***NOT1(CDC39), NOT2(CDC36), NOT3, and NOT4* encode a global-negative regulator of transcription that differentially affects TATA-element utilization**

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The yeast *HIS3* T_R and T_C TATA elements support basal transcription, but only T_R can respond to transcriptional activators. Four genes, *NOT1(CDC39)*, *NOT2(CDC36)*, *NOT3*, *NOT4*, act as general negative regulators and preferentially affect T_C-dependent transcription. Allele-specific suppression, a two-hybrid interaction, and biochemical cofractionation suggest that NOT1 and NOT2 are nuclear proteins associated in a discrete, 500-kD complex. NOT4 interacts with NOT1 and NOT3 in the two-hybrid assay, and overexpression of NOT3 or NOT4 suppresses *not1* and *not2* mutations. Repression by the NOT proteins is not attributable to inhibition of transcriptional activators, does not involve the CYC8/TUP1 negative regulatory complex, and is distinct from repression by nucleosomes or by the SPT4,5,6 proteins that affect chromatin structure. We propose that the NOT proteins inhibit the basic RNA polymerase II transcription machinery, possibly by affecting TFIID function.

[**Key Words:** *NOT* genes; negative regulators; transcription; TATA element; repression complex]

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It has become increasingly evident that RNA polymerase II transcription involves intermediary proteins that are distinct from the basic initiation factors and from specific DNA-binding activators and repressors. By definition, such intermediary proteins do not interact efficiently with DNA, but rather become associated with promoters through protein-protein interactions. Intermediary transcription factors can function by mediating interactions between activators/repressors and the basic transcription machinery, and/or by maintaining or alleviating a repressive chromatin structure. Depending on the mechanism involved, an intermediary protein might affect transcription of a small number of related genes or it might globally affect the transcription of many genes.

In the yeast *Saccharomyces cerevisiae*, a number of intermediary proteins that act as global positive and negative regulators of transcription have been identified by mutations (for review, see Struhl 1993). On the basis of phenotypic similarities and extragenic suppressor analyses, some of these regulators have been grouped functionally and proposed to form multiprotein complexes.

One potential complex includes the SNF2/SWI2, SNF5, SNF6, SWI1, and SWI3 proteins, which are important for transcriptional enhancement by a variety of DNA-bound activators (Laurent et al. 1991; Hirschhorn et al. 1992; Laurent and Carlson 1992; Peterson and Herskowitz 1992; Yoshinaga et al. 1992). The SPT4, SPT5, and SPT6 proteins carry out a negative regulatory function, probably as a complex (Swanson and Winston 1992). Phenotypic similarities between *spt4,5,6* and histone mutations suggest that these SPT proteins may affect transcription by altering chromatin structure. The CYC8/TUP1 complex (Williams et al. 1991), which is critical for repression of several transcriptional regulatory pathways, has been proposed to function by associating with specific DNA-binding proteins (Keleher et al. 1992; D. Tzamarias and K. Struhl, in prep). These putative multiprotein complexes clearly have distinct roles in transcription, but their mechanisms of action are not known.

Previously, we characterized CDC39 as an essential nuclear protein that negatively regulates transcription of many genes and differentially affects the functionally distinct TATA elements in the *HIS3* promoter region (Collart and Struhl 1993). The *HIS3* TATA elements T_C and T_R support basal transcription that depends on the TATA-binding protein (TBP), but only T_R can respond to

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transcriptional activators such as GCN4 and GAL4 (Struhl 1986; Mahadevan and Struhl 1990; Cormack and Struhl 1992). T_R contains the sequence TATAAA and behaves as a typical TATA element (Chen and Struhl 1988; Ponticelli and Struhl 1990; Wobbe and Struhl 1990), whereas T_C lacks a conventional TATA sequence and is unable to support transcription *in vitro* (Mahadevan and Struhl 1990; Ponticelli and Struhl 1990). Unlike activator proteins that function through T_R , CDC39 affects T_C -dependent transcription preferentially (Collart and Struhl 1993). Thus, T_C -dependent transcription is controlled by a global negative regulatory pathway involving CDC39. For these and other reasons, we now refer to CDC39 as *NOT1* (negative on TATA).

In this paper we identify three additional genes, *NOT2*(CDC36), *NOT3*, and *NOT4*, that behave as general negative regulators and preferentially affect T_C -dependent transcription. We present genetic and biochemical evidence for a protein complex containing NOT1 and NOT2 and for functional interactions involving NOT3 and NOT4. Finally, we show that transcriptional inhibition by the NOT proteins is mechanistically distinct from that mediated by other global negative regulators such as the CYC8/TUP1 complex, histones, and SPT6. Thus, the NOT proteins define a novel mechanism of transcriptional repression that affects TATA-element utilization. Because the NOT proteins affect transcription from T_C preferentially, the element that does not respond to activator proteins, we suggest that NOT-mediated repression involves an interaction with a component of the basic transcription machinery.

Results

Identification of four NOT genes that negatively regulate HIS3 expression

Previously, we described a genetic selection for strains that increase *HIS3* transcription by *gcn4*-C163, a derivative with a partially defective activation domain (Collart and Struhl 1993). The selection is based on the fact that the degree of GCN4 function is correlated with cell growth in medium containing aminotriazole (AT), a competitive inhibitor of the *HIS3* gene product (Hope and Struhl 1986). Using this selection, we obtained 13 recessive mutations, all of which allow growth ≤ 40 mM AT, unlike the parental strain that grows poorly at 5 mM and not at all in 10 mM AT.

Genetic analysis reveals that the 13 mutations fall into four complementation groups. As we have reported (Collart and Struhl 1993), one group is defined by a single temperature-sensitive mutation in *CDC39*, a gene implicated originally in cell-cycle control and the pheromone response (Reed 1980; Shuster and Byers 1989; de Barros Lopes et al. 1990). Our previous results argue strongly that *CDC39* is a transcriptional regulatory protein and not involved directly in cell-cycle control (Collart and Struhl 1993); for this reason and others to become apparent, we now refer to this gene as *NOT1* (negative on TATA). *NOT2* is defined by four mutations, three of

which cause slow growth at 30°C and prevent growth at 37°C; *not2-4* grows normally at both temperatures. *NOT3* is defined by seven mutations that do not cause detectable growth phenotypes on rich medium. *NOT4* is defined by a single mutation that confers a slow growth phenotype at all temperatures. Growth phenotypes, when observed, always cosegregate with the AT-resistance phenotype in tetrads.

NOT genes preferentially affect T_C -dependent transcription

In the *HIS3* promoter region, T_C is responsible for transcription from the +1 site, whereas T_R -dependent transcription is initiated almost exclusively from the +13 site (Struhl 1986; Chen and Struhl 1988; Mahadevan and Struhl 1990; Ponticelli and Struhl 1990). Basal *HIS3* transcription is initiated equally from +1 and +13 and involves both T_C and T_R . Activator proteins, such as GCN4 and GAL4 function only with T_R and stimulate +13 transcription. In contrast, loss of *NOT1* function causes a novel phenotype in which basal transcription from the +1 site is increased preferentially (Collart and Struhl 1993). Increased transcription occurs rapidly with loss of *NOT1* function, suggesting that *NOT1* acts directly on the *HIS3* promoter region. Mutational analysis indicates that *NOT1* does not affect the inherent properties of the +1 and +13 initiator elements, but rather affects T_C -dependent transcription preferentially (Collart and Struhl 1993).

