the basal transcription machinery (reviewed in Stargell and Struhl, 1996). However, modulation of chromatin structure through histone acetylation also plays an important role in transcriptional regulation of gene expression (recently reviewed in Struhl, 1998). The Gcn5 histone acetyltransferase enzyme (Brownell et al., 1996), along with its associated proteins Ada2 and Ada3, is required for transcriptional activation by some, but not all yeast activator proteins. Transcriptional activation by GCN4 and VP16, but not by HAP4, is dependent on the integrity of the Ada/Gcn5 complex (Berger et al.,...
transcriptional activation as a LexA fusion protein be responsible for Ccr4-associated conserved across widely divergent species and appears to Not2 functional domain that includes the C-terminus is complements a ability to activate transcription in yeast. [LexA–Not2 expressed as LexA fusion proteins and tested for their function. Proteins encoded by mutant alleles of not2 might play a role in transcriptional regulation by the Not proteins, we examined association between Not2 and the yeast Ada/Gcn5 histone acetyltransferase complex using a variety of functional, biochemical and genetic assays. Expression of Not2 as a LexA fusion protein (LexA–Not2) results in activation of a LexA reporter in vivo (Collart and Struhl, 1994). In this study, we demonstrate that transcriptional activation by LexA–Not2 is dependent upon the ADA2, ADA3 and GCN5 genes and that Not2 interacts with the Ada/Gcn5 complex. A not2 mutation that abrogates regulation of the HIS3 promoter and transcriptional activation as a LexA fusion protein disrupts this interaction. Agen5 and Aada2 strains also have an increased ratio of HIS3 +1 to +13 transcripts comparable with that seen in not mutants. Sequence analysis of existing not2 alleles and examination of their phenotypes suggests that the Not2 protein has two distinct domains. The N-terminal domain is unique to yeast, associates with the Ada2 component of the Ada/Gcn5 complex and regulates utilization of the HIS3 Tc promoter element. A second Not2 functional domain that includes the C-terminus is conserved across widely divergent species and appears to be responsible for Ccr4-associated NOT2 activities.

Results

The not2-4 mutation abrogates Not2 transcriptional activation as a LexA fusion protein

Expression of yeast Not2 as a LexA–Not2 fusion protein results in transcriptional activation of promoters containing LexA-binding sites (Collart and Struhl, 1994). Such effects often reflect a protein’s natural function, as is the case for both TATA box-binding protein (TBP) (Chatterjee and Struhl, 1995; Klages and Strubin, 1995; Xiao et al., 1995) and components of the RNA polymerase II holoenzyme (Barberis et al., 1995; Farrell et al., 1996; Liu et al., 1998), which activate due to interaction with components of the cellular transcription apparatus. Previously described not2 mutants had been identified as regulators of the yeast HIS3 promoter (Collart and Struhl, 1994). Abrogation of activation as LexA fusion proteins by these previously described mutant alleles would suggest that LexA–Not2 activation activity occurs as a result of a bona fide NOT2 function. Proteins encoded by mutant alleles of not2 were expressed as LexA fusion proteins and tested for their ability to activate transcription in yeast. [LexA–Not2 complements a not2 null mutation, suggesting that expression of the Not2 protein as a LexA fusion protein does not compromise its function discernibly (J.D.Benson and M.Benson, unpublished data).]

Previously described recessive not2 alleles were PCR amplified and subcloned. The protein-coding region sequence of each not2 allele was then determined. Sequencing revealed not2-1 to be a null allele containing a G→C mutation that eliminates the NOT2 initiator methionine codon. The not2-4 coding region contains two missense mutations within the N-terminus: a conservative substitution of arginine for lysine at codon 18, and substitution of arginine for glycine at codon 31. These findings are consistent with our observations that Not2 antisera did not detect any specific reactive Not2 protein in not2-1 cells, whereas a protein of identical size and abundance to wild-type Not2 protein was observed in the not2-4 strain (data not shown).

