

Trans-activation by the *Drosophila myb* gene product requires a *Drosophila* homologue of CBP

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Abstract Attempts to demonstrate *trans*-activation activity by the *Drosophila myb* gene product (D-Myb) have been unsuccessful so far. We demonstrate that co-transfection of Schneider cells with a plasmid expressing the *Drosophila* homologue of transcriptional co-activator CBP (dCBP) results in *trans*-activation by D-Myb. Using this assay system, the functional domains of D-Myb were analyzed. Two domains located in the N-proximal region, one of which is required for DNA binding and the other for dCBP binding, are both necessary and sufficient for *trans*-activation. In this respect, D-Myb is similar to c-Myb and A-Myb, but different from mammalian B-Myb. These results shed light on how the *myb* gene diverged during the course of evolution.

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Key words: *myb* proto-oncogene; *Drosophila*; Co-activator; CBP; Transcriptional activation; Functional domain

1. Introduction

Many studies indicate that the vertebrate *c-myb* nuclear proto-oncogene is primarily involved in the maintenance of the proliferative state of immature hematopoietic cells [1,2]. On the other hand, two other vertebrate members of the *myb* gene family, *A-myb* and *B-myb* [3], are thought to be important for cellular proliferation in testis and in more ubiquitous cell lineages, respectively [4–6]. The *c-myb* gene is highly conserved in many species. Although the *myb* gene has diverged into three genes in vertebrates, *Drosophila* possesses only one *myb* gene [7,8]. Comparison of the *Drosophila myb* gene product (D-Myb) with the three vertebrate *myb*-related proteins (c-Myb, A-Myb, and B-Myb) indicates the presence of two homologous regions, the N-terminal DNA-binding domain, which recognizes the specific DNA sequence, AACNG [9–11], and the C-terminal conserved region. The existence of these homologies suggests that D-Myb also functions as a transcriptional activator by binding to the same recognition sequence as vertebrate Myb. So far, however, *trans*-activation by D-Myb has not been successfully demonstrated, and the reason for this is unknown [12]. Molecular studies on the three vertebrate Myb proteins indicated that both c-Myb and A-Myb can *trans*-activate in all cells examined, and that the two domains responsible for DNA binding and transcriptional activation are sufficient for this purpose [10,13,14]. In contrast, B-Myb has *trans*-activating activity only in a limited number of types of cells, and the C-terminal region conserved between *c-myb*, *A-myb*, and *B-myb* (CR for conserved region) in the B-Myb molecule is necessary for this activity [15]. Since

the *Drosophila* system has the advantage of being accessible to genetic analysis, a study of D-Myb in this system would be expected to yield valuable information about the signal transduction pathway involving D-Myb and the molecular mechanism that regulates Myb activity. Due to the lack of an assay system to measure D-Myb activity, the analyses of the functional domains of D-Myb and the comparison of these domains with those of vertebrate Myb proteins have been impossible.

Recently, we demonstrated that the transcriptional co-activator CBP is necessary for *trans*-activation by mammalian c-Myb [16]. CBP directly binds to the transcriptional activation domain of c-Myb, which is rich in acidic amino acids, and mediates *trans*-activation by c-Myb. CBP was originally identified as a co-activator for the transcriptional activator, CREB [17]. CBP possesses intrinsic histone acetyltransferase activity [18], and also binds to another histone acetyltransferase, P/CAF [19], suggesting that the CBP/PCAF complex contributes to promoter activation by altering or disrupting inhibitory chromatin structure. Recent studies indicate that CBP is also utilized by a multitude of other transcriptional regulators including c-Jun [20], c-Fos [21], c-Myb [16,22], nuclear hormone receptors [23,24], MyoD [25], and Stat2 [26]. CBP also functions as a bridge between these regulators and the basal transcriptional machinery by binding to the basal transcriptional factor TFIIB [27] and the RNA polymerase II holoenzyme complex [28]. In mammals, the *CBP* gene family has two members, *CBP* and *p300* [29], the functions of which are indistinguishable [30,31]. Like the *myb* gene, the *CBP* gene is also conserved in many species including mammals and *C. elegans* [32]. The *Drosophila* homologue of *CBP* (dCBP) has been recently identified and shown to have striking homology with mouse *CBP* [33]. These observations suggest that the Myb–CBP interaction is conserved in *Drosophila*.

