# CoREST Represses the Heat Shock Response Mediated by HSF1

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## SUMMARY

**The stress response in cells involves a rapid and transient transcriptional activation of stress genes. It has been shown that Hsp70 limits its own transcriptional activation functioning as a corepressor of heat shock factor 1 (HSF1) during the attenuation of the stress response. Here we show that the transcriptional corepressor CoREST interacts with Hsp70. Through this interaction, CoREST represses both HSF1-dependent and heat shock-dependent transcriptional activation of the *hsp70* promoter. In cells expressing short hairpin RNAs directed against CoREST, Hsp70 cannot repress HSF1-dependent transcription. A reduction of CoREST levels also provoked a significant increase of Hsp70 protein levels and an increase of HSF1-dependent transactivation of *hsp70* promoter. Via chromatin immunoprecipitation assays we show that CoREST is bound to the *hsp70* gene promoter under basal conditions and that its binding increases during heat shock response. In conclusion, we demonstrated that CoREST is a key regulator of the heat shock stress response.**

## INTRODUCTION

CoREST was identified as a corepressor for the RE1-silencing transcription factor REST/NRSF (Andres et al., 1999), a master regulator of neurogenesis (Chong et al., 1995; Schoenherr and Anderson, 1995) that controls the expression of a large group of neural genes (Ballas et al., 2001, 2005; Lunyak et al., 2002). CoREST is an integral component of a histone deacetylase complex (Humphrey et al., 2001; You et al., 2001) that also includes the histone lysine-specific demethylase LSD1 (Shi et al., 2004). This CoREST complex can be purified in the absence of REST/NRSF, suggesting that it may bind other transcription factors to bring repressive machinery to chromatin.

CoREST comprises two SWI13/ADA2/NcoR/TFIIIB (SANT) domains (Aasland et al., 1996) and an Egl-27 and MTA1 homology 2 (ELM2) domain (Solari et al., 1999; Ding et al., 2003). The first SANT domain in CoREST is involved in its interaction with REST/NRSF (Ballas et al., 2001), and it is also necessary to recruit HDAC1 (You et al., 2001). The second SANT domain in CoREST is part of one of its repressive transcriptional domains, which shows deacetylase activity (Ballas et al., 2001). In addition, the second SANT domain mediates the binding to the nucleosomal substrate endowing LSD1 demethylation activity (Shi et al., 2005; Yang et al., 2006). Together, these domains provide a mechanism by which CoREST can target transcriptional repressive machinery to discrete places in chromatin.

CoREST orthologous have been described in *D. melanogaster* (Dallman et al., 2004), *X. laevis* (de la Calle-Mustienes et al., 2002), *C. elegans* (Jarriault and Greenwald, 2002), and mouse (Tontsch et al., 2001), sharing a high degree of structural conservation. Similar function to human CoREST has been described for *D. melanogaster* CoREST (dmCoREST). dmCoREST is a transcriptional corepressor of Tramtrack88, a potential functional homolog of REST/NRSF that limits neuronal gene expression in nonneuronal tissue in *D. melanogaster* (Dallman et al., 2004). CoREST is abundantly expressed in several tissues including the CNS. Even in neurons in which REST is absent, CoREST is expressed to high levels and exists in complexes with HDAC1 and HDAC2 (Ballas et al., 2005). Thus, CoREST is likely to constitute a predominant repressor in different cellular contexts. This high evolutionary conservation degree of CoREST and its widespread pattern of expression suggest that, in addition to serving as a corepressor for REST/NRSF, CoREST might play a similar function for other DNA-binding proteins. Following this hypothesis, we performed a two-hybrid screening and found the stress chaperone Hsp70 as a CoREST-interacting partner.

Stresses like elevated temperatures, oxidants, heavy metals, and bacterial and viral infections lead to the induction of a group of proteins called heat shock proteins (HSPs). HSP induction mainly depends on the heat shock transcription factor-1 (HSF1), which binds heat shock element (HSE), mediating transcription of the HSP genes and accumulation of HSPs (Morimoto, 1998). Under normal conditions, HSF1 exists in a transcriptionally repressed state, and it is activated by stress in a multistep process involving trimerization, acquisition of HSE binding activity, novel phosphorylation, and *trans*-activation of *hsp* genes (Sarge et al., 1993; Xia and Voellmy, 1997). Hsp70 is

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HSPA8  aa  121 SMVLTKMKEI AEAYLGKTVT NAVVTVPAYF NDSQRQATKD AGTIAGLNVL RIINEPTAAA IAYGLDKKVG AERNVLIFDL 200
clone  15           KMKEI AEAYLGKTVT NAVVTVPAYF NDSQRQATKD AGTIAGLNVL RIINEPTAAA IAYGLDKKVG AERNVLIFDL
clone  45                 AEAYLGKTVT NAVVTVPAYF NDSQRQATKD AGTIAGLNVL RIINEPTAAA IAYGLDKKVG AERNVLI.....
clone  59                                                                     AAA IAYGLDKKVG AERNVLIFDL
clone  31                                                                                KVG AERNVLIFDL

HSPA8  aa  251 GGGTFDVSIL TIEDGIFEVK STAGDTHLGG EDFDNRMVNH FIAEFKRKHK KDISENKRAV RRLRTACERA 320
Clone  15      GGGTFDVSIL TIEDGIFEVK STAGDTHLGG EDF.......................................
Clone  59      GGGTFDVSIL TIEDGIFEVK STAGDTHLGG EDFDNRMVNH FIAEFKRKHK.....................
Clone  31      GGGTFDVSIL TIEDGIFEVK STAGDTHLGG EDFDNRMVNH FIAEFKRKHK KDISENKRAV RRLRTA....

HSPA5  aa  171 WNDPSVQQDI KFLPFKVVEK KTKPYIQVDI GGGQTKTFAP EEISAMVLTK MKETAEAYLG KKVTHAVVTV PAYFNDAQRQ 250
Clone 102       DDPSVQQDI KFLPFKVVEK KTKPYIQVDI GGGQTKTFAP EEISXMVLTK *KNPLRLIWE KKVPMXVV.............
Clone  95                                                                                            R

HSPA5  aa  251 ATKDAGTIAG LNVMRIINEP TAAAIAYGLD KREGEKNILV FDLGGGTFDV SLLTIDNGVF 310
Clone  95      ATKDAGTIAG LNVMRIINEP TAAAIAYGLD KXEGEKNILV LDLGGGTF..........
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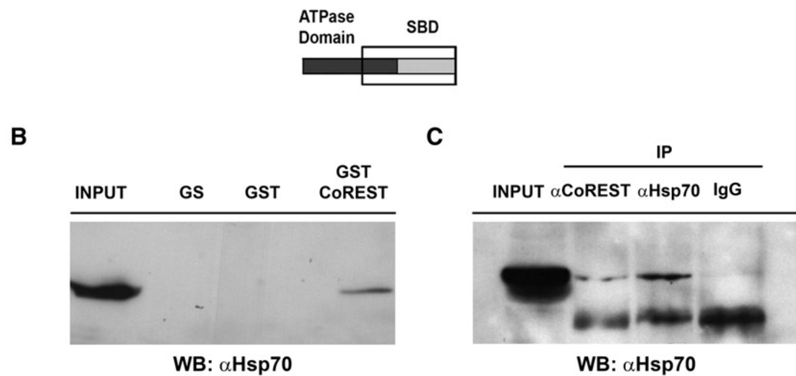


**Figure 1. CoREST Interacts with the Molecular Chaperone Hsp70**

(A) Alignment of human HSPA8 and HSPA5 sequences with the positive isolated clones obtained in the yeast two-hybrid screening performed with human $CoREST_{187–482}$ as bait.

