Repression of Promoter Activity by CNOT2, a Subunit of the Transcription Regulatory Ccr4-Not Complex*

The evolutionary conserved Ccr4-Not complex controls mRNA metabolism at multiple levels in eukaryotic cells. Genetic analysis of not mutants in yeast identifies a negative role in transcription, which is dependent on core promoter structure. To obtain direct support for this we targeted individual core subunits of the human Ccr4-Not complex to promoters in transient transfections of human cells. In this experimental setup we found that the CNOT2 and CNOT9(hRcd1/hCaf40) subunits act as repressors of reporter gene activity. Interestingly, recruitment of other Ccr4-Not subunits did not affect the reporter gene. The major repression function of CNOT2 is localized in a specialized protein motif, the Not-Box. This conserved motif is present in all CNOT2 orthologs and surprisingly also in CNOT3 orthologs. Repression by the Not-Box was sensitive to treatment with the histone deacetylase inhibitor trichostatin A. In addition, mutation of a canonical TATA-box enhanced repression. Our experiments show for the first time direct regulation of promoter activity by components of the Ccr4-Not complex.

Regulation of transcription by RNA polymerase II (pol II) requires the interplay of many transcription factors at different levels (1). This involves proteins recognizing gene-specific DNA elements, which can recruit transcriptional coactivator and corepressor complexes. Several of these cofactors mediate their effect via chromatin, either by ATP-dependent remodeling of nucleosomal structures or by altering the modification status of histone proteins. The latter involves phosphorylation, methylation, or acetylation/deacetylation of histones (2). The end point of transcriptional regulatory pathways is recruitment of the basal pol II transcription machinery. This machinery assembles at the core promoter of the gene, which often contains a combination of the TATA-box and DNA elements like the initiator and the downstream promoter element (3, 4). Within the basal machinery two large protein complexes, TFIIID and Mediator, are focal points for transcriptional regulation because they can interact with multiple gene-specific activators. Interestingly, these evolutionary conserved complexes can synergize in activation in vitro (5). The TFIIID complex, consisting of TATA-binding protein in combination with 13–14 TATA-binding protein-associated proteins (6), nucleates preinitiation complex assembly by binding to the core promoter (7, 8). The Mediator complex consisting of more than 20 subunits bridges activators with pol II (9–11). In crude extracts Mediator complexes are also required for basal levels of transcription (12–14).

It has become clear that several other regulators control the basal transcription machinery at the level of the core promoter (for reviews, see Refs. 3, 15, and 16). The Ccr4-Not complex is a key representative of this class of regulators. This complex is evolutionarily conserved and consists of a core of nine subunits (for reviews, see Refs. 17 and 18). The five NOT (negative on TATA-less) genes were isolated in the yeast Saccharomyces cerevisiae via genetic screens for an increased transcription from the TATA-less promoter of the HIS3 gene. Besides the Not proteins, the core Ccr4-Not complex of ~1.0 MDa also harbors Ccr4p, Pop2/Caf1, Caf40, and Caf130. Only Not1p is essential for yeast cell viability, but in combinations more deletions of two nonessential genes results in lethality (19). Two-hybrid interaction studies indicate that the 240-kDa Not1p protein acts as the scaffold for the complex, organizing the Ccr4p and Caf1 at a central region and the Not2p/Not3p/Not4p/Not5p module at its C terminus (17). Recent biochemical analysis revealed two different enzymatic activities associated with the core complex. First, the Ccr4p and Caf1p contain a 3′–5′ exonuclease activity involved in degradation of mRNA via poly(A) tail shortening (20–23). Second, the human ortholog of Not4p, CNOT4, contains ubiquitin protein ligase activity (24). The relevance of these findings for transcriptional regulation by the Ccr4-Not complex is yet an unresolved question.

