

## The Transcriptional Coactivator CBP Interacts with $\beta$ -Catenin to Activate Gene Expression

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**Abstract.**  $\beta$ -Catenin plays a pivotal role in the transcriptional activation of Wnt-responsive genes by binding to TCF/LEF transcription factors. Although it has been suggested that the COOH-terminal region of  $\beta$ -catenin functions as an activation domain, the mechanisms of activation remain unclear. To screen for potential transcriptional coactivators that bind to the COOH-terminal region of  $\beta$ -catenin, we used a novel yeast two-hybrid system, the Ras recruitment system (RRS) that detects protein-protein interactions at the inner surface of the plasma membrane. Using this system, we isolated the CREB-binding protein (CBP). Armadillo (Arm) repeat 10 to the COOH terminus of  $\beta$ -catenin is involved in binding to CBP, whereas  $\beta$ -catenin interacts directly with the CREB-binding do-

main of CBP.  $\beta$ -Catenin synergizes with CBP to stimulate the activity of a synthetic reporter in vivo. Conversely,  $\beta$ -catenin-dependent transcriptional activation is repressed by E1A, an antagonist of CBP function, but not by an E1A mutant that does not bind to CBP. The activation of Wnt target genes such as *siamois* and *Xnr3* in *Xenopus* embryos is also sensitive to E1A. These findings suggest that CBP provides a link between  $\beta$ -catenin and the transcriptional machinery, and possibly mediates the oncogenic function of  $\beta$ -catenin.

**Key words:** *Xenopus laevis* • CREB-binding protein • T cell factor/lymphoid enhancer factor • transcriptional activation • Wnt

### Introduction

Wnt signaling pathways regulate a variety of processes including cell growth, oncogenesis, and development (Moon et al., 1997; Miller et al., 1999). Upon Wnt signaling,  $\beta$ -catenin accumulates in the nucleus, and binds to transcription factors of the TCF/LEF<sup>1</sup> family, leading to transcriptional activation (Behrens et al., 1996). The TCF/LEF family members contain a single DNA-binding high mobility group (HMG) domain, whereas  $\beta$ -catenin provides transactivation domains (reviewed by Barker et al., 2000). In the absence of nuclear  $\beta$ -catenin, the TCF/LEF transcription factors recruit the Groucho and CtBP corepressors to

repress transcription (Roose et al., 1998; Brannon et al., 1999). An additional corepressor protein, the *Drosophila melanogaster* CREB-binding protein (dCBP), was shown to be involved in the repression of dTCF target genes in vivo (Waltzer and Bienz, 1998). In response to Wnt signaling,  $\beta$ -catenin somehow overcomes these repressive effects to activate transcription. Constitutive activation of downstream target genes, such as *c-myc* and *cyclin D1* by the TCF/LEF- $\beta$ -catenin complex is implicated in the development of cancer (He et al., 1998; Tetsu and McCormick, 1999).

$\beta$ -Catenin and its *Drosophila* ortholog, Armadillo (Arm), are composed of 12 Arm repeats flanked by unique NH<sub>2</sub> and COOH termini. Based on reporter gene assays, it has been suggested that two regions of  $\beta$ -catenin mediate transcriptional activation (Hsu et al., 1998; Hecht et al., 1999). In particular, the COOH-terminal activation domain was reported to be sufficient both for signaling and for oncogenic transformation (reviewed by Hecht et al., 1999). Genetic analysis has also demonstrated that the

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<sup>1</sup>Abbreviations used in this paper: Arm, Armadillo; CBP, CREB-binding protein; CREB, cAMP-response element binding protein; GST, glutathione S-transferase; LEF, lymphoid enhancer factor; pt, point-mutant; RRS, Ras recruitment system; RT-PCR, reverse transcriptase PCR; TCF, T cell factor.

corresponding region of Arm is required for Wingless signaling in vivo (Cox et al., 1999). However, the underlying mechanisms for transcriptional activation by  $\beta$ -catenin are poorly understood.