We report here that the recently identified dCBP binds to D-Myb. Using the co-transfection assay with the dCBP expression plasmid, we demonstrate *trans*-activation by D-Myb, and identify the functional domains involved in this activity.

2. Materials and methods

2.1. Plasmids

The D-*myb* cDNA clones were isolated from the *Drosophila* cDNA library prepared from 0–6 h embryos using the PCR-amplified DNA fragment which was based on the published sequence of D-*myb*. The plasmid pGEM-D-*myb*, which was used to synthesize D-Myb by in vitro transcription/translation, was constructed by insertion of D-*myb* cDNA into the *Bam*HI–*Sal*I sites of the pGEM vector (Promega). Using the appropriate enzyme sites, we constructed plasmids for the synthesis of various forms of D-*myb*. The CAT reporter plasmid, pADHCAT6MBS-I, was constructed by inserting six tandem repeats of the mouse c-Myb-binding site MBS-I identified in the SV40 enhancer into the *Xba*I site of pAdhCAT which contains the *Drosophila*

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alcohol dehydrogenase promoter [34]. The plasmid pA5c-D-*myb* used to express D-*myb* in the cultured cells was constructed by inserting the D-*myb* cDNA into pUchs-neoAct5c downstream of the *Drosophila* actin 5c promoter [35]. The plasmids encoding various forms of D-*myb* were constructed from pA5cD-*myb* using the appropriate enzyme sites. The plasmid used to express GST-dCBP in bacteria, the plasmid used to express dCBP in cultured cells, the control effector plasmid pA5c0, and the internal control plasmid pA5c- β -gal were described previously [33].

2.2. In vitro binding assays

GST pull-down assays were performed as described [16].

2.3. CAT co-transfection assays

Transfections in Schneider cells were done with the reporter plasmid pADCAT6MBS-I containing the six tandem repeats of the c-Myb-binding site MBS-I. Transfection assays were performed as described by Nocera and Dawid [36]. The amount of lysate used for the CAT assays was normalized by measuring β -galactosidase activity resulting from the internal control plasmid pA5c- β -gal. All CAT co-transfection experiments were repeated at least three times. Typical results are shown in the figures. The differences between each set of experiment did not vary by more than 30%.

3. Results

3.1. Binding of D-Myb to dCBP

To test for direct binding between D-Myb and dCBP, we examined whether in vitro translated D-Myb could interact with the GST-dCBP fusion protein (Fig. 1). Protein affinity resins of GST alone or GST-dCBP fusion protein (amino acids 781–1159) were used as ligands. This 379-amino-acid region of dCBP contains a 85-amino-acid region that is highly conserved between murine CBP, dCBP, and other members of the CBP family [33]. This region should be sufficient for binding D-Myb, as a 201-amino-acid region of mouse CBP containing this highly conserved region can efficiently bind to mouse c-Myb [16]. The full-length form of D-Myb was synthesized using an in vitro transcription/translation system, and mixed with the GST alone or GST-dCBP affinity resin. Approximately 12% of the input D-Myb bound to the GST-dCBP resin (Fig. 1B, lanes 1 and 6), in contrast to the GST-resin where no binding was detected (data not shown). D-Myb bound to the GST-dCBP resin without pretreatment with PKA, and PKA treatment did not increase the efficiency of D-Myb binding to dCBP (data not shown). By using the GST fusion proteins containing various regions of dCBP, we confirmed that D-Myb binds only to the 379-amino-acid region of dCBP described above (data not shown).

To examine whether dCBP interacts with the acidic region of D-Myb located downstream of the DNA-binding domain, we used deletion mutants of D-Myb in the binding assay (Fig. 1). The results of the binding assays indicate that dCBP binds to the region between amino acids 230 and 416 of D-Myb which contains the putative acidic activation domain.

