Published online March 22, 2004

1836–1847 *Nucleic Acids Research, 2004, Vol. 32, No. 5*
DOI: 10.1093/nar/gkh344

The CtBP2 co-repressor is regulated by NADH-dependent dimerization and possesses a novel N-terminal repression domain

Sharon S. C. Thio[1], Joseph V. Bonventre[3] and Stephen I-Hong Hsu[1,2,*]

[1]Department of Medicine, Faculty of Medicine, National University of Singapore, Singapore 119074, [2]Genome Institute of Singapore, Singapore 117528 and [3]Renal Unit and Department of Medicine, Massachusetts General Hospital and Harvard Medical School, Charlestown, MA 02129, USA

Received as resubmission February 9, 2004; Accepted February 27, 2004

ABSTRACT

The C-terminal binding protein 2 (CtBP2) is a 48 kDa phosphoprotein reported to function as a co-repressor for a growing list of transcriptional repressors. It was recently demonstrated that CtBP is a dimeric NAD$^+$-regulated D-isomer-specific 2-hydroxy acid dehydrogenase. However, the specific substrate(s) of CtBP enzymatic activity and the relationship of this activity to its co-repression function remain unknown. The ability of a human CtBP to bind and serve as a co-repressor of E1A has been shown to be regulated by nuclear NADH levels. Here we extend the functional characterization of CtBP by demonstrating that amino acid substitutions at Gly189 in the conserved NAD$^+$-binding fold both abrogate the ability of CtBP2 to homodimerize and are associated with a dramatic loss of co-repressor activity. Consistent with the known enzymatic activity of CtBP2, mutations at Arg272 in the substrate-binding domain and at His321 in the catalytic domain result in significant loss of CtBP2 transcriptional co-repressor activity. High resolution serial C-terminal deletion analysis of CtBP2 also revealed a novel N-terminal repression domain that is distinct from its dehydrogenase domain. Our results suggest a model in which CtBP2 co-repressor function is regulated, at least in part, through the effect of NADH on CtBP2 homodimerization.

INTRODUCTION

The recent identification and characterization of a growing list of transcriptional co-activators and co-repressors has led to a paradigm shift in our understanding of gene transcriptional regulation. Members of one such family of co-repressors, the C-terminal binding proteins (CtBPs) [reviewed in Turner and Crossley (1) and Chinnadurai (2)], have been increasingly reported to be a component of many important co-repressor complexes. CtBP is a 48 kDa cellular phosphoprotein comprised of 445 amino acids. It was originally identified through its ability to complex with the C-terminal region of the E1A adenoviral oncoprotein (3,4). Through a direct protein–protein interaction, CtBP negatively modulates the oncogenic transformation activity of the E1A protein (3,5). This member of the CtBP family has been designated human CtBP1 (hCtBP1). BLAST analysis of the expressed sequence tag (EST) database identified another homolog of CtBP, designated hCtBP2 (6). The murine ortholog of CtBP2 (mCtBP) was subsequently isolated by Turner and Crossley (7).

CtBP1 and CtBP2 are able to heterodimerize and homodimerize (8). CtBPs can repress transcription in either a histone deacetylase-dependent or -independent manner, depending on the promoter context (2). CtBP family members bind to a short sequence motif, Pro-X-Asp-Leu-Ser (PXDLS), which has been designated the CtBP interaction domain (CID) (4). The interaction of CtBP with the CID can be regulated by acetylation of residues found near the motif (9). Mutation of the CID in the E1A protein leads to a decline in transcriptional repression by CtBP and increases the ability of E1A to direct transformation (3,4).

Members of the CtBP family show a high degree of conservation among vertebrates and invertebrates. More interestingly, the CtBPs exhibit a remarkable conservation of amino acid sequence homology with various members belonging to the D-isomer-specific 2-hydroxy acid dehydrogenase (2HAD) family of bacterial enzymes. Overall sequence alignment of CtBP with the vancomycin resistance gene (VanH), an NAD$^+$-dependent 2HAD from *Enterococcus faecium* (10), showed 67% similarity overall.

hCtBP1 binding to E1A was recently reported to be dramatically regulated by nuclear NADH levels (11). The binding of NADH was also found to be responsible for hCtBP1-regulated transcriptional co-repression. Low levels of NADH, within the normal physiological range, were required to stimulate the interaction of E1A and hCtBP1. Mutational analysis revealed that Gly183 at the putative NAD$^+$-binding

*To whom correspondence should be addressed at Department of Medicine, National University Hospital, 5, Lower Kent Ridge Road, Singapore 119074. Tel: +65 68748882; Fax: +65 68748994; Email: mdchsus@nus.edu.sg
Present address:
Joseph V. Bonventre, Renal Dvision, Brigham and Women's Hospital, Medical Research Bdg, 4th floor, Boston, MA 02115, USA

domain in hCtBP1 is crucial for NAD^+ dose-dependent binding to E1A. Zhang *et al*. suggested a model whereby hCtBP1 evolved from bacterial dehydrogenases and reductases in a manner that led to a loss in enzymatic activity, but allowed the retention of the ability and the capacity to bind and be regulated by NAD^+/NADH levels (11). During the preparation of this manuscript, Kumar *et al*. reported definitive biochemical and crystallographic evidence demonstrating that functional CtBP is a dimeric NAD^+-regulated dehydrogenase, with the ability to bind and serve as a co-repressor of the E1A oncoprotein and RIP140 repressor (12).

We now show that the ability of CtBP2 to homodimerize is NADH dependent. Amino acid substitutions at conserved residue G189 in the 2HAD homology domain of CtBP2, which has been implicated in NAD^+ binding for the D-isomer-specific 2HAD family of enzymes, abrogate the dose-dependent effect of NADH on CtBP2 homodimerization and are associated with a dramatic loss of transcriptional co-repressor activity. We propose that cellular levels of NADH act to regulate CtBP2 co-repressor function by modulating the homodimerization function of CtBP2. High resolution serial C-terminal deletion analysis of CtBP2 also revealed a hitherto unreported N-terminal repression domain that is distinct from its dehydrogenase domain.

MATERIALS AND METHODS

All recombinant DNA work was performed using standard techniques (13). All PCR-generated constructs were confirmed by sequence analysis.

Plasmids

A full-length mCtBP2 cDNA (DDBJ/EMBL/GenBank accession No. AA929588) was identified by a BLAST search of the EST database. Sequence analysis revealed that this clone is completely identical to the previously reported mCtBP2 sequence (7). The full-length mCtBP2-coding region was cloned into pET21b (Novagen) by PCR using the primers CtBP-Koz (+), 5′-CCGCGGCGCATATGGCCCTTGTGGATAAGCAC-3′; and CtBP-Term (–), 5′-GTGCTCGAGTGCGGCCGCTTGCTCGTTGGGGTGCTCTCG-3′. The mCtBP-2 fragment was excised with NdeI and NotI, and subcloned into the pBXG1N [a gift from Mark Ptashne; Kakidani and Ptashne (14); Sadowski *et al*. (15)] and SV/HA epitope tag vectors (16), in-frame with the 5′ Gal4-DNA binding domain (Gal4DBD) and HA tag, respectively. The Gal4DBD–CtBP2 cassette was also subcloned into pBlueScript (Stratagene). C-terminal deletions of the full-length mCtBP2 cDNA were created by PCR (primer sequences available upon request) followed by direct cloning into pCR®2.1-TOPO (Invitrogen). Inserts were subsequently subcloned into pBXG1N.

Site-directed mutagenesis

Site-directed mutagenesis was performed using the QuikChange™ 1-day Site-Directed Mutagenesis Kit (Stratagene). All primer sequences for site directed mutagenesis are available upon request. mCtBP2 cDNA mutants were subcloned into pBXG1N.

DNA transfection and reporter gene analysis

The C33A and U2OS cell lines (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). C33A cells were transfected by calcium phosphate precipitation (17). Transfected cells were harvested 48 h post-transfection. U2OS cells were transfected using Superfect™ Transfection Reagent (Qiagen) and harvested 24 h post-transfection. The amount of SV40 promoter DNA (pBXG1N vector) transfected was kept constant. Luciferase activity was measured using the Dual-Luciferase Reporter 1000 Assay System (Promega). Transfections were typically performed in duplicate and repeated in 6–8 independent experiments. Gal4-tagged proteins and cdk2 protein levels were determined by western blot analysis with mouse monoclonal antibodies (Santa Cruz).

In vitro and *in vivo* binding studies

In vitro translation (IVT) products were synthesized using the TnT T7-coupled reticulocyte lysate system (Promega) using cold methionine, or labeled with [^{35}S]methionine. Binding reactions were performed as described (18) using [^{35}S]methionine-labeled CtBP mutants with unlabeled wild-type Gal4DBD-tagged mCtBP2 in 1× IP buffer (16). Immunoprecipitation of Gal4-tagged mCtBP2 was carried out using the mouse monoclonal antibody to the Gal4DBD (Santa Cruz). The following concentrations of NADH (Sigma), as previously employed by Zhang *et al.* (11), were used for the binding reactions and the wash buffers: 1 and 100 nM, 10, 100 and 500 μM, and 1 mM.