Results from in vitro protein-DNA cross-linking studies in yeast indicate that the Not2p, Not5p, and Ccr4p subunits associate with promoter DNA, and it was suggested that the Ccr4p activator is involved in promoter recruitment of components of the Ccr4-Not complex (25, 26). Several genetic and biochemical analyses in yeast provide links between Ccr4-Not proteins and the TFIIID and Mediator complexes (for review, see Ref. 18). For example, epitope-tagged Not5p can retain several TATA-binding protein-associated proteins from yeast extracts (26, 27). Furthermore, mutant alleles of four core subunits (NOT1, NOT3, NOT5, and CAF1) can suppress a ts allele of SBR4 (28), which encodes an essential subunit of Mediator. This finding is consistent with the proposed repressive function of the Ccr4-Not complex. However, recent analyses obtained in mammalian cells indicate that Ccr4-Not core subunits are involved in transcriptional activation. The murine CAF40 ortholog, CNOT9(mRcd1/mCaf40), is reported to be part of the core complex.
Transcriptional Repression by CNOT2

RESULTS

The Ccr4-Not Subunits CNOT2 and CNOT9 (hRcd1/hCAF40) Repress Reporter Gene Activity—To investigate the ability of the human Ccr4-Not complex to regulate transcription the individual subunits (CNOT2, CNOT3, CNOT4, CNOT8, CNOT9 (hRcd1/hCAF40)), and the C-terminal 1,290 residues of CNOT1) were targeted to a pol II promoter by fusing each subunit to the Gal4 (1–147) DBD. As a positive control a construct expressing the Sin3 interaction domain, amino acids 1–35, of Mad1 fused to the Gal4 (1–94) DBD was used (38). The Gal4 expression plasmids were cotransfected into human osteosarcoma U2OS cells with the firefly luciferase reporter plasmid 7xGalTKLuc, which contains seven Gal4 binding sites fused to the herpes simplex virus thymidine kinase (TK) promoter, or with the control reporter plasmid TKLuc lacking any Gal4 binding sites. To correct for differences in transfection efficiency a plasmid expressing the Renilla luciferase gene under control of the strong CMV promoter was also included in each transfection.

Fig. 1 shows that cotransfection of the Gal4-CNOT2 and Gal4-CNOT9 plasmids resulted in 8- and 6-fold reduction of luciferase activity, respectively. These effects were dependent on the presence of Gal4 binding sites in the promoter. Luciferase expression from the TKLuc control was only weakly affected by Gal4-CNOT2 (1.5-fold) or Gal4-CNOT9 (2.1-fold). As expected, cotransfection of Gal4-MAD plasmid reduced luciferase expression. Analysis of transfected cell lysates by immunoblotting indicated that all Gal4 fusion proteins are expressed and the expected size (data not shown). Repression by CNOT2 was also observed using ts20 (Chinese hamster lung), 911 (adenovirus-transformed human embryonic retina), and 293T (adenovirus-transformed human embryonic kidney) cell lines (data not shown).

Our experiments showed that only two of the six Ccr4-Not subunits tested here affect promoter activity. Others found that artificial recruitment of CNOT6 (hCcr4) also does not influence TK promoter activity (39). Although we did not test the CNOT7 (hCaf1) subunit, based on our results with its paralog CNOT6 (hPop2/Caf1) we expect that CNOT7 will...
also not affect promoter activity. In conclusion, we observed that promoter targeting of the CNOT2 and CNOT9 subunits of the Ccr4-Not complex resulted in a strong repression of reporter gene activity.

