Transcriptional regulation of the *Drosophila catalase* gene by the DRE/DREF system

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ABSTRACT

Reactive oxygen species (ROS) cause oxidative stress and aging. The *catalase* gene is a key component of the cellular antioxidant defense network. However, the molecular mechanisms that regulate *catalase* gene expression are poorly understood. In this study, we have identified a DNA replication-related element (DRE; 5′-TATCGATA) in the 5′-flanking region of the *Drosophila catalase* gene. Gel mobility shift assays revealed that a previously identified factor called DREF (DRE-binding factor) binds to the DRE sequence in the *Drosophila catalase* gene. We used site-directed mutagenesis and in vitro transient transfection assays to establish that expression of the *catalase* gene is regulated by DREF through the DRE site. To explore the role of DRE/DREF in vivo, we established transgenic flies carrying a catalase–lacZ fusion gene with or without mutation in the DRE. The β-galactosidase expression patterns of these reporter transgenic lines demonstrated that the *catalase* gene is upregulated by DREF through the DRE sequence. In addition, we observed suppression of the ectopic DREF-induced rough eye phenotype by a catalase amorphic CatΔ¹ allele, indicating that DREF activity is modulated by the intracellular redox state. These results indicate that the DRE/DREF system is a key regulator of *catalase* gene expression and provide evidence of cross-talk between the DRE/DREF system and the antioxidant defense system.

INTRODUCTION

Reactive oxygen species (ROS) play an important role in cell growth, differentiation, progression and death (1). At low concentration, ROS are indispensable in various biological processes such as intracellular signaling and the immune response (2). However, higher concentrations of ROS are involved in the aging process as well as in human disease states, including cancer, ischemia, and immune and endocrine system deficiencies. As a safeguard against the accumulation of ROS, several non-enzymatic and enzymatic antioxidant activities exist (1,2). The physiological level of ROS is maintained by an antioxidant defense system including the superoxide dismutase (SOD) and catalase enzymes, which convert superoxide anions to H₂O₂ and oxygen, and H₂O₂ to water and oxygen, respectively (3,4). Recent studies have shown that the induced over-expression of antioxidant enzymes such as SOD and catalase can extend the lifespan (5,6).

The *catalase* gene has been isolated from human (7), rat (8), mouse (9), *Drosophila* (10,11) and yeast (12), and it is likely that regulated expression of the *catalase* gene is critical for ROS homeostasis in many settings. Several studies have demonstrated tissue-specific expression of the *catalase* gene in mammals (13,14). Moreover, *catalase* expression is regulated at the transcriptional level by both Sp1 and CCAAT-recognizing factors in HP100 and mouse muscle cells (15). In *Drosophila*, expression of the *catalase* gene during development is responsive to ecdysone and is regulated at both transcriptional and post-transcriptional levels (16), although the molecular mechanisms of this regulation are poorly understood.

The homodimeric transcription factor DNA replication-related element (DRE)-binding factor (DREF) is known to play an important role in regulating DNA replication- and cell proliferation-related genes by binding to the DRE site (5′-TATCGATA) of target genes (17–22). The DRE–DREF interaction controls transcription of *Drosophila* mitochondrial transcription factor A gene (*D-mtTFA*) (23), and DREF associates with TRF2 to direct promoter-selective gene expression in *Drosophila* (24). Recently, a homolog of DREF (hDREF) has been identified in humans, where it may regulate genes related to cell proliferation (25).

In the present study, we have identified a DRE sequence located in the 5′-flanking region of the *Drosophila catalase* gene, and have investigated the role of DREF in transcriptional regulation of this gene. Our results indicate that the DRE/DREF system is a key regulator for *Drosophila catalase* gene expression.

MATERIALS AND METHODS

Oligonucleotides

All oligonucleotides were chemically synthesized. Sequences containing the DRE sequence or base substitutions in the