A DNA fragment containing the not2-4 coding sequence was cloned into pLex202 for expression as a LexA fusion protein. Activation by wild-type and LexA–Not2-4 fusion proteins was tested by expressing these proteins in PSY316 that contained the JK103 β-galactosidase reporter plasmid. As shown in Figure 1, the LexA–Not2-4 chimeric protein demonstrated negligible activation in comparison with LexA–Not2. Thus, the N-terminal mutations in the not2-4 missense mutant that resulted in loss of NOT2 function as a regulator of HIS3 also abolished its ability to activate transcription as a LexA fusion protein. Unlike the not2-1 null mutant, the not2-4 mutations abrogate specific functions of NOT2; the protein encoded by not2-4 retains some NOT2 functions (see below), suggesting that the lack of transcriptional activation by LexA–Not2-4 proteins is due to loss of physiologically relevant Not2 function.

The location of amino acid substitutions within the N-terminus of the Not2-4 protein suggested that this region might be required for activation in the LexA–Not2 assay. Indeed, amino acids 1–102 of Not2 were sufficient for activation when fused to LexA, whereas the same region of the Not2-4 protein did not stimulate transcription (Figure 1). Comparable expression of all LexA fusion proteins was confirmed by Western blot using anti-LexA polyclonal antisera (data not shown).

Activation by LexA–Not2 depends upon components of the histone acetyltransferase complex

The allele specificity of LexA–Not2 transcriptional activation suggested that this property could possibly be utilized to determine other activities associated with Not2 N-terminal function. That is, other yeast genes involved in normal NOT2 function might be required for activation in the LexA–Not2 assay. In consideration of the possibility that Not proteins might regulate transcription by affecting
chromatin structure (possibly through modulation of histone acetylation), we examined whether components of the Ada2/Ada3/Gcn5 histone acetyltransferase complex might be associated with Not2 function. A LexA–Not2 expression plasmid was transformed with the JK103 reporter plasmid into PSY316-derived strains with ada2, ada3 or gcn5 deletions (Figure 2). Activation by LexA–Not2 was reduced ~10-fold in cells lacking ADA2, and was negligible in yeast lacking either ADA3 or GCN5. Importantly, dependence of this activation on ADA2, ADA3 and GCN5 is not a universal feature of activated transcription, since almost no reduction of LexA–Hap4-mediated transactivation is observed in strains with deleted ada2, ada3 or gcn5 (Berger et al., 1992; Wang et al., 1995; our data not shown).

Physical interaction of yeast Not2 and Ada2

ADA2, ADA3- or GCN5-dependent transcriptional activation by LexA–Not2 and LexA–Not-N(aa1–102) suggested that interaction of the Not2 N-terminus with these gene products might be responsible for activation by LexA–Not2 and LexA–Not2-N. The dependence of transcriptional activation by LexA–Ada2 and LexA–Ada3 fusion proteins on ADA2, ADA3 and GCN5 constituted the initial evidence of a physical relationship between these components of the Ada/Gcn5 histone acetyltransferase complex (Marcus et al., 1994; Silverman et al., 1994; Horiuchi et al., 1995). Moreover, the loss of activation by LexA–Not2-4 fusion proteins predicted that if interaction between the Not2 and Ada/Gcn5 did account for LexA–Not2 activation, this interaction might be disrupted in the not2-4 mutant.

Extracts from yeast expressing hemagglutinin (HA) epitope-tagged wild-type Not2 (HA-Not2) or untagged Not2 were incubated with GST or GST–Ada2. Proteins that bound GST–Ada2 were analyzed by SDS–PAGE, followed by Western blotting and detection of HA-Not2 using the anti-hemagglutinin 12CA5 monoclonal antibody. As shown in Figure 3A, HA antibody specifically recognized a protein of the appropriate size in yeast expressing HA-Not2 (lanes 1 and 5), but not in yeast expressing untagged Not2 (lanes 2, 4 and 6). Binding of HA-Not2 to GST–Ada2 was detected (lane 5), whereas no binding of HA-Not2 to GST alone was observed (lane 3).

