Interaction of Short-Range Repressors with *Drosophila* CtBP in the Embryo

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Human CtBP attenuates transcriptional activation and tumorigenesis mediated by the adenovirus E1A protein. The E1A sequence motif that interacts with CtBP, Pro-X-Asp-Leu-Ser-X-Lys (P-DLS-K), is present in the repression domains of two unrelated short-range repressors in *Drosophila*, Knirps and Snail, and is essential for the interaction of these proteins with *Drosophila* CtBP (dCtBP). A P-element–induced mutation in dCtBP exhibits gene-dosage interactions with a null mutation in *knirps*, which is consistent with the occurrence of Knirps-dCtBP interactions in vivo. These observations suggest that CtBP and dCtBP are engaged in an evolutionarily conserved mechanism of transcriptional repression, which is used in both *Drosophila* and mammals.

Transcriptional repression is essential for establishing localized stripes (*1*), bands (*2*), and tissue-specific patterns (*3*) of gene expression in the early *Drosophila* embryo (*4*). Various modes of repression have been proposed, including competitive binding of repressors to activator elements and the local quenching (inhibition) of transcriptional activators (*5*). Repressors can be classified according to range of action (*6*). Short-range repressors work over distances of less than 100 base pairs (bp) to quench upstream activators or the core transcription complex. This form of repression allows enhancers to work independently of one another to direct complex, additive patterns of gene expression, including seven-stripe patterns of *eve* and *hairy* expression (*1*, *7*, *8*). Long-range repressors can function over distances of >1 kb to silence the transcription complex and inhibit multiple enhancers (*6*), thereby resulting in simple on/off patterns of gene expression.

Sequence-specific repressors can recruit co-repressor proteins to the DNA template (*9*). For example, two unrelated long-range repressors, Dorsal (*6*) and Hairy (*10*), recruit a common co-repressor protein, Groucho (*11*), which is related to a general repressor protein in yeast, Tup1p (*9*). Here, we show that two unrelated short-range repressors, Knirps (*12*) and Snail (*13*), recruit dCtBP, which is the *Drosophila* homolog of the human CtBP protein (*14*).

Knirps is a nuclear receptor protein that controls the segmentation of the abdomen (*12*), whereas Snail is a zinc finger protein (*13*) that establishes a boundary between the presumptive mesoderm and neurogenic ectoderm (*15*). Both protein sequences were used as bait in yeast two-hybrid assays (*16*). Knirps identified three different recombinant cDNA clones that contain overlapping versions of a common protein coding sequence; Snail identified the same coding sequence. The cDNAs that were selected by both Knirps and Snail, dCtBP, specify a putative protein of 383 amino acid residues that shares ~60% overall identity with the corresponding human CtBP protein (Fig. 1).

CtBP interacts with a conserved sequence in the adenovirus E1A protein, P-DLS-K (*14*, *17*). Mutations in this sequence eliminate E1A-CtBP interactions so that CtBP no longer inhibits E1A-mediated transcriptional activation and tumorigenesis in mammalian cell cultures (*14*). The P-DLS-K sequence is present in the repression domains of Knirps (*12*) and Snail (*13*); the latter protein also contains the related sequence P-DLS-R (*13*).

Glutathione S-transferase (GST) pull-down assays were conducted to determine whether dCtBP interacts with Knirps or Snail (*18*). These experiments involved the use of in vitro–translated, ^{35}S-labeled repressor proteins and a GST-dCtBP fusion protein produced in bacteria (*19*). Both Knirps and Snail strongly interacted with the GST-dCtBP fusion protein (Fig. 2) but exhibited little or no binding to control GST. Mutations in the Knirps P-DLS-K motif (Gal4-knirps 75-340M) abolished interactions with GST-dCtBP (Fig. 2A). Similarly, mutations in the Snail P-DLS-R motif caused a severe re-