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catalase promoter region was as follows: catalase-DRE wild-type (wt), 5'-gatccGATGGAATATCGATATCTTCGGCa-3' and 3'-gCTACCTTTATAGCTATAAGCGGcatg-5'; catalase-DRE mutant (mut), 5'-gatccGATGGAATATCGATATCTTCGGCa-3' and 3'-gCTACCTTTATAGCTATAAGCGGcatg-5'. Double-stranded oligonucleotides for site-directed mutagenesis were catalaseDREMut 5'-CTTAAATCGAAATTAGATCTTCCGGCa-3' and 3'-CCCGGCCTTCTATAGGATTTAAGCTATAATC-5'. Oligonucleotide primers for RT–PCR were designed as follows: catalase, 5'-ggtaccCTTTGAGGTGACCCACGACA-3' and 3'-gagctcACCTTTCATGTACC-5'; DREF, 5'-ctcgagATGAGCGAGGGGTACCA-3' and 3'-gagctcACCTTTCATGTCCCGAT-5'. An amplified 1756 bp fragment (pGEM-T-catalase) containing an upstream region of the catalase gene (~1030 to +18 with respect to the transcription initiation site) was inserted into pGEM-T vector (Promega). A fragment containing the catalase promoter region excised from pGEM-T-catalase was subcloned into the EcoRI site of pBluescript II KS (+) (pBScat) and sequenced. Plasmid pBScatGraf was digested with SacI and XhoI, the DNA fragment containing the catalase gene promoter region was inserted between SacI and XhoI sites of pGL2-Basic (Promega) and the resulting reporter construct was designated as pcatGraf. To construct the plasmids pcatGraf-lacZ and pcatGrafDREMut-lacZ for transgenic flies, the catalase promoter regions with and without base-substituted mutations in the DRE were inserted into the EcoRI site of the plasmid pCaSpeR-AUG-βgal, respectively.

Site-directed mutagenesis

To obtain the pcatGrafDREMut-lacZ mutant reporter plasmid carrying base substitution mutations in the DRE in the 5'-flanking region of the Drosophila catalase gene, the mutagenesis reaction was carried out on double-stranded DNA of pcatGraf-lacZ using the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene). The reaction was set up essentially as recommended by the manufacturer. The mutation and the fidelity of the remaining DNA were confirmed by sequencing.

Cell culture and DNA transfection

Drosophila Kc cells (26) were grown at 25°C in M3 (BF) medium (Sigma) supplemented with 2% fetal bovine serum and 0.5% penicillin–streptomycin (Gibco-BRL). All plasmids for transfection were prepared by using the Qiagen Plasmid Kit (Qiagen). Dimethylsulfoxide ammonium bromode- mediated transfection of Drosophila cultured cells was performed as described previously (27). Luciferase reporter assays were performed with a luminometer (TD-20/20, Turner Designs). Luciferase activities normalized to β-galactosidase activities were calculated by determining the luciferase/β-galactosidase activity ratios and by averaging the values from at least three experiments, from which means and standard errors were calculated.

Preparation of nuclear extracts

Preparation of nuclear extracts from cultured cells was performed as described elsewhere (28). Cultured Drosophila Kc cells were rinsed once with ice-cold phosphate-buffered saline (PBS). The cell pellet was collected by centrifugation at 4000 r.p.m. for 5 min and resuspended in buffer A (10 mM HEPES, 1.5 mM MgCl₂, 10 mM NaCl, 0.25% NP-40, pH 7.5) and incubated on ice for 5 min, followed by centrifugation at 4000 r.p.m. for 5 min. The supernatant (cytosolic extract) was removed and the nuclei were extracted with buffer C (20 mM HEPES, 250 mM KCl, 0.2 mM EDTA, 0.25% NP-40, pH 7.5). The nuclei were vortexed vigorously several times several over 20 min, followed by centrifugation at 14 000 r.p.m. for 5 min. The supernatant (nuclear extract) was transferred into fresh tubes, diluted 1:2 with buffer D (20 mM HEPES, 50 mM KCl, 0.2 mM EDTA, 20% glycerol, pH 7.5) and frozen at −80°C until usage.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed as described earlier (22). Kc cell nuclear extracts were incubated for 10 min at room temperature in 20 µl of reaction mixture containing 10 mM HEPES (pH 7.6), 50 mM KCl, 1 mM EDTA, 5% glycerol, 0.5 mM dithiothreitol (DTT), 1 µg of sonicated herring sperm DNA and 500 ng of poly(dI–dC). Unlabeled competitor oligonucleotides or antibody were also added at this step. After that, the 32P-end-labeled catalase-DRE oligonucleotide (1 × 10⁴ c.p.m.) were added and the mixture was further incubated for 20 min at room temperature. The retarded bands were electrophoretically resolved on a 6% non-denaturing Tris-borate-EDTA polyacrylamide gel. The gels were dried and autoradiographed on X-ray film or analyzed with a BAS 2000 imaging analyzer.