Tagged Not2-4 protein (HA-Not2-4) was also expressed and tested for interaction with GST–Ada2 (Figure 3B). HA-Not2 and HA-Not2-4 were equally expressed (lanes 2 and 3) but, in contrast to HA-Not2, HA-Not2-4 did not bind GST–Ada2 (lane 9). Thus, the not2-4 mutation abrogated interaction of Not2 with Ada2, which correlates with the inability of this allele to activate transcription as a LexA fusion protein. Interaction between Ada2 and Not2 was not detected between these in vitro translated proteins (data not shown), suggesting that this interaction is indirect, and may depend upon one or more factors or activities present in yeast extracts.

HIS3 promoter utilization in Δada2 and Δgcn5 strains recapitulates the not phenotype

Loss of interaction with the Ada/Gcn5 complex by not2-4 suggested that loss of Ada2 or Gcn5 would result in altered regulation of the HIS3 promoter that resembled the pattern of HIS3 +1/+13 transcription start site utilization found in not2 mutants. ada2 or gcn5 was knocked out in KY803, the gcn4 yeast strain originally used to identify and characterize the known NOT genes. The ratio of +1 to +13 HIS3 transcripts in these Δada2 or Δgcn5 strains was determined by nuclease protection analysis. These results are shown in Figure 4A. Lane 1 shows the ratio of +1 to +13 (+1/+13) transcripts in wild-type yeast. This ratio increased ~1.5-fold in Δgcn5 (lanes 2 and 3) and Δada2 (lanes 4 and 5) strains. This is comparable with the +1/+13 ratio observed in the not2-4 strain (lane 6; Collart and Struhl, 1994). Combination of the not2-4 allele with either Δgcn5 (lane 7) or Δada2 (lanes 8 and 9) did not significantly augment the +1 phenotype associated with each individual mutation, suggesting that
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Fig. 4. (A) HIS3 transcription patterns in Δgcn5 and Δada2 strains derived from either KY803 or KY803not2-4. RNA isolated from the indicated strains was subjected to quantitative S1 analysis. The positions of the +1 and +13 HIS3 transcripts are indicated. DED1, a polII transcript unaffected by not mutations, and tRNA(w) polIII transcripts were included as internal controls. (B) Comparison of Δspt8 (lane 4) effects on +1/+13 HIS3 ratios with those of Δada2 and Δgcn5 (lanes 2 and 3, respectively).

these genes reside within a common transcriptional regulatory pathway. The phenotypic similarity between Δada2, Δgcn5 and not2-4 strains provides independent evidence that these proteins act together to mediate GCN4-independent transcriptional regulation of the HIS3 gene.

Previous analysis has detected two separable complexes in yeast that contain histone acetylase activity, both of which also contained Ada2 and Ada3. The larger complex (termed ‘SAGA’ for Spt, Ada, Gcn5, acetylation) also contains Spt3, Spt7, Spt8 and Spt20/Ada5 (Grant et al., 1997). Not2 was not detected in the SAGA complex (P.Grant, unpublished results). In order to discern further whether components of the SAGA complex might be involved in regulation mediated by the Not2 N-terminus, we also examined HIS3 +1/+13 ratios in a KY803-derived Δspt8 strain. As shown in Figure 4B, Δspt8 had no effect on the +1/+13 HIS3 transcript ratio (compare lanes 1 and 4). Moreover, whereas ADA2 and GCN5 are required for activation by LexA–Not2, this activity does not depend on SPT8 (data not shown). Cumulatively, these results clearly distinguish the regulatory activities associated with Not2 from those associated with the SAGA complex.