To study the dimerization of CtBP2 *in vivo*, U2OS cells were co-transfected with HA-mCtBP2 wild-type and Gal4/mCtBP2 site-directed mutants. After 36 h, cells were incubated with 100–400 μM cobalt chloride ($CoCl_2$; Sigma). Whole-cell lysates were prepared 1 h post-treatment by incubating with 1× IP buffer for 30 min at 4°C. Immunoprecipitation of HA-tagged mCtBP2 was carried out using a mouse monoclonal antibody against the HA tag (Santa Cruz). Co-immunoprecipitated Gal4/CtBP2 mutants were detected with a horseradish peroxidase (HRP)-conjugated anti-Gal4DBD antibody.

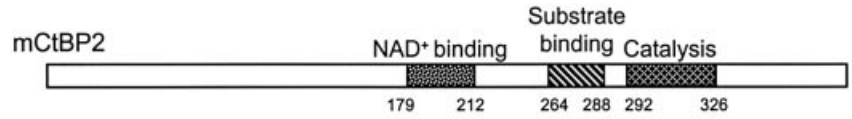
Partial proteolysis assay

In vitro translated [^{35}S]methionine-labeled wild-type and mutant CtBP2 proteins were partially digested with 0.2 μg/ml of papain (Sigma) at 37°C for 10 min in reaction buffer, as previously described (12). The digested products were separated on a 15% SDS–polyacrylamide gel, dried and exposed to film.

RESULTS

mCtBP2 is highly homologous to the 2HAD family of bacterial enzymes

A comparison of the amino acid sequence of mouse and human CtBP1 and CtBP2 with the well-characterized enzymatic functional domains of three representative members of the 2HAD family of bacterial enzymes (Fig. 1) demonstrated a high degree of amino acid sequence homology. Sequence alignment was performed using the ClustalX program (19).



A NAD$^+$ Binding βαβ Fold (NAD$^+$/NADH Binding Domain)

```
                                  βββββ  ααααααααααααααα  βββββββ          Identity to mCtBP2
mCtBP-1                           TLGIIGLGRVGQAVALRAKAFGFNVLFYDPY          81%
hCtBP-1                           TLGIIGLGRVGQAVALRAKAFGFNVLFYDPY          81%
mCtBP-2                           TLGLIGFGRTGQAVAVRAKAFGFSVIFYDPY          100%
hCtBP-2                           TLGLIGFGRTGQAVAVRAKAFGFSVIFYDPY          100%
PGDH, M. jannaschii               TLGVIGLGRIGQQVVKRAKAFGMNIIGYDPY          68%
HPR, H. methylovorum              TLGIYGFGSIGQALAKRAQGFDMDIDYFDTH          45%
VanH, E. faecium                  TVGVVGTGQIGKAVIERLRGFGCKVLAYSRS          39%
                                  * *  * *  *          #     ## #
Consensus              179        TLG-IG-GRIGQA---RAK-FG--VI-YDPY    212
```

B Substrate Binding Domain

```
                                        Substrate binding                  Identity to mCtBP2
mCtBP-1                                 GAFLVNTARGGLVDEKALAQALKEG          96%
hCtBP-1                                 GAFLVNTARGGLVDEKALAQALKEG          96%
mCtBP-2                                 GAFLVNAARGGLVDEKALAQALKEG          100%
hCtBP-2                                 GAFLVNAARGGLVDEKALAQALKEG          100%
PGDH, M. jannaschii                     NAIIVNCARGGLIDEKALYEALKEG          72%
HPR, H. methylovorum                    GAIVVNTARGDLVDNELVVAALEAG          52%
VanH, E. faecium                        GAFLINTGRGPLVDTYELVKALENG          56%
                                        #*  #* #*# *#*      **  *
Consensus                    264        GAIIVN-ARG-LVD--AL-ALKEG     288
```

C Catalysis Domain

```
                                  pH Dependence                  Catalysis          Identity to mCtBP2
mCtBP-1                           AALDVHE-SEPFSF---SQGP--------LKDAPNLICTPHAAWYS     94%
hCtBP-1                           AALDVHE-SEPFSF---SQGP--------LKDAPNLICTPHAAWYS     94%
hCtBP-2                           AALDVHE-SEPFSF---AQGP--------LKDAPNLICTPHTAWYS     100%
mCtBP-2                           AALDVHE-SEPFSF---AQGP--------LKDAPNLICTPHTAWYS     100%
PGDH, M. jannaschii               AALDVFE-EEPP-----KDNP--------LLTLDNVIGTPHQGAST     44%
HPR, H. methylovorum              AGFDVFA-GEPN-I---NEG---------YYDLPNTFLFPHIGS--     32%
VanH, E. faecium                  AALDVLEGEEEFFYSDCTQKPIDNQFLLKLQRMPNVIITPHTAYYT     43%
                                  *#** #  *#       #        #   #* # #**
Consensus              292        AALDV-E--EP---------P--------L---PN-I-TPH-----    326
```

Figure 1. Sequence alignment of the putative NAD$^+$/NADH-binding domain (**A**), the substrate-binding domain (**B**) and the regulatory domain (**C**) of mCtBP2 and three representative members of the D-isomer-specific 2-hydroxy acid dehydrogenase (2HAD) family of enzymes. A schematic representation of mCtBP2 highlighting 2HAD conserved regions (patterned boxes and denoted amino acid positions) is shown above. Detailed consensus sequences are shown below each figure. Completely conserved (five out of five) residues are denoted by an asterisk. Strict but not completely conserved (four out of five) residues are denoted by a hash sign. Amino acids that play significant roles in enzyme activity are in bold.

Human and mouse CtBP ortholog sequences were completely identical in these functional domains. Of the three bacterial 2HAD family members shown, D-3-phosphoglycerate dehydrogenase (PGDH), an enzyme from *Methanococcus jannaschii* (20), showed the highest homology to CtBP2. There was a high amino acid conservation of 68% for the NAD$^+$-binding domain (Fig. 1A), 72% for the substrate-binding domain (Fig. 1B) and 44% for the catalysis domain (Fig. 1C). The hydroxypyruvate reductase enzyme (HPR) from *Hyphomicrobium methylovorum* GM2 (21) and vancomycin resistance protein VanH from *Enterococcus faecium* (10) showed significant but lower degrees of homology with mCtBP2 (Fig. 1A–C).

In addition to a high degree of homology in the 2HAD functional domains between these proteins, key residues implicated in NAD$^+$ binding, substrate binding and catalysis were strictly conserved in mouse and human CtBPs (Fig. 1A–C). The NAD$^+$ domain [reviewed in Bellamacina *et al*. (22)] was responsible for coenzyme binding and consists of the distinctive amino acid motif GXGXXG(17 X)D, which is highly conserved in all other NAD$^+$-dependent dehydrogenases (23,24). The alternating β-strand–α-helix motif is a distinctive feature of this domain and has been named the 'Rossmann' fold (25). Not only were the three glycines (amino acid residues 184, 187 and 189) within the NAD$^+$-binding domain of CtBP found to be conserved in the same positions,

but the aspartic acid residue 17 amino acids away was also conserved in all but VanH (Fig. 1A). The function of the negatively charged aspartic acid is to form hydrogen bonds with the 2′-hydroxyl group of the adenine ribose moiety (23).

Arg272 within the substrate-binding domain of CtBP (Fig. 1B) and His321 within the catalysis domain of CtBP (Fig. 1C) have also been strictly conserved through evolution. Mutation of this catalytic histidine residue (His 296) in D-lactate dehydrogenase of *Lactobacillus plantarum* led to a complete loss of its activity (26). Thus, this specific histidine is essential for the catalysis by D-lactate dehydrogenase. This His296 is part of the aspartic acid–histidine pair, which functions as a proton relay system during catalysis (27). Notably, although mammalian CtBPs are phylogenetically distant from the 2HAD family of bacterial enzymes, amino acid residues crucial to the activity of these prokaryotic enzymes were found to be highly evolutionarily conserved in their eukaryotic CtBP counterparts.