(B) CoREST interacts with Hsp70 in vitro. Endogenous Hsp70 protein from HEK293 whole-cell extracts is specifically retained by the immobilized fusion protein GST-$CoREST_{109–293}$. GS, glutathione Sepharose.

(C) CoREST interacts in vivo with Hsp70. Whole-cell extracts from HEK293 cells were immunoprecipitated with the indicated antibodies. Precipitated immunocomplexes were fractionated by PAGE and western blot revealed with an antibody directed against inducible Hsp70.

the major inducible Hsp upon stress; however, its expression is tightly maintained at low levels under physiological conditions. Constitutive Hsp70 expression has been shown to be cytotoxic in the absence of stress in *Drosophila* (Feder et al., 1992) and in mammalian cells (Arispe et al., 2004). The regulation of Hsp70 levels after stress has been postulated to be part of a self-limiting expression mechanism (DiDomenico et al., 1982; Morimoto, 1998; Voellmy and Boellmann, 2007). It has been shown that accumulated Hsp70 protein downregulates its own promoter through its direct interaction with activated HSF1 (Mosser et al., 1993; Shi et al., 1998), indicating that the heat shock response is regulated by a negative feedback mechanism.

In this report, we show that CoREST interacts with Hsp70 and mediates the repression of the heat shock response. CoREST overexpression represses the activation of a reporter gene driven by human *hsp70* promoter induced by heat shock or by HSF1. We demonstrated that Hsp70, in order to function as a HSF1 transcriptional corepressor, requires CoREST. This is supported by the observation that reduced levels of endogenous CoREST induce a significant augmentation of HSF1-dependent induction of *hsp70* promoter as well as increased levels of endogenous Hsp70 protein. CoREST is bound to the *hsp70* gene promoter at 37°C, as demonstrated by chromatin immunoprecipitation (ChIP), and its binding increases after 1 hr of heat shock. These data support a role of CoREST suppressing Hsp70 transcription under basal conditions and during attenuation of heat shock stress response.

## RESULTS

### CoREST Interacts with Hsp70

A yeast two-hybrid screening of a HeLa cell-Gal4 activation-domain cDNA library was performed using a fragment of human CoREST (amino acids 187–482) fused to the DNA-binding domain (DBD) of LexA. A screen of $9 \times 10^6$ transformants yielded several positives clones. DNA sequencing revealed that four isolated clones aligned with the coding sequence for the *hspa8* gene, the Hsc70 protein member of the HSPs family (accession number NP_006588.1; Dworniczak and Mirault, 1987; Figure 1A). Three other isolated clones aligned with *hspa5* coding sequence, another member of Hsp70 family of proteins (accession number NP_005338.1; Hendershot et al., 1994; Figure 1A). Alignment of the positive clones obtained in the two-hybrid assay allowed us to determine that the interaction domain in the Hsp family members with CoREST starts on amino acid 192, involving mostly the substrate-binding domain (numbered according to human *hspa1a*, accession number NP_005336.2; Figure 1A). To confirm the interaction between CoREST and Hsp70 family members, we performed a GST pull-down assay using the fusion protein GST-$CoREST_{109–293}$. As revealed by western blot, the Hsp70 protein from whole-cell extract of the HEK293 cell line is effectively and specifically retained in the glutathione-agarose matrix by GST-CoREST, but not by the GST protein alone (Figure 1B). To test for in vivo interactions between endogenous Hsp70 and CoREST, HEK293 whole-cell extracts were immunoprecipitated with αCoREST antibody. Western blotting revealed the presence of Hsp70 epitopes in the αCoREST and not in the control immunoprecipitates (Figure 1C). Reverse coimmunoprecipitation assays show that αHsp70 also precipitated CoREST protein (see Figure S1 available online).

To define the domain in CoREST required for the interaction with Hsp70 proteins, we performed a yeast two-hybrid interaction assay. A family of deletion mutants of $LexA_{DBD}$-CoREST was generated and assayed for the ability to interact