CCR4-associated functions of NOT2 require a functional Not2 C-terminus

In previous analyses of not2-1 and not2-4 mutant phenotypes, both mutations resulted in increased +1/+13 transcript ratios and 3-AT resistance in the presence of a crippled Gcn4 protein. However, only the not2-1 strain demonstrated growth arrest at 37°C (Collart and Struhl, 1994). As described above, not2-1 is a null allele, whereas the mutant protein produced by not2-4 is expressed and contains two amino acid substitutions within the N-terminus. Thus, HIS3 regulation is disrupted in the not2-4 mutant while NOT2 activities associated with cell growth at restrictive temperature are retained. Both of these functions are absent in not2-1 mutants.

not2-1 yeast phenotypically resemble strains with mutations in components of the Ccr4 complex (Liu et al., 1998). However, this study did not associate specific domains of not2 with the ccr4 phenotype. In order to assess the relationship between the Not2 N-terminal domain and the ccr4 phenotype of not2-1 mutants, we compared the ccr4 phenotypes of not2-1 and not2-4 yeast. Growth of KY803 (NOT2), MY16 (not2-1) and MY22 (not2-4) under various conditions is shown in Figure 5. The ability of these strains to grow on YPD plates (Figure 5A) was
compared with growth on YPD plates containing 8 mM caffeine (Figure 5B), 0.04% (w/v) SDS (Figure 5C) and 750 mM MgCl₂ (Figure 5D), and on yeast/peptone plates containing ethanol (Figure 5E). Not2-1 failed to grow at 37°C, and not2-4 growth was unaffected by temperature (data not shown; Collart and Struhl, 1994). In these experiments, the not2-4 strain displayed none of the phenotypic characteristics of not2-1 or ccr4 strains, again suggesting that certain Not2 functions are retained in the not2-4 mutant and that these functions are separable from those relating to CCR4 function. Whereas not mutations result in resistance to 20 mM 3-AT (Collart and Struhl, 1993, 1994), deletion of CCR4 does not confer this phenotype (Liu et al., 1998), and deletion of the CCR4-associated CAF1 gene results in only very weak 3-AT resistance. These results are consistent with the conclusion that CCR4-associated NOT functions are not associated with HIS3 regulation, but that these functions are mediated by a functionally distinct domain within the Not2 C-terminus.

Both ccr4 and not2-1 mutants are impaired for maximal induction of ADH2 under non-fermentative growth conditions, as shown by their inability to grow on YEP plates (Liu et al., 1998). In contrast, the not2-4 strain could grow on YEP plates as well as the wild-type (Figure 5E), suggesting that this induction can occur in the not2-4 mutant. We also examined the effects of not2-4, Δada2 and Δspt8 mutations on ADH2 transcript levels under glucose growth conditions. ADH2 transcript levels were increased in both not2-4 and Δada2 strains grown in glucose medium (Figure 6, lanes 2 and 3), whereas Δspt8 had little or no effect on ADH2 transcript levels. Thus, in addition to the role of full-length Not2 in induction of ADH2 under non-fermentative conditions, the N-terminus of Not2 may also participate in repression of ADH2 under fermentative growth conditions.

**The Not2 C-terminus is an evolutionarily conserved and functionally discrete domain**

Analysis of not2-4 and phenotypic comparison with not2-1 suggests that the N- and C-terminal domains of yeast Not2 mediate distinguishable functions. Additional evidence for separate domains can be found by examining proteins from other species which bear homology to yeast Not2. A human cDNA has been cloned and sequenced that encodes a protein with highly significant homology to the yeast Not2 C-terminus (68% similarity and 45% identity; Figure 7A). This 534-amino-acid human Not2-like protein is much larger than yeast Not2, and has no appreciable resemblance to the yeast Not2 protein upstream of the 84-amino-acid C-terminal homologous region. A similar protein has also been identified in Drosophila as suppressor of position-effect variegation (Frolov et al., 1998). This Drosophila Not2-like protein contains the conserved C-terminal domain also present in the yeast and human proteins, whereas the N-terminus of the Drosophila protein bears similarity to the human but not the yeast protein. The concurrent divergence of the human and Drosophila N-termini from the yeast sequence, along with conservation of the C-terminal domains of these proteins, suggests that this C-terminal domain may be a discrete and evolutionarily conserved functional unit.

