2003

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## Citation/Publisher Attribution

Li, Y., Xie, M., Song, X., Gragen, S.,m Sachdeva, K., Wan, Y., & Yan, B. (2003). DEC1 Negatively Regulates the Expression of DEC2 through Binding to the E-box in the Proximal Promoter. *Journal of Biological Chemistry, 278*(19), 16899-16907. doi: 10.1074/jbc.M300596200
Available at: http://dx.doi.org/10.1074/jbc.M300596200

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 19, Issue of May 9, pp. 16899–16907, 2003
Printed in U.S.A.


# DEC1 Negatively Regulates the Expression of DEC2 through Binding to the E-box in the Proximal Promoter*

Received for publication, January 20, 2003, and in revised form, March 5, 2003
Published, JBC Papers in Press, March 6, 2003, DOI 10.1074/jbc.M300596200

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**Human DEC (differentially expressed in chondrocytes), mouse STRA (stimulated with retinoic acid), and rat SHARP (split and hairy related protein) proteins constitute a new and structurally distinct class of the basic helix-loop-helix proteins. In each species, two members are identified with a sequence identity of >90% in the basic helix-loop-helix region and ~40% in the total proteins, respectively. Recently, we have reported that DEC1 is abundantly expressed in colon carcinomas but not in the adjacent normal tissues. The present study was undertaken to extend the expression study of DEC1 and to determine whether DEC1 and DEC2 had similar expression patterns among paired cancer-normal tissues from the colon, lung, and kidney. Without exceptions, DEC1 was markedly higher in the carcinomas, whereas the opposite was true with DEC2. In stable transfectants, tetracycline-induced expression of DEC1 caused proportional decreases in the expression of DEC2. Co-transfection with DEC1 repressed the activity of a DEC2 promoter reporter by as much as 90%. The repression was observed with wild type DEC1 but not its DNA binding-defective mutants. Studies with deletion and site-directed mutants located, in the proximal promoter, an E-box motif that supported the DEC1-mediated repression. Disruption of this E-box markedly abolished the ability of the reporter to respond to DEC1. Our findings assign for DEC1 the first target gene that is regulated through direct DNA binding. DEC/STRA/SHARP proteins are highly identical in the DNA binding domain but much more diverse in other areas. DEC1-mediated repression on the expression of DEC2 provides an important mechanism that these transcription factors regulate the cellular function not only by modulating the expression of their target genes but also the expression of members within the same class.**

The basic helix-loop-helix (bHLH)[1] proteins are intimately associated with developmental events such as cell differentiation and lineage commitment (1–6). The HLH domain in the bHLH motif is responsible for dimerization, whereas the basic region mediates DNA binding (1). Based on sequence alignment and domain analysis, human DEC (differentially expressed in chondrocytes), mouse STRA (stimulated with retinoic acid), and rat SHARP (split and hairy related protein) constitute a new and structurally distinct class of bHLH proteins (7–10). These proteins are distantly related to *Drosophila* Hairy and E(spl) as well as the mammalian homologues (*e.g.* HES) with the highest sequence identity (~40%) in the bHLH region (1, 11, 12). Like Hairy/E(spl)/Hes, DEC/STRA/SHARPs contain an orange domain and a proline residue in the DNA binding domain. However, the proline is located 2 residues more toward the $NH_2$ terminus (1, 8). Another major structural difference on the functional domains is that DEC/STRA/SHARPs, unlike Hairy/E(Spl)/Hes proteins, lack the COOH-terminal WRPW tetrapeptide motif (13). Through this sequence, Hairy/E(spl)/Hes recruit corepressor Groucho to the transcription regulatory complex (13). Recruitment of Groucho is responsible for a vast array of biological activities of Hairy/E(spl)/Hes proteins including cellular differentiation and lineage commitment (14–18).

Two members of DEC/STRA/SHARP proteins are identified in each mammalian species studied with a sequence identity of >90% in the bHLH region and ~40% in the total proteins, respectively (8). They exhibit an overlapping tissue distribution, and their expression is highly elevated in response to environmental stimuli (7–10). In rats that undergo seizure induction by kainic acid, the levels of mRNA encoding SHARP1 or -2 are sharply increased within 1 h in the brain (9). In cultured human cells, both DEC1 and DEC2 are markedly induced in response to hypoxia (19). Co-transfection experiments with promoter reporters have identified functional hypoxia response elements in both DEC1 and DEC2 genes. These elements show high affinity toward hypoxia-inducible factor-1$\alpha$ and -$\beta$, providing a molecular explanation on the co-regulatory phenomena of DEC1 and DEC2 during hypoxia response (19). Rapid induction of these proteins in response to environmental stimuli suggests that DEC/STRA/SHARPs are protective against detrimental conditions.

In addition to a potential protective role against environmental stimuli, DEC/STRA/SHARPs have been implicated in cell differentiation (7, 10, 20), maturation of lymphocytes (21), and regulation of molecular clock (22). In a cell culture system, mouse STRA13 promotes neuronal but represses mesodermal and endodermal differentiation (7). Consistent with the inductive effect on neuronal differentiation, rat SHARP proteins are abundantly expressed in a subset of mature neurons (9). DEC1 has recently been shown to promote chondrocyte differentiation at the early and terminal stages (20). STRA13-deficient mice, although surviving to adulthood, develop autoimmune diseases accompanied by accumulation of spontaneously activated T and B cells (21). In addition, the mouse proteins are recently found to regulate the expression of biological clock regulator *Per* (22). Recently, we and other investigators have

* This work was supported in part by National Institute of General Medical Sciences, National Institutes of Health, Grant R01GM61988. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: bHLH, basic helix-loop-helix; HLH, helix-loop-helix; EMSA, electrophoretic mobility shift assay; RT, reverse transcription.

recently demonstrated that deregulated cell survival by DEC1 may have oncogenic significance. In paired samples, DEC1 is abundantly expressed in colon carcinomas but not in the adjacent normal tissues (23). High levels of DEC1 transcript are also detected in an array of cancer cell lines derived from a wide range of organs (24). Cells that lack the functional tumor suppressor VHL (von Hippel-Lindau) express higher levels of DEC1 (24). Forced expression of DEC1 antagonizes serum deprivation-induced apoptosis and selectively inhibits the activation of procaspases (23). These findings suggest that overexpression of DEC1 provides cells with an unusual survival mechanism and thus is oncogenic.

The present study was undertaken to extend the expression study on DEC1 and to determine whether DEC1 and DEC2 displayed similar expression patterns among paired tumor-normal tissues from the colon, lung, and kidney. Without exceptions, DEC1 was expressed markedly higher in the carcinomas, whereas DEC2 was expressed markedly higher in the adjacent normal tissues. Forced expression of DEC1 sharply decreased the expression of DEC2 and markedly repressed the activity of a DEC2 promoter reporter. Co-transfection experiments with mutant reporters and electrophoretic mobility shift assay (EMSA) located, in the proximal promoter, an E-box that supports DEC1-mediated repression. These findings provide direct evidence that DEC1 negatively regulates the expression of DEC2, which is largely achieved through direct DNA binding to the E-box in the proximal promoter of DEC2.

## MATERIALS AND METHODS

*Chemicals and Supplies*—Tri-reagent, FLAG-cytomegalovirus vector, and anti-FLAG antibody were purchased from Sigma. The goat anti-rabbit-IgG conjugated with alkaline phosphatase or horseradish peroxidase and ECL substrate were from Pierce. Dulbecco's modified Eagle's medium, LipofectAMINE, and the ThermoScript I reverse transcription-coupled PCR kit were from Invitrogen. The Dual-Luciferase reporter assay system and DNA binding buffer were from Promega. Unless otherwise indicated, all other reagents were purchased from Fisher.