In the present study, we have identified CBP as a transcriptional coactivator that binds to the COOH-terminal region of  $\beta$ -catenin.  $\beta$ -Catenin physically interacts with the CREB-binding domain of CBP. CBP then cooperates with  $\beta$ -catenin to activate transcription in mammalian cells and *Xenopus laevis* embryos.

## Materials and Methods

### Plasmids

An expression plasmid, pRas(61) $\Delta$ F- $\beta$ catR8-C, encoding the RRS bait was constructed by inserting cDNA sequences encompassing COOH-terminal region of *Xenopus*  $\beta$ -catenin (amino acids 425–781) in frame with activated Q61L c-Ha-Ras into the 3S0B-SRS (Isakoff et al., 1998). The mouse CBP expression plasmid pRc/RSV-mCBP-HA and GST-CBP 451–682 fusion plasmid were gifts from R. Goodman (Vollum Institute, Portland, OR; Chrivia et al., 1993; Kwok et al., 1996). Expression plasmids for E1A, E1A mutRB, and E1A mutCBP were provided by T. Kouzarides (Wellcome/CRC Institute, Cambridge, UK; Bannister and Kouzarides, 1995). dnLEF-1 expression plasmid was a gift from J. Behrens (Max Delbrück Center, Berlin, Germany; Behrens et al., 1996). The  $\beta$ -catenin deletion constructs shown in Fig. 1 B were amplified by PCR, digested with BamHI, and subcloned into the BamHI site of 3S0B-SRS. To generate His-tagged  $\beta$ catR10-C expression plasmid and GAL4- $\beta$ catR10-C fusion construct, an insert of the corresponding yeast expression plasmid was excised, and subcloned into pET-28c (Novagen) and pCMV-BD (Stratagene), respectively. GAL4 reporter plasmid pFR-Luc was obtained from Stratagene. For in vitro synthesis of RNA used in *Xenopus* injection experiments, the full-length mouse CBP cDNA from pRc/RSV-mCBP-HA was inserted into the HindIII and SacI sites of pXeX (Johnson and Krieg,

1994). An EcoRI fragment encoding E1A or E1A mutCBP was subcloned into pCS2+.

### RRS Screening

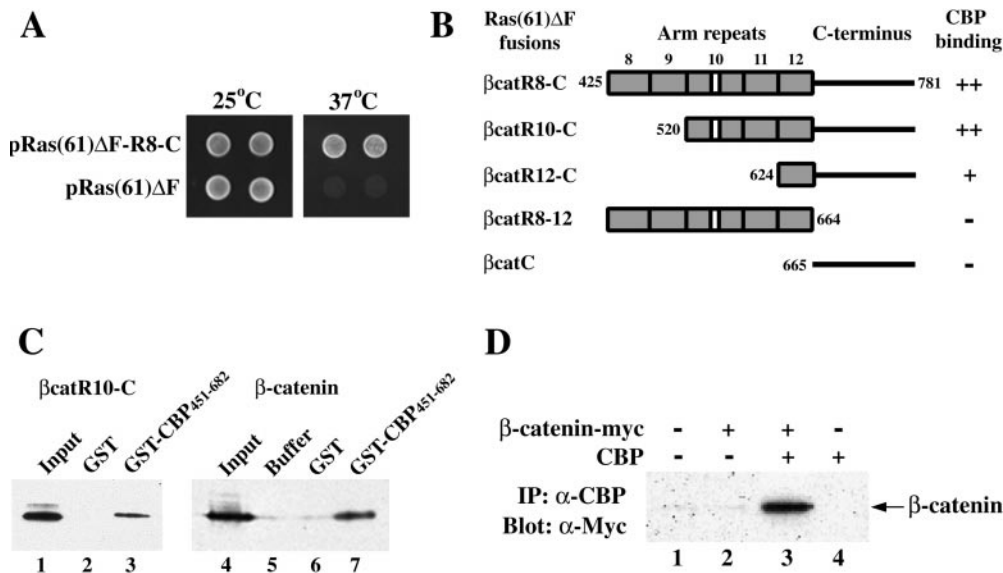
RRS screening in yeast was performed as described (Broder et al., 1998). In brief, *cdc25-2* cells were first cotransformed with the pRAS(61) $\Delta$ F- $\beta$ catR8-C bait and mGAP expression plasmid (Aronheim, 1997), and then used to transform with HeLa cell cDNA library. The expression of library cDNA was controlled by a galactose-inducible promoter. Transformants were grown on selectable minimal glucose plates for 5 d at 25°C, followed by replica plating onto minimal galactose plates, and incubated at 37°C for 5–7 d. Positive colonies exhibiting efficient growth on galactose plates at 37°C were isolated and tested for galactose-dependent growth at 37°C. Library plasmids were extracted and further analyzed by DNA sequencing and retransformation of *cdc25-2* cells to test the specificity of interaction.