Fly stocks and establishment of transgenic flies

Fly stocks were maintained at 25°C on standard food. To establish transgenic flies carrying pcatGraf-lacZ or pcatGrafDREMut-lacZ, P element-mediated germline transformation was carried out as described previously (29,30). Four independent lines were obtained with pcatGraf-lacZ and pcatGrafDREMut-lacZ constructs, respectively. The line 67 carrying pcatGraf-lacZ on the third chromosome and line 64 carrying pcatGrafDREMut-lacZ on the third chromosome were used in this study. The lines carrying the same fusion genes showed the same lacZ expression patterns. For ectopic expression of DREF using the GAL4-UAS system, Hsp70-GAL4 (hs-GAL4), GMR-GAL4 (31) lines and the transgenic flies carrying UAS-DREF on the second chromosome described previously (32) were used. The Car⁰/TM3 strain was kindly supplied by the Bloomington Stock Center. Oregon-R was used as wild type. The UAS-DREF/+;hs-GAL4/+ flies were derived from a cross of the homozygous UAS-DREF male flies to the homozygous hs-GAL4 female flies, and +/+;hs-GAL4/+ flies were derived from a cross of the Oregon-R males to the homozygous hs-GAL4 female flies.
The GMR-GAL4/+;UAS-DREF/+;Catn1/++ flies were obtained from a cross of the UAS-DREF/UAS-DREF;Catn1/TM6B females (derived from a cross of the +/SM1;Catn1/TM3 female flies to the UAS-DREF/UAS-DREF;Pre/TM6B male flies) to the males carrying pGMR-GAL4 on the X chromosome. The GMR-GAL4/+ flies derived from a cross of the GMR-GAL4/Y males to the Oregon-R females. GMR-GAL4/+;UAS-DREF/+ flies from a cross of the GMR-GAL4/Y males to the UAS-DREF/UAS-DREF females and GMR-GAL4/+;+/+;Catn1/++ flies from a cross of the GMR-GAL4/Y males to the Catn1/TM6B females were used.

RT–PCR

Total RNA from larvae was isolated with Trizol reagent (Molecular Research Center, Inc.) according to the protocol furnished by the manufacturer. cDNAs were synthesized with M-MLV-RT (Promega). The RT–PCR products were analyzed on 1.5% agarose gels stained with ethidium bromide.

X-Gal staining

The tissues were dissected and fixed for 15 min in PBS containing 1% glutaraldehyde, washed in PBS, and immersed in 0.2% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) in staining buffer containing 6.1 mM K₄Fe(CN)₆, 6.1 mM K₃Fe(CN)₆, 1 mM MgCl₂, 150 mM NaCl, 10 mM Na₂HPO₄ and 10 mM NaH₂PO₄. Incubation was in the dark at 37°C.

Quantitative measurement of β-galactosidase activity in extracts

Quantitative measurements of β-galactosidase activity in extracts prepared from Drosophila bodies were carried out as described previously (30). The β-galactosidase activity was defined as absorbance units/mg of protein/h. To correct for endogenous β-galactosidase activity, extracts from the host strain (white) were included in each experiment, and this background reading was subtracted from readings obtained with each transformant line.

Scanning electron microscopy

Adult flies were sputter-coated with platinum and observed under a Hitachi S-3500N scanning electron microscope in the low-vacuum mode.

RESULTS

DREF binds to the DRE sequence in the 5'-flanking region of the catalase gene

In the region between −85 and −78 with respect to the transcription initiation site of the Drosophila catalase gene, we identified a sequence identical to DRE (5'-TATCGATA) (Fig. 1A). Because the sequence is located near the transcription initiation site, we considered that DREF may be involved in regulation of the catalase gene promoter. We termed this DRE sequence a catalase-DRE site.

To examine whether DREF recognizes the putative DRE site located in the catalase promoter, a gel mobility shift assay was performed using Kc cell extracts and labeled catalase-DRE wt oligonucleotides as probe. Protein–DNA complexes were detected, and were efficiently competed by the inclusion of unlabeled catalase-DRE wt oligonucleotides in the binding reaction, but not by the inclusion of catalase-DREMwt oligonucleotides carrying a base substitution in the DRE sequence (Fig. 1B, lane 3). Furthermore, the addition of an anti-DREF monoclonal antibody (monoclonal antibody 4) (33) to the binding reaction resulted in a supershift of the protein–DNA complex (Fig. 1B, lane 5). These results indicate that DREF binds to the catalase-DRE site with sequence specificity.

