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

John D.Benson, Mark Benson[1,2], Peter M.Howley and Kevin Struhl[1,3]

Department of Pathology and [1]Department of Biochemistry and Molecular Pharmacology, Harvard Medical School, 200 Longwood Avenue and [2]Cardiovascular Division, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115, USA

[3]Corresponding author

J.D.Benson and M.Benson contributed equally to this work

The *NOT* genes were originally identified in a yeast genetic screen that selected mutations resulting in increased utilization of a non-consensus T_C 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* T_C 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, T_R and T_C. T_R, 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* T_C promoter element consists of a cluster of weak non-conventional TATA elements that function constitutively and respond only weakly to Gcn4. The T_C 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 T_C 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 G_1 at restrictive temperature (37°C), at which time they bore a morphological resemblance to pheromone-arrested cells (Reed, 1980; Breter *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*.,

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1992; Georgakopoulos and Thieros, 1992; Pina *et al*., 1993; Silverman *et al*., 1994; Barlev *et al*., 1995; Georgakopoulos *et al*., 1995; Horiuchi *et al*., 1995; Wang *et al*., 1995; Candau and Berger, 1996).

In order to address the possibility that histone acetylation 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. Δ*gcn5* and Δ*ada2* 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* T_C 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-bing 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).

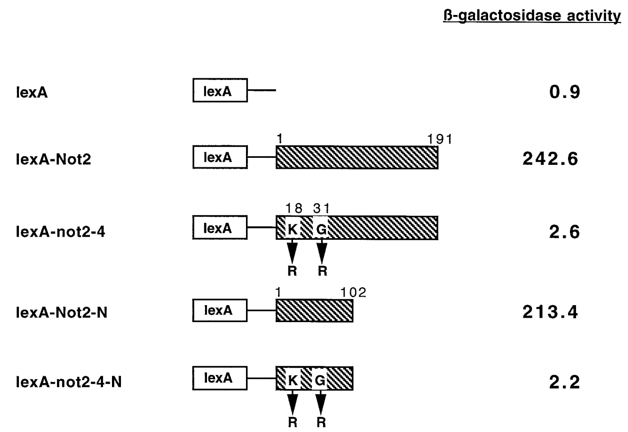

Fig. 1. Transcriptional activation by LexA–Not2 requires a functional Not2 N-terminus. Transcriptional activation of the JK103 reporter plasmid by various LexA–Not2 fusion proteins was tested in PSY316 yeast. Full-length (amino acids 1–191) N-terminal domain proteins encoded by the wild-type *NOT2* and recessive *not2-4* alleles were tested for transcriptional activation activity as LexA fusion proteins.

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

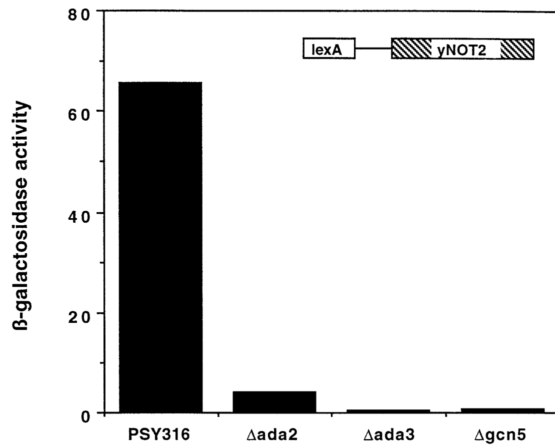

Fig. 2. Transcriptional activation by LexA–Not2 requires components of the Gcn5/Ada histone acetyltransferase complex. Activation of the JK103 by LexA–Not2 was tested in PSY316 and derived strains in which the *ADA2*, *ADA3* or *GCN5* gene was disrupted.