The Conserved C-terminal Part of CNOT2 Contains the Major Repression Function—To investigate further the strong repressive effect on transcription by CNOT2 we decided to map the region responsible for this. The human CNOT2 gene displays a strong sequence homology with the yeast ortholog Not2p in the last 190 residues (32). In addition, two short amino acid stretches (258–285 and 333–365) of CNOT2 share homology with orthologs from other metazoans but are absent in yeast orthologs. We constructed CMV promoter-based expression plasmids for CNOT2 deletions in the context of Gal4 fusions as indicated (Fig. 2A). The various Gal4-CNOT2 constructs were transiently transfected into U2OS cells together with the 7xGalTKLuc reporter plasmid. As expected full-length CNOT2 strongly repressed luciferase activity (9.1-fold). Constructs only expressing the nonconserved N-terminal part of CNOT2 did not repress the reporter. On the other hand the C-terminal constructs, spanning amino acid regions 256–540 or 334–540, repressed transcription between 8- and 10-fold. Progressive truncations indicated that the CNOT2(437–540) region is minimally required to observe the repressive effect, although it is reduced to 5-fold. Truncating 46 residues from the C-terminal end of CNOT2 completely abolished repression of the reporter as evidenced by the CNOT2(334–494) construct. Immunoblot analysis of transfected 293T cell lysates using a monoclonal antibody specific for Gal4 indicated that all proteins were expressed and of the expected size (Fig. 2B). Although some Gal4 fusions were expressed at higher levels than others, this variation did not correlate to repression properties. For example, the CNOT2(386–540) fusion is expressed to lower levels than CNOT2(1–225) or CNOT2(349–540) but showed a strong reduction in reporter activity. In conclusion, we found that the major repression function of CNOT2 is contained in the last 104 residues of the C-terminal conserved region of the protein.

Sequences Similar to the CNOT2 Region Responsible for Repression Are Also Present in CNOT3 Orthologs—Analysis of the genomic data bases for proteins containing sequences homologous to the minimal repression domain of CNOT2 revealed that this region is not unique to CNOT2 and its orthologs. Surpris-
right quadrant). Clearly, yeast Not3p and Not5p show the lowest percentage identity, and when they are excluded the lower limit is 62%. Comparison of the Not-Box of the CNOT2 and CNOT3 proteins within one species indicates that the identity ranges between 19 and 58% (depicted in bold). Clearly, Not-Boxes of CNOT2 orthologs are more similar among each other than when compared with Not-Boxes from CNOT3 orthologs and vice versa.

Taken together, we found that the region of CNOT2 capable of transcription repression is not only conserved between CNOT2 orthologs, but is also present in the C-terminal regions of CNOT3 orthologs. In addition, the sequence comparisons indicated that these Not-Boxes were separated into CNOT2 and CNOT3 types of Not-Boxes.

The Not-Box Is an Autonomous Transcription Repression Domain in Mammalian Cells—The finding that the C-terminal part of human CNOT2 can repress reporter gene activity was surprising in light of the findings of Struhl and co-workers (40), who reported that promoter targeting of yeast Not2p resulted in strong transcriptional activation of a yeast promoter. We also noted that fusion of human CNOT2 to the LexA DBD could strongly activate a promoter bearing LexA binding sites in yeast (32). To investigate this issue further we decided to test the transcriptional effect of yeast Not2p and its isolated Not-Box in human cells. In addition, we decided to analyze transcriptional properties of the isolated Not-Box of CNOT3 as we found that promoter targeting of full-length CNOT3 did not alter reporter gene activity (Fig. 1).

To this end gene fusions were constructed of the Gal4(1–147)-DBD with full-length yeast Not2p, CNOT3, the Not-Boxes of yNot2p (residues 96–191) or of CNOT3 (residues 661–753) under control of the CMV promoter. The effects of these expression plasmids on reporter gene activity were tested by transfection into U2OS cells. Interestingly, a clear repression of reporter activity, which was dependent on the presence of Gal4 binding sites, was observed for both full-length yNot2p and its isolated Not-Box (Fig. 4A, compare 7xGalTKLuc with TKLuc). In contrast to full-length CNOT3, the isolated Not-Box from CNOT3 reduces expression from the Gal4 binding site reporter gene (7xGalTKLuc) with TKLuc. We repeated this experiment in 293T human embryonic kidney cells and also observed Gal4 binding site-dependent repression by yeast Not2 (data not shown). However, in this cell line expression of the fusion of Gal4 with yeast Not2 also represses the TKLuc reporter construct lacking Gal4 binding sites. The reason for this is unclear to us. Immunoblot analysis of the transfected cell lysates indicated that proteins of the expected size were expressed (Fig. 4B). This also showed that the inability of the full-length CNOT3 fusion to repress transcription is not caused by low levels of expression as it is expressed to higher levels than the NOT2 fusion proteins (see also Supplemental Fig. 1).