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dCtBP          MDKNLMMPKRSRIDVKGNFANGPLQARPLVALLDGRDCSIEMPILKDVATVAFCDAQSTS  60
human CtBP     MGSSHLLNKGLPLGVRPPIMNGPLHPRPLVALLDGRDCTVEMPILKDVATVAFCDAQSTQ  60
               *       *     *     ****  ************  ******************

dCtBP          EIHEKVLNEAVGALMWHTIILTKEDLEKFKALRIIVRIGSGTDNIDVKAAGELGIAVCNV 120
human CtBP     EIHEKVLNEAVGALMYHTITLTREDLEKFKALRIIVRIGSGFDNIDIKSAGDLGIAVCNV 120
               *************** *** ** ****************** **** * ** ********

dCtBP          PGYGVEEVADTTMCLILNLYRRTYWLANMVREGKKFTGPEQVREAA-HG-CARIRGDTLG 178
human CtBP     PAASVEETADSTLCHILNLYRRA--TGCTRRCGRAHESRASSRSARWRPRCQDPRGD-LG 177
               *   *** ** * * *******        * *         * *     *   *** **

dCtBP          -LVGLGRIGSAVALRAKAFGFNVIFYDPYLPDGIDKSLGLTRVYTLQDLLFQSDCVSLHC 237
human CtBP     HHRTWSR-GAGSGAAGQRVGFNVLFYDPYLSDGVERALGLQRVSTLQDLLFHSDCVTLHC 236
                     * *          **** ****** **    *** ** ******* **** ***

dCtBP          TLNEHNHHLINEFTIKQMRPGAFLVNTARGGLVDDETLALALKQGRIRAAALDVHENEP- 296
human CtBP     GLNEHNHHLINDFTVKQMRQGAFLVNTARGGLVDEKALAQALKEGRIRGAALDVHESEPF 296
               ********** ** **** **************   ** *** **** ******* **

dCtBP          -Y-NGALKDAPNLICTPHAAFFSDASATELREMAATEIRRAIVGNIPDVLRNCVNKEYF- 353
human CtBP     SFSQGPLKDAPNLICTPHAAWYSEQASIEMREEAAREIRRAITGRIPDSLKNCVNKDHLT 356
                   * **************  *     * ** ** ****** * *** * *****

dCtBP          --M-RTP--PAAAAGGV-AAA-VYPEGKLQMISNQEK---------------------- 383
human CtBP     AATHWASMDPAVVHPELNGAAYRYPPGVVGVAPTGIPAAVEGIVPSAMSLSHGLPPVAHP 416
                        **        **  ** *

human CtBP     PHAPSPGQTVKPEADRDHASDQL                                       439
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Fig. 1. Conservation of CtBP in humans and *Drosophila*, as shown by alignment of the dCtBP and human CtBP protein sequences (*17*). Identical residues are denoted by asterisks. Most of the *Drosophila* sequence was derived from one of the four dCtBP cDNA clones isolated in the yeast two-hybrid assays. This particular cDNA begins at codon 8 (the proline residue); the sequence of the first seven codons was obtained from a different cDNA clone. The analysis of this latter clone suggests that the dCtBP mRNA contains a ~300-bp 5′ UTR and an optimal translation initiation sequence. The putative dCtBP protein appears to contain 383 amino acid residues, although sequence analysis of another dCtBP cDNA suggests that there may be three additional amino acid residues, VFQ, located between codons 298 and 299. Moreover, it is conceivable that the dCtBP gene encodes differentially spliced mRNAs, which encode divergent COOH-terminal sequences beginning at codon 373 (*30*). The histidine residue at position 314 of CtBP corresponds to the catalytic center of D-isomer 2-hydroxy acid dehydrogenases (*14*). The complete dCtBP sequence can be obtained from the DDBJ/EMBL/GenBank databases (accession number AB011840).

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duction in Snail-dCtBP interactions (Fig. 2B). Thus, dCtBP might interact directly with Knirps and Snail, although we cannot exclude the possibility that binding is mediated by one or more proteins in the reticulocyte lysate.

The dCtBP cDNA was used as a hybridization probe to screen an arrayed *Drosophila* genomic DNA library (Genome Systems Inc.). The smallest P1 recombinant phage that was identified, DS06433, maps to the 87D7-9 region of chromosome 3. This region contains a single P-element–induced lethal mutation, l(3)03463 (*20*). Plasmid rescue assays and polymerase chain reaction (PCR)–mediated sequence analysis suggest that this P-element maps within the 5′ untranslated region (UTR) of the dCtBP transcription unit (*21*). l(3)03463 homozygotes are lethal and embryos derived from germline clones exhibit severe patterning defects, including segment fusions and the loss of ventral tissues (*22*).