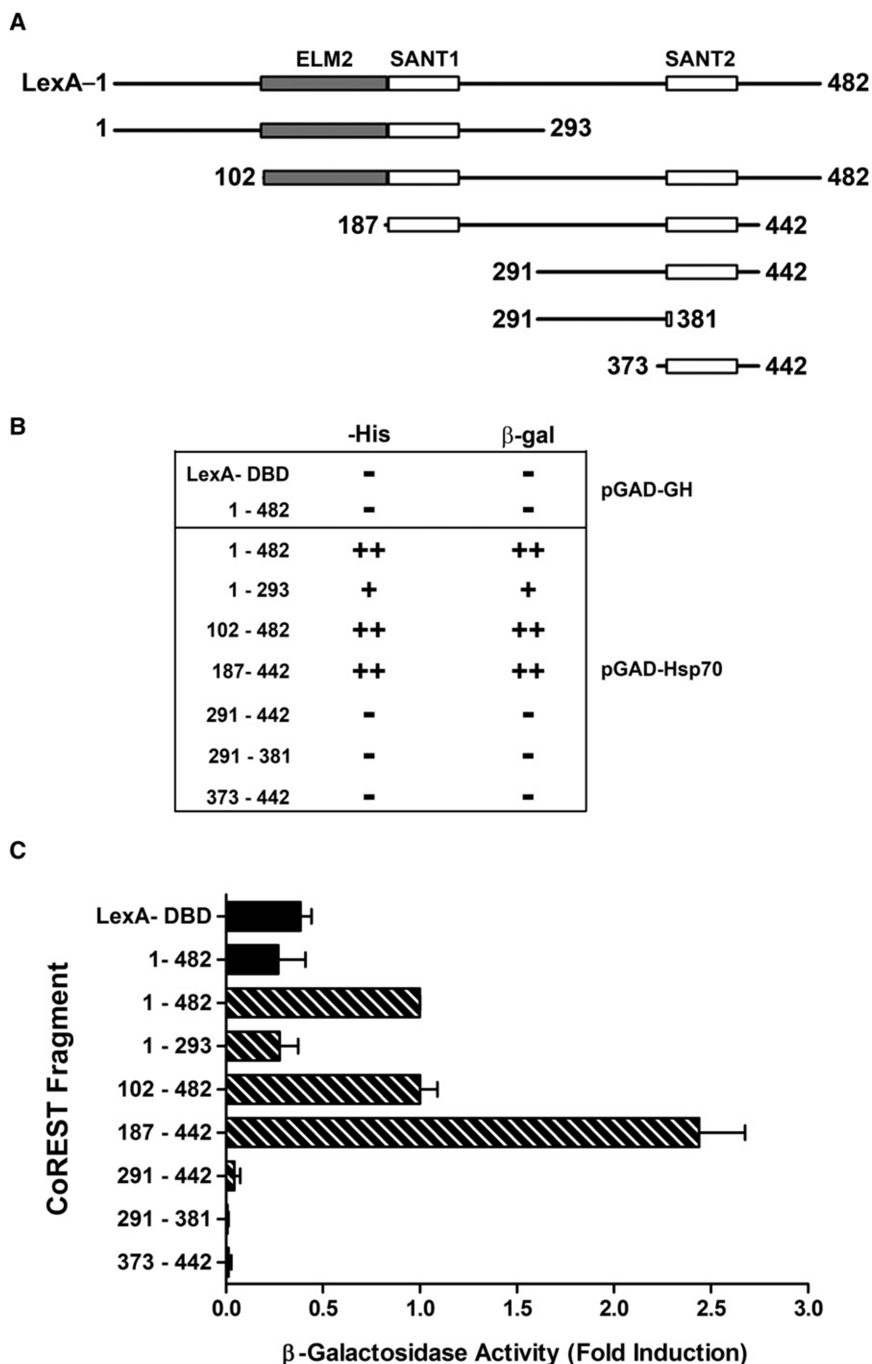


**Figure 2. The Interaction between CoREST and Hsp70 Requires CoREST SANT Domains**

(A) Scheme of the mutant deletion family of CoREST used in the yeast interaction assays with pGAD-Hsp70. Solid and white rectangles represent the ELM2 and SANT domains, respectively. CoREST (amino acids 1–482) corresponds to full-length protein.

(B) Interaction between CoREST deletion family of proteins and pGAD-Hsp70 assayed by growth on selective medium lacking histidine (−His) and β-galactosidase activity (β-gal). LexA-DBD and pGAD-GH correspond to parental vectors.

(C) Yeast two-hybrid interaction assays between CoREST deletion family of proteins and pGAD-Hsp70 (dashed columns) quantified by liquid β-galactosidase assay. Control interaction assays (black columns) correspond to LexA-DBD and pGAD-GH and LexA-CoREST and pGAD-GH. Data are expressed as fold of induction with respect to the interaction between full-length CoREST (1–482) with pGAD-Hsp70. Values correspond to the mean plus SEM of three independent experiments.

with $Gal4_{AD}$Hsp70 fusion protein in yeast (Figure 2A). Qualitative assays, i.e., growth on selective medium lacking histidine and colorimetric assay for β-galactosidase activity, show a strong interaction for CoREST constructs containing both SANT domains and weaker interaction for LexA-$CoREST_{1–293}$, which contains only the first SANT domain (Figure 2B). Quantitative β-galactosidase activity assays confirm the above results (Figure 2C) and show that the strongest interaction is produced with LexA-$CoREST_{187–442}$, the constructs that harbor both SANT domains and the inter-SANT region. Taken together, GST pull-down and two-hybrid interaction assay results indicate that the minimal domain in CoREST required to interact with Hsp70 extends from amino acids 187 to 293 containing the first SANT domain and part of the inter-SANT region. However, the strongest interaction between CoREST and Hsp70 requires both SANT domains. An interaction through SANT domains suggests modulation of CoREST repressor activity, telling that Hsp70 and CoREST may function as corepressor partners.

## CoREST Represses HSF1-Dependent Transactivation

Because Hsp70 is one of the primary induced chaperones involved in the heat shock response and is a transcriptional corepressor of HSF1, we tested the idea that CoREST could participate in the regulation of the heat shock response. Thus, we transiently overexpressed CoREST in HEK293 cells and assayed its effect over a heat shock-responsive reporter. Cells were cotransfected with the reporter plasmid p2500CAT, which contains 2.5 kb pairs of 5′-noncoding sequence from the heat-inducible human *hsp70b* gene (Schiller et al., 1988) and increasing amounts of CoREST expression plasmid. As expected, a significant increase in CAT activity is observed when cells are subjected to heat shock treatment (Figure 3A; Schiller et al., 1988). Interestingly, CoREST overexpression represses the activation of *hsp70b* gene promoter