We analyzed basal *HIS3* transcription (i.e., GCN4 was deleted) in *not* mutant backgrounds at both 30°C and 38°C (Fig. 1A). For all alleles tested, a preferential increase in the *HIS3* +1 transcript is observed, but only at 38°C for the temperature-sensitive strains. As observed previously with the *not1-2* strain (Collart and Struhl 1993), increased +1 transcription is observed within 30 min after shifting *not1-1* and *not2-1* strains to the restrictive temperature (data not shown). The levels of the +1 transcript differ among the mutant strains, with *not2-4* and *not3-2* strains showing the most dramatic increase. Thus, all four *NOT* genes regulate *HIS3* expression negatively and affect T_C -dependent transcription preferentially.

The different levels of basal *HIS3* transcription conferred by the various *not* mutations are surprising in light of the similar AT-resistance phenotypes, which are measured under conditions of transcriptional activation by *gcn4*-C163. Therefore, we analyzed *HIS3* transcription activated by *gcn4*-C163 in wild-type and *not* mutant strains (Fig. 1B). As expected, strains containing *gcn4*-C163 show more +13 transcript than corresponding *gcn4* deletion strains (e.g., cf. the +1/+13 ratio in lanes 1 and 10). Surprisingly, but in agreement with the AT-resistance phenotypes, the amount of *HIS3* transcription activated by *gcn4*-C163 is similar in all mutant strains. Thus, in the *not2-4* and *not3-2* strains, the level of +1 transcription in the presence of *gcn4*-C163 is actually lower than the basal, non-GCN4-activated level (cf. lanes 4 and 5 with lanes 13 and 14). This observation

Figure 1. Transcriptional analysis. (A) *gcn4* deletion strains containing the indicated *not* alleles were grown at the permissive (30°C) or restrictive (38°C) temperatures and analyzed for *HIS3* (+1 and +13 transcripts) and *DED1* RNAs by S1 treatment of RNA-DNA duplexes. (B) Same as A except that strains express *gcn4-C163*. (C) Same as A except that *DED1* and *HIS4* RNAs are analyzed. (D) *gcn4* deletion strains containing *not1-2*, *not2-4*, *not3-2*, and *not4-1* were grown at 30°C and analyzed for *STE4*, *BIK1*, *ORF*, *TBP*, *TFIIIB*, and *TFIIA* RNAs by Northern blotting. (E) *gcn4* deletion strains containing multicopy plasmids that express no protein (lane 1), TBP (lane 2), or TFIIIB (lane 3) were grown at 30°C and analyzed as described in A. TBP RNA levels in lane 2 are ~20-fold higher than in lane 1 (data not shown).

suggests that in some cases, increased T_C -dependent transcription with loss of *NOT* function can compete with activation by GCN4 derivatives.

The *NOT* proteins negatively regulate the same spectrum of functionally diverse genes

Previous results indicate that *NOT1* negatively regulates transcription of several unrelated genes; *not1(cdc39)-2* confers increased basal transcription of *HIS4* and *TBP* (encodes the TBP) at the restrictive temperature (Collart and Struhl 1993), and *not1(cdc39)-1* confers increased α -factor-dependent transcription of *STE4* (de Barros Lopes et al. 1990), a gene involved in the pheromone response. As shown in Figure 1, C and D, basal (i.e., not activated by GCN4 or α -factor) transcription of *HIS4*, *TBP*, *STE4*, and *BIK1* (encodes a gene involved in nuclear fusion) is increased in *not2*, *not3*, and *not4* mutant strains grown at 30°C. In contrast, RNA levels for *DED1*, the gene adjacent to *BIK1* (*ORF*; Fig. 1D), and the genes encoding TFIIIB and the small subunit of TFIIA are not increased in *not* mutant strains. We also analyzed transcription of these genes in the *not1-2* strain grown at 30°C, a condition of partial *NOT1* function (Collart and Struhl 1993). Consistent with earlier results (Collart and Struhl 1993), basal *HIS4* and *TBP* transcription is affected minimally. Basal transcription of *STE4* is only slightly increased in this circumstance, whereas *BIK1*

transcription is increased to the same extent as observed in the other *not* mutant strains. Taken together, these results indicate that the four *NOT* proteins negatively regulate a diverse set of genes.

The fact that the genes encoding TFIIIB and the small subunit of TFIIA are unaffected in *not* mutant strains indicates that *NOT*-mediated repression is not attributable to altered levels of TFIIIB or TFIIA. Although *not* mutant strains show increased levels of *TBP* RNA, this increase is unlikely to be responsible for the *not* phenotype because overexpression of *TBP* does not significantly affect the level or initiation pattern of *HIS3* transcription (Fig. 1E) or AT resistance.

NOT2 is identical to CDC36

NOT2 was cloned by complementation of the temperature-sensitive and AT-resistant phenotypes conferred by the *not2-1* mutation, and the gene was mapped to a 3-kb fragment (Sc3867; Fig. 2A). Genomic integration and genetic mapping indicate that an adjacent 2-kb DNA fragment (Sc3868) is tightly linked to the *not2-1* mutation. Hybridization of the *NOT2* gene to a collection of λ bacteriophages covering most of the yeast genome (Olson et al. 1986) indicates that it maps close to the *CDC36* locus (L. Riles, pers. comm.). Like *NOT1(CDC39)*, *CDC36* was originally identified by temperature-sensitive mutations that cause cell-cycle arrest in G_1 and constitutively ac-

Figure 2. The *NOT2*, *NOT3*, and *NOT4* loci. The structures and phenotypes (determined by complementation) of the indicated DNA fragments are shown. The location and orientation of each protein-coding sequence is indicated by the open box and arrow. Restriction sites are abbreviated as follows: (B) *Bam*HI; (Bg) *Bgl*II; (C) *Cla*I; (E) *Eag*I; (H) *Hind*III; (N) *Nde*I; (P) *Pst*I; (R) *Eco*RI; (S) *Sac*I; (Sm) *Sma*I; (Xb) *Xba*I; (X) *Xho*I.

tivate the pheromone response pathway (Reed 1980; Shuster and Byers 1989; de Barros Lopes et al. 1990). Restriction mapping and DNA sequencing confirms that Sc3867 contains *CDC36*. Moreover, a 500-bp internal deletion of *CDC36*-coding sequences (Sc3869) abolishes *NOT2* activity, whereas a 1-kb DNA fragment encompassing a minimal *CDC36* locus (Sc3870) complements both phenotypes. Hence, *NOT2* is identical to *CDC36*.