### In Vitro Binding Assays

2  $\mu$ g of GST or GST-CBP 451–682 was incubated with 1  $\mu$ g of bacterially expressed and purified His-tagged  $\beta$ catR10-C or *c-myc* epitope-tagged  $\beta$ -catenin (Yost et al., 1996) at 4°C for 1 h in 20  $\mu$ l of protein-binding buffer (PBB; 20 mM Hepes-NaCl, pH 7.9, 20% glycerol, 0.5 mM EDTA, 60 mM NaCl, 6 mM MgCl<sub>2</sub>, 0.1% NP-40, 5 mM 2-mercaptoethanol, 1 mM PMSF). After incubation, 2  $\mu$ l of glutathione-Sepharose 4B (Pharmacia Biotech) in 20  $\mu$ l of PBB was added, and the mixture was rotated at 4°C for 1 h. The beads were collected and washed with PBB, and then bound proteins were eluted for SDS-PAGE. His-tagged  $\beta$ catR10-C and  $\beta$ -catenin were detected on immunoblots by an anti-His antibody (Qiagen) and an anti-*myc* antibody, respectively.

### Cell Culture, Coimmunoprecipitation, and Luciferase Assays

HeLa and COS7 cells were maintained in MEM and DME with 10% FBS, respectively. SW480 cells were maintained in Opti-MEM I (GIBCO BRL) supplemented with 10% FBS. Transient transfections into HeLa cells were carried out by the calcium phosphate precipitation method.



**Figure 1.** Interaction between  $\beta$ -catenin and CBP. **A**, Complementation of the *cdc25-2* mutation through the interaction of  $\beta$ -catenin COOH-terminal region with CBP. The temperature-sensitive *cdc25-2* yeast cells were cotransformed with the indicated plasmids and the CBP expression plasmid isolated in the screening, and incubated on galactose containing plates either at 25 (left) or 37°C (right). **B**, Fine mapping of the  $\beta$ -catenin domain that binds to CBP in yeast. Cells were cotransformed with the indicated plasmids together with the CBP expression plasmid, and tested for the growth at 37°C as described for **A**. The numbers indicate the locations in the amino acid sequence of  $\beta$ -catenin. ++, Efficient growth; +, poor growth; -, no growth. **C**,  $\beta$ -Catenin directly binds to CREB-binding domain of CBP. Bacterially expressed and purified His-tagged  $\beta$ catR10-C or *myc*-tagged  $\beta$ -catenin was incubated with buffer (lane 5), GST (lanes 2 and 6), or GST-CBP 451–682 (lanes 3 and 7). After extensive washing, bound proteins were eluted and resolved by SDS-PAGE.  $\beta$ catR10-C and  $\beta$ -catenin were detected by an anti-His antibody and an anti-*myc* antibody, respectively. Approximately 10% of the input is shown (lanes 1 and 4). **D**, Coimmunoprecipitation of CBP and  $\beta$ -catenin. COS7 cells were transfected with expression plasmids for CBP and/or *myc*-tagged  $\beta$ -catenin as indicated. Cell lysates were immunoprecipitated with anti-CBP polyclonal antibodies 2 d after transfection. The immunoprecipitates were separated by SDS-PAGE and subjected to Western blotting with anti-*myc* antibodies.

