MOLECULAR AND CELLULAR BIOLOGY, Nov. 1997, p. 6609–6617 Vol. 17, No. 11
0270-7306/97/$04.00+0

Interaction and Functional Collaboration of p300 and C/EBPβ

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Received 20 February 1997/Returned for modification 7 April 1997/Accepted 28 August 1997

Transcriptional coactivators such as p300 and CREB-binding protein (CBP) function as important elements in the transcription factor network, linking individual transactivators via protein-protein interactions to the basal transcriptional machinery. We have investigated whether p300 plays a role in transactivation mediated by C/EBPβ, a conserved member of the C/EBP family. We show that C/EBPβ-dependent transactivation is strongly inhibited by adenovirus E1A but not by E1A mutants defective in p300 binding. Ectopic expression of p300 reverses the E1A-dependent inhibition and increases the transactivation potential of C/EBPβ. Furthermore, we show that C/EBPβ and p300 interact with each other and demonstrate that the sequences responsible for interaction map to the E1A binding region of p300 and the amino terminus of C/EBPβ. Finally, we show that the minimal C/EBPβ binding site of p300 acts as a dominant-negative inhibitor of C/EBPβ. These observations identify p300 as a bona fide coactivator for C/EBPβ. C/EBPβ is highly expressed in the myelomonocytic lineage of the hematopoietic system and cooperates with Myb to activate *mim-1*, a gene specifically expressed during myelomonocytic differentiation. Recent evidence has shown that Myb recruits CBP (and presumably p300) as a coactivator and, in contrast to C/EBPβ, interacts with the CREB binding site of p300-CBP. We show that p300 not only stimulates the activity of Myb and C/EBPβ individually but also increases the synergy between them. Thus, our results reveal a novel function of p300: in addition to linking specific transcription factors to the basal transcriptional machinery, p300 also mediates the cooperation between transactivators interacting with different domains of p300.

Initiation of transcription by RNA polymerase II involves the cooperation of transcription factors, binding to specific regulatory sequences, with the basal transcriptional machinery. The transcriptional coactivators p300 and the CREB-binding protein (CBP) have been recognized as key molecules involved in the communication between transcription factors and the transcriptional machinery and thus appear to be important elements of gene regulation networks (for a review, see reference 22).

p300 was originally identified as a cellular interaction partner of the adenovirus E1A oncoprotein (17), and inactivation of p300 by binding to E1A appears to be one of the mechanisms by which the E1A protein suppresses transcriptional activation of certain promoters (for a review, see reference 35). Since p300 has strong sequence similarity to CBP and exhibits properties similar to those of CBP, p300 and CBP are considered functional homologs (2, 33). Several transcription factors, such as CREB, Jun, Myb, Sap-1a/Elk-1, Fos, p53, MyoD, and the nuclear hormone receptors, have now been shown to require p300 and CBP as coactivators (3–5, 10, 13, 14, 21, 23, 24, 28, 31, 42, 53). p300 and CBP do not by themselves interact with specific DNA sequences; rather, they display a variety of protein interaction surfaces that are thought to link specific transcription factors via protein-protein interactions to the basal transcriptional machinery. The growing number of known interaction partners for p300 and CBP suggests that these proteins function as nodal points in the transcription factor network. Recent evidence indicates that CBP and p300 possess intrinsic histone acetyltransferase activity, raising the possibility that these coactivators cause localized changes in the chromatin structure of genes to which they are targeted via their interaction with specific transcription factors (6, 43).