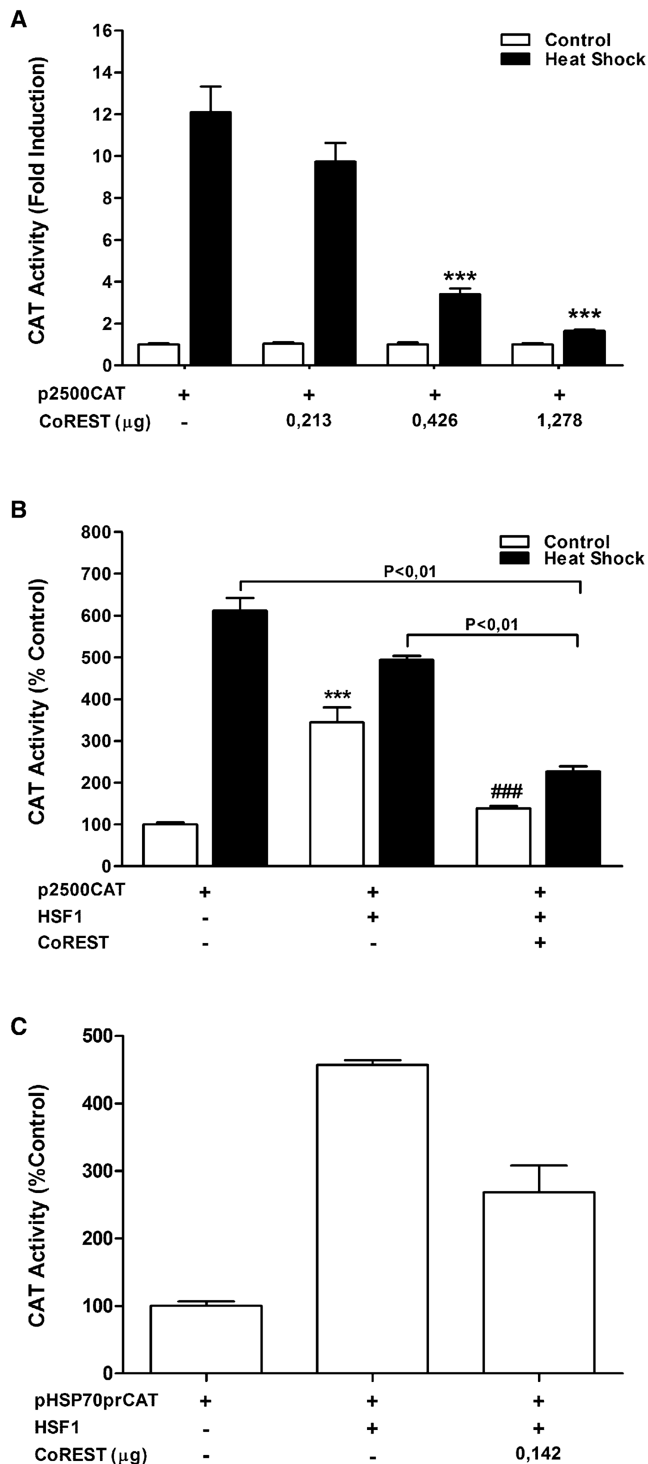


**Figure 3. CoREST Represses the Activity of *hsp70* Promoter Induced by Heat Shock and by HSF1**

(A) HEK293 cells were cotransfected with the reporter plasmid p2500CAT and increasing concentrations of pcDNACoREST (0,2 and 1,2 µg). Twenty-four hours after transfection, cells were heat shocked (43°C for 3 hr) and then returned to 37°C for another 24 hr and then harvested to perform CAT assays. Control cells were kept at 37°C for 48 hr after transfection. Data are expressed as fold of induction respective to control condition and correspond to the mean plus SEM of three independent experiments performed each by duplicate; $^{***}p < 0.001$.

(B) Cells were cotransfected with p2500CAT, Myc/His-hHSF1, and pcDNA-CoREST (0.2 µg) as indicated. HSF1 induces a significant increase of reporter activity ($^{***}p < 0.001$), which is repressed by CoREST overexpression in both control and heat shock situation ($^{\#\#\#}p < 0.001$).

(C) HEK293 cells were cotransfected with the reporter plasmids pHSP70pr-CAT, Myc/His-hHSF1, and pcDNACoREST as indicated. Forty eight hours after transfection, cells were harvested to perform CAT assay. Data are expressed as percentage of reporter activity alone and correspond to the mean plus SEM of three independent experiments performed each by duplicate. Statistical analyses were performed by one-way ANOVA followed by Tukey post hoc test (GraphPad Prism 4.0).

induced by heat shock in a dose-dependent fashion (Figure 3A). To explore if CoREST could act as a HSF1 corepressor, cells were cotransfected with p2500CAT plus an expression plasmid for HSF1, and the effect of overexpressing CoREST was analyzed under control and heat shock treatment. As reported previously, overexpressing HSF1 induces the activation of p2500CAT reporter (Figure 3B). Overexpressed HSF1 did not further increase the reporter activity already induced by heat shock. CoREST overexpression effectively repressed p2500CAT reporter activity induced by HSF1 alone as well as the activity induced by HSF1 plus heat shock (Figure 3B). Expression levels of recombinant proteins of data reported in Figures 3A and 3B were tested by western blots (Figure S2). As HSF1 overexpresion is enough to activate HSE containing promoter, we tested CoREST corepressor activity over another HSF1-responsive reporter. Hsp70prCAT reporter plasmid is driven by a region of human *hspa1a* gene (also known as *hsp70.1*), spanning from −270 to +196, containing an HSE sequence from −105 to −91 (Uenishi et al., 2005). As shown with p2500CAT, CoREST overexpression effectively reduced Hsp70prCAT activity induced by HSF1 (Figure 3C). Taken together, these results agree with the idea that CoREST represses the heat shock response by repressing HSF1-dependent transcriptional activation.

## Inhibition of CoREST Expression Results in an Increase of Both Hsp70 Protein Expression and Hsp70 Promoter Activity

To further test the idea that CoREST is a HSF1 corepressor, we investigated the effect of inhibiting CoREST expression on endogenous Hsp70 protein levels and on Hsp70 promoter activity. First, we developed stable HEK293 cell lines, pCoREST-OFF1 and pCoREST-OFF2 cells, each carrying a shRNA targeting different parts of the mRNA sequence of human CoREST. As shown in Figures 4A and 4B, the resulting stable pCoREST-OFF clones show a 50%–60% decrease in CoREST protein levels, while the control pZOFF-GFP stable clone shows CoREST protein levels similar to those of untreated HEK293 cells. The presence of the transfected vector is evidenced by GFP expression (Figures 4A and 4B). Interestingly, both pCoREST-OFF1 and pCoREST-OFF2 cells present a slight but significant increase in endogenous Hsp70 protein levels as compared to HEK293 control cells (Figures 4C and 4D), suggesting that the reduction in CoREST levels increases Hsp70 expression. This effect was further proved by analyzing the effect of decreasing CoREST

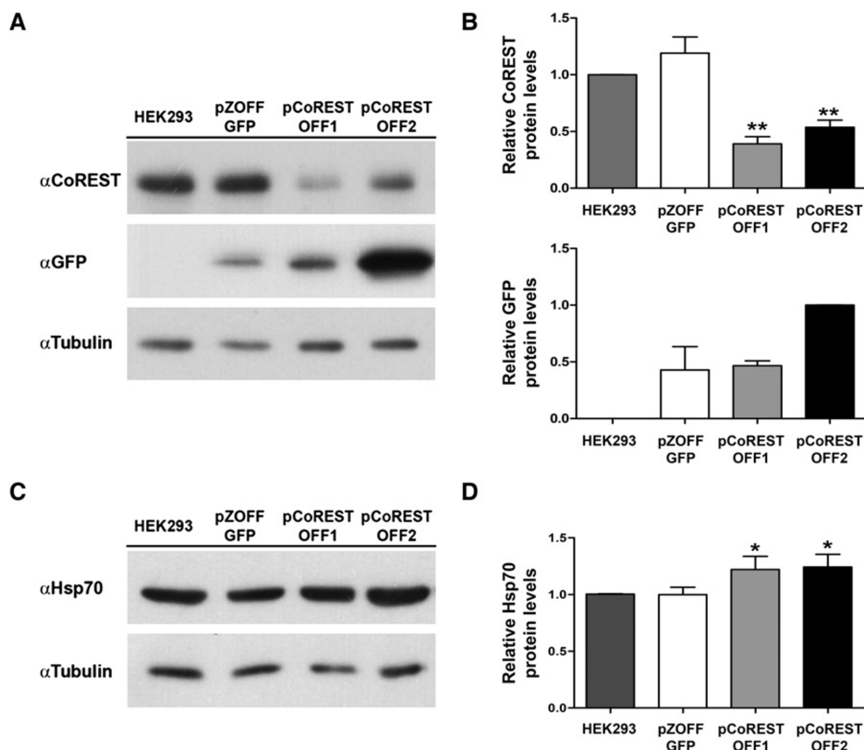


**Figure 4. Inhibition of CoREST Expression by shRNA-Mediating Silencing Results in an Increase of Hsp70 Protein Expression**