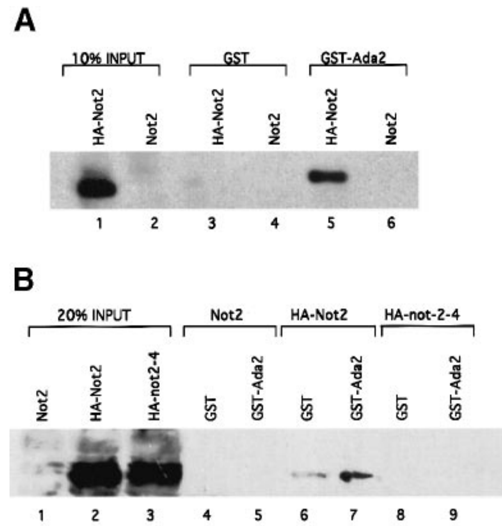

Fig. 3. (**A**) Binding of Not2 to GST–Ada2. HA-tagged Not2 (HA-Not2) or untagged Not2 were expressed in a *not2-1* derivative of KY803. Protein extracts from these cells were assayed for binding to GST–Ada2, followed by detection by Western blot using an HA monoclonal antibody. A 40 μg aliquot of total protein extract from yeast expressing tagged or untagged Not2 was loaded in lanes 1 and 2, respectively. Lanes 3 and 4 represent proteins bound to GST after incubation with 400 μg of total protein extract. GST–Ada2-associated proteins using 400 μg of total protein extract are shown in lanes 5 and 6. (**B**) The Not2-4 mutant protein does not bind GST–Ada2.

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 Δ*ada2* and Δ*gcn5* 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 Δ*ada2* or Δ*gcn5* 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 Δ*gcn5* (lanes 2 and 3) and Δ*ada2* (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 Δ*gcn5* (lane 7) or Δ*ada2* (lanes 8 and 9) did not significantly augment the +1 phenotype associated with each individual mutation, suggesting that

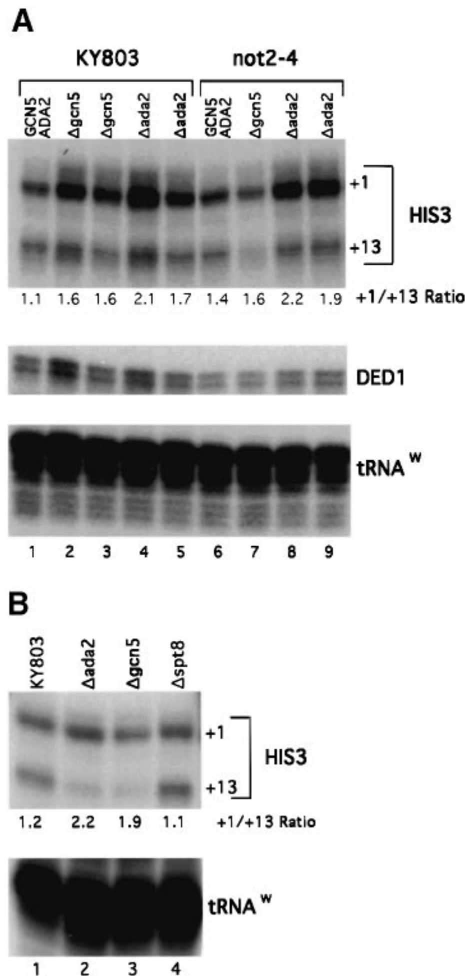

Fig. 4. (**A**) *HIS3* transcription patterns in Δ*gcn5* and Δ*ada2* strains derived from either KY803 or KY803*not2-4*. RNA isolated from the indicated strains was subjected to quantitative S1 analysis. The positions of the +1 and +13 *HIS3* transcripts are indicated. *DED1*, a polII transcript unaffected by *not* mutations, and tRNA(w) polIII transcripts were included as internal controls. (**B**) Comparison of Δ*spt8* (lane 4) effects on +1/+13 *HIS3* ratios with those of Δ*ada2* and Δ*gcn5* (lanes 2 and 3, respectively).

these genes reside within a common transcriptional regulatory pathway. The phenotypic similarity between Δ*ada2*, Δ*gcn5* 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 Δ*spt8* strain. As shown in Figure 4B, Δ*spt8* 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.