*Tissue Collection and Processing*—Samples were collected from patients who underwent surgical resection for histologically confirmed adenocarcinoma. As paired controls, specimens from the adjacent, grossly normal tissues were harvested. The samples (12 pairs) were collected from the colon, kidney, and lung with four pairs from each organ. The age of the patients was between 23 and 68 with seven male and five female. The size of tumors was generally 2–5 cm in diameter, and the degree of differentiation of tumors was moderate or poor as determined by pathological examination. Samples were freshly processed for RNA isolation and protein extraction. Total RNA was isolated with a Tri-reagent as described previously (25). For the preparation of protein extracts, tissues were homogenized in lysis buffer (20 mM Tris-HCl, pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.2 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol). The homogenates were centrifuged at 12,000 $\times$ *g* for 30 min to remove any insoluble precipitates. The protocol of using human pathological tissues was reviewed by the Institutional Review Board.

*RT-PCR*—The expression of DEC1 and DEC2 in human tissues and cultured cells was primarily determined by RT-PCR experiments with a ThermoScript I kit. Total RNA (2 $\mu$g) was subjected to the synthesis of the first strand cDNA with an oligo(dT) primer and a ThermoScript reverse transcriptase. The reactions were incubated initially at 50 °C for 30 min and then at 60 °C for 60 min after additional reverse transcriptase was added. The cDNAs were then subjected to PCR amplification with cycling parameters as follows: 95 °C for 30 s, 52 °C for 30 s, and 68 °C for 30 or 40 s for a total of 32 cycles. The primers for DEC1 amplification were 5′-GTCTGTGAGTCACTCTTCAG-3′ and 5′-GAGTCTAGTTCTGTTTGAAGG-3′. The primers for DEC2 amplification were 5′-CGCCCATTCAGTCCGACTTGGAT-3′ and 5′-TGGTTGATCAGCTGGACACAC-3′. The primers for $\beta$-actin amplification were 5′-GTACCCTGGCATTGCCGACAGGATG-3′ and 5′-CGCAACTAAGTCATAGTCCGCCTA-3′. The PCR-amplified products were analyzed by agarose gel electrophoresis.

*Plasmid*—A cDNA encoding the full-length DEC1 was isolated by a cDNA-trapping method (23, 26). Several DEC1 mutant constructs were prepared by PCR with the full-length DEC1 as the template. These mutants had a specific sequence deleted or one or more amino acids substituted. Some of the mutant constructs were prepared with the SPORT vector (the $NH_2$-terminal truncated mutants), whereas others (the COOH-terminal truncated mutants) were prepared with the FLAG vector to facilitate immunodetection. In some cases, a Kozak sequence was introduced for effective translation initiation. The DEC2 promoter reporter was prepared with the pGL3-basic luciferase vector (Promega). Human genomic DNA was isolated from the placenta with a DNA extraction kit (Qiagen) according to the manufacturer's instruction. A genomic fragment (−1,888 to +11) was generated by PCR with 5′-AACAGATGAACTGAACGGACCG-3′ and 5′-CCTCAGTGCAGTGTTGAAAGTG-3′. This PCR fragment was ligated to the pGL3 vector. Deletion mutants of this reporter were prepared by endonuclease digestion followed by ligation or PCR.

*Site-directed Mutagenesis*—The DEC2 promoter reporter had two E-box motifs that probably interact with DEC1, and the studies with deletion mutants suggested that the E-box in the proximal region supports DEC1-mediated repression. In order to definitively establish such a role, site-directed mutagenesis was performed to substitute two of the six nucleotides. The mutant construct was prepared with a QuikChange site-directed mutagenesis kit (Stratagene). Complementary oligonucleotides (5′-GATGGTACGTTCCG**A**ACG**G**GAGCTGGGTGCTGG-3′) were synthesized to target this region. To perform the substitutions, the primers were annealed to a DEC2 promoter reporter and subjected to a thermocycler for a total of 15 cycles. The resultant PCR-amplified constructs were then digested with *Dpn*I to remove the nonmutated parent construct. The mutated PCR-amplified constructs were used to transform XL1-Blue. The same approach was used to prepare three DEC1 mutants that had single or double residues substituted in the DNA binding domain (P56A, R58P, or both). The general sequence for the site-directed mutagenic oligonucleotides was 5′-GAGACCTACAAATTG**G**CG**C**AC**C**CG**C**TCATCGAGAAAAAGAG-3′ with the nucleotides in boldface type substituted individually or simultaneously. All mutated constructs were subjected to sequencing analysis to confirm the desired mutation being made without secondary mutations.

*Co-transfection Experiment*—Cells (293T) were plated in 24-well plates in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at a density of $1.6 \times 10^5$ cells/well. Transfection was conducted by lipofection with LipofectAMINE according to the manufacturer's instructions. Transfection mixtures contained DEC1 or a mutant construct (100 ng), reporter plasmid (100 ng), and the pRL-TK *Renilla* plasmid (1 ng). If a DEC1-stable line was used, DEC1 or its mutant construct was omitted from the transfection mixture. The transfected cells were cultured for an additional 24 h, washed once with phosphate-buffered saline, and resuspended in passive lysis buffer (Promega). The lysed cells were subjected to two cycles of freezing/thawing. The reporter enzyme activities were assayed with a Dual-Luciferase reporter assay system. This system contained two substrates, which were used to determine the activity of two luciferases sequentially. The firefly luciferase activity, which represented the reporter gene activity, was initiated by mixing an aliquot of lysates (20 $\mu$l) with Luciferase Assay Reagent II. Then the firefly luminescence was quenched, and the *Renilla* luminescence was simultaneously activated by adding Stop & Glo reagent to the sample wells. The firefly luminescence signal was normalized based on the *Renilla* luminescence signal. In cases where the reading on the luciferase activity was too high, the lysates were diluted, and luciferase activities were then determined to minimize the interference on the reading of the *Renilla* luciferase activity.

*EMSA*—Cells (293T) were transfected with DEC1 or a mutant, and nuclear extracts were prepared with a nuclear extraction kit (Active Motif). In some cases, DEC1-stable transfected cells were used but cultured in the presence or absence of tetracycline to modulate the expression of transfected DEC1. Nuclear proteins (10 $\mu$g) were incubated with radiolabeled double-stranded oligonucleotides (5′-CGTTCCGCACGTGAGCTGGG-3′) in a final volume of 10 $\mu$l containing 1× DNA binding buffer. For competition experiments, nuclear extracts were first incubated with a 10- or 50-fold molar excess of cold probe and then mixed with the radiolabeled probe. Oligonucleotides with a disrupted E-box were also used in the competition assays. For supershift assays, the anti-DEC1 or an anti-FLAG antibody was added either before or after the nuclear extracts were incubated with the radiolabeled probe. The protein-DNA complexes were resolved in 6% PAGE and visualized by autoradiography.

*Other Analyses*—Western analyses were conducted as described previously (27). The anti-DEC1 antibody against the COOH-terminal peptide was described elsewhere (23). Protein concentration was deter-

mined with BCA assay (Pierce) with bovine serum albumin as the standard. Data are presented as mean ± S.D. of at least four separate experiments, except where results of blots are shown, in which case a representative experiment is depicted in the figures.