High resolution serial C-terminal deletion analysis of CtBP2 reveals a novel N-terminal repression domain distinct from its dehydrogenase domain

In order to delineate the regions necessary for the transcriptional co-repressor activity of mCtBP2, 12 Gal4-tagged C-terminal deletion constructs were made using a PCR cloning approach. Consecutive serial deletion constructs differed by only 18–54 amino acid residues. A schematic diagram of all the deletion constructs, indicating a central extended region comprised of amino acid residues 20–353 reported to be conserved among all CtBPs (2), is shown in Figure 2A. Luciferase activity observed for pBXG1N/CtBP2-11 and -12 was comparable with that of the full-length protein, indicating that the last 76 amino acids are not necessary for transcriptional repression of a heterologous enhancer promoter. Further deletion of amino acids 340–368 to create pBXG1N/CtBP2-10 (1–339), resulting in the removal of 13 amino acid residues from the central conserved region, resulted in a significant increase in derepression to 23.8%. Progressive deletion to amino acid 193 within the dehydrogenase homology domain, which removed the catalysis and substrate-binding domains as well as a portion of the Rossman fold of the NAD^+-binding domain (conserved glycine residues still present), only led to a slight increase in derepression. Complete deletion of the NAD^+-binding domain in pBXG1N/CtBP2-5, which removed the conserved glycine residues essential for NAD^+ binding, led to a further 10% increase in derepression to 37.4%. Thus NAD^+ binding may play an additional role in the regulation of CtBP2 transcriptional co-repression activity, independent of the other 2HAD domains essential for dehydrogenase activity. Notably, the deletion of conserved amino acid residues 157–139 resulted in a further decrease in derepression to 59.6%, suggesting the novel finding that amino acids preceding the NAD^+-binding domain may play a role in the repression activity of CtBP2.

It was previously reported that N-terminal amino acids 1–95 were not required for hCtBP1 to repress a Gal4tkCAT heterologous reporter, nor did they exhibit independent transcriptional repression activity (28). We found that progressive deletion of residues 138–25 was associated with a stepwise increase in transcriptional repression activity to 26.6%, comparable with that of pBXG1N/CtBP2-10 (1–339). This gain-of-repression activity either suggests the presence of a novel potent N-terminal repression domain, or is the result of removal of a cryptic activation domain contained within residues 25–138. Western blot analysis of whole-cell lysates verified that the full-length and deletion mutants of mCtBP2 proteins were expressed at comparable levels in C33A cells (top panel of Fig. 2B).

In order to rule out the presence of a cryptic activation domain(s) contained within residues 25–138, we performed an N-terminal deletion analysis of the pBXG1N/CtBP2-4 constructs encoding residues 1–138 (Fig. 3A) and determined the transcriptional effects of the deletion mutants on a heterologous basal promoter in C33A cells. Western blot analysis confirmed comparable expression levels of all transfected constructs (Fig. 3B). The full-length mCtBP2 (pBXG1N/CtBP2 FL), pBXG1N/CtBP2-4 and pBXG1N/CtBP2-1 constructs exhibited levels of repression activity comparable with that previously observed for the C-terminal deletion analysis employing a heterologous enhancer promoter (compare Figs 2A and 3A). Progressive N-terminal deletion of residues 1–79 [constructs pBXG1N/CtBP2 (Δ1–24) and pBXG1N/CtBP2 (Δ1–79)] was associated with a progressive increase in repression activity (Fig. 3A). These results indicate the presence of a *bona fide* novel N-terminal repression domain consisting of residues 1–138.

Completely conserved amino acids within the 2HAD homology domain of CtBP2 play a role in transcriptional repression

In order to determine whether key conserved residues Gly189, Arg272 and His321 in the putative 2HAD enzymatic functional domains of mCtBP2 play a role in transcriptional repression activity, single amino acid substitutions were made at each of these positions by site-directed mutagenesis. A list of all the amino acid and nucleotide changes in these site-directed mutants are shown in Table 1.

To investigate the possible roles of the three completely conserved glycines in the NAD^+-binding domain, the middle glycine was mutated in an effort to disrupt NAD^+ binding. Gly189 in this middle position is an absolute requirement for NAD^+ binding, as any other amino acid residue bearing a side chain at this position would pose steric hindrance and prevent NAD^+ binding (23). G189R is a Gly→Arg mutant at amino acid position 189. Due to concerns that introducing a charged residue at this position may lead to an alteration in the secondary structure of CtBP2, we created two additional mutants (G189S and G189A), reasoning that Gly→Ser and Gly→Ala substitutions would have less of an effect, if any, on overall CtBP2 structure. The conserved arginine at amino acid position 272 was mutated to leucine (R272L). Similarly, the conserved histidine at amino acid position 321 was also changed to leucine (H321L). Since the previously published CtBP crystallography data were not made available on the public domain (12), the mutant mCtBP2 sequences were modeled against the known crystal structure of the 2HAD family member PDGH (29) using the GeneMine software (30). Results from our modeling analysis led us to infer that none of the amino acid substitutions resulted in any gross changes in the overall secondary and tertiary structure of mCtBP2 (results not shown). In order to confirm the modeling results utilizing a biochemical assay, [^{35}S]methionine-labeled

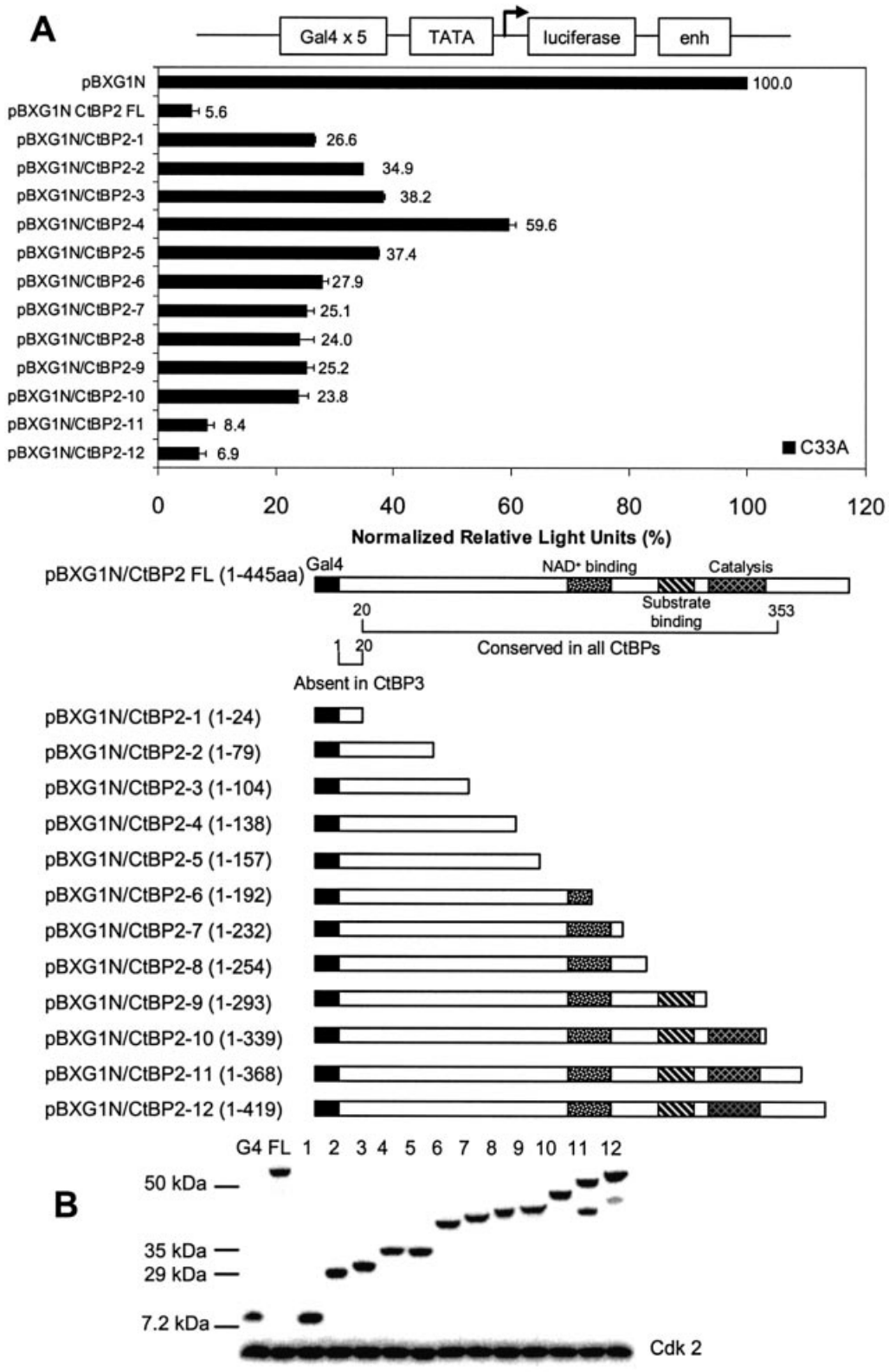


Figure 2. C-terminal deletion analysis of mCtBP2 reveals an additional N-terminal repression domain. Patterned boxes denote the functional 2HAD domains in mCtBP2. Bracketed numbers following the name of each construct indicate amino acid residues present in each construct. (**A**) C33A cells were transiently transfected with 100 ng each of Gal4-tagged full-length mCtBP2 and deletion mutants. These constructs were tested for their ability to repress a heterologous SV40 enhancer reporter plasmid pG5-GL3(SV) (3 μg). (**B**) Western blot analysis of whole-cell lysates of C33A cells transfected with 100 ng of wild-type and deletion mutants of Gal4/mCtBP2. Membranes were probed with both anti-Gal4DBD and anti-cdk2 mouse monoclonal antibodies. The top panel shows the expression levels of Gal4/mCtBP2 C-terminal deletion mutants (FL and numbers 1–12 represent full-length Gal4/mCtBP2 and mutants 1–12, respectively). Determination of the protein level of the constitutively expressed cyclin-dependent kinase 2 (cdk2) is included as a control for equal protein loading.