In conclusion, our experiments showed that the isolated Not-Boxes from yNot2 and CNOT3 also repressed gene activity upon promoter targeting.

Not-Box-mediated Repression Is Sensitive to the Histone Deacetylase Inhibitor TSA—Our experiments suggest that the Not-Box motif as present in CNOT2 and CNOT3 orthologs can actively repress transcription. At present it is not evident how the Ccr4-Not complex is recruited to promoters, but its effect is clearly dependent on the presence of Gal4 binding sites, was observed for both full-length yNot2p and its isolated Not-Box (Fig. 4A, compare 7xGalTKLuc with TKLuc). In contrast to full-length CNOT3, the isolated Not-Box from CNOT3 reduces expression from the Gal4 binding site reporter gene (7xGalTKLuc) with TKLuc. We repeated this experiment in 293T human embryonic kidney cells and also observed Gal4 binding site-dependent repression by yeast Not2 (data not shown). However, in this cell line expression of the fusion of Gal4 with yeast Not2 also represses the TKLuc reporter construct lacking Gal4 binding sites. The reason for this is unclear to us. Immunoblot analysis of the transfected cell lysates indicated that proteins of the expected size were expressed (Fig. 4B). This also showed that the inability of the full-length CNOT3 fusion to repress transcription is not caused by low levels of expression as it is expressed to higher levels than the NOT2 fusion proteins (see also Supplemental Fig. 1).

In conclusion, our experiments showed that the isolated Not-Boxes from yNot2 and CNOT3 also repressed gene activity upon promoter targeting.

Not-Box-mediated Repression Is Sensitive to the Histone Deacetylase Inhibitor TSA—Our experiments suggest that the Not-Box motif as present in CNOT2 and CNOT3 orthologs can actively repress transcription. At present it is not evident how the Ccr4-Not complex is recruited to promoters, but its effect is clearly dependent on the presence of Gal4 binding sites, was observed for both full-length yNot2p and its isolated Not-Box (Fig. 4A, compare 7xGalTKLuc with TKLuc). In contrast to full-length CNOT3, the isolated Not-Box from CNOT3 reduces expression from the Gal4 binding site reporter gene (7xGalTKLuc) with TKLuc. We repeated this experiment in 293T human embryonic kidney cells and also observed Gal4 binding site-dependent repression by yeast Not2 (data not shown). However, in this cell line expression of the fusion of Gal4 with yeast Not2 also represses the TKLuc reporter construct lacking Gal4 binding sites. The reason for this is unclear to us. Immunoblot analysis of the transfected cell lysates indicated that proteins of the expected size were expressed (Fig. 4B). This also showed that the inability of the full-length CNOT3 fusion to repress transcription is not caused by low levels of expression as it is expressed to higher levels than the NOT2 fusion proteins (see also Supplemental Fig. 1).

In conclusion, our experiments showed that the isolated Not-Boxes from yNot2 and CNOT3 also repressed gene activity upon promoter targeting.
We found that a synthetic promoter, containing five
analyzed CNOT2-mediated repression on different core pro-
ments. To investigate TATA dependence in our system we
expressions specifically repress transcription from weak TATA ele-
Genetic experiments in yeast indicate that the
Promoter — suggests involvement of histone deacetylases.
Taken together, these results showed that transcriptional re-
oved by 4–5-fold as a result of nonspecific effects by the TSA treatment. Luciferase values were corrected as described in the legend of Fig. 1. The graph presents the -fold repression by Gal4-DBD fusion proteins and is relative to the pCMVVDDB vector control transfections. Each transfection was performed in duplicate. The experiment shown is representative of at least three independent one.

Fig. 4. The isolated Not-Boxes of yNot2 and human CNOT3 are also able to repress transcription. A. U2OS cells were transiently transfected with either 7xGalTKLuc reporter plasmid or TKLuc reporter plasmid and the expression plasmids as indicated. The Not-Boxes encompass residues 96–191 of yNot2, 661–753 of CNOT3, and 457–540 of CNOT2. Luciferase values were corrected as described in the legend of Fig. 1. The graph presents the -fold repression by Gal4-DBD fusion proteins and is relative to the pcDNA3.1 control transfections. Each transfection was performed in triplicate. The experiment shown is representative of at least three independent experiments. B. 293T cells were transiently transfected with the expression plasmids for the different Gal4-DBD fusion proteins. The samples were analyzed by 12% SDS-PAGE and by immunoblotting using the RK5C1 antibody directed against the Gal4 protein. Positions of comigrating marker proteins are indicated by their molecular mass in kDa to the left of the figure.