SW480 and COS7 cells were transfected using lipofectamine Plus (GIBCO BRL). For coimmunoprecipitation experiments, COS7 cells were transfected with 0.5  $\mu$ g of *myc*-tagged point-mutant (pt)  $\beta$ -catenin expression plasmid (Yost et al., 1996) and/or 1  $\mu$ g of pRc/RSV-CBP-HA. Two days later, cells were washed and lysed in 1 ml of a lysis buffer (20 mM Tris-HCl, pH 8.0, 75 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EGTA, 0.5% NP-40, 10% glycerol, 1 mM PMSF, 10  $\mu$ g/ml each of leupeptin and aprotinin). Cell lysates were cleared and incubated with anti-CBP polyclonal antibodies (A-22; Santa Cruz Biotechnology), followed by incubation with protein A-Sepharose beads (Sigma Chemical Co.). The beads were collected and washed three times with the lysis buffer before SDS 8% PAGE. Immunoblotting was subsequently performed with an anti-*myc* mAb. For luciferase assays, a  $\beta$ -galactosidase expression plasmid was included in each transfection as an internal control. Luciferase and  $\beta$ -galactosidase activities were measured 24 h after transfection, and luciferase activity was normalized to  $\beta$ -galactosidase activity. Each transfection was performed in triplicate and repeated at least three times.

### RNA Injections and Reverse Transcriptase PCR Analysis

RNA injections and RT-PCR analysis were carried out as described previously (Brannon et al., 1999).

### Results and Discussion

To identify proteins that interact with the COOH-terminal region of  $\beta$ -catenin and that might be involved in transcriptional activation, we carried out an extensive screening using the RRS. Conventional yeast two-hybrid system was not suitable for our purpose because this region of  $\beta$ -catenin activates the reporter expression by itself when fused to a heterologous DNA-binding domain in yeast (Hecht et al., 1999). RRS is based on the ability of mammalian Ras to rescue the growth defect of the yeast temperature-sensitive *cdc25-2* strain, in which the endogenous Ras is inactive at the nonpermissive temperature (37°C) due to the lack of a functional Cdc25 guanyl nucleotide exchange factor (Broder et al., 1998). For RRS screening, a bait protein of interest is fused at the COOH terminus of mammalian activated Ras lacking its membrane localization signal [Ras(61) $\Delta$ F], whereas library cDNAs are fused to the v-Src myristoylation sequence targeted to the plasma membrane. A protein-protein interaction between the bait and library protein results in the recruitment of Ras to the membrane and complementation of the *cdc25-2* mutation.

We used the COOH-terminal region of *Xenopus*  $\beta$ -catenin extending from Arm repeat 8 to the COOH terminus,  $\beta$ catR8-C (Fig. 1 B), as a bait to cotransform *cdc25-2* yeast cells with a myristoylated HeLa cell cDNA library. After screening  $\sim 2.5 \times 10^6$  colonies, a single clone encoding an NH<sub>2</sub>-terminal fragment of CBP (amino acids 153–822) was isolated. To confirm the specificity of the interaction, the plasmid encoding CBP was recovered, and retransformed into the same yeast strain with either an expression plasmid for Ras(61) $\Delta$ F- $\beta$ catR8-C or a negative control plasmid encoding Ras(61) $\Delta$ F alone. As shown in Fig. 1 A, *cdc25-2* cells transformed with the CBP expression plasmid were able to grow at 37°C when coexpressed with Ras(61) $\Delta$ F- $\beta$ catR8-C, but not with Ras(61) $\Delta$ F, suggesting that the interaction is dependent on the  $\beta$ catR8-C bait.