3.2. Trans-activation by D-Myb in the presence of dCBP

To examine whether dCBP functions as a co-activator of D-Myb, we employed CAT co-transfection assays in *Drosophila* Schneider cells (Fig. 2). The plasmid pADHCAT6MBS-I, which contains six tandem repeats of the mouse c-Myb-binding site MBS-I linked to the *Drosophila* alcohol dehydrogenase promoter, was used as the reporter plasmid. In the absence of dCBP, an increasing amount of D-Myb expression plasmid did not activate CAT expression from this reporter plasmid (Fig. 2A). This result is consistent with a previous

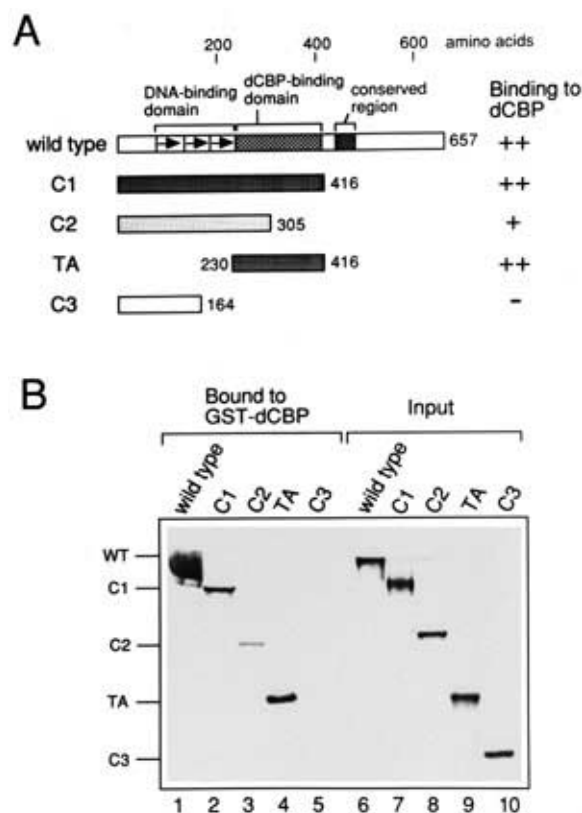


Fig. 1. Binding of D-Myb to dCBP. A: Schematic representation of the results of the binding analyses. On the top, the three regions in D-Myb comprising the DNA-binding domain, the dCBP-binding domain identified here, and the region conserved among the different members of the *myb* gene family, are indicated. The structures of the various forms of D-Myb used are shown below. Mutants that bind to dCBP are indicated by stippled bars, and a mutant that does not bind by an open bar. The results of binding assays described below are indicated on the right. The relative binding activities of the mutants are designated by ++, +, and –, which indicate, respectively, 12–16%, 5–7%, and less than 0.5% of binding of the input protein. Less than 0.5% of the input D-Myb proteins bound to the control GST resin (data not shown). B: Results of the binding analyses. The GST-dCBP fusion protein contained the 379-amino-acid region (amino acids 781–1159). In lanes 1–5, the Sepharose resin containing GST-dCBP were mixed with various derivatives of the in vitro translated [³⁵S]D-Myb as indicated above lane. After washing, the bound proteins were released and analyzed on 10% SDS-PAGE followed by autoradiography. In lanes 6–10, the various derivatives of D-Myb designated above each lane were synthesized in vitro and directly analyzed by 10% SDS-PAGE. The amount of [³⁵S]D-Myb used for the input lane was 10% of that used for the binding assays.

report of another group showing that the *trans*-activating capacity of D-Myb can not be detected under these conditions [12]. The experiment was repeated again, but this time increasing amounts of the dCBP expression plasmid were co-transfected in the presence or absence of the D-Myb expression plasmid (Fig. 2B). Co-transfection of increasing amounts of the dCBP expression plasmid increased the level of *trans*-activation by a maximum of 6.8-fold. This level was achieved at a ratio of 15 μ g of dCBP expression plasmid to 3 μ g of D-Myb expression plasmid. In the control experiment without any D-Myb expression plasmid, the addition of the dCBP expression plasmid did not affect the level of CAT activity at all. Thus, dCBP potentiates D-Myb-induced *trans*-activation.