Fig. 5. The repression by CNOT2 is sensitive to TSA. A. 293T cells were transiently transfected with the luciferase reporter plasmids and expression plasmids for the indicated proteins. Cells were incubated with dimethyl sulfoxide and 100, 400, or 1,000 nM TSA for 17 h prior to harvesting. Treatment with 400 or 1,000 nM TSA caused clear morphological changes in the cells. In the mock and Gal4-DBD transfections the firefly and Renilla luciferase values increased 2–4-fold as a result of nonspecific effects by the TSA treatment. Luciferase values were corrected as described in the legend of Fig. 1. The graph presents the -fold repression by Gal4-DBD fusion proteins and is relative to the pCMVVDDB vector control transfections. Each transfection was performed in duplicate. The experiment shown is representative of at least three independent ones. B. 293T cells were transiently transfected with the expression plasmids for the different Gal4-DBD fusion proteins. Cells were treated with TSA as indicated above. The samples were analyzed by 12% SDS-PAGE and by immunoblotting using the RK5C1 antibody directed against the Gal4 protein. The pCMVWT1Gal could not be included because the shorter Gal4-DBD(1–94) lacks the epitope for the RK5C1 antibody. Positions of comigrating marker proteins are indicated by their molecular mass in kDa to the left of the figure.

Gal4 binding sites fused to the consensus TATA-box from the adenovirus major late promoter and the retinoic acid receptor-β
initiator element, was also repressed by CNOT2 (Fig. 6). Mutation of the second residue of the TATA-box (5xGal.TGTA-
A.A.A.Luc) enhanced repression about 2-fold by full-length CNOT2, MAD, or the isolated Gal4-DBD. However, repression
by the Not-Box of CNOT2 increased 8-fold. Interestingly, repression by the isolated Not-Box became as strong as repression by full-length CNOT2. We also analyzed repression of other core promoters by targeting of CNOT2 and found that the adenovirus E1B core promoter was also repressed, but that, for
example, the T-cell receptor Vβ 8.1 core promoter was not affected (data not shown). Whereas E1B contains a canonical TATA-box, the Vβ 8.1 core promoter has a nonconsensus GATAAAA sequence around position ~30 (42), which indicates that elements outside the TATA-box may also contribute to the effect of CNOT2.

Taken together, these results indicate that core promoter

The mutant TATA luciferase construct is comparable with the wild type. for the Gal4 fusion proteins as indicated. Luciferase values were correlated with repression by CNOT2 protein.

293T cells were transiently transfected with luciferase reporter construct, containing either wild type or repressed by CNOT2 protein.

transfection was performed in triplicate. The experiment shown is relative to the pcDNA3.1 empty vector control transfection. Each samples play an important role in repression by the Not-Box of CNOT2 in mammalian cells.

The basal activity of the mutant TATA luciferase construct is comparable with the wild type. The graph presents the fold repression by Gal4-DBD fusion proteins and is relative to the pcDNA3.1 empty vector control transfection. Each transfection was performed in triplicate. The experiment shown is representative of at least three independent ones.

Components of the Ccr4-Not complex regulate cellular mRNA metabolism at the levels of synthesis and degradation (for reviews, see Refs. 17 and 18). The initial genetic identification of the Not components indicated that they act as repressors of RNA pol II-mediated transcription. At present it is not known how the Ccr4-Not complex is recruited to pol II promoters. To study transcriptional repression by Ccr4-Not we circumvented this issue by fusing individual subunits of the human complex to the DBD of the Gal4 transcription factor and tested them for transcription regulation in human cells. We showed that components of the Ccr4-Not complex could actively repress gene expression. In transient transfection assays using various human cell lines a strong repression by the CNOT2 and CNOT9(hRed1aCaF40) subunits was observed (Fig. 1). We found that its major repression function was localized in the conserved C-terminal 104 residues of CNOT2 (Fig. 2). We named this protein motif the Not-Box and found that its transcriptional repression function was sensitive to the histone deacetylase inhibitor TSA and was dependent on core promoter sequences (Figs. 5 and 6).