Although *NOT2*(*CDC36*) has been defined by temperature-sensitive mutations, it is not essential for vegetative growth; cells carrying a chromosomal deletion grow

very slowly at 30°C and not at all at 37°C on rich medium (S. Reed, pers. comm.). Three of our *not2* alleles have the same phenotype as a null allele, whereas *not2-4* grows normally on rich medium at all temperatures tested. Surprisingly, *not2-4* confers the strongest T_C-dependent transcriptional effect, indicating that temperature sensitivity and transcriptional phenotypes are not strictly correlated. None of our *not2* mutations are the result of severe alterations in the gene, as determined by genomic hybridization, and the 0.9-kb *NOT2* RNA is expressed at equivalent levels in parental and *not2* mutant strains (not shown). Interestingly, a high-copy plasmid containing *NOT2* weakly suppresses the temperature-sensitive phenotype of *not1-1* (Table 1).

NOT3 is a high-copy suppressor of not1 and not2 mutations

To clone *NOT3*, we took advantage of the observation (see below) that *not3* mutations exacerbate the growth phenotypes of *not2* temperature-sensitive mutations such that the double mutants grow very poorly at 25°C and 30°C. We obtained one plasmid containing ~15 kb of yeast DNA (Sc3876) that complements the slow-growth phenotype at 30°C but not the temperature-sensitive phenotype of a *not2-1 not3-5* strain. Sc3876 contains *NOT3* because it directs genomic integration to a site tightly linked to the *not3-1* locus. By testing restriction fragments phenotypically, we located a *Sac*I site that may be within the complementing gene (Fig. 2B). Partial DNA sequencing reveals that the region around the *Sac*I site is identical to a sequence reported to be that of *CDC39* (Ferguson et al. 1986) but clearly different from the correct sequence of *NOT1*(*CDC39*) (Collart and Struhl 1993). To prove that *NOT3* was equivalent to this previously identified gene, we cloned a minimal DNA fragment encompassing the 835-residue open reading frame (Sc3875) and showed that it complements both the slow-growth phenotypes of the *not2 not3* double mutants and the AT-resistance phenotypes of the *not3* mutants. *NOT3* maps to the left arm of chromosome 9 as

Table 1. High-copy suppression

<i>not</i> allele	Overexpressed NOT proteins			
	NOT1	NOT2	NOT3	NOT4
<i>not1-1</i>	++	±	++	++
<i>not1-2</i>	++	-	-	+
<i>not2-1</i>	-	++	++	+
<i>not2-2</i>	N.T.	++	N.T.	N.T.
<i>not2-3</i>	-	++	++	N.T.
<i>not4-1</i>	-	-	-	++

Growth at 37°C for strains containing the indicated *not* alleles and high-copy plasmids containing the indicated *NOT* genes. (++) Wild-type growth; (+) slower than wild-type; (±) barely detectable; (-) none. *not3* mutant strains grow normally at this temperature and hence were not examined (N.T.) Not tested.

determined by hybridization to the collection of λ bacteriophages.

Because *NOT3* was originally identified in an attempt to clone *NOT1* by complementation of the *not1(cdc39)-1* allele (Ferguson et al. 1986), it seemed likely that *NOT3* would act as a high-copy suppressor of *not1* mutations. A multicopy plasmid containing *NOT3* suppresses the temperature-sensitive growth phenotype of the *not1-1* strain (Table 1), a finding supported by others (S. Reed, pers. comm.). However, overexpression of *NOT3* does not suppress any of the phenotypes caused by a *not1-2* strain. *NOT3* overexpression also suppresses the temperature-sensitive phenotype of two different *not2* mutations, but it has no effect on the slow-growth phenotype conferred by *not4-1* (Table 1).

None of the *not3* mutations are the result of severe alterations in the gene, as determined by genomic hybridization, and the 2.8-kb RNA is expressed at equivalent levels in the parental and *not3* mutant strains. We constructed a null allele of *NOT3* (Sc3877) and found it to grow normally at all temperatures tested on rich medium. This strain has the same AT-resistance phenotype as the originally isolated *not3* mutant strains, and it shows the same preferential increase in T_C -dependent transcription (data not shown).

NOT4 is identical to SSF1 and is a high-copy suppressor of not1 and not2 mutations

NOT4 was cloned by complementation of the slow growth phenotype associated with the *not4-1* mutation, and the gene was mapped to a 3.7-kb fragment (Sc3882; Fig. 2C). Restriction mapping and partial DNA sequencing revealed that *NOT4* is identical to *SSF1*, a gene identified by mutations that suppress the mating defect in a *ste4* strain (K. Irie and K. Matsumoto, pers. comm.). The defining phenotype of *ssf1* mutations presumably reflects constitutive activation of the pheromone response pathway and, hence, is reminiscent of phenotypes conferred by the original *cdc39(not1)* and *cdc36(not2)* mutations. A null allele of *NOT4* (Sc3883) confers the same growth and AT-resistance phenotypes as the *not4-1* mutation, and it causes the same preferential increase in

T_C -dependent transcription (data not shown). As determined by hybridization to the collection of λ bacteriophages, *NOT4* lies centromeric to the ribosomal DNA locus on the right arm of chromosome 12. In confirmation of a result obtained by K. Irie and K. Matsumoto (pers. comm.), a multicopy plasmid containing *NOT4* suppresses the temperature-sensitive phenotypes of *not1* and *not2* mutations (Table 1). Growth of the resulting strains at 37°C is slower than that of the parental strain, indicating that *NOT4* overexpression does not compensate fully for loss of *NOT1* or *NOT2* function.

not2-4 is an allele-specific suppressor of not1-2

To test for genetic interactions among the *not* mutations, we constructed all double mutant strains involving *not1-2* and *not2-1* by standard genetic crosses. All of the double mutants are viable and, with one clear exception, grow similarly on 40 mM AT. In general, the double mutants grow slightly more slowly in rich medium than the corresponding single mutants. However, unlike strains containing individual mutations, all *not2-1 not3* double mutant strains grow very poorly at 25°C and 30°C.

The exceptional double mutant, *not1-2 not2-4*, behaves similarly to the parental (*NOT1 NOT2*) strain on AT. Moreover, this double mutant strain grows well at 37°C, although the *not1-2* single mutant is temperature sensitive for growth. This allele-specific suppression is also observed at the transcriptional level (Fig. 3). In a typical double mutant, such as *not1-2 not2-1*, the basal level of the +1 transcript is slightly higher than that observed in the single mutants (cf. lanes 8 and 9 with 11). However, in the *not1-2 not2-4* double mutant, the basal *HIS3* levels are similar to those in the parental strain and much lower than in the *not2-4* strain at 30°C (cf. lanes 1, 4, and 6); at 38°C, the level of the +1 transcript is slightly higher than in the parental strain (lanes 7,12) but much lower than in the *not2-4* mutant (lane 10). These observations indicate that *not2-4* and *not1-2* are mutual suppressor mutations that largely restore a wild-type phenotype, and they strongly suggest a direct interaction between *NOT1* and *NOT2*.