## RESULTS

*DEC1 and DEC2 Are Inversely Expressed in Paired Carcinomas and Adjacent Normal Tissues*—We have reported that DEC1 is abundantly expressed in colon carcinoma but not in the adjacent normal tissues (23). The initial focus of the present study was to extend the expression study on DEC1 and to determine whether DEC1 and DEC2 shared similar expression patterns among paired cancer-normal tissues from the colon, kidney, and lung. RT-PCR experiments with primers specific to DEC1 and DEC2 were performed. As shown in Fig. 1, without exceptions, the levels of DEC1 mRNA were markedly higher in the carcinomas, whereas the levels of DEC2 mRNA were markedly higher in the adjacent normal tissues. Between paired samples, the levels of β-actin mRNA were comparable. The carcinoma-related increase in DEC1 expression was also detected by Western blot (*top* of each depicted figure), suggesting that mRNA levels are indicative of the overall expression of these two genes.

*Forced Expression of DEC1 Proportionally Decreases the Expression of DEC2*—The inversed expression patterns between DEC1 and DEC2 suggest that DEC1 negatively regulates the expression of DEC2 or *vice versa*. In order to directly test this possibility, DEC1-stable transfected lines were used to study the expression relationship between DEC1 and DEC2. Two clonal stable lines were included: one expressing DEC1 (wild type) and the other expressing DEC1-M, which lacked the DNA binding domain. The stable lines were prepared with 293T cells and the pcDNA6/TR-pcDNA4 expression system; therefore, the expression of DEC1 and DEC1-M was inducibly regulated by tetracycline as described previously (23). As expected, the addition of tetracycline caused a concentration-dependent increase on the levels of DEC1 as determined by Western blots (Fig. 2*A*, *top*). Consistent with the inducible increase in the levels of DEC1 protein, the levels of DEC1 mRNA were proportionally increased (data not shown). In contrast to the increased expression of DEC1, the levels of DEC2 mRNA were proportionally decreased (Fig. 2*A*). However, such inversed expression patterns were observed only in the cells expressing wild-type DEC1 (Fig. 2*A*) and not the cells expressing the DEC1 mutant, although the levels of DEC1-M were markedly induced by tetracycline (Fig. 2*B*).

*The E-box in the Proximal Promoter of DEC2 Is the Sequence Targeted by DEC1*—The inability of DEC1-M to down-regulate the expression of DEC2 suggests that DEC1-mediated repression is achieved through a DNA-binding mechanism. In order to directly test this possibility, reporter experiments and EMSA were conducted. A DEC2 promoter reporter (pLuc-1888) was constructed to contain the basal promoter and other potential regulatory sequences of the DEC2 gene (−1,888 to +11). This region was chosen because it contained two E-box motifs that commonly serve as target sequences for bHLH transcription factors (1). A series of 5′ deletion mutants of this reporter was also prepared and designed to specify the location of DNA sequence that is targeted by DEC1 (Fig. 3*A*, *left*). Co-transfection experiments were conducted to test these reporters for their ability to support DEC1-mediated activity. The stable transfected line (wild-type DEC1 only) was transfected again with a reporter construct and cultured in the presence or absence of tetracycline to modulate the expression of DEC1. The pRL-TK *Renilla* plasmid was also included in the transfection mixture to normalize transfection efficiency. As described in Fig. 3*A* (*right*), the addition of tetracycline decreased the activity of the pLuc-1888 reporter by as much as 90%. Similar repression was observed with the reporters that had the sequence deleted up to nucleotide −535. In contrast, reporter pLuc-125, which had a further deletion from nucleotide −535 to −125, simultaneously lost the basal transcription activity and the ability to respond to DEC1, suggesting the importance of this region (−535 to −125) in both basal and regulatory transcription.

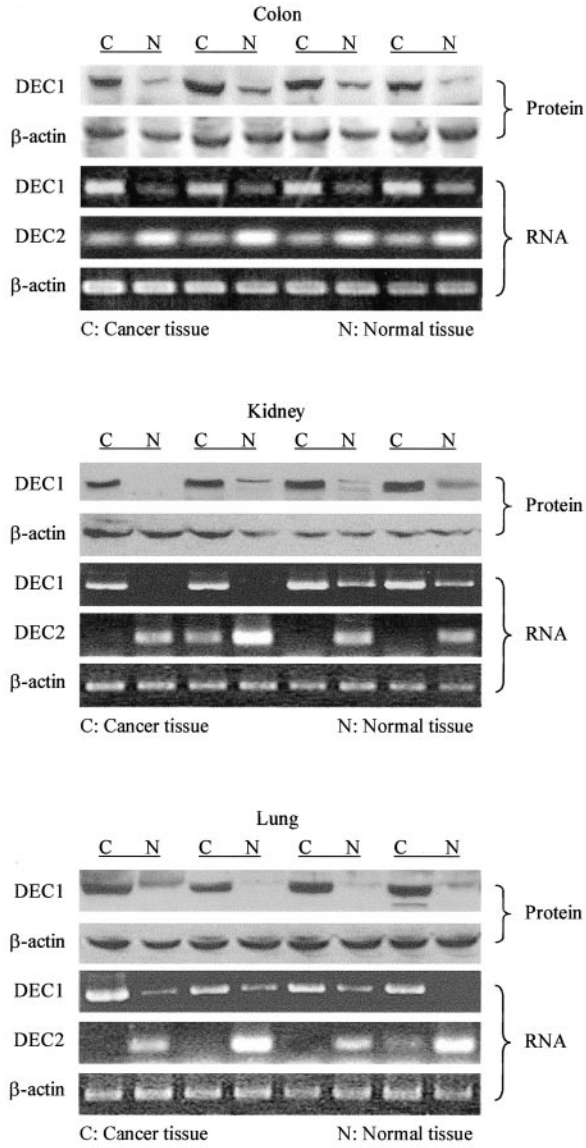


FIG. 1. **Inversed expression patterns of DEC1 and DEC2 in the carcinoma and the adjacent normal tissues from the colon, kidney, and lung.** Total RNA (5 μg) of carcinoma-normal paired samples from the colon, kidney, and lung was subjected to RT-PCR analyses with a ThermoScript I kit. For PCR amplification, a master tube containing all common reagents was prepared and equally distributed to individual PCR tubes (DEC1, DEC2, and β-actin). PCR amplification was conducted with cycling parameters as follows: 95 °C for 30 s, 52 °C for 30 s, and 68 °C for 30 or 40 s for a total of 32 cycles. The PCR-amplified products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. For Western blotting analysis, homogenates (10 μg) were subjected to SDS-PAGE. The immunoblot was incubated with the antibody against DEC1. The primary antibody was then located by horseradish peroxidase-conjugated goat anti-rabbit IgG and visualized with chemiluminescent substrate.

We next examined whether responsiveness to DEC1 could be separated from the basal transcription activity in the DEC2 promoter reporter. Given the fact that this region (−535 to −125) contains a single E-box that is probably targeted by