Repression and Activation by Not2 Proteins—Although we observed strong transcriptional repression by Not2 proteins in human cells, the situation is clearly different in yeast cells. Promoter targeting of human CNOT2 or yeast Not2p by the LexA DBD leads to strong transcriptional activation in yeast (32, 40, 43). In this respect it is important to note that the relatively weak TK promoter used in most of our experiments would allow detection of both promoter activation and repression, whereas only activation can be observed in the yeast experiments. Possibly, the activation function is not revealed in the cell cycle. In this way the observed TSA sensitivity could represent an indirect effect on CNOT2-mediated repression rather than direct involvement of histone deacetylases. Clearly, further experiments are required to establish a direct connection between CNOT2-dependent repression and histone deacetylase function.

The graph presents the fold repression by Gal4-DBD fusion proteins and is relative to the pcDNA3.1 empty vector control transfection. Each transfection was performed in triplicate. The experiment shown is representative of at least three independent ones.

FIG. 6. Mutation of the TATA-box affects the transcriptional repression by CNOT2 protein. 293T cells were transiently transfected with luciferase reporter construct, containing either wild type or mutated adenovirus major late TATA-box (indicated here as 5xGal.TATAAAA.Luc and 5xGal.TGTAAAA.Luc) and expression constructs for the Gal4 fusion proteins as indicated. Luciferase values were correlated with repression by CNOT2 protein.

The basal activity of the mutant TATA luciferase construct is comparable with the wild type. The graph presents the fold repression by Gal4-DBD fusion proteins and is relative to the pcDNA3.1 empty vector control transfection. Each transfection was performed in triplicate. The experiment shown is representative of at least three independent ones.

Repression and Activation by Not2 Proteins—Although we observed strong transcriptional repression by Not2 proteins in human cells, the situation is clearly different in yeast cells. Promoter targeting of human CNOT2 or yeast Not2p by the LexA DBD leads to strong transcriptional activation in yeast (32, 40, 43). In this respect it is important to note that the relatively weak TK promoter used in most of our experiments would allow detection of both promoter activation and repression, whereas only activation can be observed in the yeast experiments. Possibly, the activation function is not revealed in the cell cycle. In this way the observed TSA sensitivity could represent an indirect effect on CNOT2-mediated repression rather than direct involvement of histone deacetylases. Clearly, further experiments are required to establish a direct connection between CNOT2-dependent repression and histone deacetylase function.

Components of the Ccr4-Not complex regulate cellular mRNA metabolism at the levels of synthesis and degradation (for reviews, see Refs. 17 and 18). The initial genetic identification of the Not components indicated that they act as repressors of RNA pol II-mediated transcription. At present it is not known how the Ccr4-Not complex is recruited to pol II promoters. To study transcriptional regulation by Ccr4-Not we circumvented this issue by fusing individual subunits of the human complex to the DBD of the Gal4 transcription factor and tested them for transcription regulation in human cells. We showed that components of the Ccr4-Not complex could actively repress gene expression. In transient transfection assays using various human cell lines a strong repression by the CNOT2 and CNOT9(hRed1aCaF40) subunits was observed (Fig. 1). We found that its major repression function was localized in the conserved C-terminal 104 residues of CNOT2 (Fig. 2). We named this protein motif the Not-Box and found that its transcriptional repression function was sensitive to the histone deacetylase inhibitor TSA and was dependent on core promoter sequences (Figs. 5 and 6).