The CCAAT-box/enhancer binding proteins (C/EBPs) are members of the basic region/leucine zipper class of transcription factors and have been implicated in the differentiation of certain cell types, such as liver and fat cells, macrophages, and granulocytes (1, 7–9, 12, 15, 19, 25, 29, 32, 40, 44, 47, 48, 50, 52). In the hematopoietic system, C/EBP family members are highly expressed in the myelomonocytic lineage and have been shown to be directly involved in the transcription of several genes specifically expressed during the differentiation of macrophages and granulocytes, such as *mim-1* and the lysozyme gene. Activation of these genes requires cooperation of C/EBP family members with c-Myb (or its oncogenic derivative v-Myb), a transcription factor which is primarily expressed in immature hematopoietic cells (8, 40; for a review of Myb, see reference 20). Myb and C/EBP activate the *mim-1* gene via a composite Myb-C/EBP response element, consisting of adjacent Myb and C/EBP binding sites (34). The cooperation of Myb and C/EBP is sufficient to direct expression of the *mim-1* gene to heterologous cell types (7, 36). This finding has raised the intriguing possibility that the interplay of Myb and C/EBP provides the crucial signal for switching on specific genes during myelomonocytic differentiation.

The molecular mechanism by which Myb and C/EBP cooperate is not yet fully understood. We have recently demonstrated a direct interaction between Myb and C/EBPβ (34). In addition, c-Myb has been identified as an interaction partner of CBP (14, 42). Because of the intimate functional relationship of Myb and C/EBP, we were interested to determine, as a first step, whether p300/CBP also plays a role in C/EBP-mediated gene activation. The data presented here show that p300 indeed functions as a coactivator for C/EBPβ, a C/EBP family member highly expressed in myelomonocytic cells. Interestingly, Myb and C/EBP interact with different domains of p300. Whereas Myb interacts with the CREB binding domain of

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p300/CBP, the interaction of C/EBPβ with p300 is mediated by the E1A binding domain of p300. Furthermore, our data show that p300 not only stimulates Myb and C/EBP individually but also stimulates the synergy between Myb and C/EBPβ. Our results thus provide evidence that, in addition to linking specific transcription factors to the basal transcriptional machinery, p300/CBP also mediates the cooperation between different transcription factors.

MATERIALS AND METHODS

Eukaryotic expression vectors. The v-Myb expression vector pVM134 and the v-Myb frameshift vector pVM111 have been described elsewhere (26). The E1A expression plasmids pRSV-E1A12S, pRSV-E1A12SΔCR1, pRSV-E1A12SΔCR2, pRSV-E1A12SmutCBP, and pRSV-E1A12SmutRB have been described elsewhere (5).

The chicken C/EBPβ expression vector pCDNA3-CCR (encoding full-length C/EBPβ) was generated by cloning an *Eco*RI/*Xba*I fragment of pCRNC-CCR (8) between the *Eco*RI and *Xba*I sites of pcDNA3 (Invitrogen). pCRNC-chC/EBPα encodes full-length chicken C/EBPα and was described previously (34). pCMV-Gal-CCR encodes a fusion protein of full-length chicken C/EBPβ and the Gal4 DNA binding domain and was constructed by cloning an *Eco*RV/*Xba*I fragment from pBluescript-CCR (8) between the *Sma*I and *Xba*I sites of pCMV-Gal1. pCMV-Gal1 is a derivative of pSG424 (46), which was obtained by subcloning a *Hin*dIII/*Xba*I fragment from pSG424 (encoding the Gal4 DNA binding domain) between the *Hin*dIII and *Xba*I sites of pcDNA3. pCMV-Gal-CCRΔN49, pCMV-Gal-CCRΔN83, and pCMV-Gal-CCRΔN110 encode deletion mutants of chicken C/EBPβ lacking 49, 83, or 110 amino acids, respectively, from the N terminus, fused in frame to the Gal4 DNA binding region. The truncated C/EBPβ coding regions were generated by PCR with appropriate primers and cloned into pCMV-Gal1. pGal-CCR-ΔDBD encodes a fusion protein containing the Gal4 DNA binding domain and the first 236 amino acids of C/EBPβ. This plasmid was generated by C-terminal truncation of the C/EBPβ coding region at an *Nar*I site and cloning of the truncated coding region into pSG424 (46), with appropriate restriction sites. pCDNA3-CJ encodes a hybrid protein consisting of the amino terminus of C/EBPβ and the DNA binding domain of c-Jun and has been described before (34).