Figure 3. Allele-specific suppression. *gcn4* deletion strains containing the indicated *not* alleles were grown at the temperatures indicated and analyzed for *HIS3* (+1 and +13 transcripts) and *DED1* RNAs. The specific activity of the *DED1* probe relative to the *HIS3* probe was twofold higher at 30°C (left) as compared with 38°C (right).

Two-hybrid interactions

To provide independent evidence for interactions between NOT proteins, we used the two-hybrid assay (Fields and Song 1989) as modified by Zervos et al. (1993). NOT proteins were expressed as nuclearly localized, fusion proteins with either the LexA DNA-binding domain or the B42 acidic activation region; the fusion proteins confer NOT function as assayed by complementation. These proteins were analyzed, singly or in combination, for their ability to activate transcription of *LEU2* or *lacZ* reporter genes containing LexA operators upstream of a TATA element (Table 2). Two-hybrid interactions are clearly detected between NOT1 and NOT2 (9-fold increase in β -galactosidase activity), NOT1 and NOT4 (20-fold increase), and NOT3 and NOT4 (25-fold increase). These interactions are specific; other combinations of B42-hybrid proteins and LexA-NOT1 or LexA-NOT3 showed no increase in β -galactosidase activity. However, the combination of LexA-NOT3 and B42-NOT1 leads to a *Leu*⁺ phenotype (Fig. 4), suggesting the possibility of a weak two-hybrid interaction between NOT3 and NOT1. LexA-NOT2 activates transcription on its own and, hence, is unsuitable for the two-hybrid assay. These results indicate that NOT1-NOT2, NOT1-NOT4, NOT3-NOT4, and perhaps NOT3-NOT1 are associated physically.

NOT1 and NOT2 are nuclear proteins that are associated in a large complex

The two-hybrid experiments establish that NOT1 and NOT2 can interact when both proteins are localized nuclearly. However, although NOT1 is a nuclear protein (Collart and Struhl 1993), the localization of NOT2 is unknown. Therefore, we analyzed total, nuclear, and cytoplasmic protein extracts by Western blotting with antibodies raised against NOT2 (kind gift from S. Reed, Scripps Institute, La Jolla, CA). As shown in Figure 5, NOT2 is detected as a protein with an apparent molecular mass of 22 kD, consistent with its expected size. The protein is detected in total and nuclear extracts but not in cytoplasmic extracts or in total extracts of strains deleted for NOT2 (kind gift from S. Reed). Thus, NOT1 and NOT2 are nuclear proteins and, hence, potentially able to interact in normal cells.

To provide biochemical evidence for a complex con-

Figure 4. Two-hybrid interactions. EGY42 strains expressing LexA-NOT3 and the indicated B42 activation domain derivatives were plated on either 2% glucose (Glu) or 2% galactose (Gal) minimal medium lacking leucine and selective for plasmid maintenance. Colony growth requires activation of the *LEU2* gene that is driven by a promoter containing six LexA operators upstream of the *CYC1* TATA element.

taining NOT1 and NOT2, we fractionated proteins from cells expressing NOT2 and epitope-tagged NOT1 on a Superose 6 gel filtration column. Western blots reveal that NOT1 and NOT2 are not detected as monomeric species but, instead, cofractionate at a position corresponding to a molecular mass of 500 kD (Fig. 6). Several observations indicate that this 500-kD species represents a discrete multiprotein complex and is not attributable to nonspecific aggregation. First, the sharpness of the NOT1 and NOT2 protein peaks are similar to those of purified proteins run through the column as molecular mass markers. Second, the column was run in moderately high ionic strength (0.35 M) and in the presence of detergent (0.1% Tween), conditions that do not favor aggregation. Third, NOT1 and NOT2 are separated easily from the vast majority of proteins in the extract that fractionate as species of lower molecular mass. Fourth, the sharpness of the peaks and the fact that the extracts contain <1% of total cellular DNA make it highly unlikely that NOT1 and NOT2 cofractionate as a 500-kD species as a result of their association with DNA. Thus, NOT1 and NOT2 are components of a large, multiprotein complex.

Figure 5. Subcellular localization of NOT2. Protein from total (Tot), nuclear (Nuc), and cytoplasmic (Cyt) extracts prepared from *NOT2* (MY340) or *not2::TRP1* (MY94) strains analyzed by Western blotting using specific antibodies to the NOT2 protein. The position of the 22-kD NOT2 protein is indicated.

Table 2. Two-hybrid assays

LexA hybrid proteins	B42 activation domain hybrid proteins				
	B42	B42-NOT1	B42-NOT2	B42-NOT3	B42-NOT4
LexA-NOT1	38	34	330	41	780
LexA-NOT2	870	1060	630	N.T.	N.T.
LexA-NOT3	21	15	17	19	550

β -Galactosidase activities (Miller units/mg protein) of EGY42 derivatives expressing the indicated proteins. (N.T.) Not tested.

Figure 6. NOT1 and NOT2 are associated in a complex. Proteins from KY340 cells, which express NOT2 and a HA1-tagged NOT1 were separated on a Superose 6 column by fast protein liquid chromatography. Fractions (defined by ml eluted from the column) were assayed for protein content by the Bradford assay (A) and for HA1–NOT1 and NOT2 by Western blotting (B). Indicated on the graph are the fractions containing the molecular mass (in kD) markers and the HA1–NOT1 and NOT2 (boxes; darker shading indicates stronger signal in Western blot). The positions corresponding to HA1–NOT1 and NOT2 on the Western blot are indicated; additional bands detected in HA1 probing are cross-reactive species that do not depend on expression of HA1–NOT1 (Collart and Struhl 1993).

Negative regulation by the NOT proteins is distinct from repression by the Cyc8/Tup1 complex

The CYC8/TUP1 protein complex represses transcription of many genes regulated by cell type, glucose, and oxygen (Williams et al. 1991; Keleher et al. 1992). To examine the relationship between the CYC8/TUP1 complex and the NOT proteins, we analyzed basal *HIS3* transcription in strains deleted for *CYC8* or for *TUP1*. The levels of the +1 and +13 transcripts in *cyc8* or *tup1* mutant strains are indistinguishable from those in the wild-type strain (Fig. 7), indicating that negative regulation of *HIS3* transcription by the NOT proteins is not mediated by the CYC8/TUP1 complex.