In order to determine the functional conservation of this human C-terminal domain, the C-terminal domain of the human cDNA was substituted for the corresponding region of yNot2. The resulting yeast–human chimeric

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**Fig. 6.** Effects of mutations associated with the Not2 N-terminus on ADH2 transcript levels. ADH2 transcripts from yeast grown in glucose were characterized by quantitative S1 analysis, with tRNA(w) as an internal control. Ratios of ADH2 transcripts in not2-4, Δada2 and Δgen5 mutant strains (lanes 2, 3 and 4, respectively), relative to that of the parental KY803 strain (lane 1), were quantitated by phosphorimager analysis and normalized for loading relative to tRNA.

**Fig. 7.** (A) Schematic diagram and alignment of the conserved Not2 C-terminal domain. Residues 107–191 of yNot2 are highly homologous to residues 441–534 encoded by a human cDNA. (-) denotes identical or conserved amino acid residues. (B) Functional conservation of the Not2 C-terminal domain. Chimeric proteins fusing amino acids 1–106 of yNot2 to amino acids 441–534 of hNot2 were expressed in yeast and tested for their ability to complement the not2-1 null mutation, by assaying growth on YPD plates at 38°C.
Not2 protein was tested for its ability to complement the not2-1 null mutant. The yeast–human chimera in these experiments encoded the first 102 amino acids of yNot2 fused to codons 437–534 of the human Not2 cDNA. The yNot2–yNot2 control in these experiments recombinated the natural sequences found within the native yNot2 protein, but incorporated the few junctional amino acid alterations that were necessary for creation of the yeast–human chimeric Not2 protein. Expression of the Not2 proteins in these experiments utilized a single-copy yeast vector (RS316) containing the endogenous NOT2 promoter and terminator sequences to ensure expression comparable with that of endogenous Not2. Equivalent expression was confirmed by Western blot using anti-Not2 polyclonal antiserum (data not shown).

As shown in Figure 7B, expression of either yNot2–yNot2 or yNot2–hNot2 (yeast–human Not2) proteins complemented the not2-1 growth phenotype. Neither the yeast Not2 N-terminus (amino acids 1–102), the yeast Not2 C-terminus nor the C-terminal domain of the human Not2 cDNA complemented not2-1 when expressed under control of the ADH promoter (Figure 7B; J.Benson and M.Benson, unpublished data). Taken together, these results suggest that the Not2 C-terminus is required for normal Not2 activity in yeast, and that this function is conserved within the analogous region of Not2-like coding sequences found in higher eukaryotes.

**Discussion**

The yeast Not proteins originally were identified as components of a global transcriptional repressor complex (Collart and Struhl, 1994), and later as components of the CCR4 transcriptional regulatory complex (Liu et al., 1998). The original screen for not mutants (Collart and Struhl, 1994) yielded not2 mutant alleles with two distinguishable phenotypes. not2-4 was characterized as a recessive 3-A T-resistant mutant that displayed increased utilization of the +1 HIS3 transcription start site, whereas the recessive not2-1 null mutant was both 3-A T-resistant and growth defective. (not2-1 strains grow poorly at 30°C and not at all at 37°C.) Not2 also activated transcription as a LexA fusion protein (Collart and Struhl, 1994). In the experiments presented here, this activation capacity of LexA–Not2 was utilized to define further Not2 functions that mediate transcriptional regulation. A LexA fusion protein expressing an independently derived allele of Not2 (LexA–Not2-4) did not activate the LexA reporter, strongly suggesting that activation by LexA–Not2 depends on a normal function of the Not2 N-terminus, and is not the result of spurious activation activity such as that observed for acidic peptides derived from random *Escherichia coli* sequences (Ma and Ptashne, 1987). This LexA–Not2 activation is dependent on ADA2, ADA3 and GCN5. We have also demonstrated binding of Not2 to Ada2. This function is also abrogated by the not2-4 mutation. not2, ada2, ada3 and gcn5 mutations have a common effect on regulation of HIS3, resulting in increased utilization of the +1 start site. This phenotype provides additional evidence of meaningful association between these proteins. Thus, in addition to their role in activator-mediated induction of transcription, the Gcn5 histone acetylase and its associated proteins may also participate in Gcn4-independent regulation of the HIS3 promoter. ADA2, ADA3 and GCN5 appear to collaborate with NOT2 in regulation of the HIS3 gene, but other functions of these genes appear to be independent of each other. For example, whereas ada2 or gcn5 mutant strains tolerate overexpression of Gal4-VP16 (Berger et al., 1992), not2 mutants do not (M.Benson, unpublished data). Also, Δada2, Δada3 and Δgcn5 strains grow more slowly than wild-type, but do not display the temperature-sensitive growth arrest phenotype associated with not2-1 (Collart and Struhl, 1994).