pCoREST-OFF cell lines were generated by stable transfection of HEK293 cells with pCoREST-OFF plasmids and selected by resistance to G-418 as described in the Experimental Procedures. (A and C) Representative immunoblots of the expression levels of CoREST, GFP, Hsp70, and tubulin in pCoREST-OFF and control cell lines. (B) Quantification of relative CoREST and GFP protein levels in HEK293, pZOFF-GFP, and pCoREST-OFF cell lines. Tubulin was used as load control. Data are expressed as mean plus SEM of at least three independent cultures. Statistical analyses were performed by Mann-Whitney test. **$p < 0.01$ respective to HEK293 and pZOFF-GFP controls. (D) Quantification of relative Hsp70 protein levels in HEK293, pZOFF-GFP, and pCoREST-OFF cell lines. Tubulin was used as load control. Statistical analysis was performed by Mann-Whitney test. *$p < 0.05$ respective to HEK293.

expression over *hsp70* promoter activity. HSF1 overexpression induced a significantly higher p2500CAT activity in pCoREST-OFF cells than in pZOFF-GFP control cells (Figure 5A and Figure S3A). Taken together, the results further confirm the corepressor role of CoREST over HSF1 transactivation and suggest that CoREST may regulate basal expression of Hsp70.

Finally, we evaluated the repressor role of Hsp70 in the stable pCoREST-OFF clones by cotransfecting, in the same conditions described above, an Hsp70 expression plasmid. Overexpressing Hsp70 represses HSF1-dependent transactivation of p2500CAT in pZOFF-GFP control cells (Figure 5B). This Hsp70-repressive effect is further increased when CoREST is overexpressed (Figure 5B). However, the repression exerted by Hsp70 is lost in cells with decreased CoREST protein levels. As shown in Figure 5B, Hsp70 overexpression has no effect over HSF1-dependent transactivation in both pCoREST-OFF1 and pCoREST-OFF2 stable clones. In order to test if increasing CoREST levels allow recovery of the Hsp70-repressive effect, stable pCoREST-OFF clones were transiently transfected with CoREST expression plasmids. Recombinant CoREST was observed by western blot of whole extracts from transfected pCoREST-OFF clones (Figure S3B). Increased levels of CoREST allowed the recovery of the repressive effect induced by Hsp70 (Figure 5B). These results demonstrate that CoREST represses the heat shock response induced by HSF1 and point out that Hsp70-repressive action rests on its interaction with CoREST.

## CoREST Is Bound to the Endogenous *hsp70* Gene Promoter

To further define the role of CoREST in the regulation of heat shock response, we carried out ChIP assays over the *hspa1a* promoter in unstressed and at 60 and 240 min of heat shock-stressed HEK293 cells at 42°C. Together with CoREST, we followed the binding of HSF1 and Hsp70 to characterize the relationship among these factors during the stress response. Chromatin from control and heat-shocked HEK293 cells was sonicated to obtain fragments below 600 bp (Figure S4A) and immunoprecipitated using policlonal antibodies against CoREST, Hsp70, and HSF1. Quantitative real-time PCR analysis was carried out with primers specific for the *hspa1a* gene promoter encompassing the HSE element ($^{-}$195 to $^{-}$13). Specificity of binding was controlled by amplifying sequences of the *hspa1a* coding region from the precipitated chromatin with each antibody and preimmune IgG. Significant binding of HSF1, CoREST, and Hsp70 was observed under control conditions only in the promoter region of hspa1a gene (Figure S4C).

Experimental data (Kline and Morimoto, 1997) and mathematical modeling (Rieger et al., 2005) of the stress response show that shifting HeLa cells from 37°C to 42°C induces a rapid increase of HSF1 binding to heat shock gene promoters followed by an attenuation step characterized by a sustained decrease of HSF1 binding, even though cells are kept at high temperature. Effectively, we observed that binding of HSF1 to *hspa1a* promoter increases more than ten times at 60 min of heat shock, decreasing to 4.5 times at 240 min of heat shock as compared to basal binding (Figure 6A). This HSF1 binding profile resembles experimental data obtained by Kline and Morimoto (1997) using gel shift assays. Surprisingly, under the same experimental conditions, Hsp70 binds to the *hspa1a* gene promoter similarly in unstressed cells as well as during the different steps of heat shock response (Figure 6B). Our ChIP results using anti-CoREST antibodies demonstrate that CoREST binds specifically to the endogenous *hspa1a* gene promoter (Figure S4B). Similarly to HSF1 and Hsp70, significant binding of CoREST is observed at 37°C (Figures S4B and S4C), supporting a role of CoREST regulating Hsp70 expression

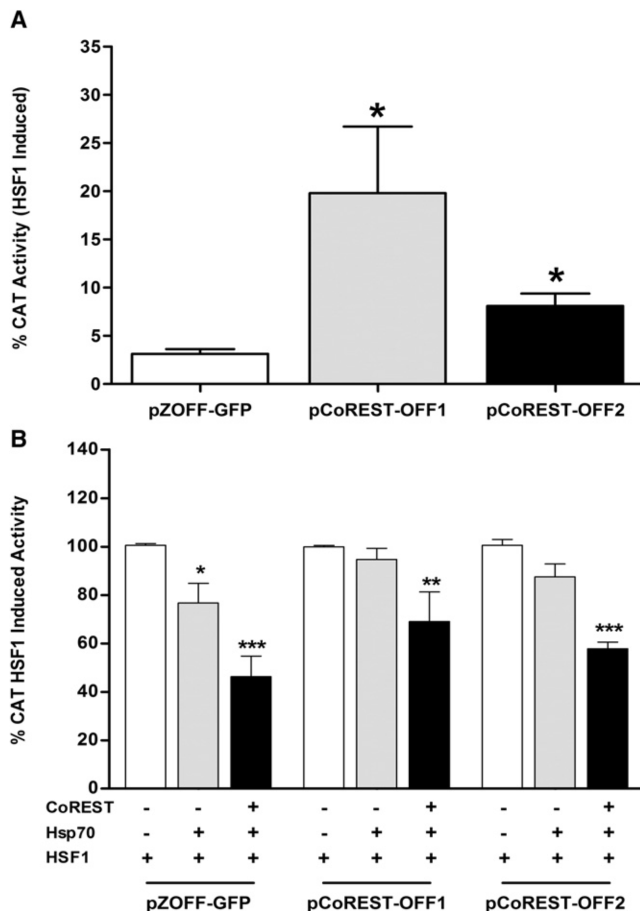


**Figure 5. pCoREST-OFF Cell Lines Show an Increase in the *hsp70* Promoter Activity Induced by HSF1, which Is Not Repressed by Overexpressing Hsp70**

(A) pZOFF-GFP and pCoREST-OFF stable cell lines were cotransfected with the reporter plasmid p2500CAT and Myc/His-hHSF1. Forty eight hours after transfection, cells were collected to carry out CAT assays. Data correspond to the mean plus SEM of three independent experiments, each performed by duplicate. Statistical analysis by Mann-Whitney test, *p < 0.05 respective to control (pZOFF-GFP).