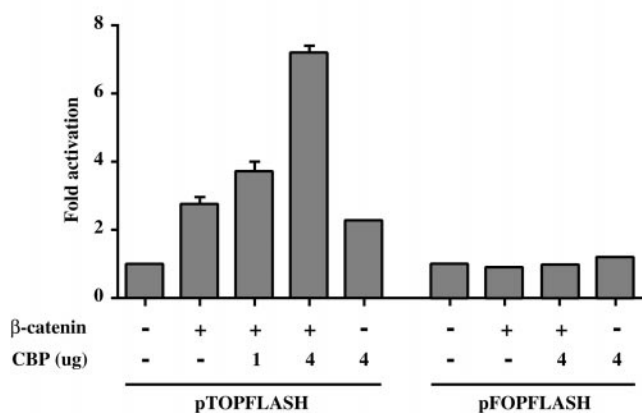
To further narrow down the CBP interaction domain in  $\beta$ -catenin, a series of Ras(61) $\Delta$ F- $\beta$ -catenin fusion constructs spanning the COOH-terminal region were transfected into *cdc25-2* cells, together with the CBP expres-

sion plasmid, and tested for growth at 37°C (Fig. 1 B). Although Arm repeats 8 and 9 are dispensable, deletion to Arm repeat 11 diminished the interaction with CBP. No interaction was seen with Arm repeats 8–12 or the COOH terminus alone. Western blot experiments with anti-Ras mAbs verified that all the  $\beta$ -catenin fusion proteins were expressed at similar levels in yeast (data not shown). Taken together, these results show that Arm repeat 10 to the COOH terminus of  $\beta$ -catenin is involved in the interaction with CBP.

The CBP clone isolated in our screening contains a CREB-binding domain, also called the KIX domain (Parker et al., 1996). This domain has been demonstrated to contact a large number of transcription factors such as CREB, c-Jun, Tax, and c-Myb (reviewed by Goldman et al., 1997). To investigate whether the CREB-binding domain of CBP could bind to  $\beta$ -catenin, GST pull-down assays were performed (Fig. 1 C). We found that a GST fusion protein with the CREB-binding domain of CBP (amino acids 451–682) was able to bind to Arm repeat 10 to the COOH terminus of  $\beta$ -catenin (Fig. 1 C, lane 3), as well as full-length  $\beta$ -catenin (Fig. 1 C, lane 7). The binding was specific because neither  $\beta$ catR10-C nor full-length  $\beta$ -catenin showed any interaction with GST alone (Fig. 1 C, lanes 2 and 6). These data demonstrate that  $\beta$ -catenin directly binds to the CREB-binding domain of CBP.

We next examined whether  $\beta$ -catenin and CBP would interact in vivo by coimmunoprecipitation experiments. CBP and *myc*-tagged pt  $\beta$ -catenin were expressed either individually or in combination in COS7 cells. Cell lysates from each culture were immunoprecipitated with anti-CBP antibodies, and immunoblotted with anti-*myc* antibodies. As shown in Fig. 1 D,  $\beta$ -catenin coimmunoprecipitates with CBP (Fig. 1 D, lane 3). Thus, we conclude that  $\beta$ -catenin and CBP exist as a complex in vivo.

CBP and the closely related homologue p300 are known to function as transcriptional coactivators by connecting a