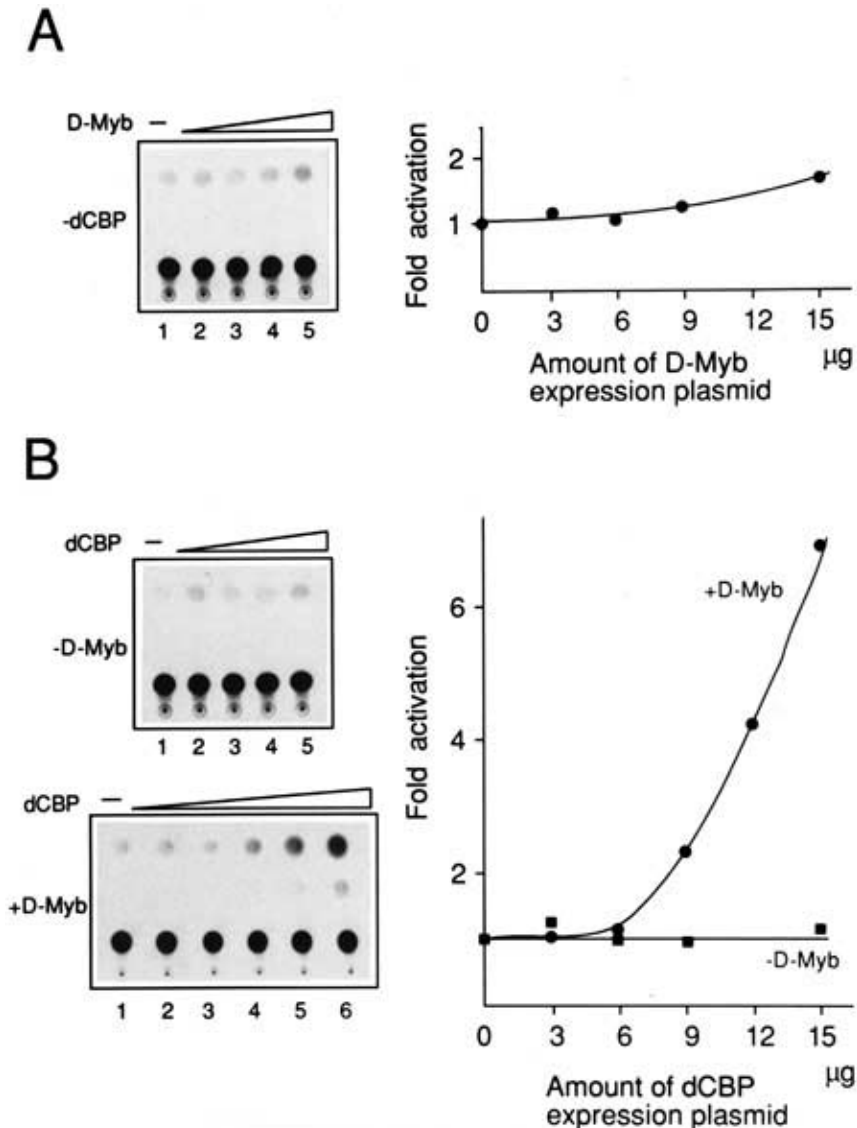


Fig. 2. Potentiation of D-Myb-dependent *trans*-activation by dCBP. A: D-Myb fails to *trans*-activate without the dCBP expression plasmid. A mixture of 3 µg of the CAT reporter plasmid pADHCAT6MBS-I, increasing amounts of the D-Myb expression plasmid pact5c-D-*myb* or the control plasmid pact5c0 lacking the D-Myb-coding region, and 1 µg of the internal control plasmid pact5c-β-gal was transfected into Schneider cells. The total amount of DNA was adjusted to 19 µg by adding the control plasmid pact5c0. CAT assays were done, and a set of typical results is shown on the left. The degree of *trans*-activation (compared to samples without the c-Myb expression plasmid) is shown on the right. B: Effect of dCBP on D-Myb-dependent *trans*-activation. A mixture of 3 µg of a CAT reporter plasmid pADHCAT6MBS-I, increasing amounts of the dCBP expression plasmid pact5c-dCBP, 3 µg of the D-Myb expression plasmid pact5c-D-*myb* or the control plasmid pact5c0 lacking the D-Myb coding region, and 1 µg of the internal control plasmid pact5c-β-gal was transfected into Schneider cells. The total amount of DNA was adjusted to 19 µg by adding the control plasmid pact5c0. The CAT assays were done, and the results are indicated as described above.