(B) pZOFF-GFP and pCoREST-OFF cell lines were cotransfected with the reporter plasmid, p2500CAT, plus Myc/His-hHSF1, pCI-Hsp70, and pcDNA-CoREST as indicated. CAT activity induced by HSF1 in each cell line was set as 100%. Data correspond to the mean plus SEM of three independent experiments, each performed by duplicate. Statistical analysis by two-way ANOVA followed by Bonferroni post hoc test. *p < 0.05, **p < 0.01, and ***p < 0.001 respective to control.

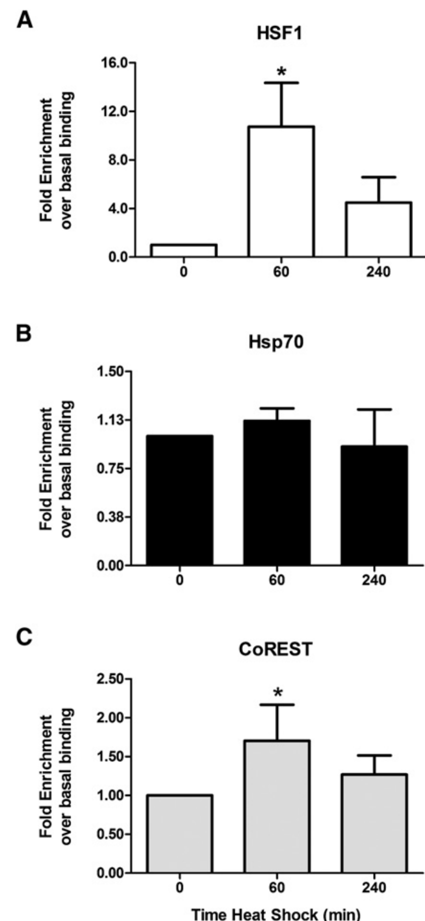


**Figure 6. CoREST Is Bound to the *hspa1a* Gene Promoter under Basal and Heat Shock Stress Conditions**

HEK293 cells were heat shocked at 42°C during 60 and 240 min or kept unstressed (0 min). Chromatin was immunoprecipitated with anti-HSF1 (A), anti-Hsp70 (B), and anti-CoREST (C) antibodies and amplified by quantitative real-time PCR using primers flanking HSE element (⁻195 to ⁻13) of hspa1a gene promoter. Data are expressed as fold enrichment over basal condition (unstressed, t = 0 min), and correspond to the mean plus SEM of four independent experiments. Statistical analysis by Kruskal-Wallis one-way ANOVA was followed by Dunn's multiple comparison test; *p < 0.05.

under basal conditions. A significant increase of CoREST occupancy of the *hspa1a* gene promoter is observed at 60 min, which slightly decreases at 240 min of heat shock (Figure 6C). Taken together, these data demonstrate that CoREST binds to heat shock promoter under basal conditions and that its binding significantly increases during the initiation of the attenuation of HSR.

## DISCUSSION

The stress response involves a rapid transcriptional activation of stress genes in cells exposed to several kinds of stressors. The induction of stress genes is transient, even though the stressor—for instance, heat—is still present. The attenuation of the stress response allows returning of heat shock genes to basal levels after a rapid and efficient induction. The mechanism responsible for the stress response attenuation started to emerge when it was discovered that the chaperone Hsp70 behaves as a corepressor of HSF1 (Mosser et al., 1993; Shi et al., 1998). Here we show that the transcriptional corepressor CoREST is an interacting partner of Hsp70. Through this interaction, CoREST represses both HSF1-dependent transcriptional activation and heat shock-dependent activation of the *hsp70* promoter. According to this, we propose that CoREST is a key player of the regulatory mechanism of stress gene expression.

Heat shock transcriptional activation of heat shock genes is induced by binding of activated HSF1 trimers to HSE elements,

triggering the increase of chaperone expression-like Hsp70, a hallmark of HSR. Attenuation of HSR involves several steps, one of which is the rapid repression of heat shock gene transcription, before HSF1 leaves HSE elements (Abravaya et al., 1991; Kline and Morimoto, 1997). In a series of experiments, Morimoto's team demonstrated that the molecular chaperone Hsp70 interacted directly with HSF1 when it is still bound to promoters and repressed the heat shock gene transcription (Shi et al., 1998). Since neither HSF1 DNA binding nor phosphorylation of HSF1 was affected by Hsp70, it was proposed that Hsp70 acted by blocking the activation domain (AD) of HSF1, and indeed they demonstrated that Hsp70 interacted with the transcriptional AD of HSF1 (Shi et al., 1998). At first sight, this would mean that blocking the interaction of HSF1 with transcriptional coactivators and/or basal transcriptional machinery would be enough to prevent transcription of heat shock genes. Our experiments with shRNA against CoREST clearly establish that this is not the case. The transcriptional repressive effect of overexpressed Hsp70 over HSF1-dependent transactivation shown by us here and by others (Shi et al., 1998) is lost in cells with reduced CoREST levels. The repressive effect of Hsp70 was recovered when CoREST was reintroduced in cells (Figure 5). These data suggest that the mechanism of attenuation of heat shock stress is an active transcriptional repression process mediated by CoREST. This possibility is supported by the fact that CoREST binding to the *hsp70* gene promoter increases significantly at the initiation of the attenuation of HSR, as shown by ChIP assays. Interestingly, a complex relationship among HSF1, Hsp70, and CoREST emerged from the ChIP assays. Under basal conditions (no stress) there is a significant binding of HSF1, Hsp70, and CoREST to the *hsp70* promoter. After 1 hr of heat shock stress, corresponding to the initial phase of the attenuation of the stress response (Rieger et al., 2005), the highest binding of HSF1 is observed, which exactly matches previous results (Kline and Morimoto, 1997). CoREST binding to *hsp70* promoter is also highest at the initiation of the attenuation phase of the stress response. Then, at the end of the attenuation, characterized by a significant decrease of HSF1 binding to the *hsp70* promoter, CoREST and Hsp70 binding are maintained. We propose that two complexes regulate the expression of Hsp70. One complex consists of HSF1 inactive with a low affinity for Hsp70 and CoREST that keeps a restricted expression of heat shock genes during basal conditions, and another complex consists of HSF1 active with a high affinity for Hsp70 and CoREST, which represses the expression of heat shock genes during the attenuation of the stress response. This model also explains the increment of endogenous Hsp70 protein levels (Figure 4) as well as the increment in basal activity of *hsp70* promoter (Figure S3) provoked in cells with decreased levels of CoREST. Further experiments will clarify whether CoREST-Hsp70-repressive complex leaves the *hsp70* promoter at the initiation of the stress response or if the repressive activity of CoREST-Hsp70 complex is regulated through the different phases of the stress response by posttranslational modifications.