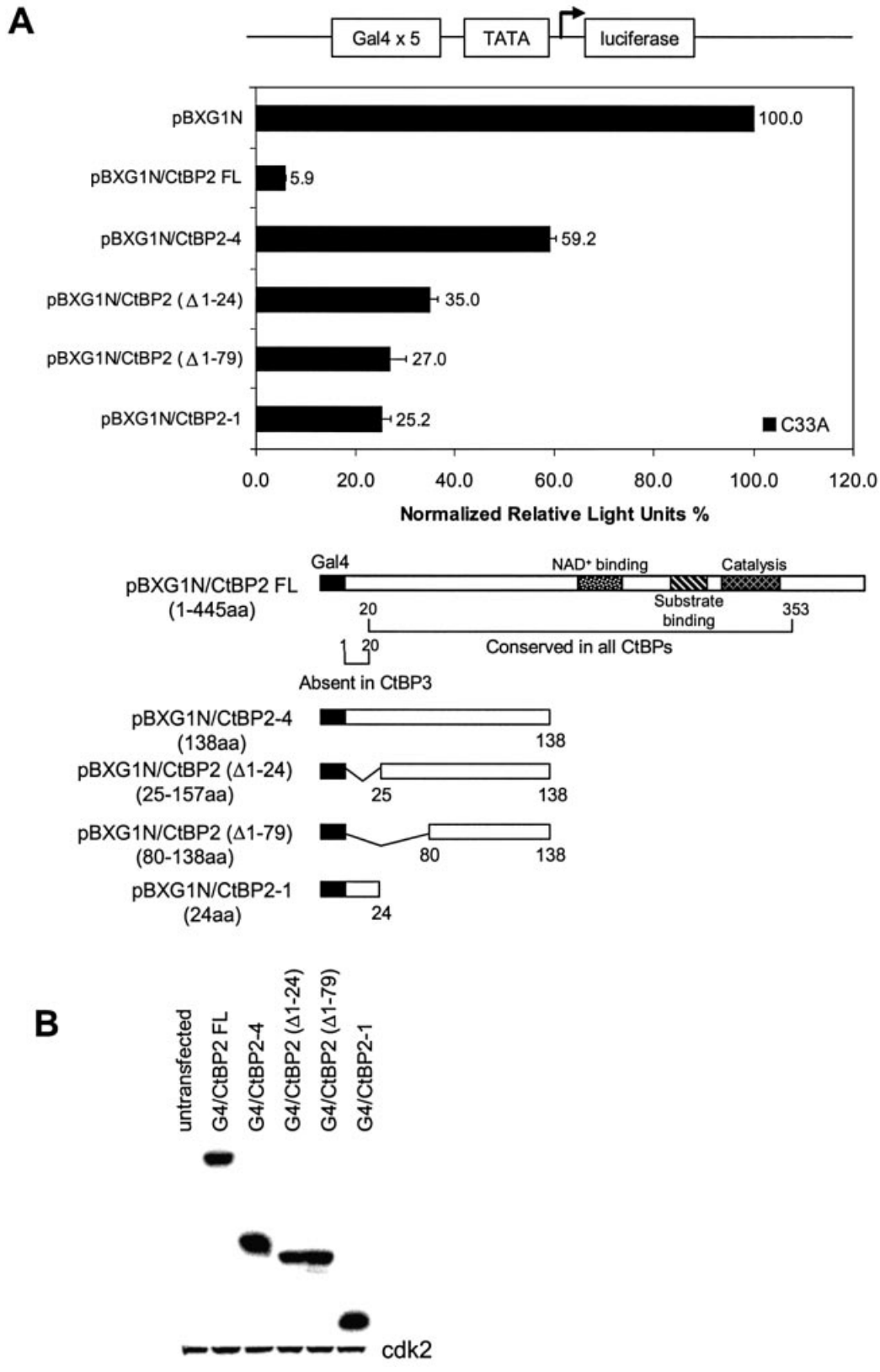


Figure 3. Identification of a novel N-terminal transcriptional repression domain. Patterned boxes denote the functional 2HAD domains in mCtBP2. Bracketed numbers following the name of each construct indicate amino acid residues present in each construct. (**A**) C33A cells were transiently transfected with 100 ng each of Gal4-tagged full-length mCtBP2 and mCtBP2 deletion constructs. These constructs were tested for their ability to repress a heterologous basal reporter plasmid pG5-GL3 (3 μg). (**B**) Western blot analysis of whole-cell lysates of C33A cells transfected with 100 ng of full-length and deletion constructs of Gal4/mCtBP2. Membranes were probed with both anti-Gal4DBD and anti-cdk2 mouse monoclonal antibodies.

Table 1. Site-directed mutagenesis of conserved amino acids found within the proposed enzymatic domains of mCtBP2

Proposed functional domain	Name of mutant	Wild type to mutant amino acid change	Wild type to mutant nucleotide change
NAD^+ binding	G189R	Gly→Arg	GGC→**C**GC
	G189S	Gly→Ser	GGC→G**C**C
	G189A	Gly→Ala	GGC→**T**GC
Substrate binding	R272L	Arg→Leu	CGA→C**T**A
Catalysis	H321L	His→Leu	CAC→CTC

wild-type and mutant full-length mCtBP2 *in vitro* translated proteins were subjected to partial proteolytic digestion with papain. Results reveal that all the mCtBP2 mutants (including the G189A/R and S) showed a pattern of two closely separated bands, indistinguishable from that observed with wild-type mCtBP2 (Fig. 4A). Thus, results obtained using two independent methodologies suggest that substitution of single amino acid residues in the mCtBP2 site-directed mutants are unlikely to result in gross changes in protein structure.

As has been previously reported (7), we first confirmed that the expression of wild-type Gal4-tagged mCtBP2 led to a potent dose-dependent repression of a heterologous SV40 enhancer–activated reporter construct in transfected C33A cells (results not shown). Transfection of 100 ng of the Gal4/CtBP2 wild-type construct repressed transcription to ~20%, compared with the vector control set arbitrarily at 100% (Fig. 4C). We next examined the transcriptional repression activity of the mCtBP2 site-directed mutants. All three G189 mutants led to a quantitatively similar and dramatic loss of transcriptional repressor activity (~85% reporter activity compared with control), consistent with our observations from structural modeling studies that the various amino acid substitutions at position 189 did not affect the overall structure of mCtBP2. Mutations at position R272L and H321L were also associated with significant loss of transcriptional repression activity, while a double mutant G189R/R272L essentially abolished transcriptional repression (Fig. 4C). Western blot analysis of whole-cell lysates verified that the wild-type and mutant mCtBP2 proteins were expressed at similar levels in C33A cells (Fig. 4B). Determination of cdk2 protein levels served as a control for equal protein loading. Our results strongly implicate a functional role for key conserved amino acid residues within the 2HAD homology domain of mCtBP2 in mediating transcriptional repression activity. Notably, mutagenesis of the single evolutionarily conserved amino acid at G189 alone, predicted to cause a functional disruption to the NAD^+-binding domain of mCtBP2, was associated with near complete loss of repression activity.