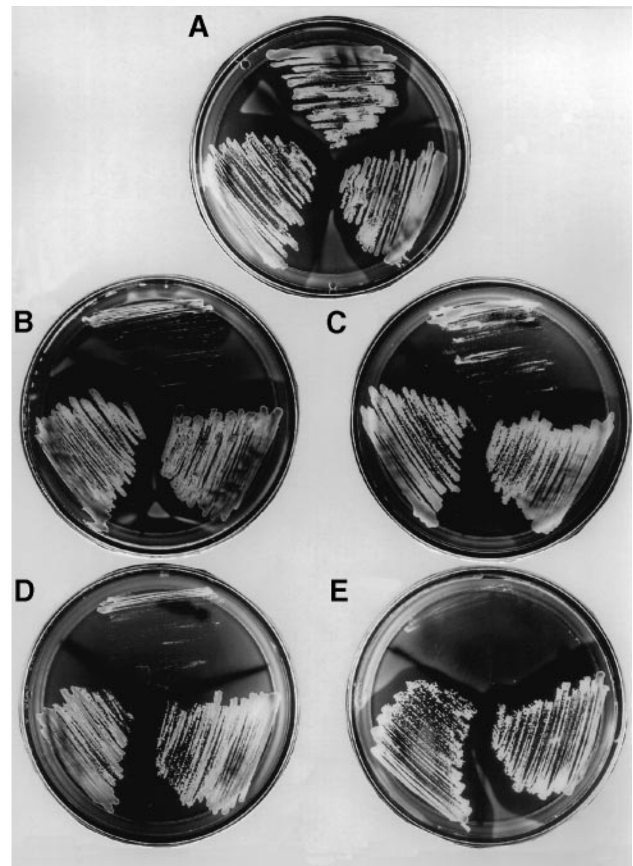

Fig. 5. Unlike *not2-1* strains, *not2-4* yeast do not have a *ccr4* phenotype. The KY803 parental strain (left quadrant) was streaked with derived strains containing the *not2-1* allele (top quadrant) or the *not2-4* allele (right quadrant). Growth was tested under the following conditions: (**A**) YPD/30°C; (**B**) YPD/0.4% SDS; (**C**)YPD/37°C; (**D**) YPD/8 μM caffeine; (**E**) YEP.

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 $MgCl_2$ (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),

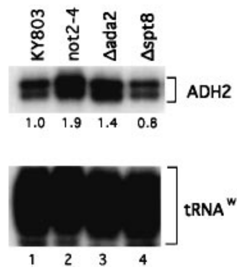

Fig. 6. Effects of mutations associated with the Not2 N-terminus on *ADH2* transcript levels. *ADH2* transcripts from yeast grown in glucose were characterized by quantitative S1 analysis, with tRNA(w) as an internal control. Ratios of *ADH2* transcripts in *not2-4*, Δ*ada2* and Δ*gcn5* mutant strains (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.

suggesting that this induction can occur in the *not2-4* mutant. We also examined the effects of *not2-4*, Δ*ada2* and Δ*spt8* mutations on *ADH2* transcript levels under glucose growth conditions. *ADH2* transcript levels were increased in both *not2-4* and Δ*ada2* strains grown in glucose medium (Figure 6, lanes 2 and 3), whereas Δ*spt8* had little or no effect on *ADH2* transcript levels. Thus, in addtion 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