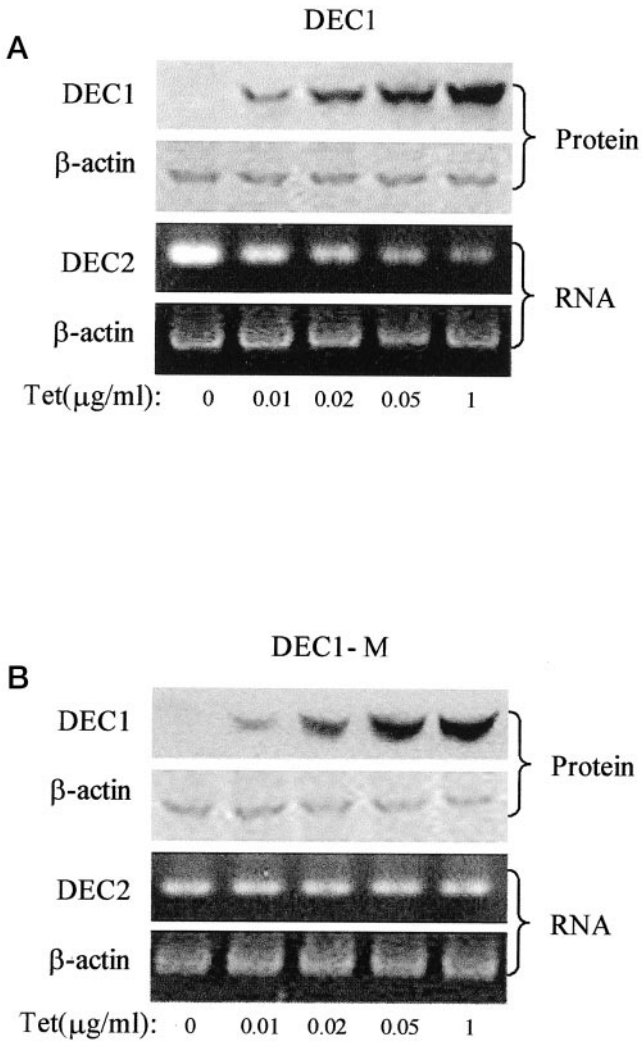


FIG. 2. **Repressed expression of DEC2 by DEC1 in stable transfected cells.** Stable transfected cells by DEC1 (*A*) or DEC1-M (*B*) were seeded in six-well plate. After reaching ~80% confluence, cells were treated with tetracycline at various concentrations (0–1 μg/ml) for 24 h. Total RNA and homogenates were prepared and analyzed for the expression of DEC1 by Western blots or DEC2 by RT-PCR as described in the legend for Fig. 1. Similarly, the expression of β-actin was determined and served as control.

DEC1, a reporter with this E-box disrupted was tested for the ability to confer basal transcription. Reporter pLuc-535 was subjected to site-directed mutagenesis to selectively disrupt the E-box (**C**ACG**T**G to **A**ACG**G**G). Similarly, co-transfection experiments were performed. As shown in Fig. 3*A* (*bottom*), disruption of this E-box (pLuc-535-M) caused little change in the basal activity (cultured without tetracycline), suggesting that this E-box contributes little to basal transcription. In contrast, the reporter mutant (pLuc-535-M) exhibited only ~35% repression in response to DEC1 (Fig. 3*A*, *lane 8*), which contrasts strikingly with 90% repression observed with the corresponding nonmutagenic reporter (Fig. 3*A*, *lane 5*). These findings suggest that the proximal E-box is largely responsible for DEC1-mediated repression. It should be emphasized that a similar observation was made with a substitution mutant reporter prepared from the longest reporter pLuc-1888, and the expression levels of DEC1 were comparable among all cells as determined by Western blots (data not shown).

We next examined whether this E-box interacted directly with DEC1. The DEC1-stable line was cultured in the presence or absence of tetracycline, and nuclear extracts were prepared. Double-stranded oligonucleotides harboring this E-box were synthesized and radiolabeled. The labeled probe was incubated with the nuclear extracts and analyzed by EMSA. As shown in Fig. 3*B*, incubation with the extracts from the cells cultured in the presence of tetracycline yielded a shifted band (*lane 8*). This band was not detected when incubation was performed with the extracts from the cell cultured without tetracycline (*lane 7*). The shifted band was competed completely by 50× (*lane 1*) or partially by 10× excess cold probe (*lane 3*). However, the oligonucleotides (50×) that harbored a mutated E-box (E-box-M) showed no competitive activity (*lane 2*). In addition, the shifted band was supershifted by the anti-DEC1 but not the anti-FLAG antibody. The supershifted band appeared whether the antibody was added before or after the DEC1-DNA complexes were formed (*lanes 5* and *6*), suggesting that the antibody binding does not interfere with interactions between DEC1 and its element (the antibody directed against the COOH-terminal sequence of DEC1).

*DNA Binding Is Required to Effectively Repress the DEC2 Promoter Reporter*—Disruption of the proximal E-box caused drastic but incomplete loss of responsiveness to DEC1 (Fig. 3*A*), suggesting that DNA binding is not the only mechanism involved in DEC1-mediated repression on the DEC2 reporter or that an additional DEC1 binding site exists in this region. We next tested whether DEC1 mutants, defective of DNA binding, had any repressive activity. These mutants had one or more residues in the DNA binding domain substituted or one or more structural domains deleted (Fig. 4*A*). A total of three deletion mutants (DEC1-M, $DEC1_{105–412}$ and $DEC1_{237–412}$) were prepared, and all of them lacked the DNA binding domain. As shown in Fig. 4*A*, additional sequences were also deleted in $DEC1_{105–412}$ (the HLH motif) and $DEC1_{237–412}$ (the HLH motif and orange domain). The HLH motif and the orange domain are shown in other bHLH proteins to mediate dimerization and protein interactions, respectively (1). Similarly, three substitution mutants were prepared, including $DEC1_{P56A}$, $DEC1_{R58P}$, and $DEC1_{P56A/R58P}$. The rationale for preparing the substitution mutants was that proline 56 was assumed to be critical in DNA binding based on studies with other bHLH proteins (1). However, there is a major difference regarding the location of this proline. In other bHLH proteins, the proline is located 2 residues more carboxyl terminal (corresponding to residue 58 in DEC1) (7, 8). Therefore, the mutants represented substitution of proline 56 with an alanine ($DEC1_{P56A}$), arginine 58 with a proline ($DEC1_{R58P}$), or both ($DEC1_{P56A/R58P}$).

Co-transfection experiments were conducted to test these DEC1 mutants for their ability to repress the DEC2 reporter (pLuc-1888). As shown in Fig. 4*A* (*top*), all deletion mutants (DEC1-M, $DEC1_{105–412}$, and $DEC1_{237–412}$) exhibited little repressive activity toward this reporter. In contrast, all substitution mutants repressed the DEC2 reporter, but the overall repressive activity varied markedly among them. The $DEC1_{P56A}$ mutant showed a similar potency as the wild-type DEC1 (~90% repression), whereas the other two mutants ($DEC1_{R58P}$ and $DEC1_{P56A/R58P}$) caused only ~65 and ~50% repression, respectively. The expression of DEC1 and its mutants was comparable with the exception of DEC1-M that was expressed to a higher extent (Fig. 4*B*), excluding the possibility that lack of expression was a contributing factor to the weaker repression by some of the mutants (*e.g.* $DEC1_{P56A/R58P}$). In order to determine whether these mutants, particularly the mutants $DEC1_{R58P}$ and $DEC1_{P56A/R58P}$, indeed lost DNA binding ability, nuclear extracts from the respective transfected cells were incubated with the radiolabeled E-box oligonucleotides, and the corresponding DNA-protein complexes were analyzed by EMSA. As predicted, all deletion mutants (DEC1-M,