p300 expression vectors pCMV-p300, pCMV-p300CHA, pCMV-p300del30CHA, and pCMV-p300del33CHA were gifts from R. Eckner (17). pCMV-p300 and pCMV-p300CHA encode full-length human p300 lacking or containing a C-terminal hemagglutinin tag. In pCMV-p300del30CHA amino acids 1737 to 1809 are missing, and in pCMV-p300del33CHA amino acids 1737 to 1836 of p300 are missing. pCDNA300-ATG-Xba encodes a polypeptide derived from the N terminus of p300 (amino acids 1 to 1279). To construct this vector, the sequence at the 5′ end of the p300 open reading frame was altered by PCR such that a hemagglutinin tag was introduced immediately downstream of the start codon and a *Bam*HI site was created upstream of the start codon. p300 sequences were then cloned as an approximately 4-kb *Bam*HI/*Xba*I fragment between the *Bam*HI and *Xba*I sites of pCDNA3. pCDNA300-SN13G/3, pCDNA300-SN1G/3, and pCDNA300-SN1G/2 encode amino acids 1515 to 1998, 1752 to 1998, and 1752 to 1859 of p300, respectively, and were generated by PCR amplification of the corresponding fragments of the p300 cDNA. 5′ primers used for PCR contained an in-frame start codon and an additional *Bam*HI site, and 3′ primers introducing a stop codon contained additional *Hin*dIII and *Eco*RI sites. The PCR fragments were cloned between the *Bam*HI and *Eco*RI sites of pCDNA3. pCDNA300-BAE encodes amino acids 1752 to 2414 of p300 and was generated by cloning a *Bam*HI/*Apa*I fragment from pCDNA300-SN1G/3 and an *Apa*I/*Eco*RI fragment from pCMVβ-p300-CHA between the *Bam*HI and *Eco*RI sites of pCDNA3. pCDNA300-BPE encodes amino acids 1853 to 2414 of p300 and was generated by exchanging a *Bam*HI/*Pml*I fragment of plasmid pCDNA300-BAE (corresponding to amino acids 1752 to 1942) with a PCR-amplified fragment corresponding to amino acids 1853 to 1942. The 5′ primer used for PCR contained an in-frame start codon and an additional *Bam*HI site to facilitate cloning. pCDNA300-Gal-SN1G/2 encodes a Gal4 fusion protein containing amino acids 1752 to 1859 of p300 and was generated by cloning a *Bam*HI/*Xba*I fragment of pCDNA300-SN1G/2 between the *Bam*HI and *Xba*I sites of pCMV-Gal1. pCDNA3-VP16 encodes the VP16 transactivation domain (49) cloned between the *Hin*dIII and *Xba*I sites of pCDNA3. pCDNA300-SN1G/3-VP16 is a derivative of pCDNA3-VP16 and encodes amino acids 1752 to 1998 of p300 fused to the VP16 transactivation domain.

