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_R-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 TFID 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.

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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
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 +13 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
suggests that in some cases, increased Tc-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 TFIIA 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 TFIIA and the small subunit of TFIIA are unaffected in not mutant strains indicates that NOT-mediated repression is not attributable to altered levels of TFIIA 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 G1 and constitutively ac-
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A

| Sc3867 | Sc3868 | Sc3869 | Sc3870 |
|--------|--------|--------|--------|
| +      | -      | -      | +      |

37°C AT

B

| Sc3876 | Sc3871 | Sc3872 | Sc3873 | Sc3874 | Sc3875 | Sc3877 |
|--------|--------|--------|--------|--------|--------|--------|
| +      | -      | -      | +      | +      | +      | -      |

1 kb

C

| Sc3878 | Sc3879 | Sc3880 | Sc3881 | Sc3882 | Sc3883 |
|--------|--------|--------|--------|--------|--------|
| +      | -      | -      | -      | -      | -      |

Growth AT

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) BamHI; (Bg) BglII; (C) ClaI; (E) EcoRI; (H) HindIII; (N) NdeI; (P) PstI; (R) EcoRI; (S) SacI; (Sm) SmaI; (Xb) XbaI; (X) Xhol.

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volve 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.].

Table 1. High-copy suppression

| not allele | NOT1 | NOT2 | NOT3 | NOT4 |
|------------|------|------|------|------|
| not1-1     | +    | ±    | +    | +    |
| not1-2     | +    | -    | -    | +    |
| not2-1     | -    | +    | +    | +    |
| not2-2     | N.T. | +    | N.T. | N.T. |
| not2-3     | -    | -    | +    | +    |
| not4-1     | -    | -    | -    | +    |

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.
NOT proteins differentially affect TATA utilization
determined by hybridization to the collection of \( \lambda \) bacterial phages.

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 [Sc3887] and found it to grow normally at all temperatures tested on rich medium. This strain has the same A1-resistance phenotype as the originally isolated not3 mutant strains, and it shows the same preferential increase in Tc-dependent transcription [data not shown].

NOT4 is identical to SSFl 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 SSFl, 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 ssfl 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 Tc-dependent transcription [data not shown]. As determined by hybridization to the collection of \( \lambda \) bacterial phages, 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 not1-1 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](image-url)
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 /3-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 p-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 containing 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 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.

Table 2. Two-hybrid assays

| LexA hybrid proteins | B42 activation domain hybrid proteins |
|----------------------|--------------------------------------|
| LexA-NOT1            | B42-NOT1 B42-NOT2 B42-NOT3 B42-NOT4 |
| LexA-NOT2            | 1060 630 21 17 |
| LexA-NOT3            | 870 1060 21 15 |

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

![Table 2](image)

Figure 5. Subcellular localization of NOT2. Protein from total (Tot), nuclear (Nuc), and cytoplasmic (Cyt) extracts prepared from not2Δ [MY340] or not2::TRPI [MY94] strains analyzed by Western blotting using specific antibodies to the NOT2 protein. The position of the 22-kD NOT2 protein is indicated.

![Figure 5](image)

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.
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 DEDI 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 Tc-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 +1 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 +1 transcript but does not affect the level of +1 transcription. Thus, alterations in chromatin structure affect TR transcription preferentially, whereas NOT-mediated repression affects TC-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

| LexA hybrid proteins | CYC1 [MY276] | lexAop–CYC1 [MY277] |
|----------------------|--------------|---------------------|
| 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, TFIIF] 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 TC-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 TFII B and the small subunit of TFIIA are unaffected in not mutant strains (Fig. 1D), and overexpression of TBP or TFII B 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 Tc-dependent transcription. Preferential effects on Tc-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, TFII B, and TFIIA is not. Finally, the original not1(cdc39) and not2(cdc36) mutations have other common phenotypes including cell-cycle arrest in G1, 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(ssl1) also activates the pheromone response pathway (K. Irie and K. Matsumoto, pers. comm.). These striking similarities of the various not mutant strains strongly suggest 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 Tc-dependent transcription preferentially, whereas activator proteins function almost exclusively through Tc. 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 simplify reflect the proximity of the GCN4-binding site and Tc. Competition is not observed in most not strains, and GCN4 can activate transcription when Tc is located at the position normally occupied by Tc (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 spI 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 +13 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 \( T^+ \) and TR, which functions together with SPT4 and SPT5, possibly by affecting chromatin structure because spI 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 +13 transcript is unaffected, suggesting that \( T_R \)-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.