CYC8 (Keleher et al. 1992), TUP1 (D. Tzamarias and K. Struhl, in prep), and SIN3 (Wang and Stillman 1993) can repress transcription when bound upstream of the intact *CYC1* promoter by the heterologous LexA DNA-binding domain. To test whether the NOT proteins could repress transcription in this manner, we introduced LexA–NOT1, LexA–NOT2, and control proteins into strains containing *CYC1* promoters that did or did not have upstream LexA operators (Table 3). LexA–NOT1 does not affect expression of either reporter gene, whereas LexA–NOT2 slightly increases transcription

from the promoter containing the LexA operators. As expected, expression from the promoter containing LexA operators is increased by the transcriptional activator LexA–MyoD, whereas it is repressed by LexA–CYC8. Thus, unlike CYC8, TUP1, and SIN3, neither NOT1 nor NOT2 can repress transcription when brought to DNA

Figure 7. *CYC8* and *TUP1* are not required for NOT-mediated repression. *gcn4*-deleted strains containing *cyc8::LEU2* (MY96) or *tup1::LEU2* (MY97) were analyzed for *HIS3* (+1 and +13 transcripts) and *DED1* RNAs.

by a heterologous DNA-binding domain. In fact, NOT2 increases transcription in this artificial situation, in contrast to its effect on many native yeast genes.

NOT function is distinct from general chromatin-based repression

Because nucleosomes repress transcription globally, we considered the possibility that the NOT proteins are involved in maintaining normal chromatin structure. Previously, we argued against such a role (Collart and Struhl 1993) on the basis of the observation that loss of nucleosomes, obtained by repressing histone H4 expression, leads to an induction of T_R -dependent transcription (Durrin et al. 1992), an effect in contrast to that observed with loss of NOT function. However, as nucleosome loss severely disrupts chromatin structure, we examined basal *HIS3* transcription in two other situations involving less dramatic alterations.

First, we examined the effect of two temperature-sensitive mutations in *SPT6*, a gene encoding a nonhistone protein implicated in maintaining normal chromatin structure (Swanson and Winston 1992), at permissive and restrictive temperatures (Fig. 8). Loss of *SPT6* function at the restrictive temperature leads to increased transcription from the +13 site but does not affect transcription from the +1 site. Conversely, *not* mutations do not have an Spt^- phenotype (not shown). Thus, negative regulation by the NOT proteins is mechanistically distinct from that mediated by *SPT6* and the putative *SPT4,5,6* complex.

Second, we analyzed basal *HIS3* transcription in a strain containing a derivative of histone H4 that lacks the amino-terminal tail and affects transcriptional activation and silencing (Kayne et al. 1988; Durrin et al. 1991). In the presence (Fig. 8B) or absence (not shown) of GCN4, this amino-terminally deleted H4 protein leads to a severe loss of the +13 transcript but does not affect the level of +1 transcription. Thus, alterations in chromatin structure affect T_R transcription preferentially, whereas NOT-mediated repression affects T_C -dependent transcription preferentially. These results suggest that the NOT proteins are not simply involved in maintaining the general repressive chromatin structure.

Table 3. Transcriptional repression by LexA hybrid proteins

	Promoter (strain)	
	<i>CYC1</i> (MY276)	<i>lexAop-CYC1</i> (MY277)
LexA hybrid proteins		
LexA-NOT1	182	172
LexA-NOT2	248	428
LexA-MyoD	110	1573
LexA-CYC8	174	7

β -Galactosidase activities (Miller units/mg protein) of strains containing the indicated LexA hybrid proteins and *CYC1-lacZ* promoters.

Figure 8. *SPT6* and the amino-terminal tail of histone H4 are not involved in NOT-mediated repression. *gcn4*-deleted strains containing *SPT6* (KY803), *spt6-14* (MY433), and *spt6-140* (MY432) analyzed 0 and 90 min after a shift to 37°C, or GCN4 containing strains that do (PKY501) or do not (PKY813) contain the amino-terminal tail of histone H4 were analyzed for *HIS3* (+1 and +13 transcripts) and *DED1* RNAs.

Discussion

The NOT proteins are global negative regulators

The basic observation that recessive, presumably loss-of-function *not* mutations cause increased transcription formally defines the NOT proteins as negative regulators. The NOT proteins inhibit the expression of genes that carry out diverse biological functions (histidine biosynthesis, pheromone response, nuclear fusion, RNA polymerase II transcription). However, the NOT proteins are unlikely to encode basic components of the RNA polymerase II transcription machinery because several genes (*DED1*, *ORF*, *TFIIB*, *TFIIA*) are unaffected in *not* mutant strains and because null alleles of *NOT2*, *NOT3*, and *NOT4* are viable.

Results presented here and elsewhere strongly support a direct role of the NOT proteins in inhibiting transcription. First, it is very unlikely that the NOT proteins inhibit transcription indirectly by activating a repressor. Increased T_C -dependent transcription is observed within 30 min of shifting a *not1-2* strain to the restrictive temperature (Collart and Struhl 1993), and similar results are obtained in *not1-1* and *not2-1* strains (data not shown). The rapid induction with loss of NOT function strongly supports a direct transcriptional effect of the NOT proteins, and it indicates that a putative repressor activated by the NOT proteins would have to be highly unstable. However, the possibility of a highly unstable repressor is inconsistent with the observation that *HIS3* transcription is not affected for several hours after treatment with cycloheximide (Cormack and Struhl 1992). Finally, transcriptional analysis of nine genes provides no evidence for positive regulation by the NOT proteins.

Second, it is also unlikely that the NOT proteins exert their effects indirectly by affecting differentially the expression of the basic transcription factors that are required for initiation by RNA polymerase II. The genes

encoding TFIIB and the small subunit of TFIIA are unaffected in *not* mutant strains (Fig. 1D), and overexpression of TBP or TFIIB does not confer the transcriptional initiation pattern (Fig. 1E) or the AT-resistance phenotype characteristic of *not* mutant strains. Furthermore, the *HIS3* initiation pattern is not altered in numerous TBP mutant strains that show a general increase in RNA polymerase II transcription because they are defective in RNA polymerase III transcription (Cormack and Struhl 1993). Conversely, reduced levels of TBP [obtained by blocking transcription of the *TBP* gene] do not affect the relative levels of the +1 and +13 transcripts (Cormack and Struhl 1992).

The NOT proteins are related functionally

The *not* mutant strains arose from the same genetic selection and have additional phenotypic similarities. Most important, mutations in all four *NOT* genes increase *HIS3* expression in the absence of GCN4 and preferentially affect T_C -dependent transcription. Preferential effects on T_C -dependent transcription are unique to NOT proteins, having never been observed in numerous other situations involving the *HIS3* promoter region described here and elsewhere. The *not* mutations also affect the same spectra of genes; transcription of *HIS4*, *TBP*, *STE4*, and *BIK1* is increased, but transcription of *DED1*, *ORF*, *TFIIB*, and *TFIIA* is not. Finally, the original *not1(cdc39)* and *not2(cdc36)* mutations have other common phenotypes including cell-cycle arrest in G_1 , pachytene arrest in meiosis, and inappropriate activation of the mating pheromone response (Reed 1980; Shuster and Byers 1989; de Barros Lopes et al. 1990); *not4(ssf1)* also activates the pheromone response pathway (K. Irie and K. Matsumoto, pers. comm.). These striking similarities of the various *not* mutant strains strongly suggests that the four NOT proteins regulate transcription negatively through the same pathway.