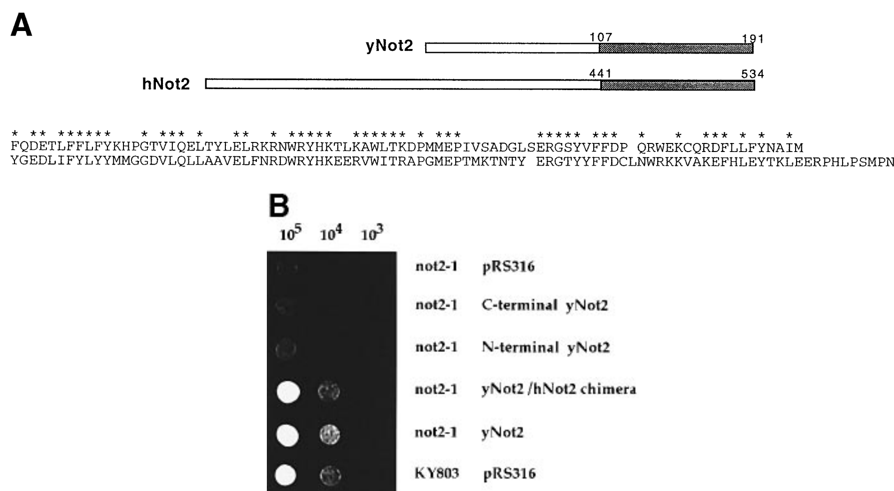

Fig. 7. (**A**) Schematic diagram and alignment of the conserved Not2 C-terminal domain. Residues 107–191 of yNOT2 are highly homologous to residues 441–534 encoded by a human cDNA. (∗) denotes identical or conserved amino acid residues. (**B**) Functional conservation of the Not2 C-terminal domain. 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 assessing 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 recombined 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-AT-resistant mutant that displayed increased utilization of the +1 *HIS3* transcription start site, whereas the recessive *not2-1* null mutant was both 3-AT 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, Δ*ada2*, Δ*ada3* and Δ*gcn5* 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-AT 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 must also have distinct functions. *not2-4* and *not2-1* are both 3-AT 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 Δ*ada2* 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 acetylation 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* T_C and T_R 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 *Eco*RI–*Xho*I PCR products into the *Eco*RI–*Sal*I sites of pLex202 (Brent and Ptashne, 1985). DNA from yeast strain KY803 (***a*** *ura3-52 trp1-Δ1 leu2::PET56 gal2 gcn4-Δ1*) was used as a PCR template for wild-type *NOT2*. DNA from MY22 (***a*** *ura3-52 trp1-Δ1 leu2::PET56 gal2 gcn4-Δ1 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 Δhis3-200 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 (Guarente, 1983). In experiments examining the dependence of LexA–Not2 trancriptional activation on Δ*ada2*, Δ*ada3*, Δ*gcn5* or Δ*spt8*, 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 *Eco*RI–*Sal*I sites of pUC19 and sequenced by dideoxyribonucleotide 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 OD_{600} of 1.0 in selective medium. Cells were then pelleted and placed on ice. Four pellet volumes of Tris phosphate buffer (25 mM Tris base adjusted to pH 6.7 with phosphoric acid) containing 2 mM phenylmethylsulfonyl fluoride (PMSF), and one pellet volume of glass beads (Sigma G-8772) were added. Each tube was vortexed for 15 s, then returned to ice for several seconds for a total of 10 cycles. Lysates were then centrifuged at 12 000 *g* for 10 min at 4°C. Total protein concentration of the supernatant was determined by Bradford assay. Protein extracts were quick-frozen with liquid nitrogen and stored at –70°C until use in binding assays.

Interaction between GST–Ada2 and Not2

GST–Ada2 (generously provided by S.Berger and N.Barlev) was prepared as described previously (Frangioni and Neel, 1993). Yeast protein extract (400 μg) was incubated with 5 μg of GST–Ada2 on glutathione beads in 0.7 ml of binding buffer [20 mM Tris pH 7.4, 50 mM NaCl, 10mM $MgCl_2$, 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 *SDS3* 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 polIII 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 trp1-Δ1 leu2::PET56 gal2 gcn4-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 OD_{600}, and 3 μl spots containing 10^5, 10^4 or 10^3 cells were placed on casamino acid plates lacking uracil. yNot2-C was expressed from the *ADC1* promoter using pTY316 (Yasugi and Howley, 1996).

Analysis of ccr4 phenotypes of not2 mutant strains

Growth of KY803 (wild-type NOT2), MY16 (***a*** *ura3-52 trp1-Δ1 leu2::-PET56 gal2 gcn4-Δ1 Δnot2*) and MY22 (***a*** *ura3-52 trp1-Δ1 leu2::PET56 gal2 gcn4-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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