NOT2 was cloned by complementation of the not1-1 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 ura3-52 trp1-Δ1 leu2::PET56 gal2 gcn4-Δ1 | 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 not1-2                    | Collart and Struhl [1993] |
| MY27     | KY803 not2-1                    | this work               |
| MY22     | KY803 not2-4                    | this work               |
| MY25     | KY803 not3-2                    | this work               |
| MY21     | KY803 not3-4                    | this work               |
| MY18     | KY803 not3-5                    | this work               |
| MY20     | KY803 not4-1                    | this work               |
| MY508    | KY803 not3::URA3                | this work               |
| MY509    | KY803 not1-1                    | this work               |
| MY520    | KY803 not4::URA3                | this work               |
| MY146    | KY803 not1-2 not3-5             | this work               |
| MY42     | KY803 not1-2 not2-1             | this work               |
| MY210    | KY803 not1-2 not2-4             | this work               |
| MY340    | MY804 not1-2 (bc9b-HA1–NOT1)    | this work               |
| MY55     | KY804 not1-2 his3::TRP1         | Collart and Struhl [1993] |
| MY66     | KY803 not3-2 his3::TRP1         | this work               |
| KY94     | KY803 not1-1 his3::TRP1         | this work               |
| ELY54    | a not1-1 ac66 his4 lys2 trp1    | Steve Reed [pers. comm.]|
| ECY42    | a trp1 ura3 his3 LEU2::pLexAop6–LEU2 | Zervos et al. [1993]    |
| MY94     | a ade1 his2 leu2 trp1 ura3 not2::TRP1 | Steve Reed              |
| MY96     | KY804 Δcyc8::LEU2               | this work               |
| MY97     | KY804 Δtup1::LEU2               | this work               |
| MY276    | a his3-Δ200 ura3-52 trp1-Δ3 ura3::CYC–lacZ | D. Tzamaris and K. Struhl |
| MY277    | isogenic to MY276 except ura3::lexAop–CYC–lacZ | D. Tzamaris and K. Struhl |
| MY432    | KY803 spot6-140                 | this work               |
| MY433    | KY803 spot6-14                  | this work               |
| PKY01    | a ade2-101 arg4-1 his3-Δ200 leu2-3, 112 lys-2801 | Kayne et al. [1988] |
| PKY813   | PKY01 except [pURA3 CEN3 ARS1 hhj2-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 GCGGAATTCATGCGACAAATAACCTTAAAGCGTGATACCGC and GCCCTCGAGCTCGACATGCAATTCTCCCTGCAATCCCAAAACGTAACTACCCACTCATCATCCCTTTGTTTCTGACGCGAACACTATCC, and an EcoRI–SalI fragment generated by PCR amplification of Sc3873 using the forward primer and GCGGAATTCATGCTGAGGAAGTTTGATTTCACAACGAC and GGTGAGCTCTTATTGTTGGAGGTATTGGAGATTGGAGGCTAATG, 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–BanHI-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 GCCGAATTCATGCTGAGGAAGTTTGATTTCACAACGAC and GGTGAGCTCTTATTGTTGGAGGTATTGGAGATTGGAGGCTAATG, 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–BanHI-digested YEplac112 [Gietz and Sugino 1988].

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 GCCCTCGAGCTCGACATGCAATTCTCCCTGCAATCCCAAAACGTAACTACCCACTCATCATCCCTTTGTTTCTGACGCGAACACTATCC, and an EcoRI–SalI fragment generated by PCR amplification of Sc3873 using the forward primer and GCGGAATTCATGCTGAGGAAGTTTGATTTCACAACGAC and GGTGAGCTCTTATTGTTGGAGGTATTGGAGATTGGAGGCTAATG, 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–BanHI-digested YEplac112 [Gietz and Sugino 1988].

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–Eagl fragment from the initial clones containing a 6-kb HindIII–EcoRI fragment of genomic DNA into pRS316. Partial sequencing around the Smal, BamHI, and EcoRI sites revealed that this gene was identical to SSFI, 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 GCCGGTTCGACCAACCAACACTGCAGAAGATTTGTTG and GCCGAAATTCGATGAAATTCC; artificially generated restriction fragments containing the entire TBP–TfIIA–BP1–TfIIA–Sphl–coding sequences; an U-kb Smal, BamHI, EcoRI fragment of genomic DNA into pRS316. Partial sequencing during the course of the experiments. This work was supported by a postdoctoral fellowship to M.A.C. from the Swiss National Fund and by grant GM30186 to K.S. from the National Institutes of Health.

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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 phosphatase response [E. Leberer, D. Dignard, D. Harcus, M. White, and D.Y. Thomas, unpubl.; accession number M96736].

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