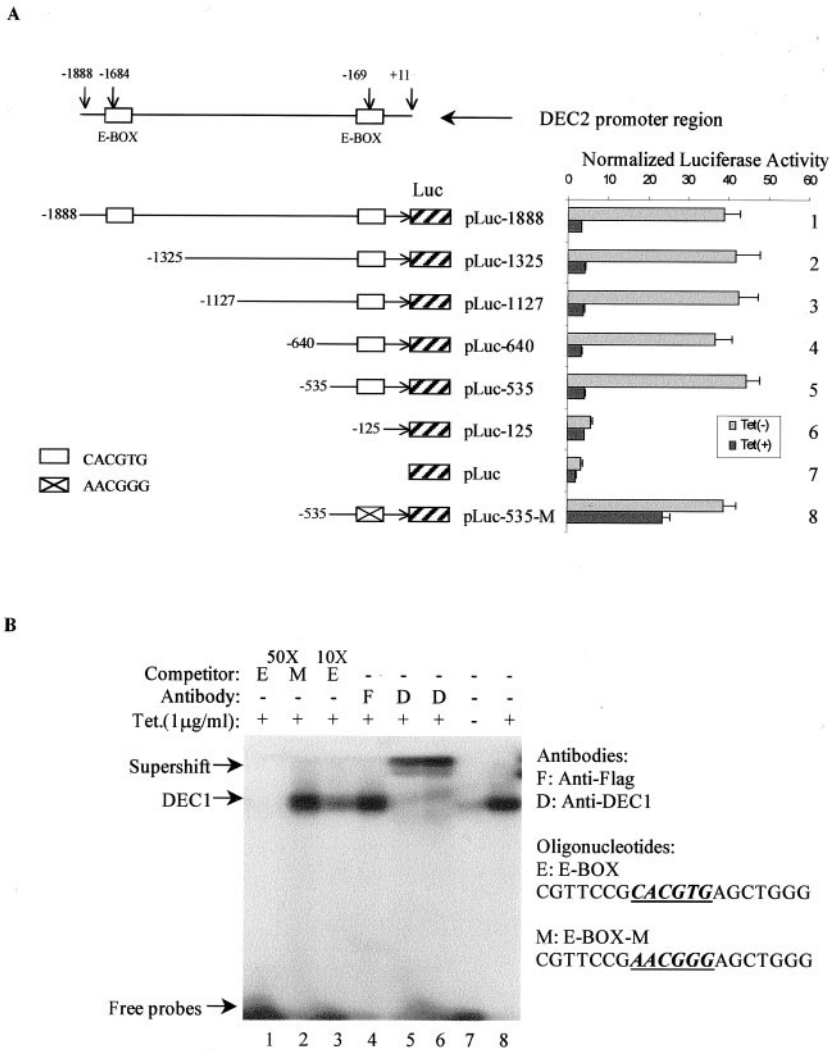


FIG. 3. **DEC1-mediated repression on the DEC2 promoter reporter and binding to the proximal E-box.** *A*, DEC1-mediated repression on the DEC2 promoter reporter. Deletion and site-directed mutants of the DEC2-promoter reporter (pLuc-1888) were prepared by endonuclease digestion followed by ligation or by PCR with a QuikChange site-directed mutagenesis kit. DEC1-stable transfected cells were cultured in 24-well plates at ~80% confluence and transfected again with a reporter construct (100 ng) and the pRL-TK *Renilla* (1 ng). The retransfected cells were cultured in the presence or absence of tetracycline (1 $\mu$g/ml) for 24 h. The cells were collected, washed once with phosphate-buffered saline, and resuspended in passive lysis buffer. The reporter enzyme activities were assayed with a Dual-Luciferase reporter assay system. The firefly luminescence signal was normalized based on the *Renilla* luminescence signal. *B*, EMSA DEC1-stable transfected cells were cultured in the presence or absence of tetracycline (*tet*; 1 $\mu$g/ml) for 24 h, and nuclear extracts were prepared with a nuclear extraction kit (Active Motif). Nuclear proteins (10 $\mu$g) were incubated with radiolabeled double-stranded oligonucleotides harboring the proximal E-box in a final volume of 10 $\mu$l containing 1× DNA binding buffer. For competition experiments, nuclear extracts were first incubated with excess cold probe (50× in *lane 1* or 10× in *lane 3*) and then mixed with the radiolabeled probe. Oligonucleotides (*M*) with the E-box disrupted were also used in the competition assays (50× in *lane 2*). For supershift assays, the anti-DEC1 antibody (*D*) was added either before (*lane 5*) or after (*lane 6*) the nuclear extracts were incubated with the radiolabeled probe. As a control, the anti-DEC1 antibody was replaced by an anti-FLAG antibody (*F*, *lane 4*). The protein-DNA complexes were resolved in 6% polyacrylamide gel electrophoresis and visualized by autoradiography.

$DEC1_{105-412}$, and $DEC1_{237-412}$) showed no DNA binding activity (result shown for DEC1-M only) (Fig. 4*C*). In contrast, DNA binding activity varied among the substitution mutants. $DEC1_{P56A}$ showed a similar binding ability as DEC1, whereas $DEC1_{R58P}$ and $DEC1_{P56A/R58P}$ had no DNA binding activity, consistent with the fact that $DEC1_{P56A}$ was the only substitution mutant that effectively repressed the promoter activity of DEC2 (Fig. 4*A*).

*DNA Binding Is Not Sufficient to Confer Repressive Activity*—The studies with DNA binding defective mutants clearly demonstrated the importance of DNA binding in repressing the DEC2 promoter. We next examined whether DNA binding was sufficient to exert repression. In order to directly test this possibility, DEC1 mutants were prepared to keep the bHLH motif intact (DNA binding) but have sequences with various lengths deleted from the COOH terminus (Fig. 5*A*). These COOH-terminal truncated mutants were subcloned in the FLAG vector to facilitate immunodetection. Similarly, co-transfection experiments were performed with DEC1 or a mutant along with the DEC2 reporter (pLuc-1888). As shown in Fig. 5*A*, deletion of the COOH-terminal 65 residues (FLAG-$DEC1_{1-347}$) caused no changes in the repressive activity (1). In contrast, deletions of additional COOH-terminal sequence caused a partial or a complete loss of repressive ability. As a matter of fact, FLAG-$DEC1_{1-150}$ no longer had any repressive activity. Western analyses were performed to confirm that the mutants were actually expressed slightly higher than the wild type DEC1 (Fig. 5*B*).

Next, we examined whether these mutants actually retained DNA binding activity. EMSA was performed with the nuclear extracts from the cells used for reporter assays. As shown in Fig. 5*C*, a shifted band was detected with all COOH-terminal