Bacterial expression vectors and preparation of GST fusion proteins. Bacterial expression vectors encoding glutathione *S*-transferase (GST) fusion proteins were generated by using the pGEX vector system (Pharmacia). pGEX-2T-TFIID1-163 contains the N terminus of TFIID and was obtained from T. Kouzarides. pGEX-20/21 encoded a GST fusion protein with amino acids 1838 to 1927 of p300 and was generated by PCR with appropriate primers. pGEX-SN13G/3, pGEX-SN1G/3, and pGEX-SN1G/2 encode subfragments of the E1A binding region of p300 (amino acids 1515 to 1998, 1752 to 1998, and 1752 to 1859, respectively) and were generated by subcloning *Bam*HI/*Eco*RI insert fragments of pCDNA300-SN13G/3, pCDNA300-SN1G/3, and pCDNA300-SN1G/2, respectively, between the *Bam*HI and *Eco*RI sites of pGEX-3X. GST fusion protein expression was induced in logarithmically growing cultures of transformed bacteria (*Escherichia coli* N15) by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM, and growing the bacteria for an additional 6 h. Bacteria were lysed by lysozyme digestion, sonification, and three freeze-thaw cycles, and an extract of soluble protein was prepared by centrifugation for 10 min at 14,000 rpm. An extract containing 5 to 10 μg of GST fusion protein was mixed with 500 μl of glutathione-Sepharose (Pharmacia) and incubated at 4°C under constant agitation for 30 min. The Sepharose beads were then washed three times with phosphate-buffered saline (PBS) and either used directly or stored in the presence of 1 mM phenylmethylsulfonyl fluoride and 5 μg each of aprotinin and leupeptin per ml at 4°C for several days.

In vitro protein binding assays. GST pull-down assays were performed as follows. C/EBPβ was translated in vitro with the TNT translation system (Promega) and pCDNA3-CCR as template. All subsequent steps were carried out on ice. The translation reaction mixture was then diluted 10-fold with GEX incubation buffer (20 mM HEPES [pH 7.8], 75 mM KCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1% Nonidet P-40 [NP-40], 500 μg of bovine serum albumin per ml) and cleared by centrifugation for 10 min at 14,000 × *g*. A total of 50 μl of supernatant was then mixed with 500 ng of bacterially expressed GST fusion protein, coupled to 50 μl of glutathione-Sepharose, and incubated under constant agitation for 1 h at 4°C. The Sepharose beads were washed once with GEX incubation buffer, once with PBS supplemented with 1% Triton X-100, and once with PBS. Finally, the proteins were eluted from the beads with sodium dodecyl sulfate (SDS) sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography.

Reporter genes, transfections, luciferase, and β-galactosidase assays. The reporter plasmids p-240Luc (41), pDel130/33TRE-Luc (34), pG5E4-38Luc (34), and CMVβ (Clontech) have been described elsewhere. DNA transfection with the quail fibroblast cell line QT6 (36) was performed as described elsewhere (34). The amounts of DNA used for transfection of cells in a 10-cm tissue culture dish are indicated in the figure legends. The cells were harvested 24 h after transfection. Cell extracts were prepared and luciferase and β-galactosidase assays were performed as described elsewhere (34).

Antibodies and Western blotting. Polyclonal rabbit antiserum against chicken C/EBPβ has been described elsewhere (34). p300-specific and Gal4-specific antisera were obtained from Upstate Biotechnology, Inc. and Santa Cruz, respectively. Immunostaining of proteins transferred to nitrocellulose was performed as described previously (34).

Northern blotting. Polyadenylated RNA was prepared and Northern blotting and detection of glyceraldehyde-3-phosphate dehydrogenase were performed as described previously (8). Quail 126 mRNA was detected with a 126-specific probe (39).

In vivo coimmunoprecipitation. To detect complexes of p300 and C/EBPβ in vivo, we transfected the quail fibroblast cell line QT6 (36) with expression vectors for different subdomains of p300 and C/EBPβ. Twenty-four hours after transfection, the cells were radiolabeled for 2 h with 200 μCi of [^{35}S]methionine (specific activity, >800 Ci/mmol) per ml, washed with PBS, and lysed in ice-cold lysis buffer (10 mM Tris-HCl [pH 7.8], 50 mM NaCl, 0.5% sodium deoxycholate, 0.5% NP-40, 0.1% SDS). All subsequent steps were carried out on ice. After the DNA was sheared with a syringe, insoluble material was removed by centrifugation for 20 min at 4°C and 14,000 × *g*. The supernatant was divided into two aliquots, and rabbit antiserum against chicken C/EBPβ (34) or monoclonal antibodies against p300 (Upstate Biotechnology Inc.) or against the hemagglutinin epitope for the N-terminal p300 fragment p300 1515-1998 (12CA5) were added, followed by incubation overnight. Finally, *Staphylococcus aureus* cells (Pansorbin; Calbiochem) were added, incubated for 1 h, and washed five times with lysis buffer. Before the last washing step, the material was transferred to a fresh tube. Finally, the immunoprecipitated proteins were analyzed by SDS-PAGE and autoradiography.