Mutations at conserved amino acid G189 affect CtBP2 dimerization

Functional CtBP is known to exist as a dimer (8). The presence of NADH increases the binding of CtBP to E1A (11). Here, we investigated whether increasing levels of NADH had an effect on the ability of mCtBP2 to homodimerize and the role of residue G189 in this process. Wild-type and G189 mutants of mCtBP2 were *in vitro* translated and labeled with [^{35}S]methionine. A Gal4-tagged mCtBP2 was also *in vitro* translated with and without the addition of [^{35}S]methionine. Analysis of all radioactively labeled IVT products by SDS–PAGE confirmed that both the tagged and untagged proteins were transcribed and translated at the same efficiency (Fig. 5A). Larger molecular weights species were consistently seen in all four lanes. These most probably represent phosphorylated forms of the *in vitro* translated mCtBP2 wild-type and mutant proteins (3,4). We next demonstrated that the radioactively labeled Gal4-tagged mCtBP2 IVT product, but not untagged mCtBP2, could be immunoprecipitated with a mouse monoclonal antibody against the Gal4DBD (Fig. 5B). We devised a co-immunoprecipitation assay to investigate the direct effect of the G189 mutations on the efficiency of mCtBP2 dimerization (Fig. 5C). Unlabeled Gal4-tagged mCtBP2 was mixed with [^{35}S]methionine-labeled untagged wild-type and G189 mCtBP2 mutant IVT products in binding reactions, and dimerization efficiencies were assessed by immunoprecipitation with anti-Gal4DBD antibody. Detection of dimerization resulting from Gal4/CtBP–CtBP/Gal4 or CtBP/Gal4–Gal4/CtBP interactions (bottom two complexes in Fig. 5C) was effectively ruled out because the Gal4-tagged proteins were not [^{35}S]methionine labeled. Since the Gal4 tag alone was also unable to interact and co-immunoprecipitate [^{35}S]methionine-labeled mCtBP2 (Fig. 5D), all bands visualized were solely due to dimerization arising from [^{35}S]methionine-labeled wild-type or mutant mCtBP2 with cold Gal4/CtBP2. Dimerization efficiencies for all mCtBP2 G189 mutants with Gal4-tagged mCtBP2 were significantly lower compared with wild-type mCtBP2 homodimerization (Fig. 5E, top panel). Dimerization of the R272L and H321L mutants to wild-type mCtBP2 served as excellent positive controls. Dimerization efficiencies for mCtBP2 R272L mutants with Gal4/CtBP2 were comparable with wild-type mCtBP2 homodimerization. The H321L mCtBP2 mutant was able to bind even better than the wild-type mCtBP2 (Fig. 5E, bottom panel).

Next, we determined whether NADH levels affected the dimerization efficiencies of mCtBP2 *in vitro*. By using the co-immunoprecipitation method as described above, we observed that increasing levels of NADH dramatically increased the efficiency of mCtBP2 homodimerization (Fig. 6A). Physiologically relevant NADH levels as low as 1 nM led to an observable increase in mCtBP2 homodimerization. It has been reported that while increasing levels of NADH lead to enhancement of binding between wild-type hCtBP1 and E1A binding, a G183A mutant of hCtBP1 still binds to E1A at background levels but in an NADH-independent manner (11). By using the co-immunoprecipitation method as described above, we also observed that increasing amounts of NADH did not lead to a significant increase in homodimerization of all three [^{35}S]methionine-labeled G189 mCtBP2 mutants to unlabeled Gal4-tagged mCtBP2. However, the dimerization efficiencies of both the R272L and H321L mutants were found to be responsive to NADH, although dimerization efficiencies

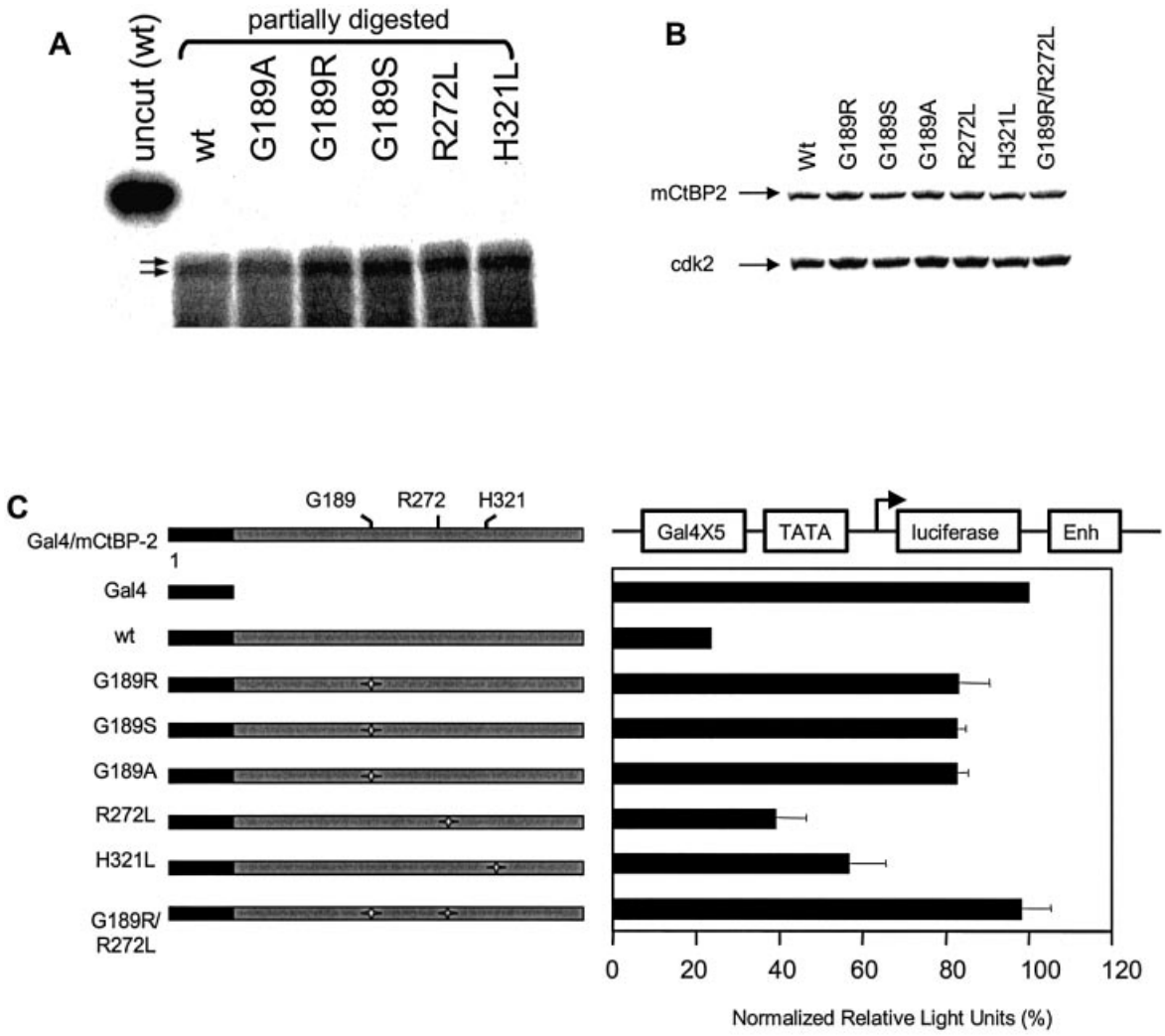


Figure 4. Gal4-tagged mCtBP2 mutants display loss of transcriptional repression activity. (**A**) Partial protease digestion of wild-type and mutant mCtBP2. [^{35}S]Methionine-labeled *in vitro* translated mutant proteins with papain. (**B**) Western blot analysis of whole-cell lysates of C33A cells transfected with 100 ng of wild-type and mutant Gal4-tagged mCtBP2. Membranes were probed with both anti-Gal4DBD and anti-cdk2 mouse monoclonal antibodies. (**C**) C33A cells were transiently transfected with 100 ng each of Gal4-tagged mCtBP2, Gal4-tagged mCtBP2 single and double mutants, together with 3 μg of the SV40 enhancer reporter plasmid pG5-GL3(SV).

were not as strong as observed with the wild-type mCtBP2 (Fig. 6B). We conclude that mCtBP2 homodimerization occurs through both NADH-dependent and -independent mechanisms. The presence of NADH significantly enhances the dimerization efficiencies of mCtBP2. During the preparation of this manuscript, exclusion chromatography was used to independently demonstrate that CtBP exists in a dimeric form in the presence of NAD$^+$/NADH (31).

A similar co-immunoprecipitation experiment was carried out to test the dimerization efficiencies of wild-type and mutant mCtBP2 *in vivo* (Fig. 7). U2OS cells were co-transfected with HA/mCtBP2 wild-type and the respective Gal4/mCtBP2 wild-type and site-directed mutants. Similar to what was observed *in vitro*, G189 mutants dimerize less efficiently to wild-type mCtBP2, as compared with wild-type mCtBP2 and the R272L and H321L mutants (Fig. 7B). Since the key determinant of the cellular redox state within the cell is the ratio NADH:NAD$^+$ rather than the absolute values of NADH or NAD$^+$, we used $CoCl_2$ to alter these ratios. $CoCl_2$ is known to alter the chemical redox state of the cell by increasing the cellular ratio of NADH to NAD$^+$. Increasing doses of $CoCl_2$ led to a concomitant increase in dimerization between wild-type mCtBP2 proteins. as well as between wild-type mCtBP2 and the R272L and H321L mutants, but was not observed in the case of G189 mutants (Fig. 7C).