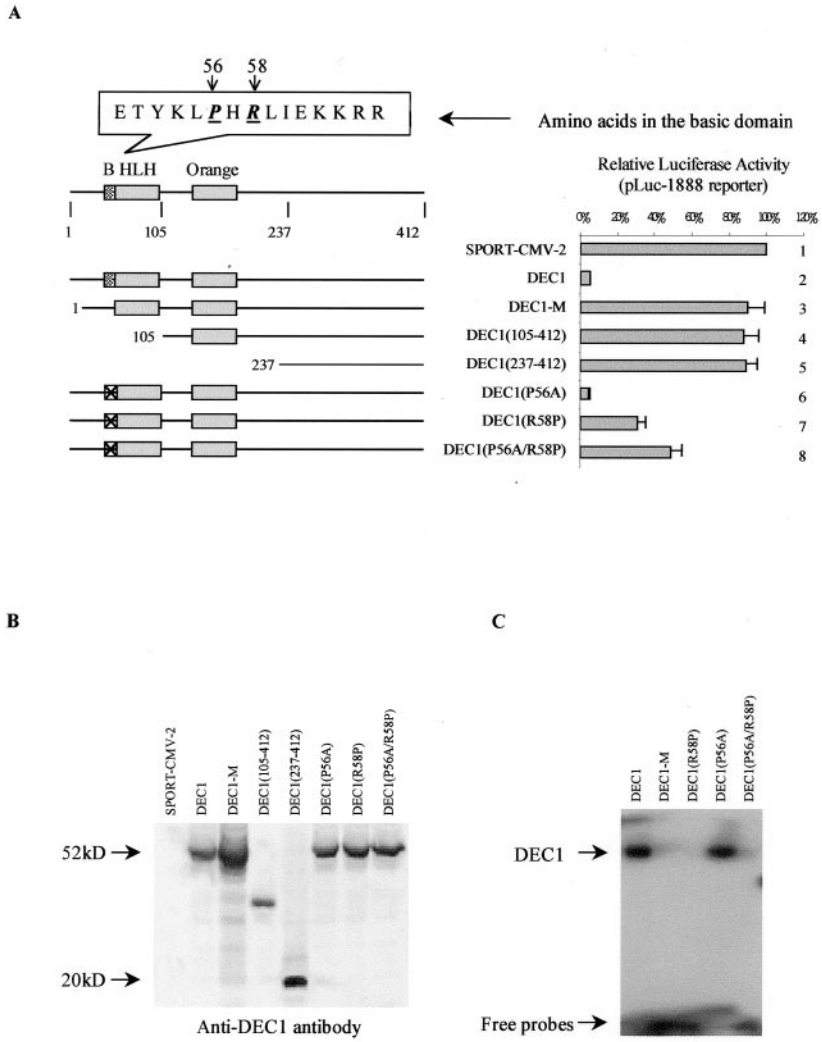


FIG. 4. **Essentiality of DNA binding for DEC1 to repress DEC2 promoter activity.** *A*, co-transfection experiment. Cells (293T) were cultured in 24-well plates and transiently transfected with DEC1 or a DNA binding defective mutant (100 ng), DEC2 promoter reporter (pLuc-1888; 100 ng) and the pRL-TK *Renilla* (1 ng). After a 24-h incubation, cells were collected and analyzed for luciferase activities. Similarly, firefly luminescence signal was normalized based on the *Renilla* luminescence signal, and the ratios from the cells transfected with the vector were calculated as 100%. *B*, immunoblotting analysis. The cell lysates (10 $\mu$g) from the cells used for reporter activity were analyzed for the expression of DEC1 or its mutants by anti-DEC1 antibody as described in the legend to Fig. 1. *C*, EMSA. Nuclear contracts were prepared from cells transiently transfected with DEC1 or a mutant and incubated with the radiolabeled proximal E-box probe. The DNA-protein complexes were resolved by PAGE.

truncated mutants. The relative electrophoretic mobility was generally associated with the size of a mutant. For example, FLAG-DEC1$_{1–150}$ was the shortest among the mutants, and the shifted band with this mutant exhibited the fastest mobility. More importantly, the addition of an anti-FLAG antibody into the binding reactions resulted in the appearance of a supershifted band accompanied by the disappearance of the original shifted band, providing direct evidence that the observed protein-DNA interactions were highly specific. These findings also suggest that DNA binding, although essential, is not sufficient to confer repressive effect.

*The HLH Motif Is Required for Dominant Interfering Regulation*—The inability of FLAG-DEC1$_{1–150}$ to exert repression, although it bound effectively to DNA, points to two important possibilities: the deleted region from residue 150 to 347 has intrinsic repressive activity, or this region is responsible for recruiting protein(s) that causes repression. Apparently, comprehensive experiments are required to definitively establish the involvement of each possibility. However, we examined the second possibility by testing mutants that contained part or the entire sequence of this region for the ability to function as a dominant interfering regulator. Co-transfection experiments were conducted with DEC1 in the presence and absence of a mutant. Among mutants DEC1-M, DEC1$_{105–412}$, and DEC1$_{237–412}$, only DEC1-M effectively reversed DEC1-mediated repression (Fig. 6*A*), although they all shared two important features; they lacked the entire DNA binding domain and lacked repressive activity themselves (Fig. 4*A*). Among the substitution mutants, DEC1$_{R58P}$ and DEC1$_{P56A/R58P}$ but not DEC1$_{P58A}$ partially but significantly reversed DEC1-mediated repression, consistent with the fact that DEC1$_{P58A}$ was a potent repressor itself (as potent as wild type DEC1), whereas DEC1$_{R58P}$ and DEC1$_{P56A/R58P}$ were much less repressive (Fig. 4*A*). It should be emphasized that the expression patterns in the cells co-transfected with DEC1 and a mutant were consistent with what was predicted; a band with more intensified staining was detected if a mutant co-migrated with DEC1 (*e.g.* DEC1$_{P56A/R58P}$); otherwise, an additional band (*e.g.* DEC1$_{105–412}$) was detected if a mutant and DEC1 were electrophoretically distinct.

We also tested all COOH-terminal truncated mutants for the ability to function as dominant interfering regulators. Generally, these mutants either partially or completely antagonized DEC1-mediated repression, depending on the relative potency to act as a repressor by its own (Fig. 6*B*). Mutants with less intrinsic repressive activity exhibited a higher potency to reverse the repression by DEC1. For example, FLAG-DEC1$_{1–150}$ itself had no repressive activity (Fig. 4*A*) but completely reversed DEC1-mediated repression (Fig. 6*B*). Among all mutants that were less repressive than wild type DEC1, only DEC1$_{105–412}$ and DEC1$_{237–412}$ failed to reverse the repression by DEC1 (Fig. 6), and they were the only mutants that did not contain the HLH domain (Figs. 4*A* and 5*A*), suggesting that the dominant interfering regulatory activity is achieved through the HLH domain. The HLH domain is known to mediate dimerization (1), and mutants with an intact HLH domain probably

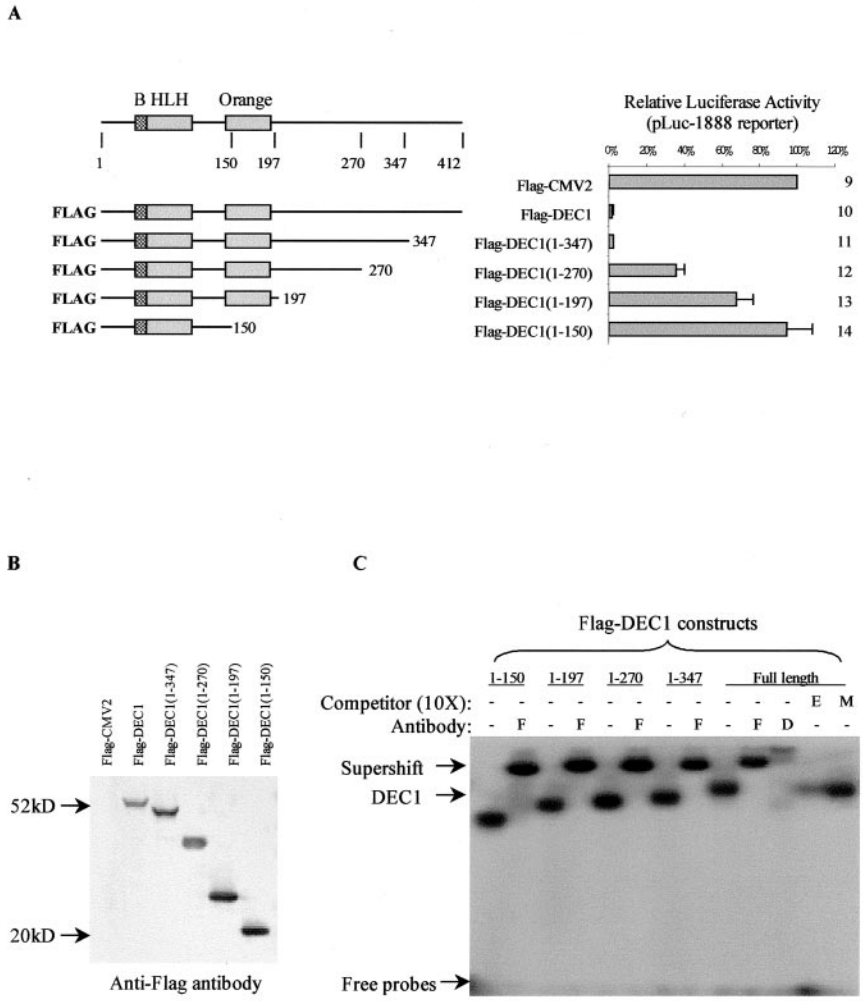


FIG. 5. **DNA binding is not sufficient for DEC1 to repress DEC2 promoter activity.** *A*, co-transfection experiment. Cells (293T) were cultured in 24-well plates and transiently transfected with DEC1 or a COOH-terminal truncated mutant (100 ng), DEC2 promoter reporter (pLuc-1888; 100 ng), and the pRL-TK *Renilla* (1 ng). Determination and calculation of the luciferase activities were described in the legend to Fig. 4. *B*, immunoblotting analysis. The cell lysates (10 $\mu$g) from the cells used for reporter activity were analyzed for the expression of DEC1 or its mutants by an anti-FLAG antibody as described in the legend to Fig. 1. *C*, EMSA. Nuclear contracts were prepared from cells transiently transfected with DEC1 or a mutant and incubated with radiolabeled oligonucleotides harboring the proximal E-box. Similarly, competition experiments were performed with excess cold probe (*E*) or a mutant probe (*M*) as described in the legend to Fig. 3*B*. For supershift assays, an anti-FLAG (*F*) or the anti-DEC1 (*D*) was added to the incubation mixtures before being analyzed by PAGE.