To detect complexes of endogenous p300 and C/EBP, HeLa cells were labeled for 4 h with 500 μCi of [^{35}S]methionine (specific activity, >800 Ci/mmol) per ml, washed with PBS, and lysed in ice-cold NETN lysis buffer (50 mM Tris-HCl [pH 7.5]; 5 mM EDTA; 1 mM dithiothreitol; 0.1% NP-40; 300 mM NaCl; 0.2 mM phenylmethylsulfonyl fluoride; and 10 μg each of protease inhibitors leupeptin, aprotinin, and pepstatin per ml). After lysis, the salt concentration was reduced to 100 mM NaCl. Immunoprecipitation was carried out as described above, with the p300-specific monoclonal antibody AC238 (18), C/EBPβ-specific rabbit antiserum, or preimmune serum. Immunoprecipitates were dissolved by being boiled in a small volume of SDS sample buffer, diluted with NETN lysis buffer, and reprecipitated with the AC238 antibody. Immunoprecipitates were finally analyzed by SDS-PAGE and autoradiography.

RESULTS

Inhibition of C/EBP-mediated transactivation by the adenovirus E1A protein. To examine the possibility that p300-CBP acts as a coactivator for the members of the C/EBP family, we studied the effect of the adenovirus E1A protein on the transactivation potential of C/EBPβ. An expression vector for