Not2 and other members of the Not complex were identified recently as components of the Ccr4 transcriptional regulatory complex (Liu et al., 1998). ccr4 mutations or mutations in genes associated with ccr4 (i.e. caf1, dbf2) result in cell wall integrity defects and temperature-sensitive growth. not2-1 yeast, as well as strains bearing mutations in several other NOT genes, share these phenotypes (Liu et al., 1998). However, ccr4 yeast do not have increased 3-A T resistance (Liu et al., 1998), the hallmark of the known not mutants (Collart and Struhl, 1993, 1994; Oberholzer and Collart, 1998). This characteristic distinguishes CCR4 from NOT functions, and suggests that although components of these respective complexes may collaborate to perform certain regulatory functions, they also have distinct functions. not2-4 and not2-1 are both 3-A T resistant, but only not2-1 has a slow growth phenotype associated with ccr4 mutations. Since neither the not2-1 nor the not2-4 alleles produce a protein that interacts with Ada/Gcn5, the Ccr4-associated functions of NOT2 do not depend on such an interaction. This raises the possibility that the yeast Not2 protein consists of at least two functional domains: an N-terminal domain that regulates HIS3 through interaction with the Ada/Gcn5 complex, and a functionally separable domain that is required for Ccr4-associated functions.

Identification of a human cDNA that encodes a protein with sequences highly homologous to the C-terminal 84 amino acids of yeast Not2 supports our proposal of a Not2 domain structure. An analogous C-terminal domain was also found in a *Drosophila* protein. Interestingly, a *Drosophila* NOT2-like gene has been identified in a screen for suppressors of position effect variegation (Frolov et al., 1998). The function of this domain appears to be conserved, since chimeric proteins containing the N-terminus of yeast Not2 fused to the human C-terminal Not2 domain complements not2-1 in yeast. Homologs of the yeast NOT1, CCR4 and CAF1 genes have been identified in higher eukaryotes, further suggesting that this C-terminal domain may act through a conserved transcriptional regulatory pathway (M.Benson and J.Benson, unpublished; Draper et al., 1995; Green and Besharse, 1996).

Liu et al. have demonstrated that both NOT- and CCR4-related genes negatively regulate FUS1–lacZ, a function consistent with the previously observed repressive properties of the NOT genes (Liu et al., 1998). However, it appears that these factors may also affect transcription in a positive manner under certain circumstances, since the CCR4 and the NOT genes were also required for activation of the ADH2 promoter under non-fermentative growth conditions. Specifically, both ccr4 and not2-1 mutations...
impaired ADH2 response to ethanol induction and a concomitant inability to grow under non-fermentative conditions on YEP plates. These observations suggested that Not and Ccr4 complexes are physically and functionally associated, and that these proteins collaborate to regulate transcription positively or negatively (Liu et al., 1998). The ability of KY803 containing the not2-4 N-terminal mutation to grow on YEP plates suggests that induction of ADH2 under non-fermentative growth conditions depends upon the integrity of the Not2 C-terminus. Moreover, the increase in ADH2 transcript levels in not2-4 and Δada2 strains suggests that N-terminal-associated Not2 activities (i.e. association with Ada2) may also play a role in repression of ADH2 under fermentative growth conditions.