Aside from these phenotypic similarities, functional relationships among the NOT proteins are inferred from a variety of genetic observations. First, *not1-2* and *not2-4* are allele-specific suppressors. Second, *not2 not3* double mutant strains grow much more poorly than either of the corresponding single mutant strains. Third, *NOT3* is a high-copy and allele-specific suppressor of *not1-1*. Fourth, *NOT4* is a high-copy suppressor of *not1* and *not2* mutations. These observations do not reflect transcriptional cross-regulation because *NOT1-4* RNA levels are not affected by mutations in any of the four *NOT* genes (not shown). The functional relationships reinforce the conclusion that the NOT proteins act together in regulating transcription negatively.

Evidence for a NOT complex

Genetic and biochemical experiments demonstrate that NOT1 and NOT2 are components of a large, multiprotein complex. NOT1 and NOT2 are nuclear proteins and they cofractionate biochemically as a discrete 500-kD species. This complex is relatively resistant to ionic

strength and detergent, is separable from bulk protein in the extract, and is unlikely to contain DNA. The existence of a complex containing NOT1 and NOT2 is confirmed by the two-hybrid assay. From these results, it cannot be determined whether NOT1 and NOT2 interact directly with each other or with other proteins in the complex. However, the allele-specific suppression between the *not1-1* and *not2-4* mutations argues in favor of a direct NOT1–NOT2 interaction.

Our results suggest, but do not prove, that NOT3 and NOT4 are also part of the complex. The strongest evidence is that NOT4 interacts with NOT1 and NOT3 in the two-hybrid assay (Table 2); NOT1 and NOT3 may also interact weakly in this assay. Supporting evidence comes from the observations that overexpression of NOT3 or NOT4 suppresses the temperature-sensitive phenotypes of *not1* and *not2* mutations (Table 1). The degree of suppression depends on the gene that is overexpressed and on the mutant allele; in at least one case (high-copy *NOT3* and *not1-2*), suppression is not observed. Interestingly, *not1-2* maps between residues 371 and 1318 (M. Collart and K. Struhl, unpubl.), whereas *not1-1* is a tryptophan to arginine change of codon 1753 (Y.-K. Hong and E.O. Schuster, pers. comm.). A complex containing one molecule of each of the four NOT proteins would have a predicted molecular mass of 430 kD, a size consistent with that of the biochemically detected complex containing NOT1 and NOT2. However, we cannot exclude the possibility of multiple complexes that contain different subsets of NOT (and perhaps other) proteins.

NOT proteins define a novel transcriptional regulatory pathway

Our results suggest strongly that the NOT proteins function by a mechanism that is distinct from all previously described examples of repression in yeast. NOT-mediated repression is not attributable to inhibition of activator proteins because loss of NOT function increases T_C -dependent transcription preferentially, whereas activator proteins function almost exclusively through T_R . Consistent with this view, *not* mutations increase basal *HIS3* transcription, which occurs in the absence of known activator proteins. In *not2-4* and *not3-2* strains, the level of +1 transcription is actually reduced in the presence of *gcn4-C163*, suggesting that increased transcription upon loss of NOT function can compete with GCN4 activation. This competition is unlikely to simply reflect the proximity of the GCN4-binding site and T_C . Competition is not observed in most *not* strains, and GCN4 can activate transcription when T_R is located at the position normally occupied by T_C (Harbury and Struhl 1989). Thus, NOT-mediated repression and transcriptional activation are likely to occur by distinct pathways.

Repression by the CYC8/TUP1 complex and by SIN3 has been proposed to occur by their association with specific DNA-binding proteins that interact with subsets of promoters (Keleher et al. 1992; Wang and Stillman 1993;

D. Tzamarias and K. Struhl, in prep). This hypothesis is based primarily on the observations that *CYC8*, *TUP1*, and *SIN3* can repress transcription when bound upstream of the *CYC1* promoter by a heterologous DNA-binding domain. In contrast to these negative regulators, *NOT1* and *NOT2* are unable to repress transcription in this assay. Moreover, basal *HIS3* transcription is unaffected in *cyc8* and *tup1* deletion strains indicating that *NOT*-mediated repression does not require either *CYC8* or *TUP1*. These results suggest that the *NOT* proteins do not regulate transcription negatively by associating with promoter-specific, DNA-binding proteins. Consistent with this view, *NOT1* regulates transcription negatively from a minimal *HIS3* promoter derivative containing T_C and T_R but lacking all upstream sequences (Collart and Struhl 1993).

Negative regulation by the *NOT* proteins also appears to be mechanistically distinct from global repression mediated through chromatin. As discussed previously (Collart and Struhl 1993), nucleosome loss in vivo leads to increased *HIS3* transcription that is entirely mediated by T_R (Durrin et al. 1992). Similarly, loss of *SPT6* function leads to a *GCN4*-independent increase in the level of +13 transcript. *SPT6* is a negative regulatory protein that functions together with *SPT4* and *SPT5*, possibly by affecting chromatin structure because *spt* mutant strains are phenotypically similar to strains lacking one of the two histone H2A–H2B gene pairs (Swanson and Winston 1992). Finally, a histone H4 derivative lacking the amino-terminal tail, which is defective in activation and silencing of various genes (Kayne et al. 1988; Durrin et al. 1991), leads to a nearly complete loss of +13 transcription. In all of these situations, the level of the +1 transcript is unaffected, suggesting that T_C -dependent transcription is not controlled by the state of chromatin structure. Thus, it seems unlikely that the *NOT* proteins are involved in maintaining the normal repressive chromatin structure, although more complex effects on chromatin cannot be excluded.

NOT proteins may affect the RNA polymerase II transcription machinery

We suggest that the *NOT* proteins inhibit a basic transcription factor, thereby making it limiting for initiation. The TBP is an attractive candidate because the *NOT* proteins distinguish between T_C and T_R , which differ with respect to TBP binding and TBP-dependent transcription in vitro (Ponticelli and Struhl 1990) yet require TBP for transcription in vivo (Cormack and Struhl 1992). Competition between *NOT*-mediated inhibition of TBP and specific binding of TBP to the TATA element might explain why T_C , a nonconsensus and poor binding site (Mahadevan and Struhl 1990; Ponticelli and Struhl 1990), is affected more strongly than T_R , a consensus and efficient binding site (Chen and Struhl 1988; Wobbe and Struhl 1990). Alternatively, the *NOT* proteins might act through TBP-associated proteins that are part of the yeast TFIID complex (Thompson et al. 1993) or through TFIIA, which interacts with TBP and affects TATA-ele-

ment binding (Buratowski and Zhou 1992; Lee et al. 1992). Biochemical activities that inhibit TFIID function in vitro have been described (Meisterernst and Roeder 1991; Meisterernst et al. 1991; Inostroza et al. 1992; Auble and Hahn 1993), but their biological roles are unknown.