Figure 1. Complex formation between the DRE in the 5'-upstream region of the catalase gene and the Kc cell nuclear extract. (A) Structure of the 5'-upstream region of the catalase gene and base substitutions in the DRE. The transcription initiation site is indicated by the arrowhead and numbered +1. The closed box indicates the DRE sequence in the catalase gene promoter. The nucleotide sequences of the catalase-DRE site and its base-substituted mutants are shown in boxes below, with lower case letters for substituted nucleotides. (B) 32P-Labeled catalase-DRE oligonucleotides (Probe) were incubated with Kc cell nuclear extracts in the absence of competitor (lane 2), in the presence of excess unlabeled catalase-DRE competitor (lane 3, wild-type; lane 4, mutant; see Materials and Methods) or in the presence of anti-DREF monoclonal antibody (MAb) (lane 5) (33). Nuclear extract was omitted from the assay shown in lane 1.
Role of DRE in catalase gene expression

To examine the role of the DRE in catalase gene promoter activity, we constructed reporter plasmids containing the catalase promoter region (−1030 to +18 with respect to the transcription initiation site) with or without mutation in the DRE fused to a luciferase reporter (Fig. 1A). The plasmids were then transfected into Drosophila Kc cells and luciferase expression levels were determined. As shown in Figure 2, mutation in the DRE resulted in extensive reduction (95%) of luciferase expression.

To investigate the role of the DRE in catalase expression in living flies, we established transgenic flies carrying the catalase promoter region with or without base-substituted mutation in the DRE fused to lacZ. Quantitative β-galactosidase activities of the transgenic larvae and adults bearing catalase–lacZ or catalaseDREmut–lacZ fusion genes were examined. The mutation in the DRE reduced the β-galactosidase expression to 85% in larvae and to 84–85% in adults as shown in Figure 3A. We examined the expression of catalase–lacZ and catalaseDREmut–lacZ fusion gene in transgenic larvae and adults by X-gal staining. In larval tissues, the β-galactosidase expression was detected in the brain lobes, ganglions, guts and gonads of third instar larvae bearing catalase–lacZ. β-Galactosidase expression in the same tissues bearing catalaseDREmut–lacZ was significantly reduced (data not shown). In adult tissues, β-galactosidase expression was detected in the foregut, hindgut, muscle and reproductive system (Fig. 3B). β-Galactosidase expression in the same tissues of adult flies carrying catalaseDREmut–lacZ were significantly reduced (Fig. 3B). These results indicate that the DRE is required for tissue-specific expression of the catalase gene.

Figure 2. Effect of base substitution mutations in the DRE sequence on catalase gene promoter activity in Kc cells. Wild-type catalase-luc (300 ng) or selected point mutant catalaseDREmut-luc (300 ng) reporter plasmids were transiently transfected into Kc cells. After incubation for 48 h, cells were harvested for analyses of luciferase and β-galactosidase activity. All values were normalized for co-transfected β-galactosidase activity. The luciferase activity of the wild-type reporter gene alone was set at 1. Average values obtained from four independent experiments with SE values are given as luciferase activity relative to that of the wild-type reporter gene.

Figure 3. Effect of the DRE sequence on catalase gene promoter activity in vivo. (A) Quantitative β-galactosidase activities of the transgenic flies bearing two copies of a catalase–lacZ or catalaseDREmut–lacZ fusion gene. Crude extracts were prepared from third instar larvae and 3-day-old adult transgenic flies as described in Materials and Methods. The β-galactosidase activities are expressed as absorbance units at 574 nm/h/mg of protein. Averaged values obtained from four independent experiments with SE values are shown. (B) The histochemical staining of β-galactosidase activity in 3-day-old adult transgenic flies having one copy of a catalase–lacZ or catalaseDREmut–lacZ fusion gene. The adult tissues were dissected and stained with 0.2% X-gal solution in the dark. Foregut (a and b), hindgut (c and d), muscle (e and f), testis (g and h), ovaries (i and j).
DREF over-expression stimulates catalase gene transcription

To investigate a role for DREF in catalase gene expression in vivo, ectopic expression of DREF in living flies was performed with the GAL4-UAS system (34,35). Transgenic flies carrying UAS-DREF (32) were crossed with transgenic flies carrying GAL4 cDNA placed under control of the hsp70 gene promoter (hs-GAL4). Quantitative analysis of β-galactosidase activity in total crude extracts of the larvae of a UAS-DREF/+; catalase-lacZ/hs-GAL4 line was carried out. The level of β-galactosidase activity in the heat-shocked larvae UAS-DREF/+; catalase-lacZ/hs-GAL4 was higher than that of the heat-shocked larvae +/-; catalase-lacZ/hs-GAL4 (Fig. 4A). This result indicates that DREF can directly stimulate Drosophila catalase gene promoter activity in vivo.

To confirm the upregulation of catalase expression by DREF, ectopic DREF expression in third instar larvae carrying single copies of both hs-GAL4 and UAS-DREF was examined by RT-PCR (Fig. 4B). The level of catalase mRNA in the third instar larvae at 6 h after heat shock was higher than that in third instar larvae carrying a single copy of hs-GAL4 (Fig. 4B). This result suggests that DREF can upregulate catalase gene expression, although we cannot exclude possible post-transcriptional effects on catalase mRNA.