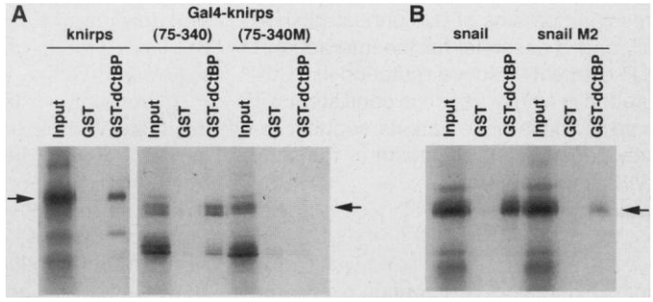

Fig. 2. In vitro binding assays using a GST-dCtBP fusion protein. Full-length Knirps and Snail proteins were translated with a rabbit reticulocyte lysate and labeled with [^{35}S]methionine. Each protein was incubated with either a GST nonfusion protein or a GST-dCtBP protein containing dCtBP amino acid residues 8 to 383. (**A**) Knirps. Aliquots containing 10 μl of each of the indicated ^{35}S-labeled proteins were incubated with 6 μg of either GST or the GST-dCtBP fusion protein bound to glutathione-agarose beads. Bound proteins were fractionated by SDS-PAGE and visualized by autoradiography. The input lanes contain 20% (2 μl) of each ^{35}S-labeled protein used in the binding assays. The full-length Knirps protein exhibited efficient binding to the dCtBP-GST fusion protein (arrow). A Gal4-Knirps fusion protein containing amino acid residues 75 to 340 also exhibited efficient binding. A mutagenized derivative of this protein, 75-340M, containing alanine residues in place of the DLS sequence within the P-DLS-K motif did not exhibit specific binding to GST-dCtBP. (**B**) Snail. The full-length Snail protein exhibited efficient binding to the GST-dCtBP fusion protein (arrow). However, there was a substantial reduction in binding when P-DLS-R was mutagenized to A-AAA-R (to produce the Snail M2 protein). Presumably, the residual binding that is observed (arrow) is mediated by the P-DLS-K motif, which was left intact. Removal of both motifs abolished dCtBP interactions (*30*).

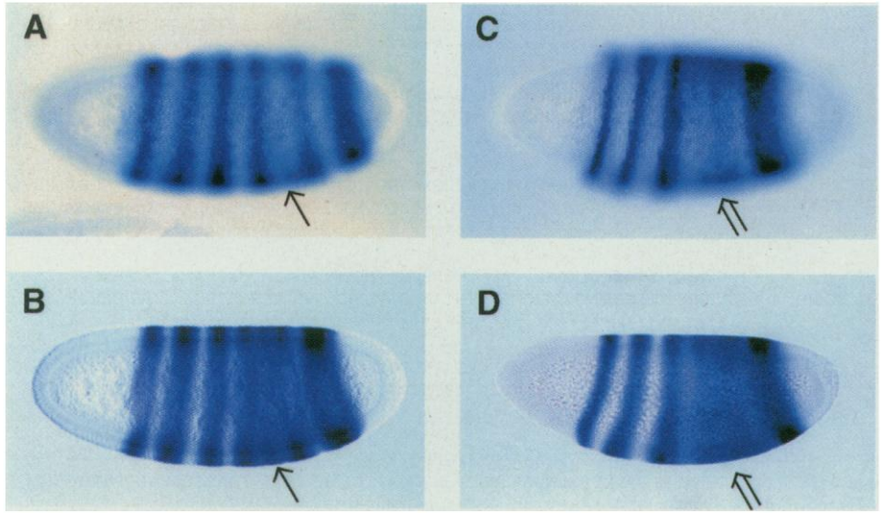

Fig. 3. Genetic interactions between dCtBP and *knirps*. Heterozygous females carrying the P-element–induced mutation in the dCtBP gene [l(3)03463/TM3, Sb] were mated with heterozygous males carrying a null mutation in *knirps* (*knirps*^{9}/TM3, Ser). Embryos were hybridized with a digoxigenin-labeled *eve* antisense RNA probe and visualized by histochemical staining (*3*). The embryos are undergoing cellularization (anterior is to the left; dorsal is up). (**A**) Control embryo obtained from the mating of *knirps*^{9}/TM3, Ser males with normal females. There are occasional reductions in *eve* stripe 5. This particular embryo lacks stripe 5 expression (arrow), which is the most extreme phenotype that is observed for such heterozygotes. (**B**) F_1 embryo obtained by mating dCtBP heterozygous females with *knirps*^{9} heterozygous males. This mutant phenotype [fusions of *eve* stripes 3, 4, and 5 (arrow)] represents the most common class of defects that were observed. (**C**) A different *knirps*^{9}/dCtBP transheterozygous embryo. There is almost a complete loss of *eve* stripes 4, 5, and 6 (arrow). About 5 to 10% of the mutant embryos exhibited this severe phenotype. (**D**) A *knirps*^{9}/*knirps*^{9} homozygous mutant embryo showing an *eve* staining pattern. Stripes 4, 5, and 6 are fused and reduced (arrow), similar to the pattern observed in *knirps*^{9}/dCtBP transheterozygotes [compare with (C)].