DISCUSSION

The members of the mammalian family of CtBP co-repressor proteins have been identified as components of a growing list of transcriptional co-repressor complexes, which have been implicated in diverse biological functions and roles such as gene transcriptional regulation, development and tumorigenesis [reviewed in Turner and Crossley (1) and Chinnadurai (2)]. We have been particularly intrigued by the possibility that the CtBPs may be the mammalian orthologs of the

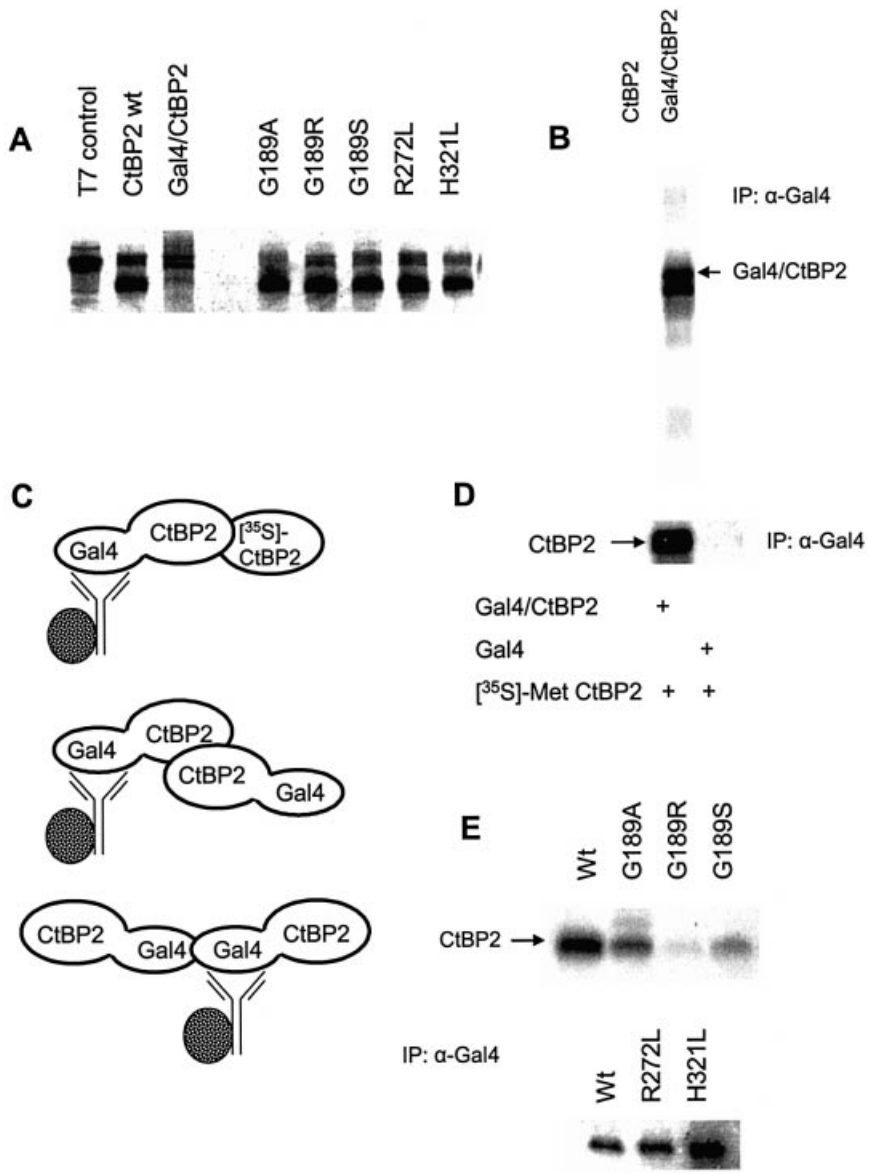


Figure 5. Mutants of conserved amino acid G189 in mCtBP2 show a reduced ability to dimerize with wild-type mCtBP2. (**A**) Gal4-tagged and untagged mCtBP2 IVT products were synthesized. A 1 µl aliquot of each IVT product was loaded per lane. (**B**) [^{35}S]Methionine-labeled Gal4/mCtBP2, but not mCtBP2, is specifically immunoprecipitated by a mouse monoclonal antibody against Gal4DBD. (**C**) A schematic diagram illustrating the co-immunoprecipitation and dimerization assays. The shaded sphere represents the protein A bead that binds to the anti-Gal4DBD monoclonal antibody. The antibody recognizes the Gal4DBD tag on the unlabeled, cold Gal4/mCtBP2, which in turn dimerizes to non-tagged [^{35}S]methionine-labeled wild-type or mutant mCtBP2 (upper complex). Homodimerization of Gal4/CtBP2 through protein–protein interactions mediated by either CtBP2 (middle complex) or the Gal4DBD (lower complex), which is known to homodimerize (e.g. CtBP2/Gal4–Gal4/CtBP2), cannot be detected by autoradiography since Gal4DBD proteins are not [^{35}S]methionine labeled. (**D**) Co-immunoprecipitation of [^{35}S]methionine-labeled mCtBP2 by Gal4/CtBP2 is specific. '+' indicates IVT products present in each binding reaction. (**E**) Co-immunoprecipitation of [^{35}S]methionine-labeled mutant G189A, G189R and G189S mCtBP2 IVT products with unlabeled Gal4/mCtBP2 using a mouse monoclonal antibody against Gal4DBD. A 5 µl aliquot of labeled and unlabeled IVT products was included in each binding reaction.

D-isomer-specific 2HAD family, and thus represent a novel family of redox-regulated and enzymatically active transcriptional co-repressors. During the preparation of this manuscript, Kumar *et al*. reported definitive evidence that functional CtBP is a dimeric NAD$^+$-regulated dehydrogenase, with the ability to bind and serve as a co-repressor of the E1A and RIP140 proteins (12).

We now provide the first evidence that homodimerization of mammalian CtBP2 is regulated by the NADH level and is functionally mediated, at least in part, by the completely conserved Gly189 residue in the NAD-binding domain known as the 'Rossmann' fold (25). Notably, site-directed mutagenesis of the evolutionarily conserved Gly189 residue alone leads to both a dramatic decline in homodimerization efficiency and transcriptional repressor activity. A double mutant G189R/R272L, which also introduces an amino acid substitution at a highly evolutionarily conserved arginine residue implicated in substrate binding for bacterial 2HAD

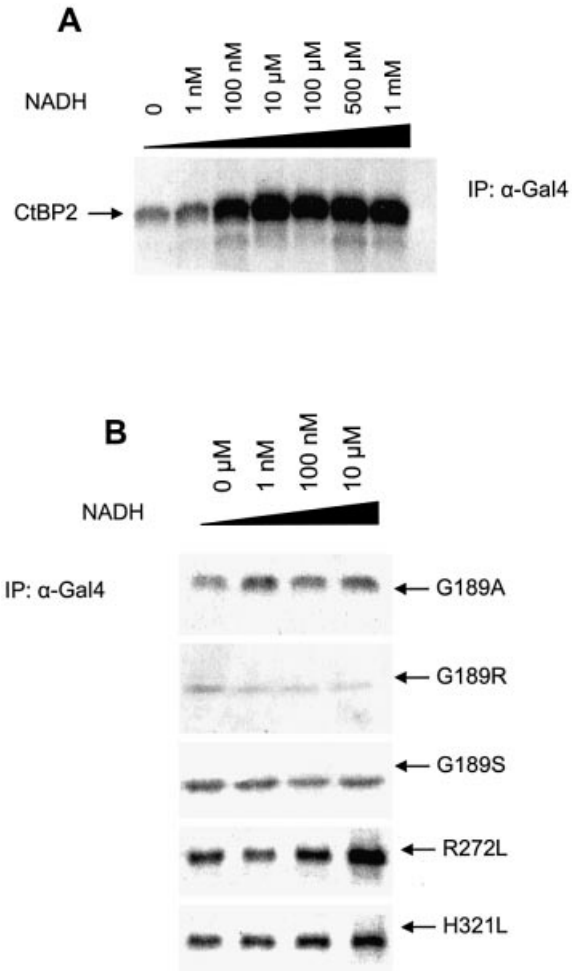


Figure 6. NADH regulates homodimerization of mCtBP2 *in vitro*. (**A**) NADH enhances homodimerization of wild-type mCtBP2 in a dose-dependent manner. (**B**) Increasing concentrations of NADH do not affect dimerization of G189 mutants of mCtBP2 with wild-type mCtBP2. Dimerized R272L and H321L mutants are still responsive to increasing doses of NADH. Co-immunoprecipitations of [^{35}S]methionine-labeled G189A, G189R, G189S, R272L and H321L proteins with cold Gal4/mCtBP2 were performed using a mouse monoclonal antibody against Gal4DBD in the presence of increasing concentrations of NADH. In both (A) and (B), 5 µl of labeled and unlabeled IVT products were included in each binding reaction.