Several reports have shown that the inducible transcription of Hsp70 is enhanced by treating with inhibitors of histone deacetylases (Chen et al., 2002; Ovakim and Heikkila, 2003; Zhao et al., 2005, 2006), demonstrating that histone deacetylases are involved in *hsp70* gene transcriptional regulation. CoREST complex brings to chromatin histone deacetylases activity (Ballas et al., 2001) by forming a stable complex with HDAC1 and HDAC2 (You et al., 2001; Hakimi et al., 2002). It has been shown that CoREST has a main role promoting H3 deacetylation in nucleosomes (Lee et al., 2005), a feature that could be related to the fluctuated acetylation level of histone H3 at *hsp70* promoter exhibited during heat shock (Zhao et al., 2006). Thus, we propose that the interaction with CoREST allows a wide set of transcriptional regulatory events to be coupled to the transcription of heat shock genes, ranging from repression to silencing. These CoREST-mediated responses have been demonstrated for transcriptional regulation of neuronal genes (Ballas et al., 2005).

Similarly to the interaction between HSF1 with Hsp70 (Shi et al., 1998), the domain in Hsp70 required to interact with CoREST comprises mainly the substrate binding domain of Hsp70, thus predicting a substrate-chaperone complex. Further studies will be required to determine if CoREST-Hsp70 complexes are dissociated by ATP. On the other hand, the domain in CoREST required to interact with Hsp70 includes both SANT domains and the inter-SANT domain region. The first SANT domain by itself is able to maintain a rather weak stable interaction with Hsp70 (GST pull-down results). SANT domains are conserved protein motifs present in several transcription factors, which have been proposed to interact with histone tails and promote histone deacetylation. Thus, we suggest that a stabilized interaction between CoREST and Hsp70 including both SANT domains will bring HDACs to heat shock gene promoters. A role of molecular chaperones in transcriptional activation and repression has been described in other scenarios (Johnson et al., 2002; Freeman and Yamamoto, 2002). It is worth noting that Hsp70 was shown to form an integral part of the LSD1 histone demethylase-CoREST complex (Shi et al., 2005), suggesting that the interaction between CoREST and Hsp70 can have functional meaning beyond heat shock gene transcriptional repression. On other hand, it has been substantiated that HSF1 functions as a transcriptional repressor regulating the expression of several genes (Chen et al., 1997; Xie et al., 2002; Ciocca et al., 2006). Interestingly, it was demonstrated that Hsp70 and HSF1 cooperate to repress Ras-induced transcriptional activation of the *c-fos* gene (He et al., 2000). These data allow the suggestion that the association with CoREST-Hsp70, and probably with HSF1, plays regulatory roles in the control of transcription of several sets of genes.

In summary, we provide evidence that the transcriptional corepressor CoREST represses basal as well as heat shock- and HSF1-dependent transcriptional induction of Hsp70. Based on these observations, we suggest that the attenuation process of the HSR is an active transcriptional repressive process mediated by the CoREST complex. Importantly, this study uncovers a new set of genes, besides neuronal genes, that is regulated by CoREST.

## EXPERIMENTAL PROCEDURES

### Cell Culture Conditions

Human embryonic kidney cell line (HEK293) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and maintained at 37°C and 5% $CO_2$.

### Antibodies

Antibodies that recognize Hsp70 and GFP were purchased from Santa Cruz Biotechnology (K-20 and B-2, respectively). Polyclonal anti-CoREST antibody was described previously (Andres et al., 1999). Antibody that recognizes α-tubulin was obtained from Sigma (T5168).

### Plasmids

LexA-CoREST full-length, the mutant deletion family of CoREST fragments fused to LexA; GST-CoREST; and pcDNACoREST were described previously (Andres et al., 1999; Battaglioli et al., 2002). pGAD-Hsp70 corresponds to clone 31 obtained from the yeast two-hybrid screening assay, and encodes amino acids 188–646 of human Hsp70, HSPA8, fused to the GAL4 AD in pGAD-GH. Expression vectors Myc/His-hHSF1 and pCI-Hsp70 encoding full-length human HSF1 and Hsp70, respectively, were described previously (Hietakangas et al., 2003; Chávez Zobel et al., 2003). The plasmid p2500CAT (Stressgen Biotechnologies Corp) encodes for the chloramphenicol acetyltransferase (CAT) gene driven by 2.5 kb pairs of 5′-noncoding sequence from the heat-inducible human *hsp70b* gene (Schiller et al., 1988). pHsp70prCAT was kindly provided by Dr. Shingi Koizumi (Uenishi et al., 2005).

### Yeast Two-Hybrid Screening

Yeast two-hybrid screening was performed essentially as described (Andres et al., 1999). Briefly, L-40 yeasts were cotransformed with LexA-CoREST$_{(187–482)}$, used as bait, and together with a HeLa cell cDNA library fused to the AD of Gal4 in pGAD-GH. An estimated 9 × $10^6$ transformants were screened. Clones interacting with CoREST were identified as previously described (Hollenberg et al., 1995) by growth on selective medium lacking histidine and confirmed by assaying for β-galactosidase activity. Specificity of the interaction was tested by a mating assay between the positive L-40 transformants and an AMR-70 strain expressing either LexA-CoREST$_{(187–482)}$ and several LexA fusion proteins unrelated to CoREST. Positives clones were characterized by sequencing analysis. Direct interaction assays were performed by using yeast transformed with either LexA-CoREST full length or smaller fragments of CoREST (Ballas et al., 2001) with pGAD-Hsp70 fused to Gal4-AD. Interaction was evaluated by growth on medium lacking histidine followed by qualitative and quantitative β-galactosidase assays as described (Hollenberg et al., 1995; Galleguillos et al., 2004).

### GST Pull-Down Assay

Protein (200 μg) from HEK293 whole-cell extract (obtained as described bellow) was incubated with either GST-CoREST$_{(109–293)}$ or GST (20 μg) bound to glutathione-agarose beads (Pharmacia) for 1 hr at 4°C. After extensive washing with PBS 0.3% Tween-20, retained proteins were eluted by boiling the beads in Laemmli sample buffer containing 5% β-mercaptoethanol. Eluted proteins were fractionated by SDS-PAGE and visualized by western blot with a goat polyclonal antibody against human Hsp70.