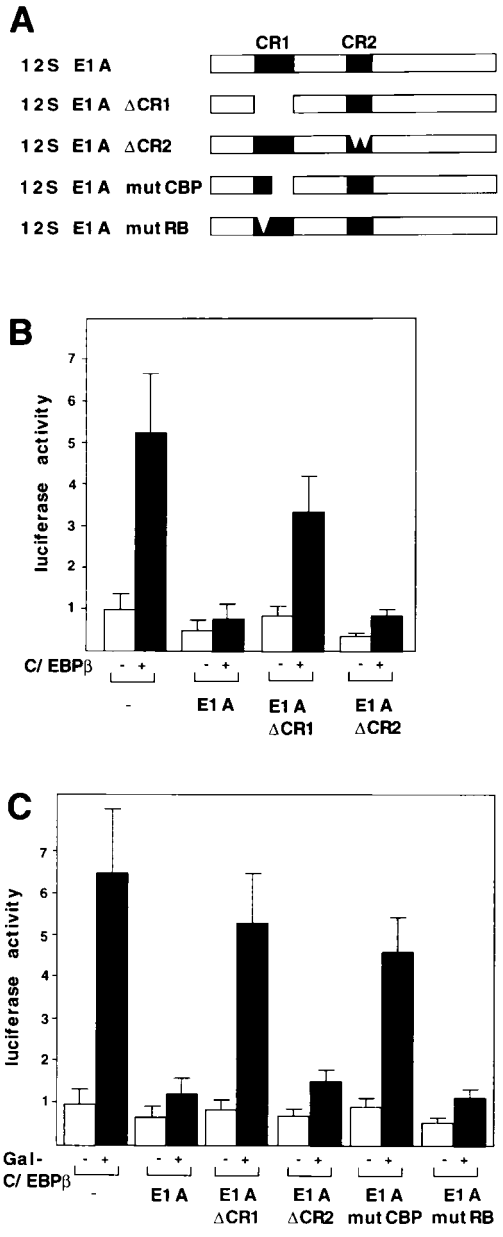

FIG. 1. Inhibition of C/EBPβ-dependent transactivation by E1A and E1A mutants. (A) Schematic illustration of wt and mutant E1A proteins. (B) QT6 cells were transfected with the *mim-1* reporter gene p-240Luc (3 μg), the β-galactosidase plasmid pCMVβ (0.2 μg), the C/EBPβ expression vector pCDNA3-CCR (1 μg, black bars) or the same amount of empty expression vector (white bars), and the indicated expression vectors for wt or mutant E1A (0.1 μg each). The columns show the average luciferase activity, normalized with respect to the cotransfected β-galactosidase plasmid. Thin lines show standard deviations. (C) QT6 cells were transfected with the Gal4-responsive reporter plasmid pG5E4-38Luc (3 μg), the β-galactosidase plasmid pCMVβ (0.2 μg), the Gal4-C/EBPβ expression vector pCMV-Gal-CCR-ΔDBD (1 μg, black bars) or the same amount of expression vector encoding only the Gal4 DNA binding domain (pCMV-Gal-1, white bars), and the indicated E1A expression vectors (0.1 μg each). Luciferase activities are illustrated as in panel B.

chicken C/EBPβ was cotransfected with a C/EBP-dependent reporter gene derived from the chicken *mim-1* promoter (41) and expression vectors for wild-type (wt) or mutant 12s E1A proteins. As shown in Fig. 1, wt E1A strongly suppressed C/EBPβ-mediated transactivation of the *mim-1* reporter gene. Control Western blotting experiments confirmed that the amount of C/EBPβ was not decreased in the presence of E1A (data not shown). The inhibitory effect was abolished by deletion of the conserved region 1 (CR1) of E1A but was not affected by a deletion of conserved region 2 (CR2), suggesting that the inhibition might be due to a titration of p300. E1A also strongly repressed transactivation mediated by a Gal4-C/EBPβ fusion protein (Fig. 1C). Furthermore, a point mutant of CR1 which is defective in binding of the E1A protein to p300 (E1A-mutCBP) failed to inhibit Gal4-C/EBPβ-mediated transactivation. By contrast, a different mutant of CR1 (E1A-mutRB), which lacks retinoblastoma protein binding activity but shows normal binding to p300, had no effect on the E1A-mediated repression of Gal4-C/EBPβ activity. Additional experiments showed that E1A also strongly inhibits transactivation mediated by C/EBPα, another member of the C/EBP family (data not shown). Taken together, these experiments provide strong evidence for a role for p300 in C/EBP-dependent transactivation.

Overexpression of p300 increases the transactivation potential of C/EBP and neutralizes E1A-mediated repression. To show more directly that the E1A-mediated inhibition of C/EBP activity was due to inactivation of p300 or of related factors, we examined whether overexpression of p300 could restore the activity of C/EBPβ in the presence of E1A. As illustrated in Fig. 2, ectopic expression of p300 completely reversed the inhibitory effect of E1A, suggesting that the inhibition by E1A was indeed due to titration of endogenous p300 (or of related factors, such as CBP).

To further demonstrate that p300 is involved in C/EBP-mediated transactivation and acts as a coactivator of C/EBPβ, we studied the effect of p300 overexpression on the transactivation potential of C/EBPβ and C/EBPα. As illustrated in Fig. 3, p300 expression increased the activation of a C/EBP-dependent reporter gene by C/EBPβ and C/EBPα. Interestingly, the p300 deletion mutant del33, which is defective in E1A binding, failed to increase transactivation by C/EBPβ. By contrast, the