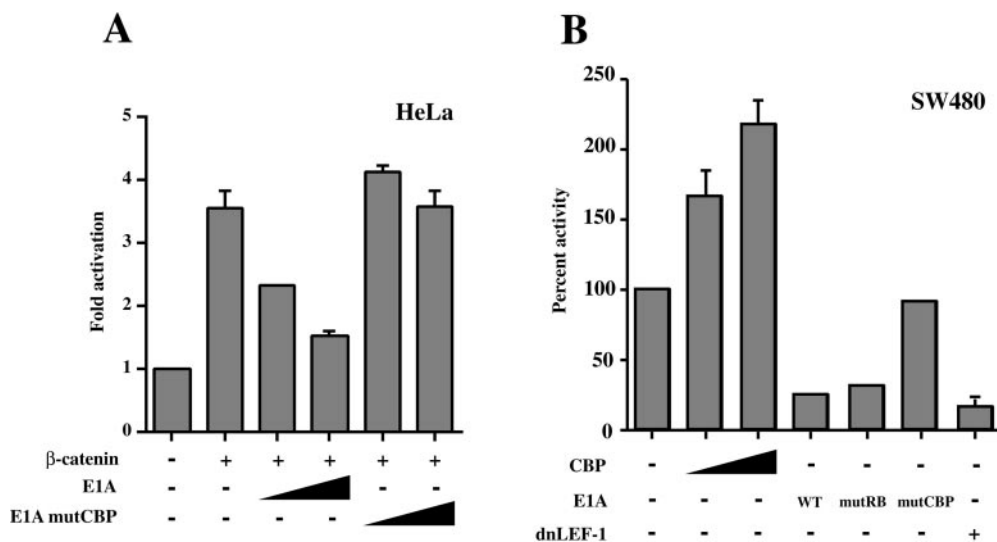


**Figure 2.** CBP synergizes with  $\beta$ -catenin to activate transcription. HeLa cells were transiently transfected with 1  $\mu$ g of pTOPFLASH or pFOPFLASH reporter, indicated amounts of CBP expression plasmid, either with or without 1  $\mu$ g of pt  $\beta$ -catenin-encoding plasmid. Transfections were carried out in triplicate and the means  $\pm$  SD are shown. Where they are not evident, this is due to minimal variability between transfections.

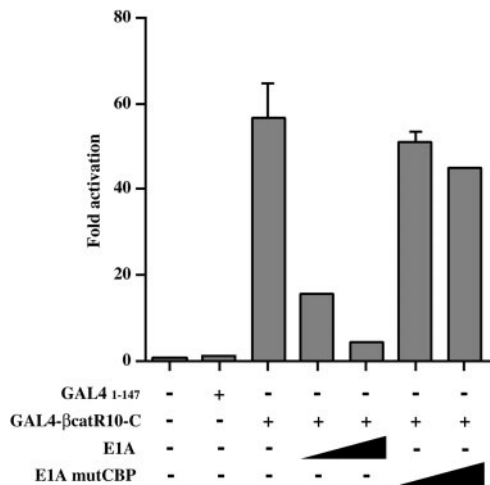
variety of transcription factors to the basal transcription machinery (reviewed by Goldman et al., 1997). Additionally, CBP/p300 possesses histone acetyltransferase activity, suggesting that it potentiates transcription by loosening the chromatin structure (Ogryzko et al., 1996). On the other hand, in *Drosophila* dCBP acetylates dTCF in the Arm-binding domain, and lowers the affinity of dTCF for Arm, thereby negatively regulating Wnt signaling (Waltzer and Bienz, 1998). In an attempt to evaluate the physiological significance of the interaction between CBP and  $\beta$ -catenin, we performed transient transfections in HeLa cells with the TCF reporter plasmid pTOPFLASH containing three optimal TCF binding sites, or the mutant negative control plasmid pFOPFLASH (Korinek et al., 1997). A stabilized  $\beta$ -catenin was used in which four serine and threonine residues at NH<sub>2</sub> terminus were changed to alanine (pt  $\beta$ -catenin; Yost et al., 1996). Fig. 2 shows that expression of pt  $\beta$ -catenin resulted in about a threefold increase in luciferase activity compared with pTOPFLASH alone. The level of  $\beta$ -catenin was saturated under this condition since we did not observe a further increase in luciferase activity with a larger amount of pt  $\beta$ -catenin expression plasmid (data not shown). The  $\beta$ -catenin-dependent transcription was further activated by cotransfecting a vector expressing CBP in a dose-dependent manner, more than doubling the activity observed with saturating levels of  $\beta$ -catenin alone (Fig. 2). Similarly, the activation by wild-type  $\beta$ -catenin was also stimulated by CBP (data not shown). These data are consistent with the observation that the amount of CBP in cells is limiting (Kwok et al., 1994). In contrast, minimal stimulation by CBP was observed in the absence of  $\beta$ -catenin (Fig. 2). In separate experiments using human embryonic kidney 293 cells, we also observed that when we had reached saturating levels of  $\beta$ -catenin, and then cotransfected CBP, there