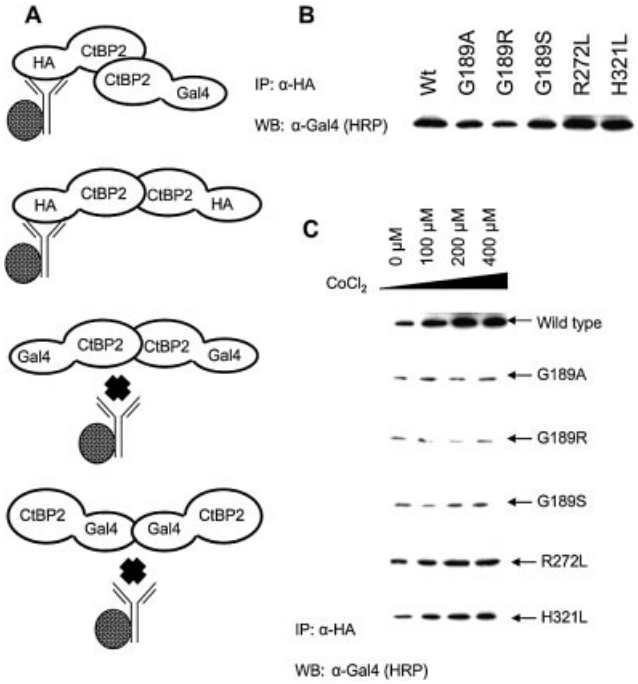


Figure 7. NADH regulates homodimerization of mCtBP2 *in vivo*. (**A**) A schematic diagram illustrating the *in vivo* co-immunoprecipitation and dimerization assays is shown. The shaded sphere represents the protein A agarose bead that binds to the anti-HA monoclonal antibody. This antibody recognizes the HA tag on HA/mCtBP2, which in turn dimerizes to Gal4/mCtBP2 wild-type or mutant proteins. Homodimerization of HA/CtBP2 through protein interactions mediated by CtBP2 (second complex) cannot be detected by the Gal4DBD monoclonal antibody used for the western blot. Similarly, homodimerization of Gal4/CtBP2 through protein–protein interactions mediated by either CtBP2 (third complex) or the Gal4DBD (fourth complex), which is known to homodimerize (e.g. CtBP2/Gal4–Gal4/CtBP2), cannot be detected since Gal4DBD proteins cannot be immunoprecipitated by the anti-HA antibody. (**B**) Co-immunoprecipitation of mutant G189A, G189R, G189S, R272L and H321L mCtBP2 proteins using a mouse monoclonal antibody against HA. (**C**) Co-immunoprecipitations of G189A, G189R, G189S, R272L and H312L proteins with HA/mCtBP2 were performed using a mouse monoclonal antibody against HA in the presence of increasing concentrations of $CoCl_2$.

family members, completely abolished mCtBP2 transcriptional repression activity. Our results support the findings of Kumar *et al*., in which triple mutants of the NAD$^+$-binding and catalysis domains led to a loss of transcriptional activity (12).

All three domains within the 2HAD homology region of CtBP that play a role in enzymatic activity in bacterial orthologs have been reported to be important for homo- and heterodimerization of mammalian CtBPs (8), suggesting that these domains play dual functional roles. Our observations from high resolution C-terminal serial deletion analysis of mCtBP2 provide further support for this model. Constructs pBXG1N/CtBP2-10 through pBXG1N/CtBP2-6 displayed similar levels of transcriptional repression activity, thus defining a functional 'repression domain' within the 2HAD homology region that includes the catalysis and substrate-binding domains, and the C-terminal portion of the Rossman fold. Further deletion of residues 158–192 containing the three conserved glycines in the NAD$^+$-binding domain resulted in a further loss of transcriptional repression activity, suggesting a functional role for the NAD$^+$-binding domain independent of its role in mediating dehydrogenase activity. Our studies (Figs 3–6) indicate that one such role could be to mediate NADH-regulated homodimerization of CtBP2. Serial deletion analysis also revealed the existence of a novel CtBP2 repression domain comprised of amino acid residues 1–138. Notably, CtBP3 lacks residues 1–20, which are conserved among all vertebrate CtBP1 and CtBP2 family members (2), thus highlighting the potential structural and perhaps functional differences between family members.

Although CtBP is abundant in the nucleus, it is not exclusive to it (1). In order to rule out the possibility that increasing concentrations of NADH might have a regulatory effect on the subcellular location of CtBP (i.e. translocation from cytoplasm to nucleus), we performed immunofluorescence studies in C33A and U2OS cells. Cells were separately treated with increasing doses of NADH (0.1–10 µM), $CoCl_2$

(100–200 μM) or NaN_3 (another redox-inducing chemical; 5–20 mM). The location of endogenous CtBP as determined by immunofluorescence remained principally nuclear in both cell lines under all three treatments. Thus, while NADH regulates the homodimerization and repression activities of CtBP2, it does not appear to achieve this through alterations in the cellular distribution of CtBP.

We propose that the CtBP2 homodimerization function serves as a cellular redox sensor for its co-repressor function. In such a role, CtBPs can detect the readiness of a cell to proceed with various cellular processes by the levels of cellular NAD^+/NADH present. The ability of CtBP co-repressors to sense the cellular redox state is likely to be specific to NAD^+/NADH. Zhang *et al*. reported that the binding of hCtBP1 to E1A was insensitive to $NADP^+$, NADPH and FAD^+ (11). This is not surprising since the NAD^+-binding domain of CtBPs contain the conserved GXGXXG motif (where X is any amino acid), characteristic of enzymes that use NADH as a cofactor. The nucleotide-binding domains of enzymes that use NADPH as a cofactor contain a distinct GXGXXA motif (23). The nucleotide-binding domain in the 2HAD family of bacterial enzymes moderates enzyme activity by determining the availability of the active site. The active site is found in a crevice between the nucleotide-binding and substrate-binding domains, which are connected by a flexible hinge region (29). Cofactor binding causes a conformational change of the dimer, thus closing the active site cleft. This effectively brings the NAD^+ cofactor into a favorable position relative to the substrate. Once the hydride transfer from substrate to NAD^+ is completed, the cleft opens again to allow the release of the enzymatic product (29). The inability to bind NAD^+/NADH, as in the case of the Gly189 mutation in mCtBP2, would leave the active site closed once the substrate is bound, thus rendering the enzyme incapable of carrying out any further catalytic reactions.

Our proposal is supported by a well-established paradigm of redox-regulated transcriptional regulation. The silent information regulator 2 protein (Sir2) in yeast mediates transcriptional silencing (32) by promoting the deacetylation of histones in a reaction that absolutely depends on the presence of NAD^+ (33). A detailed mechanism for the reaction catalyzed by Sir2 has recently been proposed (34). The proto-oncogenes c-*fos* and c-*jun* are known to be regulated by the cellular redox state. Their protein products, Fos and Jun, function cooperatively by forming a heterodimeric complex known as activator protein-1 (AP-1). AP-1 binds to the DNA regulatory element known as the AP-1-binding site. The ability of AP-1 to bind DNA is regulated by the reduction–oxidation of a single conserved cysteine residue of the DNA-binding domain of these two proteins (35). The reduction of this residue is carried out by redox factor-1 (Ref-1), which is itself subject to redox-mediated regulation (36). The oxidized or reduced state of cells would thus trigger a redox cascade involving Ref-1, AP-1 and possibly many other proteins, which would eventually lead to the transcriptional repression or activation of downstream target genes (37). NADPH has been reported to reverse the oxidation of the yeast homolog of AP-1 (YAP-1) (38).

The demonstration that CtBP is an NAD^+-regulated dehydrogenase that mediates transcriptional repression (12), and our finding that the level of NADH controls and regulates CtBP2 co-repressor function through its effect on CtBP2 homodimerization, provides further evidence for a broader functional role of enzymatic activity in general, and NAD^+-dependent dehydrogenase activity in particular, in gene transcriptional regulation.

ACKNOWLEDGEMENTS

We thank Dr Jeremiah Stanson Joseph (Genome Institute of Singapore) for his assistance in the structural modeling of CtBP mutant proteins, and Khe Guan Sim (Department of Medicine, National University of Singapore) for his assistance with the partial protease assays. This research was supported by National Medical Research Council (Singapore) grant 0332/1999 to S.I.-H.H.