### Coimmunoprecipitation Assay

Whole-cell extracts from HEK293 cells were obtained by lysing cells in lysis buffer (50 mM HEPES [pH 7.9], 150 mM NaCl, 1 mM EDTA, 2 mM $MgCl_2$, 1% Triton X-100, 10% glycerol, and 3 mM PMSF plus proteases inhibitors). Whole-cell extracts were precleared with protein A Sepharose beads, preblocked overnight at 4°C in Curran's blocking buffer (CBB, 10 mM $MgCl_2$, 100 mM KCl, 1 mM $CaCl_2$, 10 mM imidazole [pH 7.0], 5% BSA, and 0.3% Tween-20). After preclearing, extracts were incubated with 2 μg of the appropriate antibody overnight at 4°C. Immunocomplexes were precipitated by adding 20 μl of fresh preblocked protein A Sepharose beads incubated in rotation for 3 hr at 4°C. Finally, after extensive washing with wash buffer (20 mM HEPES [pH 7.9], 250 mM NaCl, 1.5 mM $MgCl_2$, 2 mM EDTA, and 1 mM PMSF), bound proteins were eluted by boiling the beads in Laemmli sample buffer containing 5% β-mercaptoethanol, separated by SDS-PAGE, and visualized by western blot with an Hsp70 polyclonal antibody.

### Transient Transfections and Reporter Gene Assays

HEK293 cells (5,5 × $10^5$cells/well, 6-well plate) were transfected with 1 μg of total DNA by using Lipofectamine 2000 reagent (Invitrogen). p2500CAT reporter plasmid (0,42 μg) was used in 2:1 molar ratio respective to expression plasmids Myc/His-hHSF1, pcDNACoREST, and pCI-Hsp70. Additionally, Hsp70prCAT reporter plasmid (0,267 μg) was used in 3:1 molar ratio respective to expression plasmids. Control experiments were carried out by using molar equivalent amounts of empty vectors. DNA was kept constant by adding pBLUescript SK (Stratagene), and in every experiment 0,25 μg of pCMX-β-gal reporter vector was cotransfected as control of transfection efficiency. CAT assays were performed 48 hr after transfection as described (Tapia-Ramirez et al., 1997). CAT activity was quantified in a Cyclone System from Perkin Elmer. Heat shock treatment was applied 24 hr after transfection.

### shRNA Vector Construction

pZOFF-EGFP (Terry-Lorenzo et al., 2005) was used to coexpress shRNA against CoREST along with EGFP in order to identify transfected cells and to analyze the cellular effects of reducing endogenous CoREST protein levels by RNA interference. pCoREST-OFF (1 and 2) constructs were created by ligating the annealed oligos 5′-GATCCCCGGTAATTGGTTTCAGTCAAATTTCTCTTGAAAATTTGACTGAAACCAATTCCTTTTTGGAAA-3′ and 5′-AGCTTTTCCAAAAAGGAATTGGTTTCAGTCAAATTTTCAAGAGAAATTTGACTGAAACCAATTCCGGG-3′ for pCoREST-OFF1 and 5′-GATCCCCCGACGCCGCTTCAACATAGTTTCTCTTGAAAACTATGTTGAAGCGGCGTCGTTTTTGGAAA-3′ and 5′-AGCTTTTCCAAAAACGACGCCGCTTCAACATAGTTTTCAAGAGAAACTATGTTGAAGCGGCGTCGGGG-3′ for pCoREST-OFF2 into HindIII- and BglII-digested pZOFF-EGFP. The resulting shRNAs targeted human CoREST base pairs 1020–1038 and 1273–1291, respectively (accession number NM_015156.1).

### Generation of pCoREST-OFF Stable Cell Lines

HEK293 cells were transfected with 5 μg of either pZOFF-GFP, pCoREST-OFF1, or pCoREST-OFF2 plasmids by using the calcium phosphate method. Forty-eight hours after transfection, media was removed and replaced with fresh media containing 600 μg/ml of G-418 (Sigma) for selection. Six days later, resistant cell clones were isolated by trypsinization and transferred to polypetri dishes for further selection. Isolated clones were maintained with 200 μg/ml of G-418. Efficacy of pCoREST-OFFs was analyzed by monitoring protein expression by western analysis with mouse anti-GFP antibody (Santa Cruz Biotech) and with rabbit polyclonal anti-CoREST antibody. Hsp70 protein levels were compared and quantified in pCoREST-OFF and pZOFF-GFP (control) stable cell lines in western blot assays using tubulin as load control. Quantification of protein levels was performed by using ImageJ software (National Institutes of Health). Transient transfection and CAT experiments with pZOFF-GFP and pCoREST-OFF stable cell lines were conducted as described previously in this section.

### Chromatin Immunoprecipitation Assay

ChIP was performed as described previously (Ballas et al., 2001) with some modifications. For detailed protocol description, see the Supplemental Experimental Procedures. Briefly, DNA was crosslinked to protein with formaldehyde. Cells were harvested and lysed in cell lysis buffer (CLB). Isolated nuclei were resuspended in nuclear lysis buffer (NLB) and sonicated to shear chromatin. Immunoprecipitations were carried out with the following antibodies (5.0 μg): anti-CoREST, anti-HSF1, and anti-Hsp70. Preimmune IgG (5.0 μg) and no antibody were utilized as control of specificity of immunoprecipitation. Subsequently, crosslink were reverted and bound DNA was purified by phenol:chloroform extraction. Quantitative real-time PCR analysis was performed using a LightCycler (Roche), and 1 μl of each sample was subjected to PCR using the following primers: Hspa1apr-F (5′-GGAAGGTGCGGGAAGGTTCG-3′) and Hspa1apr-R (5′-TTCTTGTCGGATGCTGGA-3′), Hspa1ac.1-F (5′-AAGGACATCAGCCAGAACAAGCGA-3′) and Hspa1ac.1-R (5′-ACGTGTAGAAGTCGATGCCCTCAA-3′), and Hspa1ac.2-F (5′-TGGAGTCCTACGCCTTCAACATGA-3′) and Hspa1ac.2-R (5′-TCCTCTTGTGCTCAAACTCGTCCA-3′). Standard curves were generated by using serial dilutions of previously quantified genomic DNA. The amount of immunoprecipitated DNA in each sample was calculated using fit point analysis with no baseline adjustment.

### Heat Shock Protocols

Heat shock was performed by incubating HEK293 cells for 3 hr at 43°C and then returned to 37°C for another 24 hr. For ChIP assays, HEK293 cells were heat shocked at 42°C during 1 or 4 hr.

## SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures and four figures and can be found with this article online at http://www.molecule.org/cgi/content/full/31/2/222/DC1/.

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