form dimers with wild type DEC1, but the resultant dimers have no DNA binding activity or are transcriptionally inactive. In support of the first possibility, we performed EMSA and found that $DEC1_{R58P}$ and $DEC1_{P56A/R58P}$ (DNA binding-defective mutants) markedly abolished the DNA binding ability of DEC1 when cells were co-transfected together with DEC1 and $DEC1_{R58P}$ or $DEC1_{P56A/R58P}$ (data not shown).

## DISCUSSION

The bHLH proteins are intimately associated with developmental events such as cell differentiation and lineage commitment (1). Based on sequence alignment and functional domain analyses, human DEC proteins, along with mouse STRA and rat SHARP, constitute a new class of bHLH transcription factors (7–10). These proteins are shown to play important roles in cell differentiation, regulation of molecular clock, immune response, and xenobiotic response (7, 10, 19–22, 28). Recently, we have reported that DEC1 is abundantly expressed in colon carcinomas, antagonizes serum deprivation-induced apoptosis, and selectively inhibits the activation of procaspases (23). In this report, we describe inversed expression patterns between DEC1 and DEC2 among paired tumor-normal samples from the colon, lung, and kidney. Experimentally forced induction of DEC1 causes proportional decreases in the expression of DEC2. Given the fact that DEC/STRA/SHARP proteins are highly identical (>90%) in the DNA binding region, but very diverse in other areas (<40%), our findings described in this report provide an important mechanism by which the cellular function of target genes probably shared by these proteins can be coordinately affected by members within the same class.

DNA binding is probably the primary mechanism responsible for DEC1-mediated repression on the expression of DEC2, although members of DEC/STRA/SHARP protein family have been shown to use non-DNA binding mechanism(s) (29–31). Several lines of evidence presented in this study support this notion. First, studies with deletion and site-directed reporter mutants identify the proximal E-box that supports the repression by DEC1. This E-box exhibits a high affinity toward DEC1, and disruption of this E-box markedly reduces its responsiveness to DEC1 (Fig. 3), suggesting that DNA binding is involved in the DEC1-mediated repression. Second, DEC1 deletion mutants (DEC1-M, $DEC1_{105-412}$, and $DEC1_{237-412}$), which lack the entire DNA binding domain, show neither DNA binding ability nor repressive activity (Fig. 4*A*), providing direct evidence that DNA binding is required for DEC1 to repress the DEC2 promoter. Finally, among the substitution mutants, $DEC1_{P58A}$ binds to the E-box as much as wild type DEC1 and is equally active in repression, whereas $DEC1_{R58P}$ and $DEC1_{P56A/R58P}$ show no DNA binding ability and are much less repressive (Fig. 4*A*), further supporting the notion that DEC1-mediated repression is largely achieved through DNA binding. It remains to be determined whether $DEC1_{R58P}$ and $DEC1_{P56A/R58P}$, although lacking DNA binding ability, cause some repression. It is likely that these two mutants retain some DNA binding ability within the cells, but the conditions employed for EMSA fail to support such interactions. Alternatively, they exert repression through non-DNA binding mechanisms (29–31).

DNA binding, although required to exert effective repres-

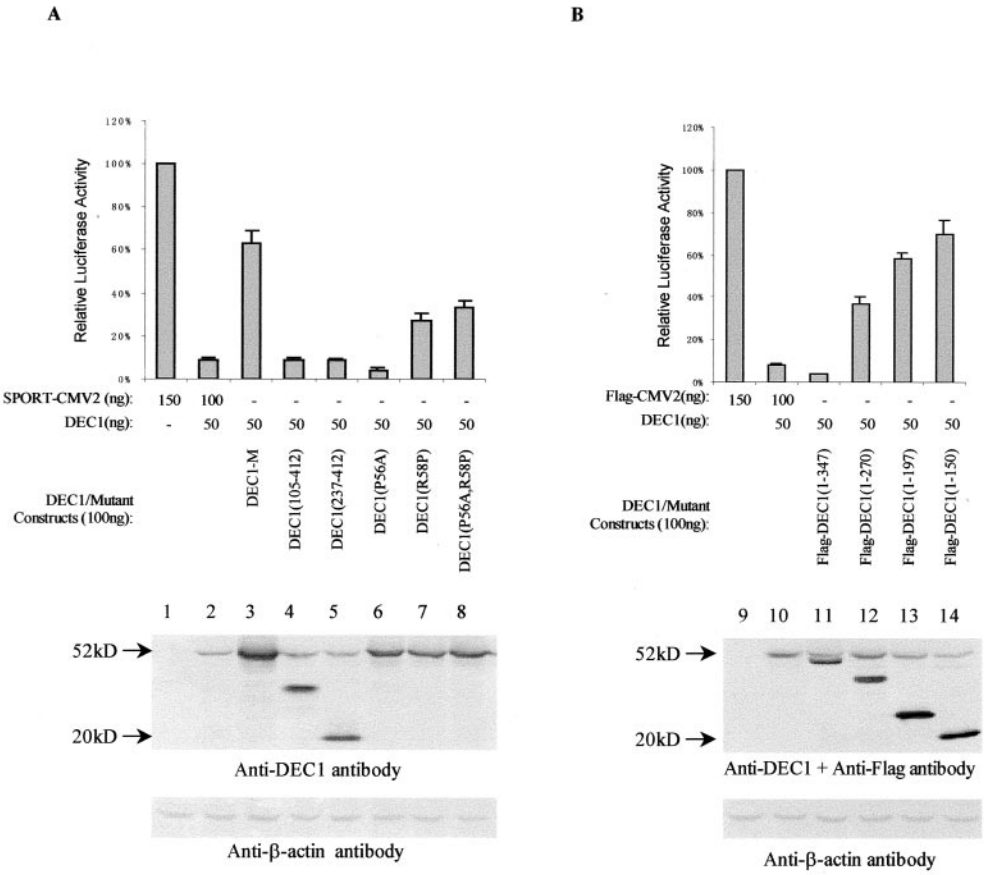


FIG. 6. **Dominant interfering regulation on DEC1-mediated repression.** *A*, effects of DNA binding-defective mutants on the repressive activity by DEC1. Cells (293T) were cultured in 24-well plates and transiently transfected with DEC1 (50 ng) in the presence or absence of a DNA binding-defective mutant (100 ng). Vector construct was used to equalize the amount of plasmid in each transfection. Similarly, the pRL-TK *Renilla* plasmid (1 ng) was included in the transfection mixture to normalize transfection efficiency. Determination and calculation of the luciferase activities were described in the legend to Fig. 4. To determine the expression levels of transfected constructs, cell lysates (10 $\mu$g) from the cells used for reporter activity were analyzed for the expression of DEC1 and its mutants by the anti-DEC1 antibody (specific to the COOH terminus of DEC1). *B*, effects of the COOH-terminal truncated mutants on the repressive activity by DEC1. The co-transfection and immunodetection were performed as described in the legend to Fig. 6*A*. However, both anti-DEC1 and anti-FLAG antibodies were simultaneously used for the immunodetection, because the COOH-terminal truncated constructs were prepared with the FLAG-cytomegalovirus vector.