Gene dosage assays suggest that *knirps* and dCtBP interact in vivo. Embryos that are heterozygous for the *knirps*^{9} (*23*) null mutation (*knirps*^{9}/+) exhibited occasional defects in the *eve* expression pattern, including reduced staining of stripe 5 (Fig. 3A). Combining the dCtBP and *knirps*^{9} mutations [mating l(3)03463/+ females with *knirps*^{9}/+ males] resulted in more severe disruptions in the *eve* pattern, including the fusion (Fig. 3B) or loss (Fig. 3C) of stripes 4 to 6. The latter *knirps*^{9}/dCtBP transheterozygous phenotype (Fig. 3C) is virtually indistinguishable from that observed for *knirps*$^-$ embryos (Fig. 3D).

The Knirps repression domain (amino acid residues 255 to 429) contains a copy of the P-DLS-K motif. A Gal4-Kni255-429 fusion protein was expressed in ventral regions of transgenic embryos by means of a mesoderm-specific enhancer derived from the *twist* gene (Fig. 4A) (*3*, *24*). An *eve* stripe 2–*lacZ* reporter gene was introduced into embryos expressing the Gal4-Kni255-429 fusion protein (see diagram above Fig. 4A). The reporter gene contained two tandem copies of the yeast Gal4 operator site (UAS) and is normally expressed equally in dorsal and ventral regions. However, the Gal4-Kni255-429 fusion protein bound to the UAS sites and repressed stripe 2–*lacZ* staining in ventral regions (arrowhead, Fig. 4A). A mutant form of the Gal4-Kni255-429 protein, which contained the sequence AAAA in place of P-DL in the P-DLS-K motif, failed to mediate repression of the stripe 2 pattern in ventral regions (Fig. 4B). This result suggests that P-DLS-K is essential for Knirps-mediated repression in the *Drosophila* embryo.

To determine whether dCtBP is sufficient for transcriptional repression, we expressed a Gal4-dCtBP fusion protein in ventral regions of transgenic embryos (Fig. 4C). There was a slight reduction in the ventral expression of the stripe 2–*lacZ* reporter gene, although repression was not as complete as that observed with the Gal4-Kni255-429 fusion protein (Fig. 4A). These results raise the possibility that dCtBP is part of a larger co-repressor complex that assembles on the Knirps/DNA template.

Taken together, in vitro binding assays, gene dosage studies, and transgenic repression assays suggest that dCtBP is essential for Knirps-mediated repression in the early *Drosophila* embryo. Snail might also require dCtBP (see Fig. 2), because embryos derived from dCtBP^{-} oocytes exhibit dorsoventral patterning defects (*22*). The COOH-terminal repression domain of a third short-range repressor, Kruppel, contains a sequence that is related to the CtBP and dCtBP interaction sequence: P-DLS-H (*25*). Mutations in this sequence nearly abolish Kruppel-mediated repression in human osteocarcinoma cells (*25*). Thus, short-range and long-range repressors may recruit distinct co-repressor complexes; dCtBP is essential for short-range repression, whereas Groucho mediates long-range repression.

Recent studies suggest that repression involves changes in chromatin structure (*26*). For example, certain mammalian regulatory factors, including the thyroid hormone receptor, interact with a protein complex that includes histone deacetylases. CtBP and dCtBP may mediate repression through the enzymatic modification of chromatin because both proteins are related to D-isomer 2-hydroxy acid dehydrogenases (*14*). Despite this rather unexpected homology, immunolocalization assays indicate that the dCtBP protein accumulates in nuclei (Fig. 4D). Perhaps CtBP and dCtBP cause local changes in chromatin structure by introducing subtle changes in core histones. Alternatively, it is possible that CtBP and dCtBP are components of an enzymatic cascade that modulates the activities of histone deacetylases or other co-repressor proteins.