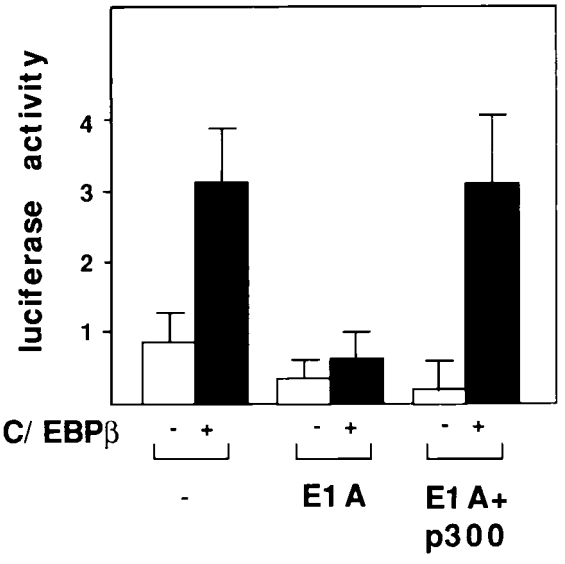

FIG. 2. p300 reverses the E1A-mediated inhibition of C/EBPβ-dependent transactivation. QT6 cells were transfected with the *mim-1* reporter gene p-240Luc (3 μg), the β-galactosidase plasmid pCMVβ (0.2 μg), C/EBPβ expression vector pCDNA3-CCR (1 μg, black bars) or the same amount of empty control vector (white bars), and expression vectors for wt E1A (pRSV-E1A12S, 0.2 μg) and p300 (pCMV-p300CHA, 5 μg), as indicated below the columns. The columns show the average luciferase activity relative to the activity of the co-transfected β-galactosidase plasmid. Thin lines indicate standard deviations.

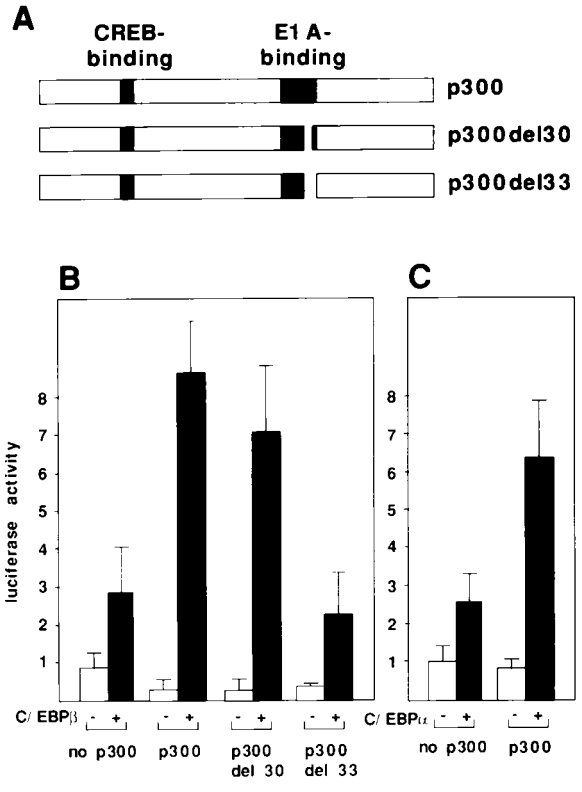

FIG. 3. p300 stimulates C/EBPα- and C/EBPβ-dependent transactivation. (A) Schematic illustration of full-length and mutant p300 and the positions of the CREB and E1A binding regions. (B) QT6 cells were transfected with the *mim-1* reporter gene p-240Luc (3 μg), the β-galactosidase plasmid pCMVβ (0.2 μg), C/EBPβ expression vector pCDNA3-CCR (1 μg, black bars) or the same amount of empty control vector (white bars), and expression vectors for wt (5 μg) or mutant p300 (2 μg each), as indicated below the columns. Control transfections lacking p300 contained equivalent amounts of the empty expression vector pCDNA3. The columns show the average luciferase activity, normalized with respect to the cotransfected β-galactosidase expression plasmid. Thin lines show standard deviations. (C) Similar transfection as in panel B except that C/EBPα expression vector pCRNC-cC/EBPα (0.2 μg, black bars) or the appropriate empty expression vector (pCRNC-M, white bars) was used.

related mutant del30, carrying a slightly smaller deletion, has retained the ability to stimulate C/EBPβ. These findings suggested that sequences responsible for stimulating C/EBP-mediated transactivation are located in the C-terminal half of p300 and partially overlap with the E1A binding domain of p300.