sion, is not sufficient to repress the DEC2 promoter. Mutant FLAG-DEC1$_{1-150}$, for example, binds effectively to DNA but shows no repressive activity (Fig. 5*A*). As a matter of fact, mutants, with a deletion in the region from residue 150 to 347, all bind to DNA as effectively as wild type DEC1 but are markedly less repressive (Fig. 5*A*). In this region, several helical structures and particularly an orange domain are located (7, 8). These structures are assumed to mediate protein-protein interactions based on studies with other bHLH proteins (1, 7, 8). It is likely that this region recruits proteins that cause repression. However, the necessity of protein recruitment to repress DEC2 is unlikely, because mutants such as DEC1$_{105-412}$ contain the entire sequence of this region but show no dominant interfering activity against wild type DEC1 (Fig. 6*A*), suggesting that this region has intrinsic repressive activity. Alternatively, proteins assumed to be recruited are abundantly expressed in the cells employed in this study. Although we have not provided sufficient data to support protein recruitment in repressing DEC2, it cannot be excluded that such events are involved in the regulation of other genes by DEC1, particularly given the fact mouse STRA13, a highly identical protein to DEC1, has been shown to interact directly with TFIIB through this region (29).

DEC/STRA/SHARPs differ significantly from other bHLH proteins in terms of binding to DNA. Most bHLH proteins bind to E-box (CANNTG) or N-box (CANNAG). Binding preference is specified by the sequence in the basic region. Generally, proline-containing basic regions have higher affinity toward the N-box (1, 32), whereas the basic regions without a proline preferentially recognize the E-box. DEC/STRA/SHARPs contain a proline; however, this proline (residue 56 based on DEC1) is located 2 residues more amino-terminal (8). Instead, DEC/STRA/SHARPs have an arginine (residue 58) that substitutes for the conserved proline among N-box binding bHLH proteins. Although initial studies suggest that STRA13 has no binding activity toward classic E- or N-box (7), PCR-based site selection experiments have recently identified a class B type E-box (CACGTG) that is effectively bound by DEC1 and STRA13 (28, 33). In this study, we have demonstrated that the contribution of Pro$^{56}$ to DNA binding is insignificant because mutant DEC1$_{P58A}$ is equally effective as wild type DEC1 in DNA binding (Fig. 4*C*). In contrast, introduction of a proline by substituting Arg$^{58}$ completely eliminates DNA binding activity (Fig. 4*C*), suggesting that residue in this location is indeed important for E-box binding. It would be interesting to test whether DEC1$_{R58P}$ and DEC1$_{P56A/R58P}$ show an increase in binding to an N-box sequence. In addition, the DEC2 reporter contains two identical E-box sequences (proximal and distal) (Fig. 3*A*); however, only the proximal E-box is required for responding to the repression by DEC1. The precise mechanism for such a difference remains to be determined. It is likely that the genomic context rather than an E-box alone determines

intracellular DNA binding. In support of this possibility, STRA13 has been shown to preferably bind to an E-box flanked with certain nucleotides.

DEC1 and DEC2 share the DNA binding domain with an exception of a single residue (aspartate *versus* glutamate, the farthest $NH_2$-terminal residue of this domain) (8); therefore, DEC2 probably acts as an autoregulator. This possibility is further supported by their highly identical sequences flanking the DNA binding domain. Immediately COOH-terminal to the DNA binding domain is the helix-loop-helix domain that is identical between DEC1 and DEC2, and $NH_2$-terminal to this domain is an acidic residue-rich stretch in both proteins (8). The DNA binding domain and its highly identical flanking sequences suggest that DEC1 and DEC2 have overlapping target genes, particularly those that are regulated through direct DNA binding. In support of this notion, mouse proteins (STRA13 and DEC2) have been recently shown to repress Clock/Bmal1-induced activation of the *Per* promoter (22), a gene that is involved in the regulation of the molecular clock. Therefore, it is likely that DEC/STRA/SHARP proteins are functionally redundant on some target genes, and such a redundant mechanism provides a possible explanation that STRA13 knockout mice develop to adulthood and show no discernible phenotypic differences compared with their wild-type littermates (21). It should be emphasized, however, that DEC1 and DEC2 may not necessarily exert the same biological activity on all target genes and in all cell types, particularly given the fact that they have a minimal sequence identity (<40%) in the COOH-terminal half and exhibit several major structural differences (8). Both DEC1 and DEC2 have an orange domain (two helical structures spanned by ~50 residues); however, the overall sequence identity in this domain is only moderate (~50%). In addition, an alanine/glycine-rich region is present in DEC2 but absent in DEC1. Previous studies with STRA13 as well as the findings described in this study have demonstrated that the region harboring the orange domain is required to exert effective repression by both proteins (Fig. 5*A*) (7). Amino acid repeats, on the other hand, are implicated in protein folding, protein-protein interactions, and degradation (34).

DEC1-mediated repression is probably responsible for the differences on cell and tissue distributions between DEC1 and DEC2. Although Northern analyses have shown that DEC1 and DEC2 have an overlapping tissue distribution (8, 10), it remains to be determined whether they are actually expressed in the same cell type and to a similar extent (8, 10). Some organs with high levels of DEC1 (*e.g.* liver) express lower levels of DEC2 (10). Very recently, DEC1 and DEC2 were found to regulate the mammalian molecular clock, but they exhibit distinct and area-dependent expression patterns in the brain (21). In this report, we have demonstrated that these two proteins exhibit inversed expression patterns among the paired tumor-normal tissues, and forced expression of DEC1 causes proportional decreases in the expression of DEC2 (Figs. 1 and 2), providing direct evidence that increased expression of DEC1 is at least in part responsible for decreased expression of DEC2 in a given cell context. DEC1-mediated repression, although profound, may not always dictate the expression of DEC2. For example, DEC1 and DEC2 are both up-regulated in response to hypoxia induction (19). Acute hypoxia is considered severe cytotoxic stimulus, and rapid induction of both genes maximizes the cellular survival mechanism based on our recent report that DEC1 is antiapoptotic (23), although it remains to be determined whether DEC2 is actually antiapoptotic as well.

In summary, we have demonstrated that DEC1 is a negative regulator on the expression of DEC2. These two proteins exhibit inversed expression patterns among paired samples from the colon, kidney, and lung. An inducible expression system demonstrates that increased expression of DEC1 proportionally decreases the expression of DEC2. The DEC1-mediated repression is primarily achieved by binding to the E-box in the proximal promoter of DEC2. Site-directed mutagenesis studies show that arginine 58 in the DNA binding domain is critical for DEC1 to interact with this E-box. Given the fact that DEC/STRA/SHARP proteins are emerging as very important regulators in a vast array of cellular events including cell differentiation, maturation of lymphocytes, oncogenesis, molecular clock, and xenobiotic response, our findings described in this study provide an important mechanism by which these proteins regulate the cellular function by not only modulating the expression of their target genes but also the expression of the members within the same class.

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# DEC1 Negatively Regulates the Expression of DEC2 through Binding to the E-box in the Proximal Promoter

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*J. Biol. Chem. 2003, 278:16899-16907.*
*doi: 10.1074/jbc.M300596200 originally published online March 6, 2003*

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