Catalase amorphic Catn1 mutation suppresses the DREF over-expression-induced rough eye phenotype

In a recent study, we found that the DNA binding activity of DREF is directly modulated by elevated ROS through the effect of ROS on two cysteine residues located in the DNA-binding domain of DREF (36). One prediction of a functional relationship between DREF and catalase expression is that reducing catalase expression might suppress a phenotype associated with ectopic DREF over-expression. To test this hypothesis, we investigated whether a catalase amorphic allele Catn1 (11,37) could suppress the rough eye phenotype that results from ectopic expression of DREF in eye imaginal discs (32). Under scanning electron microscopy, all 13 eyes of the GMR-GAL4/+; UAS-DREF/+; +/- flies examined exhibited a severe rough eye phenotype (Fig. 5B and F). This is consistent with the report that adult flies expressing DREF under a GMR-GAL4 driver exhibit a severe rough eye phenotype (32). Fifteen of 18 eyes of GMR-GAL4/+; UAS-DREF/+; Catn1/+ flies exhibited a suppression of the DREF-induced rough eye phenotype (Fig. 5C and G). This indicates that the rough eye phenotype caused by ectopic expression of DREF is suppressed by the Catn1 mutation.

DISCUSSION

The catalase gene is a key component of a cellular antioxidant defense network. Several studies have shown that catalase expression is regulated at the transcriptional level by both Sp1 and CCAAT-recognizing factors in mammals (13,15). The 5′-upstream region of the Drosophila catalase gene contains an ecdysone response element (ERE), a TATA-like sequence, as well as CCAAT-like box and GC boxes (38). However, the molecular mechanisms of Drosophila catalase gene regulation are not well defined. In the present study, we identified a DREF-binding sequence (catalase-DRE) in the 5′-flanking region of the Drosophila catalase gene (Fig. 1A) and demonstrated that this element is required for in vitro and in vivo expression of the catalase gene. We have also shown that DREF binding to the catalase-DRE site positively regulates the catalase gene at the transcriptional level. These findings indicate that the DRE/DREF system is a key regulator of catalase gene expression in Drosophila.

In mammals, it has been reported that the expression of the catalase gene is regulated in a tissue-specific manner (13,14). The catalase mRNAs are found in liver, kidney, heart, brain, spleen, lung and muscle in mice (39). In Drosophila, it has been known that the expression of the catalase gene during development is responsive to ecdysone (16,38). In this study,
the expression of catalase–lacZ containing a 1.03 kb 5’-flanking region of the catalase gene (−1030 to +18 with respect to the transcription initiation site) fused to the lacZ reporter showed the highest lacZ signal at the adult stage among various developmental stages (data not shown). This is inconsistent with major peaks of the endogenous catalase mRNA level in late third larvae and pupal stages during development (38), suggesting the existence of a regulatory element(s) in the upper region than 1.03 kb of the 5’-flanking region. Although it is the tissue-specific patterns of catalase expression that need to be confirmed by in situ hybridization, the expression of the catalase–lacZ fusion in transgenic adults was detected in the foregut, hindgut, muscle and reproductive system (Fig. 3B). In addition, the expression of catalaseDREmut–lacZ in the same tissues indicated that DRE is required for the expression of the catalase gene in the foregut, hindgut, muscle and reproductive system (Fig. 3B), suggesting a novel function for DREF in ROS homeostasis of these tissues.

It has been well documented that the proliferative potential decreases as the tissues age (40,41). However, the molecular mechanism for the age-related decrease in proliferation remains unknown. Several studies reported that the intracellular ROS level increased with age (42,43). We found that the catalase allele Cat1 mutation suppressed the characteristic rough eye phenotype induced by DREF (Fig. 5). This indicates that DREF function was altered by the Cat1 mutation and that the expression of catalase is required for DREF function. Since DREF is known to play an important role in regulating cell proliferation-related genes (17–22), this suggests that expression of catalase enzymes may convey an advantage for cell proliferation. A recent study has reported a significant correlation between expression of an antioxidant enzyme and cell proliferation (44). In addition, it has been reported that the extended longevity phenotypes of Drosophila are correlated with upregulation of the antioxidant defense system genes and enzymes including SOD and catalase (45). The upregulation of catalase expression by DREF and the alteration of DREF function by redox—indicating a cross-talk between the DRE/DREF system and the antioxidant defense system—may contribute to the underlying molecular mechanism for the age-related decrease in proliferation.

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