3.3. Functional domains of D-Myb

Using the co-transfection assays with the dCBP expression plasmid, we then attempted to identify the regions of D-Myb required for *trans*-activation (Fig. 3). The effector plasmids used to express various forms of D-Myb were individually co-transfected with the reporter plasmid pADH6MBS-I and the dCBP expression plasmid, and CAT activity was measured. The amino acid sequence of DNA-binding domain in the N-terminal region is conserved between mammalian c-Myb and D-Myb, and this region contains the nuclear localization signals [10]. Consistent with this, all forms of D-Myb used here were localized in the nuclei, and the level of each

form was almost similar by judging with the Western blotting (data not shown). The results obtained using a series of C-truncated D-Myb mutants indicate that the N-terminal 416-amino-acid region, which contains the DNA-binding domain and the dCBP-binding domain, is both necessary and sufficient for *trans*-activation by D-Myb. These results show that the dCBP-binding domain located downstream of the DNA-binding domain functions as a transcriptional activation domain. In addition, our results show that the C-terminal region located downstream of the dCBP-binding domain, which contains a region conserved among members of the *myb* gene family, does not affect the *trans*-activating activity of D-Myb.

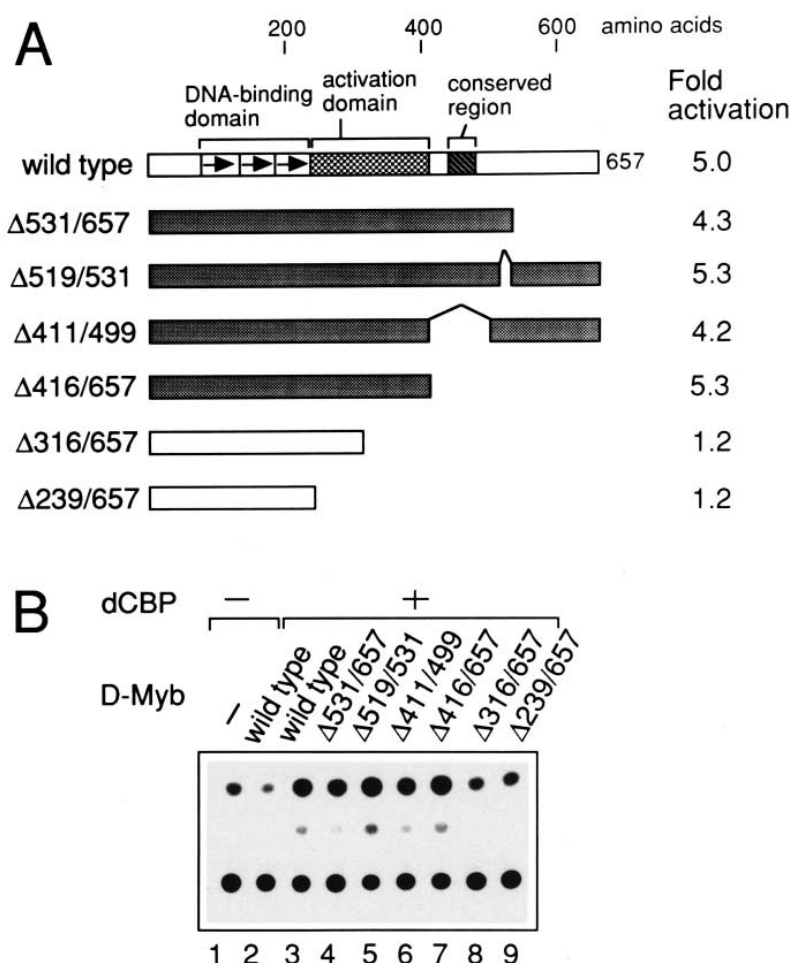


Fig. 3. Domain analyses of D-Myb. A: Schematic representation of the CAT assays. On the top, the three regions in D-Myb are indicated. The structures of various forms of D-Myb are shown below. Mutants that possess and lack the *trans*-activating capacity are indicated by *stippled bars*, and *open bars*, respectively. The degree of *trans*-activation by each mutant is indicated on the right. B: Results of CAT assays. *Trans*-activation of various forms of D-Myb were examined as described in Fig. 2. A mixture of 7 μ g of a CAT reporter plasmid pADH-CAT6MBS-I, 9 μ g of the dCBP expression plasmid pact5c-dCBP, 4 μ g of the D-Myb expression plasmid encoding the protein indicated above each lane or the control plasmid pact5c0 lacking the D-Myb coding region, and 2 μ g of the internal control plasmid pact5c- β -gal was transfected into Schneider cells. The total amount of DNA was adjusted to 22 μ g by adding the control plasmid pact5c0. The CAT assays were done, and the results are indicated as described above.

4. Discussion

By realizing co-transfection assays with the dCBP expression plasmid, we have been able to demonstrate D-Myb-induced *trans*-activation in Schneider cells. Multiple parameters including the endogenous levels of transcription factors and their associated co-factors are known to affect the level of *trans*-activation observed in co-transfection assays using specific cell lines. In the case of mammals, many cell lines are currently available and thus, the optimal cell line for the co-transfection assays can be chosen. However, only a limited number of *Drosophila* cell lines such as Schneider and Kc cell lines have been established. So far, we and other groups have not been succeeded to demonstrate *trans*-activation by D-Myb in these cell lines [12], probably as a result of limiting amounts of dCBP. A reduced endogenous level of dCBP in *Drosophila* may be due to competition for a limited pool of CBP protein, as in vertebrates a large number of transcription factors are known to use CBP as a co-activator.

We have shown that the region downstream of the DNA-binding domain in D-Myb binds to dCBP. Like the activation

domain of mammalian c-Myb and A-Myb, this region contains a region rich in acidic amino acids. Our domain analyses of D-Myb indicate that both the DNA binding and dCBP binding domains are necessary and sufficient for *trans*-activation. This is similar to the case of mammalian A-Myb and c-Myb, but unlike that of B-Myb which requires an additional C-terminal conserved region for *trans*-activation [15]. Although D-Myb also possesses this C-terminal conserved region, deletion of this region did not affect its *trans*-activating capacity. D-Myb is also different from mammalian A-Myb and c-Myb with respect to the role of the C-terminal half of the molecule. Removal of a region downstream of the CBP-binding domain in mammalian c-Myb and A-Myb increases their *trans*-activating activity [10,14], whereas deletion of a similar region in D-Myb did not affect *trans*-activating activity. In the case of c-Myb, the leucine zipper is located in the C-terminal proximal region, and disruption of this motif leads to increased activity [37]. However, D-Myb does not have the leucine zipper motif. In addition, the EVES motif in the C-terminal region of c-Myb binds to the DNA-binding domain intramolecularly [38], but this EVES motif is not present in

D-Myb. Although these results suggest that the regulation of the mammalian Myb activity by the C-proximal region was an additional element acquired during evolution, we can not completely exclude that D-Myb activity is modulated by the C-proximal region under certain *in vivo* conditions. For instance, c-Myb activity is known to be regulated by proline-directed serine/threonine protein kinase [39], and the putative phosphorylation sites of this protein kinase are also present in the C-proximal region of D-Myb. Furthermore, the modulation of c-Myb activity by phosphorylation varies depending on the target promoter [40]. These results raise the possibility that D-Myb activity may also be affected by phosphorylation within the C-terminal domain depending on the nature of the target promoter.

Recently, *Drosophila myb* mutants have been isolated. Genetic analyses of these mutants indicated that D-*myb* is important for both embryonic and imaginal development [41]. The system described here should be useful for the further study of the relationship between the biochemical characteristics and functional activity of D-*myb* *in vivo*.

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