was a greater than doubling of the luciferase activity (data not shown). As expected, the synergistic activation by  $\beta$ -catenin and CBP was not observed with the control pFOPFLASH (Fig. 2). These functional assays clearly indicate that CBP acts as a coactivator for  $\beta$ -catenin in mammalian cells.

The adenovirus E1A oncoprotein is known to bind and inhibit the function of CBP (reviewed by Goldman et al., 1997). If our hypothesis that CBP is a coactivator with  $\beta$ -catenin were true, one would predict that E1A would inhibit pTOPFLASH expression in our assays. We tested this prediction, and found that E1A significantly inhibited transcription in a dose-dependent manner (Fig. 3 A). In contrast, expression of an E1A mutant that lacks the ability to interact with CBP (E1A mutCBP; Bannister and Kouzarides, 1995) even slightly enhanced reporter gene activity. To further demonstrate the cooperation between CBP and  $\beta$ -catenin, we carried out transfection assays with the human colon carcinoma cell line, SW480 (Fig. 3 B). In these cells, the level of  $\beta$ -catenin is increased as a result of inactivating mutations in the tumor suppressor gene APC, leading to constitutive transcriptional activation by endogenous  $\beta$ -catenin (Korinek et al., 1997). CBP is also limiting in SW480, since expression of CBP further increased pTOPFLASH activity (Fig. 3 B). Expression of E1A or E1A mutRB, which interacts with CBP like E1A, but is deficient in binding to the retinoblastoma protein (RB; Bannister and Kouzarides, 1995), resulted in a three- to fourfold decrease in reporter activity (Fig. 3 B). In contrast, E1A mutCBP did not significantly affect reporter gene expression. We also tested a dominant negative form of LEF-1 (dnLEF-1), which lacks  $\beta$ -catenin binding domain, and found that dnLEF-1 reduced reporter activity to an extent similar to E1A or E1A mutRB (Fig. 3 B). These observations suggest that endogenous CBP is required for



**Figure 3.** E1A represses  $\beta$ -catenin-mediated transcriptional activation. **A**, The reporter plasmid pTOPFLASH (1  $\mu$ g) was transfected into HeLa cells with or without 1  $\mu$ g of pt  $\beta$ -catenin-encoding plasmid. The expression vector encoding E1A or E1A mutCBP (100 or 500 ng) were cotransfected as indicated. **B**, SW480 cells were cotransfected with 100 ng of the pTOPFLASH reporter, and increasing amounts (100 or 400 ng) of CBP expression plasmid, 5 ng each of the plasmids encoding E1A WT, E1A mutRB, or E1A mutCBP, or 50 ng of dnLEF-1 expression plasmid as indicated. The activity of the pTOPFLASH reporter in the absence of exogenous CBP, E1A, or dnLEF-1 was set at 100%.



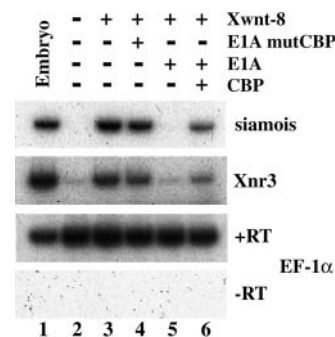
**Figure 4.** Endogenous CBP is required for transactivation by GAL4-βcatR10-C fusion protein. HeLa cells were transfected with 1 μg of GAL4 reporter construct, pFR-Luc, 50 ng of GAL4 1-147 or GAL4-βcatR10-C, and increasing amounts of EIA or EIA mutCBP expression plasmid (25 or 500 ng) in combinations as indicated.