Repression and Activation by Not2 Proteins—Although we observed strong transcriptional repression by Not2 proteins in human cells, the situation is clearly different in yeast cells. Promoter targeting of human CNOT2 or yeast Not2p by the LexA DBD leads to strong transcriptional activation in yeast (32, 40, 43). In this respect it is important to note that the relatively weak TK promoter used in most of our experiments would allow detection of both promoter activation and repression, whereas only activation can be observed in the yeast experiments. Possibly, the activation function is not revealed in the cell cycle. In this way the observed TSA sensitivity could represent an indirect effect on CNOT2-mediated repression rather than direct involvement of histone deacetylases. Clearly, further experiments are required to establish a direct connection between CNOT2-dependent repression and histone deacetylase function.

Although genetic characterization of Ccr4-Not subunits in-
Transcriptional Repression by CNOT2

While it is surprising that repression is not observed with all core subunits, CNOT7 and CNOT6 are involved in mRNA degradation in the cytoplasm (20, 50). However, this does not exclude a nuclear role for these proteins. A significant portion of CNOT7 and CNOT6 proteins is associated with the nuclear form of the human Ccr4-Not complex (30, 51). Also, overexpression of human CNOT5 and CNOT7 has been shown to support transcriptional repression (30). Several explanations are possible for the observations that individual Ccr4-Not components do not show a similar behavior in different assays. The individual proteins may have distinct functional properties outside of the Ccr4-Not complex. In fact, gel filtration analyses of both yeast and human cell extracts indicate that significant amounts of CNOT6/Ccr4p and CNOT7/Caf1p proteins do not coelute with Not proteins (30, 52). Second, it is also possible that the Ccr4-Not complex can exert control both at the mRNA synthesis level by promoter interactions and at the mRNA degradation level by direct associations with mRNA itself (18). Alternatively, the core Ccr4-Not complex may represent a regulatory platform, which associates with other proteins to carry out specific functions, as with the Dhh1p subunit of the decapping platform, which associates with other proteins to support mRNA degradation.2

How does this help an understanding of the selective repression by the CNOT2 and CNOT9 subunits in our assays? Either these proteins carry out specific functions independently of the other core subunits or CNOT2 and CNOT9 are the limiting components in our transient transfections. In this latter scenario targeting of these subunits would result in recruitment of the complete Ccr4-Not complex(es) to the promoter. This predicts that cotransfection of other subunits may either enhance or abolish repression. This was not observed (data not shown), which suggests that CNOT2 and CNOT9 mediate transcriptional repression without involvement of other core subunits. Rigorous testing of this hypothesis requires development of in vitro assays. The results presented in this study provide the framework for these in vitro studies, which should lead to elucidation of the mechanism of repression by CNOT2 and CNOT9 and a more detailed understanding of transcription regulation by the Ccr4-Not complex.

Acknowledgments—We are grateful to Drs. D. Reinberg and G. Folkers for the 7xGalTKLuc and TKLuc reporter plasmids, respectively. The pBSIIKS(+)Gal4(1–14/200) and B42-yNot2 plasmids were provided by Dr. H. G. Stunnenberg for the 5xGal-M2-Luc and 5xGal-M1-Luc plasmids. The pBSIIKS(+)Gal4(1–14/200) and B42-yNot2 plasmids were provided by Dr. H. G. Stunnenberg for the 5xGal-M2-Luc and 5xGal-M1-Luc plasmids. The pBSIIKS(+)Gal4(1–14/200) and B42-yNot2 plasmids were provided by Dr. H. G. Stunnenberg for the 5xGal-M2-Luc and 5xGal-M1-Luc plasmids. The pBSIIKS(+)Gal4(1–14/200) and B42-yNot2 plasmids were provided by Dr. H. G. Stunnenberg for the 5xGal-M2-Luc and 5xGal-M1-Luc plasmids. The pBSIIKS(+)Gal4(1–14/200) and B42-yNot2 plasmids were provided by Dr. H. G. Stunnenberg for the 5xGal-M2-Luc and 5xGal-M1-Luc plasmids. The pBSIIKS(+)Gal4(1–14/200) and B42-yNot2 plasmids were provided by Dr. H. G. Stunnenberg for the 5xGal-M2-Luc and 5xGal-M1-Luc plasmids.