Although differential gene regulation is largely the result of the individual and combinatorial properties of specific DNA-binding activators and repressors, functional distinctions between TATA elements also play a role (Struhl 1986; Homa et al. 1988; Simon et al. 1988; Harbury and Struhl 1989; Wefald et al. 1990). In yeast, where unrelated genes are often closely packed, functionally distinct TATA elements are likely to be important for independently regulating genes that are transcribed divergently and share upstream promoter elements; such a situation occurs in the *HIS3*–*PET56* promoter region (Struhl 1986). Thus, negative regulation by the *NOT* complex might represent part of the mechanism by which yeast cells distinguish functionally among TATA elements at various promoters.

Materials and methods

Yeast strains

The yeast strains used in this work are listed in Table 4 and were generated by standard genetic techniques. The *not* mutations were isolated from strains KY1600 (Collart and Struhl 1993) and KY1601, derivatives of KY803 (Hope and Struhl 1986) and KY804 that contain YCp88–Sc4363, a *URA3* centromeric vector that expresses *gcn4*–C163 from the *DED1* promoter, and YCp87–Sc3866, a *LEU2* centromeric vector containing a *his3*–*lacZ* fusion under the control of a promoter with an optimal AP-1 site upstream of the *HIS3* TATA region. KY804 is an isogenic α derivative of KY803, which was obtained by *HO*-mediated mating-type switching (Herskowitz and Jensen 1991). The *not* mutant strains were generated as spontaneous revertants that grew on glucose minimal plates containing 20 mM AT and were blue on X-gal indicator plates (Collart and Struhl 1993). To analyze basal *HIS3* transcription in the *not* strains, both plasmids were cured. Disruption of the *HIS3* locus was performed by one-step gene replacement with Sc3445, which carries the *his3*::*TRP1* allele (Struhl 1987). To eliminate *TUP1* and *CYC8* function, *tup1*::*LEU2* (lacks *TUP1* codons 70–262) and *cyc8*::*LEU2* (lacks *CYC8* codons 99–862) substitution alleles were introduced into KY803 by one-step gene replacement. Iso-genic derivatives of KY803 carrying the *spt6*–140, *spt6*–14, and *not1*–1 alleles were generated by two successive gene replacement events with pAB56 and pAB75 DNAs (obtained from A. Bortvin, Harvard Medical School) or with Ylp5–*not1*–1 DNA (obtained from Y.-K. Hong and E.O. Schuster). The *not3*::*URA3* and *not4*::*URA3* derivatives of KY803 were obtained by one-step gene replacement with Sc3877 and Sc3883.

DNA manipulations

NOT2 was cloned by complementation of the *not2*–1 mutation. A YCp50 library containing partial *Sau3A* fragments of yeast genomic DNA (obtained from M. Rose, Princeton University, NJ) was introduced into MY27 and selected for growth at 37°C. Four plasmids containing 15–20 kb of yeast DNA were recovered from such transformants and shown to complement both phenotypes. The following fragments were subcloned into

Table 4. Strain list

Strain	Genotype	Source
KY803	a <i>ura3-52 trp1-Δ1 leu2::PET56 gal2 gcn4-Δ1</i>	Hope and Struhl (1986)
KY804	isogenic to KY803 except α	this work
KY1600	KY803 (YCp88-gcn4-C163 + YCp87-Sc3866)	Collart and Struhl (1993)
KY1601	isogenic to KY1600 except α	this work
MY28	KY804 <i>not1-2</i>	Collart and Struhl (1993)
MY27	KY803 <i>not2-1</i>	this work
MY22	KY803 <i>not2-4</i>	this work
MY25	KY803 <i>not3-2</i>	this work
MY21	KY803 <i>not3-4</i>	this work
MY18	KY803 <i>not3-5</i>	this work
MY20	KY803 <i>not4-1</i>	this work
MY508	KY803 <i>not3::URA3</i>	this work
MY509	KY803 <i>not1-1</i>	this work
MY520	KY803 <i>not4::URA3</i>	this work
MY146	KY803 <i>not2-1 not3-5</i>	this work
MY42	KY803 <i>not1-2 not2-1</i>	this work
MY210	KY803 <i>not1-2 not2-4</i>	this work
MY340	MY804 <i>not1-2</i> (bc9b-HA1-NOT1)	this work
MY55	KY804 <i>not1-2 his3::TRP1</i>	Collart and Struhl (1993)
MY66	KY803 <i>not3-2 his3::TRP1</i>	this work
MY32	KY803 <i>not4-1 his3::TRP1</i>	this work
ELY54	a <i>not1-1 ace6 his4 lys2 trp1</i>	Steve Reed (pers. comm.)
EGY42	a <i>trp1 ura3 his3 LEU2::plexAop6-LEU2</i>	Zervos et al. (1993)
MY94	a <i>ade1 his2 leu2 trp1 ura3 not2::TRP1</i>	Steve Reed
MY96	KY804 Δ <i>cyc8::LEU2</i>	this work
MY97	KY804 Δ <i>tup1::LEU2</i>	this work
MY276	a <i>his3-Δ200 ura3-52 trp1-Δ63 ura3::CYC-lacZ</i>	D. Tzamarias and K. Struhl
MY277	isogenic to MY276 except <i>ura3::lexAop-CYC-lacZ</i>	D. Tzamarias and K. Struhl
MY432	KY803 <i>spt6-140</i>	this work
MY433	KY803 <i>spt6-14</i>	this work
PKY501	a <i>ade2-101 arg4-1 his3-Δ200 leu2-3, 112 lys2-801 trp1-Δ1 ura3-52 thr tyr Δhhf1::HIS3 Δhhf2::LEU2</i> (pURA3 CEN3 ARS1 HHF2)	Kayne et al. (1988)
PKY813	PKY501 except (pURA3 CEN3 ARS1 hhf2-Δ4-28)	Kayne et al. (1988)

pRS306 or pRS316 (Sikorski and Hieter 1989): Sc3867, 3-kb *EcoRI* fragment present in all four clones; Sc3868, an adjacent *EcoRI*-*BglII* fragment; Sc3869, an internal *NdeI* deletion of Sc3867; Sc3870, a *SacI*-*PstI* fragment of Sc3867. For overexpression, Sc3870 was cloned into YEplac195 (Gietz and Sugino 1988). For two-hybrid assays, *NOT2* protein-coding sequences from the initiation codon were obtained by PCR amplification of Sc3870 with GCCGAATTCATGGAATAATTTGGTTTAAAGCGCTAGTACCGC and CCGTCGACCTGCAGACCTTTCCCTATCGCC, digested by *EcoRI* and *SalI*, and ligated into Lex202 and pJG4-5 (Zervos et al. 1993).