The Ccr4 and Caf1 proteins have been detected in complexes of 1.2×10^6 and 1.9×10^6 Da. Not proteins have been observed in both complexes. The Not proteins were also identified as components of a 6×10^5 Da complex (Collart and Struhl, 1994). Direct interaction between Not1 and Not2 was suggested previously by two-hybrid interaction and allele-specific suppression of not1-2 by the not2-4 allele. However, it now appears likely that the Not proteins form a variety of complexes with potentially distinct regulatory activities. Further characterization of the sizes, constituents, activities and regulatory properties of the yeast protein complexes containing Not2 may be necessary to understand fully the functions of the Not proteins and their relationship with histone acetylase activity and Ccr4-related activities.

It is unclear whether histone acetylase activity per se mediates Not2 regulation of HIS3. We have not examined Not complexes directly for associated histone acetyltransferase activity. The fact that Not2 is not associated with known complexes that contain histone acetyltransferase activity may suggest that the normal function of Not2 involves aspects of Ada or Gcn5 function not associated with this enzymatic activity. Alternatively, Not2 could be an auxiliary component of a particular Gcn5 HAT complex that does not remain stably associated throughout purification.

Previous models for the mechanism of transcriptional regulation by the Not complex proposed that it might act through TBP, perhaps by determining the ability of TBP to bind high versus low affinity sites (Collart and Struhl, 1993, 1994; Collart, 1996). This type of model for NOT function is not mutually exclusive of our suggestion that Not2 may act on the HIS3 promoter by affecting chromatin structure through interaction with components of the Gcn5 histone acetylase complex. Indeed, individual components of the Not complex could collaborate to modulate gene expression through both chromatin modification and regulation of TBP binding. The accessibility of specific promoter regions like HIS3 TC and Tg to transcription factors like TBP would almost certainly be influenced even by subtle changes in the pattern of localized histone acetylation. The Not proteins and their associated complexes may participate in the intricate interplay between these modes of transcriptional regulation both by acting through the basal transcription machinery and by affecting chromatin structure.

Materials and methods

Transcriptional activation by LexA–Not2 proteins in yeast

Cloning of full-length Not2 and Not1 for expression as a LexA fusion proteins was described previously (Collart and Struhl, 1994). The Not2- and Not2-4-coding sequences were cloned as EcoRI–XhoI PCR products into the EcoRI–SalI sites of pLex202 (Brent and Passmore, 1985). DNA from yeast strain KY803 (a ura3-52 trpl-Δ leu2::PET56 gal2 gcn4-ΔI) was used as a PCR template for wild-type NOT2. DNA from MY22 (a ura3-52 trpl-Δ leu2::PET56 gal2 gcn4-ΔI not2-4), a strain derived from KY803 that contains the not2-4 allele (Collart and Struhl, 1994), was used as a template for cloning of not2-4-derived fragments. Not2-N and Not2-4-N correspond to amino acids 1–102 of each respective Not2 protein. pLex202 plasmids were transformed into PSY316 (a ade2-101 his3-200 leu2-3,112 lys2 ura3-53) with the JK103 plasmid containing the LexA-responsive lacZ reporter (Kamens and Brent, 1991). β-galactosidase assays of yeast liquid cultures were performed as described previously (Guarante, 1983). In experiments examining the dependence of LexA–Not2 transcriptional activation on Δada2, Δada3, Δgcn5 or Δspt8, derivatives of PSY316 in which these genes were knocked out individually were transformed with pLex202-Not2 and JK103.

Cloning and sequencing of not2 mutant alleles

DNA from KY803 isogenic strains containing the not2-1 or not2-4 mutant allele was amplified by PCR using the same primers described above for cloning of full-length Not2 into pLex202. These fragments were subcloned into the EcoRI–SalI sites of pUC19 and sequenced by dideoxynucleotide sequencing using Sequenase (United States Biochemical) and appropriate primers. Both strands of two independent clones of each allele were sequenced.