Note added in proof: dCtBP has been shown to interact with Hairy (*32*).

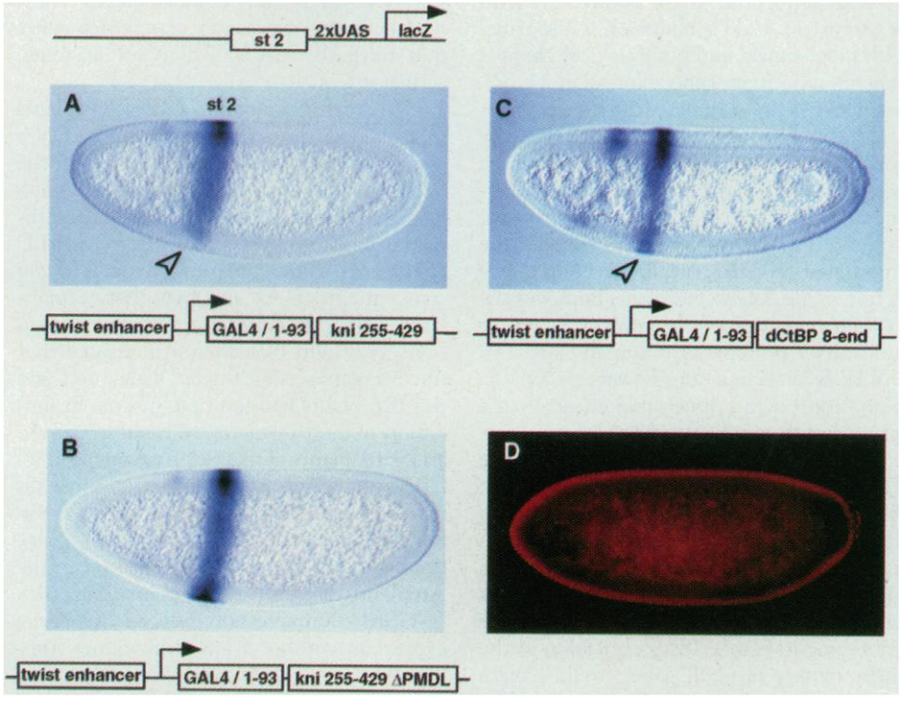

Fig. 4. The Knirps P-DLS-K motif is essential for repression in vivo. (**A** to **C**) Transgenic embryos express an *eve* stripe 2–*lacZ* reporter gene that contains two Gal4 (UAS) binding sites [see diagram above (A)]. The reporter gene was introduced into strains that express different Gal4-Knirps fusion proteins [(A) and (B)] or a Gal4-dCtBP fusion protein (C) under the control of a mesoderm-specific enhancer from the *twist* promoter region (see diagrams below the panels). Reporter gene expression was visualized by hybridizing the embryos with a digoxigenin-labeled *lacZ* antisense RNA probe. Embryos are oriented as in Fig. 3. In (A), a lateral view of a cellularizing embryo that expresses a wild-type Gal4-Knirps 255-429 fusion protein, stripe 2 staining is selectively repressed in ventral regions (arrowhead). In (B), the conditions are as in (A) except that the Knirps fusion protein was mutagenized to convert the P-DLS-K (PMDLSMK) motif into the sequence AAAASMK. The mutant protein fails to repress *eve* stripe 2, so that there is uniform staining in ventral and dorsal regions [compare with (A)]. In (C), a stripe 2–lacZ staining pattern is seen in a transgenic embryo that expresses a Gal4-dCtBP fusion protein (containing amino acid residues 8 to 383 of dCtBP). Staining is reduced in ventral regions (arrowhead), although the repression is less complete than that observed for the Gal-Knirps 255-429 fusion protein [compare with (A)]. The staining in anterior, dorsal regions is attributable to vector sequences in the P-element transformation vector (*7, 8*). (**D**) Normal embryo stained with a rat dCtBP antiserum (*31*). Staining is primarily detected in nuclei.