REFERENCES

1. Turner,J. and Crossley,M. (2001) The CtBP family: enigmatic and enzymatic transcriptional co-repressors. *Bioessays*, **23**, 683–690.
2. Chinnadurai,G. (2002) CtBP, an unconventional transcriptional corepressor in development and oncogenesis. *Mol. Cell*, **9**, 213–224.
3. Boyd,J.M., Subramanian,T., Scheaper,U., La Regina,M., Bayley,S. and Chinnadurai,G. (1993) A region in the C-terminus of adenovirus 2/5 E1A protein is required for association with a cellular phosphoprotein and important for the negative modulation of T24-ras mediated transformation, tumorigenesis and metastasis. *EMBO J.*, **12**, 469–478.
4. Scheaper,U., Boyd,J.M., Verma,S., Uhlmann,E., Subramanian,T. and Chinnadurai,G. (1995) Molecular cloning and characterization of a cellular phosphoprotein that interacts with a conserved C-terminal domain of adenovirus E1A involved in negative modulation of oncogenic transformation. *Proc. Natl Acad. Sci. USA*, **92**, 10467–10471.
5. Subramanian,T., La Regina,M. and Chinnadurai,G. (1989) Enhanced ras oncogene mediated cell transformation and tumorigenesis by adenovirus 2 mutants lacking the C-terminal region of E1A protein. *Oncogene*, **4**, 415–420.
6. Katsanis,N. and Fisher,E.M.C. (1998) A novel C-terminal binding protein (CTBP2) is closely related to CTBP1, an adenovirus E1A-binding protein and maps to human chromosome 21q21.3. *Genomics*, **47**, 294–299.
7. Turner,J. and Crossley,M. (1998) Cloning and characterization of mCtBP2, a co-repressor that associates with basic Kruppel-like factor and other mammalian transcriptional regulators. *EMBO J.*, **17**, 5129–5140.
8. Sewalt,R.G.A.B., Gunster,M.J., van der Vlag,J., Satijn,D.P.E. and Otte,A.P. (1999) C-terminal binding protein is a transcriptional repressor that interacts with a specific class of vertebrate Polycomb proteins. *Mol. Cell. Biol.*, **19**, 777–787.
9. Zhang,Q., Yao,H., Vo,N. and Goodman,R.H. (2000) Acetylation of adenovirus E1A regulates binding of the transcriptional corepressor CtBP. *Proc. Natl Acad. Sci. USA*, **97**, 14323–14328.
10. Arthur,M., Molinas,C., Dutka-Malen,S. and Courvalin,P. (1991) Structural relationship between the vanomycin resistance protein VanH and 2-hydroxycarboxylic acid dehydrogenases. *Gene*, **103**, 133–134.
11. Zhang,Q., Piston,D.W. and Goodman,R.H. (2002) Regulation of corepressor function by nuclear NADH. *Science*, **295**, 1895–1897.
12. Kumar,V., Carlson,J.E., Ohgi,K.A., Edwards,T.A., Rose,D.W., Escalante,C.R., Rosenfeld,M.G. and Aggarwal,A.K. (2002) Transcription corepressor CtBP is an NAD^+-regulated dehydrogenase. *Mol. Cell*, **10**, 857–869.
13. Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
14. Kakidani,H. and Ptashne,M. (1988) Gal4 activates gene expression in mammalian cells. *Cell*, **52**, 161–167.
15. Sadowski,I., Ma,J., Triezenberg,S. and Ptashne,M. (1988) Gal4-VP16 is an unusually potent transcription activator. *Nature*, **335**, 563–564.
16. Hsu,S.I.-H., Yang,C.M., Sim,K.G., Hentschel,D.M., O'Leary,E. and Bonventre,J.V. (2001) TRIP-Br: a novel family of PHD zinc finger- and

bromodomain-interacting proteins that regulate the transcriptional actvity of E2F-1/DP-1. *EMBO J.*, **20**, 2273–2285.
17. Martin,K., Trouche,D., Hagemeier,C., Sorenson,T.S., La Thangue,N.B. and Kouzarides,T. (1995) Stimulation of E2F/DP1 transcriptional activity by MDM2 oncoprotein. *Nature*, **375**, 691–694.
18. Marcus,G.A., Silverman,N., Berger,S.L., Horiuchi,J. and Guarente,L. (1994) Functional similarity and physical association between GCN5 and ADA2: putative transcriptional adaptors. *EMBO J.*, **13**, 4807–4815.
19. Thompson,J.D., Gibson,T.J., Plewniak,F., Jeanmougin,F. and Higgins,D.G. (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.*, **25**, 4876–4882.
20. Bult,C.J., White,O., Olsen,G.J., Zhou,L., Fleischmann,R.D., Sutton,G.G., Blake,J.A., FitzGerald,L.M., Clayton,R.A., Gocayne,J.D. *et al*. (1996) Complete genome sequence of the methanogenic archeon *Methanococcus jannaschii*. *Science*, **273**, 1058–1073.
21. Yoshida,T., Yamaguchi,K., Hagishita,T., Mitsunaga,T., Miyata,A., Tanabe,T., Toh,H., Ohshiro,T., Shimao,M. and Izumi,Y. (1994) Cloning and expression of the gene for hydroxypyruvate reductase (D-glycerate dehydrogenase) from an obligate methylotroph *Hyphomicrobium methylovorum* GM2. *Eur. J. Biochem.*, **223**, 727–732.
22. Bellamacina,C.R. (1996) The nicotinamide dinucleotide binding motif: a comparison of nucleotide binding proteins. *FASEB J.*, **10**, 1257–1269.
23. Weirenga,R.K., De Maeyer,M.C.H. and Hol,W.G.J. (1985) Interaction of pyrophosphate moieties with α-helixes in dinucleotide binding proteins. *Biochemistry*, **24**, 1346–1357.
24. Weirenga,R.K., Terpstra,P. and Hol,W.G.J. (1986) Prediction of the occurence of the ADP-binding βαβ-fold in proteins, using an amino acid sequence fingerprint. *J. Mol. Biol.*, **187**, 101–107.
25. Rossman,M.G., Moras,D. and Olsen,K.W. (1974) Chemical and biological evolution of a nucleotide-binding protein. *Nature*, **250**, 194–199.
26. Taguchi,H. and Ohta,T. (1993) Histidine 296 is essential for the catalysis in *Lactobacillus plantarum* D-lactate dehydrogenase. *J. Biol. Chem.*, **268**, 18030–18034.
27. Kochhar,S., Hunziker,P.E., Leong-Morgenthaler,P. and Hottinger,H. (1992) Evolutionary relationship of NAD(+)-dependent D-lactate dehydrogenase: comparison of primary structure of 2-hydroxy acid dehydrogenases. *Biochem. Biophys. Res. Commun.*, **184**, 60–66.
28. Koipally,J. and Georgopoulos,K. (2000) Ikaros interactions with CtBP reveal a repression mechanism that is independent of histone deacetylase activity. *J. Biol. Chem.*, **275**, 19594–19602.
29. Schuller,D.J., Grant,G.A. and Banaszak,L.J. (1995) The allosteric ligand site in the V_{max}-type cooperative enzyme phosphoglycerate dehydrogenase. *Struct. Biol.*, **2**, 69–76.
30. Lee,C. and Irizarry,K. (2001) The GeneMine system for genome/proteome annotation and collaborative data mining. *IBM Systems J.*, **40**, 592–603.
31. Balasubramanian,P., Zhao,L.-J. and Chinnadurai,G. (2003) Nicotinamide adenine dinucleotide stimulates oligomerization, interaction with adenovirus E1A and an intrinsic dehydrogenase activity of CtBP. *FEBS Lett.*, **537**, 157–160.
32. Imai,S.-I., Armstrong,C.M., Kaeberlein,M. and Guarente,L. (2000) Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. *Nature*, **403**, 795–800.
33. Landry,J., Sutton,A., Tafrov,S.T., Heller,R.C., Stebbins,J., Pillus,L. and Sternglanz,R. (2000) The silencing protein SIR2 and its homologs are NAD-dependent protein deacetylases. *Proc. Natl Acad. Sci. USA*, **97**, 5807–5811.
34. Sauve,A.A., Celic,I., Avalos,J., Deng,H., Boeke,J.D. and Schramm,V.L. (2001) Chemistry of gene silencing: the mechanism of NAD^+-dependent deacetylation reactions. *Biochemistry*, **40**, 15456–15463.
35. Abate,C., Patel,L., Rauscher,F.J.,III and Curran,T. (1990) Redox regulation of Fos and Jun DNA-binding activity *in vitro*. *Science*, **249**, 1157–1161.
36. Xanthoudakis,S. and Curran,T. (1992) Identification and characterization of Ref-1, a nuclear protein that facilitates AP-1 DNA-binding activity. *EMBO J.*, **11**, 653–665.
37. Xanthoudakis,S., Miao,G., Wang,F., Pan,Y.E. and Curran,T. (1992) Redox activation of Fos-Jun DNA binding activity is mediated by a DNA repair enzyme. *EMBO J.*, **11**, 3323–3335.
38. Delaunay,A., Isnard,A.-D. and Toledano,M.B. (2000) H_2O_2 sensing through oxidation of the Yap1 transcription factor. *EMBO J.*, **19**, 5157–5166.