β-catenin-mediated transcriptional activation. The experiments with EIA thus confirm the prior data supporting the hypothesis that CBP is a coactivator with β-catenin.

The previous finding that dCBP could bind to mouse LEF-1 *in vitro* (Waltzer and Bienz, 1998) raised the possibility that CBP might be recruited through the interaction with TCF/LEF factors, but not through β-catenin. To analyze the functional importance of the binding of the βcatR10-C domain to CBP more directly, we performed transient transfection assays in HeLa cells using the βcatR10-C domain fused to the DNA-binding domain of the yeast GAL4 protein (GAL4-βcatR10-C). As previously reported (Hecht et al., 1999), expression of GAL4-βcatR10-C fusion was sufficient to activate the pFR-Luc reporter harboring five GAL4-binding sites (Fig. 4). Coexpression of EIA efficiently abrogated the reporter gene activity. In contrast, little effect was observed with coexpression of EIA mutCBP. These results clearly indicate that β-catenin cooperates with CBP to activate transcription.

To examine whether the activation of endogenous target genes by β-catenin also involves CBP, we analyzed the effects of EIA on the expression of Wnt target genes in *Xenopus* animal cap explants by RT-PCR (Fig. 5). Consistent with the results described above, coexpression of EIA strongly reduced the expression of *siamois* and *Xnr3* induced by Xwnt-8 (Fig. 5, lane 5 compared with Xwnt-8 alone, lane 3), whereas EIA mutCBP showed a weaker inhibition (Fig. 5, lane 4 compared with lane 3). Furthermore, coexpression of CBP overcame the inhibitory effect of EIA (Fig. 5, lane 6 compared with lane 5). These data suggest that CBP is required for the activation of Wnt-responsive genes in *Xenopus* embryos.

Our results provide compelling evidence that CBP is a coactivator for β-catenin. In *Drosophila*, it has been shown that dCBP negatively regulates Wingless signaling (Waltzer and Bienz, 1998). However, overexpression of



**Figure 5.** The activation of endogenous Wnt-responsive genes is sensitive to EIA. Both blastomeres of two-cell stage *Xenopus* embryos were injected with 20 pg of Xwnt-8, 0.5 ng of EIA mutCBP, or EIA, and 3.2 ng of CBP RNAs as indicated. Animal cap explants were dissected at stage 8 to 9 and analyzed for gene expression by RT-PCR.

CBP had no repressive effect on reporter expression in our assays. Moreover, we could not detect an interaction between human LEF-1 and the C/H3 domain of CBP using RRS (data not shown), although the corresponding region of dCBP was shown to bind to mouse LEF-1 (Waltzer and Bienz, 1998). It is possible that dCBP functionally differs from its vertebrate orthologs. In vertebrates, CBP functions as a transcriptional coactivator through the interaction with β-catenin, whereas dCBP is recruited to a promoter by dTCF to repress transcription. Alternatively, CBP may behave as a repressor in the absence of Wnt signaling, but it turns into a coactivator of β-catenin upon signaling.

The mechanism by which CBP stimulates β-catenin-mediated activation is yet to be established. CBP might help to recruit the basal transcription machinery. In addition, CBP was shown to possess intrinsic histone acetyltransferase activity (Ogryzko et al., 1996), and to associate with multiple acetyltransferases, such as P/CAF (Yang et al., 1996). On the other hand, Groucho has been demonstrated to directly interact with the histone deacetylase Rpd3 in *Drosophila*, suggesting that Groucho-mediated repression may involve the modulation of local chromatin structure (Chen et al., 1999). Thus, it remains possible that the histone acetyltransferase activities are required for remodeling chromatin structure, alleviating the repression by Groucho factors.

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