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16. A *Drosophila* cDNA expression library (MATCHMAKER, Clontech) was screened with a Gal4-Snail expression vector containing the entire repression domain (amino acid residues 1 to 244); dCtBP was identified among 16 randomly sequenced clones. A Gal4-Knirps expression vector, containing amino acid residues 255 to 429, was used to screen a pACT cDNA expression library (*27*). A total of 14 clones were identified, and all correspond to one of three different dCtBP cDNA clones. The Snail and Knirps repression domains were inserted into the pGBT9 or pAS2-1 expression vectors (Clontech) and transformed into the Y190 strain of *Saccharomyces cerevisiae*. Each reporter strain was then transformed with the *Drosophila* cDNA libraries described above. A total of 1.5×10^7 transformants were screened with the pG/Sna bait, and 1.2×10^6 transformants were screened with pAS2/Kni. His$^+$ colonies exhibiting β-galactosidase activity were sequenced using the Ladderman dideoxy sequencing kit (Takara Shuzo, Japan). The four different dCtBP cDNA clones (one using Gal4-snail and three using Gal4-knirps) were not in the same reading frame as the Gal4 activation domain in the expression vectors. However, yeast has an intrinsic frame-shifting activity, which would be expected to result in the expression of small amounts of in-frame fusion proteins (*28*). Retransformation of yeast Y190 strains with in-frame dCtBP expression vectors resulted in reduced growth but specific interaction with the Knirps and Snail expression vectors.
17. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
18. Coding sequences for *snail* and *knirps* were cloned into the pBluescript SK(+) plasmid (Stratagene), whereas Gal4-Knirps fusion proteins (Gal4-kni75-340 and Gal4-kni75-340M) were prepared with the pRSETA expression vector (Invitrogen). Coupled in vitro transcription and translation reactions were performed by incubating 1 μg of each plasmid DNA in a TNT-coupled reticulocyte lysate system (Promega) using T7 RNA polymerase exactly as described by

the manufacturer. The coding sequence for dCtBP, containing codons 8 to 383, was inserted into the pGEX-5X-3 expression plasmid (Pharmacia) and transformed into strain BL21::DE3 pLysS of *Escherichia coli*. Protein expression was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside at 37°C for 3 hours. Cells were then sonicated in buffer A [20 mM tris-HCl (pH 7.9), 0.2 mM EDTA, 0.1 M NaCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.2% Nonidet P-40], and total lysates containing the GST and GST-dCtBP proteins were immobilized onto glutathione-agarose beads (Sigma) in buffer A. Binding assays were done as described (*29*). ^{35}S-labeled proteins were fractionated on a 10% SDS-polyacrylamide gel and visualized by autoradiography.

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21. Genomic DNA was extracted from l(3)03463 heterozygous stocks, annealed with various primers, and then subjected to polymerase chain reaction (PCR) amplification using standard methods. A 550-bp DNA fragment was obtained using a 23-nucleotide primer from the 5′ end of the dCtBP cDNA (TGAAAGCTGCGAGTGGAATTTGG) and a 28-nucleotide primer from the 3′ region of the P-element (CTTGCCGACGGGACCACCTTATGTTATT). The 5′ end of this PCR product contains 37 bp of perfect identity to the 5′ end of the dCtBP UTR. The remaining sequence does not contain any discernible homology with dCtBP, which suggests that the dCtBP UTR is interrupted by an intron located 37 bp downstream of the 5′ end of the largest dCtBP cDNA. It would appear that the P-element maps within this intron.
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24. P-elements were introduced into the *Drosophila* germline by injection of yw^{67} or w^{1118} embryos as described (*3*). In situ hybridizations on whole mount preparations of staged, transgenic embryos were also done as described (*3*) using a digoxigenin–uridine triphosphate–labeled lacZ antisense RNA probe. The stripe 2 reporter gene and Gal4 expression vector are identical to those used by Arnosti *et al.* (*8*).
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31. A GST-dCtBP fusion protein containing amino acid residues 8 to 383 was injected into a rat (Pocono Rabbit Farm, PA). The preimmune serum did not detectably cross-react with fixed embryos. The GST-dCtBP antiserum specifically stained nuclei in mixed-stage embryos. Reduced staining was detected in l(3)03463 homozygous embryos. Embryos were fixed and preincubated in bovine serum albumin as described (*15*). The rat serum was diluted 1:1000, and dCtBP was visualized using a 1:200 dilution of tetramethyl rhodamine isothiocyanate–conjugated antibodies to rat immunoglobulin (Jackson Labs).
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Arabidopsis CBF1 Overexpression Induces *COR* Genes and Enhances Freezing Tolerance