Epitope-tagged Not2 protein expression and preparation of yeast protein extracts

Not2-coding sequences were engineered to express an epitope-tagged Not2 protein under control of its natural flanking sequences. The Not2-coding sequence, followed by three tandem HA epitopes, nine histidine residues and a stop codon, was inserted into a single-copy pRS316-derived vector under control of the endogenous NOT2 promoter and terminator sequences. The resulting HA-Not2 protein could complement not2 mutations. The HA-Not2-4 expression construct was identical, but incorporated a fragment from the not2-4 allele containing the two point mutations that are characteristic of this mutant allele.

Protein extracts from not2-1 mutant yeast expressing Not2, HA-Not2 or HA-Not2-4 from the RS316 plasmid were prepared after growth to an OD600 of 1.0 in a defined medium. Cells were then pelleted and placed on ice. Four pellet volumes of Trit phosphate buffer (25 mM Tris pH 7.4, 50 mM NaCl, 10 mM MgCl2, 5 mM EDTA, 10% (v/v) glycerol, 1 mM PMSF) at 4°C for 1 h with rotation. Following four washes with 0.7 ml of ice-cold binding buffer, samples were loaded on a 12% SDS–PAGE gel. Following Western transfer, membranes were blocked for 1 h in 1× TBS containing 2.5% (w/v) non-fat powdered milk and 0.05% (v/v) Tween-20. Antibody reactions with 1:400 ascites fluid containing mouse 12CA5 anti-HA mAb were carried out in the same buffer at room temperature for 2 h. The membrane was rinsed quickly, washed twice for 10 min with 1× TBS/0.05% Tween-20, incubated for 1 h with a 1:400 dilution of biotinylated anti-mouse antibody (Amersham RPN 1001), and washed as above. This was followed by incubation for 1 h with a 1:1000 solution of streptavidin–horseradish peroxidase-conjugated antibody (Amersham RPN 1231), two 10 min washes with TBS/Tween and visualization using the ECL system (Dupont/NEN).
Gene disruption of GCN5, ADA2 and SPT8

The GCN5, ADA2 and SIDS genes were disrupted in KY803 or KY803 containing not2-4 (Collart and Struhl, 1994) by one-step gene disruption-deletion using LEU2 as the disrupting marker. Gene disruptions were verified by Southern blot.

S1 analysis

HIS3 and ADH2 transcripts were quantitated by S1 nuclease digestion, electrophoresis and phosphorimager analysis as described previously (Cormack et al., 1994; Iyer and Struhl, 1995). Probes to an unrelated polII gene (DED1) or to a polII transcript (tRNA-w) were included as internal controls (Collart and Struhl, 1994).

Complementation of not2-1 slow growth phenotype

KY803 or MY27 (a ura3-52 trpl-1Δ1 leu2::PET56 gal2 gen4-Δ1 not2-1) were transformed with empty RS316 or RS316 expressing various forms of Not2 and grown overnight in casamino acids medium lacking uracil. Cells were pelleted by centrifugation and resuspended in disaggregation buffer (10 mM Tris, pH 7.5, 10 mM EDTA). Cell density was determined by OD600, and 3 µl spots containing 105, 104 or 103 cells were placed on casamino acid plates lacking uracil. YNot2-C was expressed from the ADCl promoter using pTY316 (Yasugi and Howley, 1996).

Analysis of cre4 phenotypes of not2 mutant strains

Growth of KY803 (wild-type NOT2), MY16 (a ura3-52 trpl-1Δ1 leu2::PET56 gal2 gen4-Δ1) and MY22 (a ura3-52 trpl-1Δ1 leu2::PET56 gal2 gen4-Δ1 not2-4) was tested as indicated on YPD plates containing 0.04% (w/v) SDS, 8 mM caffeine or YEP plates containing 3% (v/v) ethanol.

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References


Association of yeast Not2 with Ada2/Gcn5 and Ccr4


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