NOT3 was cloned by complementation of the *not3-5* allele by introducing the library described above into MY146 and selecting transformants that grew better than untransformed cells at 25°C but did not grow at 37°C. A single plasmid containing 15 kb of yeast DNA (Sc3876) was recovered and shown to complement the growth and AT-resistance phenotypes. YCp50 derivatives containing Sc3871, Sc3872, and Sc3874 were obtained by digesting YCp50-Sc3876 with *EagI*, *SacI*, and *Clal*, respectively, and religating the largest fragments. Sc3873, a *Clal*-*SacI* fragment of Sc3874, was partially sequenced. For two-hybrid assays, *NOT3* protein-coding sequences from the initiation codon were obtained by cloning the *SacI*-*SalI* fragment obtained by PCR amplification of Sc3874 with CCGTCGACAGTTTATGCCCATTCGCTATTCAACATGTTTGGTGG and CGGGAGCT-

CCTCACATCATCCTTTAGTTTCCTTGGCGACAAATCC, and an *EcoRI*-*SacI* fragment generated by PCR amplification of Sc3873 using the forward primer and GCCGAATTCATGGCTCATAGAAAATTACAGCAGGAGGTCGATAGGG, into Lex202 and pJG4-5 (Zervos et al. 1993). For *NOT3* overexpression, the *Clal*-*SacI* fragment of Sc3873 and the *SacI*-*NdeI* fragment of Sc3875 were cloned into *SalI*-*BamHI*-digested YEplac112 (Gietz and Sugino 1988).

To generate LexA-NOT1 and B42-NOT1, *NOT1* sequences were PCR amplified from Sc3863 (Collart and Struhl 1993) with GCCGAATTCATGCTATCGGCCACATACCGTGATTGAACACAGC and GTTGAGCTCTTATTTGTTCCGAGGTTAGGATTTGGGTAGGC, and digested with *EcoRI* and *SacI*. This fragment was combined with *SacI*-*BglII*-cleaved Sc3861 (Collart and Struhl 1993) and then cloned into Lex202 and pJG4-5 digested with *EcoRI* and *BamHI* (Zervos et al. 1993). *cyc8::LEU2* was generated by cloning the *XhoI*-*SalI* *LEU2* fragment between the *PstI* sites within the *CYC8* gene. *tup1::LEU2* was generated by cloning the same *LEU2* fragment between the *EcoRI* and *BamHI* sites in *TUP1*.

NOT4 was cloned by complementation of the *not4-1* mutation by introducing the library described above into MY20 and selecting transformants that grew better than untransformed cells. Two plasmids containing 10 kb of yeast DNA were recovered and shown to complement the AT-resistant phenotype.

pRS316–Sc3878 was obtained by cloning a 6.3-kb *HindIII*–*EagI* fragment from the initial clones containing a 6-kb *HindIII*–*EcoRI* fragment of genomic DNA into pRS316. Partial sequencing around the *SmaI*, *BamHI*, and *EcoRI* sites revealed that this gene was identical to *SSF1*, a gene isolated by K. Irie and K. Matsumoto. For overexpression of *NOT4*, the 6-kb *HindIII*–*EcoRI* fragment of Sc3878 was cloned into YEplac112 and YEplac181. For two-hybrid assays, *NOT4* protein-coding sequences from the initiation codon were amplified by PCR amplification of Sc3878 with GCCCGTCGACCAACCAACT-GCGCAAGAGATTTTCGTTG and GCCGAATTCATGATGA-ATCCACACGTTCAAGAAATTTGC, digested by *EcoRI* and *SalI* and cloned into Lex202 and pJG4-5.

RNA analysis

Total RNA (30 µg) from cells grown under appropriate conditions were hybridized to completion with an excess (2 ng) of the appropriate oligonucleotides, and the products were digested with S1 nuclease and electrophoretically separated as described previously (Collart and Struhl 1993). All hybridization reactions contained multiple probes to ensure that the determinations were controlled internally. Northern blots were performed as described previously (Collart and Struhl 1993) using the following probes: a 6-kb *HindIII* fragment containing *BIK1* and *ORF* (Trueheart et al. 1987); a *TFIIA* fragment obtained by PCR amplification of genomic DNA with GATCGAATTCATGAAC-TGAATTGTCAATAC and GAAGGATTCCATTATTTTCAT-GACACTGTTCC; artificially generated *NdeI*–*BamHI* and *EcoRI*–*SalI* restriction fragments containing the entire *TBP*- and *TFIIB*-coding sequences; an 11-kb *BamHI*–*SphI* fragment from EB125 (Collart and Struhl 1993) for *STE4*.

Subcellular localization of *NOT2*

Total cellular, nuclear, and cytoplasmic extracts were prepared as described previously (Collart and Struhl 1993). Total protein from each extract (50 µg as quantitated by the Bio-Rad/Bradford assay) was separated on a 12% SDS–polyacrylamide gel, and electroblotted on to polyvinylidene difluoride membranes. *NOT2* was detected with a polyclonal antibody (7412 kindly supplied by S. Reed) used at a 1:500 dilution, followed by a 1:5000 dilution of goat anti-rabbit IgG-specific antibody conjugated to alkaline phosphatase.

β -Galactosidase assays

For two-hybrid assays, stationary cells were diluted 50-fold in minimal medium containing 2% raffinose, grown for 12 hr, washed twice, and grown 4–5 hr in minimal medium containing 4% galactose. For experiments not involving galactose induction, cells were grown to log phase in glucose minimal medium. For experiments testing repression, cells were grown from inoculation of a single colony to an OD < 1. In all cases, cells were spun down and lysed with glass beads in 0.1 M Tris (pH 8), 20% glycerol, 1 mM DTT and 2 mM PMSF; β -galactosidase activities were normalized to protein concentration.

Gel filtration

Cells (100 ml) were grown overnight in glucose medium containing casamino acids to late log phase and washed once in buffer [40 mM HEPES (pH 7.2), 5% glycerol, 350 mM NaCl, 0.1% Tween, and protease inhibitors]. The cell pellet was resuspended in buffer at 100–200 OD/ml (2.5 ml) and glass beads were added. Cells were broken by vortexing four times for 20

sec, and the extract was clarified by centrifugation at 100,000g for 1 hr. The supernatant was filtered through 0.45-µm pores, and 250 µl of the extract was loaded on a fast protein liquid chromatography Superose 6 column. The column was washed with buffer, and 250 µl fractions were collected. Proteins from these fractions were precipitated with TCA and analyzed by Western blotting using rabbit polyclonal antibodies to *NOT2* (7412 from S. Reed), or the HA1-specific monoclonal antibody that recognizes epitope-tagged *NOT1* (Collart and Struhl 1993). A mixture of five proteins (0.5 mg/ml of blue dextran, 1 mg/ml of human IgG, 1 mg/ml of BSA, 1 mg/ml of cytochrome *c*, and 4 mg/ml of aprotinin) was analyzed under the same conditions to determine the elution volumes of proteins with known molecular weights. To determine the extent of DNA contamination, 125 µl of total cell extract, with or without RNase A treatment, was analyzed by agarose gel electrophoresis; DNA was not detected.

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Note added in proof

We recently found that the *NOT4* sequence has been described previously as *SIG1*, a gene involved in G-protein-mediated pheromone response (E. Leberer, D. Dignard, D. H Marcus, M. White-way, and D.Y. Thomas, unpubl.; accession number M96736).

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