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Many plants, including *Arabidopsis*, show increased resistance to freezing after they have been exposed to low nonfreezing temperatures. This response, termed cold acclimation, is associated with the induction of *COR* (cold-regulated) genes mediated by the C-repeat/drought-responsive element (CRT/DRE) DNA regulatory element. Increased expression of *Arabidopsis* CBF1, a transcriptional activator that binds to the CRT/DRE sequence, induced *COR* gene expression and increased the freezing tolerance of nonacclimated *Arabidopsis* plants. We conclude that CBF1 is a likely regulator of the cold acclimation response, controlling the level of *COR* gene expression, which in turn promotes tolerance to freezing.

Studies of the molecular basis of plant tolerance to freezing have focused primarily on the cold acclimation response, the process by which plants increase their tolerance to freezing in response to low nonfreezing temperatures (*1*). Cold acclimation is associated with biochemical and physiological changes and alterations in gene expression (*1*, *2*). Studies of genes stimulated by low temperature have revealed that many, including the *Arabidopsis COR* genes, encode hydrophilic polypeptides that potentially promote tolerance to freezing (*1–3*). Indeed, constitutive expression of *COR15a* (which encodes the chloroplast-targeted polypeptide COR15am) in transgenic *Arabidopsis* plants improves the freezing tolerance of chloroplasts frozen in situ and of protoplasts frozen in vitro (*4*). Unlike cold acclimation, however, *COR15a* expression has no discernible effect on the survival of frozen plants (*2*, *5*).

Genetic analyses indicate that multiple genes are involved in cold acclimation in plants (*6*). Several *COR* genes are coordinately stimulated along with *COR15a* in response to low temperature (*2*, *7*), which suggests that *COR15a* might act in concert with other *COR* genes to enhance tolerance to freezing in plants. If so, expression of the entire battery of *COR* genes would have a greater effect on freezing tolerance than *COR15a* expression alone. To test this hypothesis, we attempted to induce expression of the *COR* gene "regulon" with the *Arabidopsis* transcriptional activator CBF1 (CRT/DRE binding factor 1) (*8*), a putative *COR* gene regulator. CBF1 binds to the cis-acting CRT (C-repeat)/DRE (drought-responsive element) sequence (*9*, *10*), a DNA regulatory element that stimulates transcription in response to both low temperature and water deficit (*9*). The element is present in the promoters of multiple *COR* genes including *COR15a*, *COR78* (also known as RD29A and LTI78), and *COR6.6* (*10–12*). Expression of *CBF1* in yeast (*Saccharomyces cerevisiae*) activates expression of reporter genes that have the CRT/DRE as an upstream activator sequence (*8*).

We created transgenic *Arabidopsis* plants that overexpress *CBF1* by placing a cDNA encoding CBF1 under the control of the strong cauliflower mosaic virus (CaMV) 35S RNA promoter and transforming the chimeric gene into *Arabidopsis* ecotype RLD plants (*13*). Initial screening gave rise to two transgenic lines, A6 and B16, that accumulated *CBF1* transcripts at high concentrations. Southern blot analysis indicated that the A6 plants had a single DNA insert and the B16 plants had multiple inserts. Examination of fourth generation homozygous A6 and B16 plants indicated that amounts of *CBF1* transcript were higher in nonacclimated A6 and B16 plants than they were in nonacclimated RLD plants (Fig. 1A). Quantities of *CBF1* transcript were greater in the A6 plants than in the B16 plants (Fig. 1A).

CBF1 overexpression induced *COR* gene expression without a low-temperature stimulus (Fig. 1A). Specifically, greater than normal amounts of *COR6.6*, *COR15a*, *COR47*, and *COR78* transcripts were detected in nonacclimated A6 and B16 plants. In nonacclimated A6 plants, *COR* transcript concentrations approximated those found in cold-acclimated RLD plants. In nonacclimated B16 plants, they were less than in cold-acclimated RLD plants. Immunoblot analysis indicated that the amounts of the COR15am (Fig. 1B) and COR6.6 polypeptides were also elevated in the A6 and B16 plants, with a higher level of expression in A6 plants. We were unable to identify the CBF1 protein in either RLD or transgenic plants (*5*). Overexpression of *CBF1* did not affect transcript concentrations of *eIF4A*

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