Synergistic activation of an endogenous C/EBP-dependent gene by p300 and C/EBPβ. To confirm under more physiological conditions that p300 acts as a coactivator for C/EBPβ, we studied the effect of C/EBPβ and p300 on the expression of an endogenous C/EBP-dependent gene. The gene referred to as *126* had originally been isolated by virtue of its specific expression in E26-transformed chicken myeloblasts (39). Subsequently, it was shown that this gene is not expressed in fibroblasts but can be activated in these cells by ectopic expression of C/EBPβ (27). As illustrated in Fig. 4, transfection of an expression vector for C/EBPβ into the quail fibroblast cell line QT6 was sufficient to induce the transcription of endogenous *126* mRNA, confirming that *126* is a C/EBP-inducible gene. Interestingly, cotransfection of expression vectors for C/EBPβ and p300 resulted in strongly increased expression of the gene, while p300 alone had no effect. As expected, the p300 deletion mutant del33 did not significantly stimulate the activation of the *126* gene by C/EBPβ. Thus, this experiment confirms that p300 also acts as a coactivator of C/EBPβ in the context of a physiological C/EBP target gene.

C/EBPβ interacts with p300 in vivo and in vitro. To demonstrate an interaction between p300 and C/EBPβ, we performed in vivo coimmunoprecipitation and in vitro protein binding experiments. In the experiment whose results are shown in Fig. 5, we used the quail QT6 cell line to express C/EBPβ together with different regions of p300. The transfected cells were labeled with [^{35}S]methionine and analyzed by immunoprecipitation with C/EBPβ- and p300-specific antisera. As shown in Fig. 5B and C, C-terminal fragments of p300 which contain the E1A binding region (p300/1752-2414 and p300/1515-1998) were coprecipitated efficiently by the C/EBPβ-specific antiserum. By contrast, a C-terminal fragment lacking the E1A binding site (p300/1853-2414) or the N-terminal region of p300 encompassing the CREB binding domain (p300/1-1279) was not coprecipitated. Furthermore, as shown in Fig. 5D, a C-terminal fragment of p300 was coprecipitated by C/EBP-specific antiserum only in the presence, not in the absence, of C/EBPβ, excluding the trivial explanation that the observed coprecipitation was due to cross-reactivity of the antiserum or to insufficient washing of the immunoprecipitates. This experiment also showed that C/EBPβ was coprecipitated by p300-specific antiserum, confirming the interaction of both proteins. C/EBPβ was coprecipitated less efficiently than p300, presumably because there was an excess of C/EBPβ under these conditions. Again, the controls showed that coprecipitation of C/EBPβ by p300-specific antiserum was dependent on the presence of p300. Taken together, the experiments whose results are shown in Fig. 5 indicate that C/EBPβ specifically interacts with amino acids 1515 to 1998 of p300. This domain of p300 encompasses the E1A binding site.

To provide additional evidence for an interaction of C/EBPβ and p300 and to more precisely map the sequences of p300 which are involved in this interaction, we expressed different parts of p300 as bacterial GST fusion proteins and subjected them to in vitro binding assays. Figure 6A schematically depicts the GST fusion proteins, and Fig. 6B illustrates a typical binding experiment using radiolabeled C/EBPβ. It is evident that a GST-p300 fusion protein containing amino acids 1515 to 1998 of p300 binds to C/EBPβ in vitro, in agreement with the results of the in vivo coprecipitation studies presented above. Additional binding assays using GST-p300 fusion proteins that con-

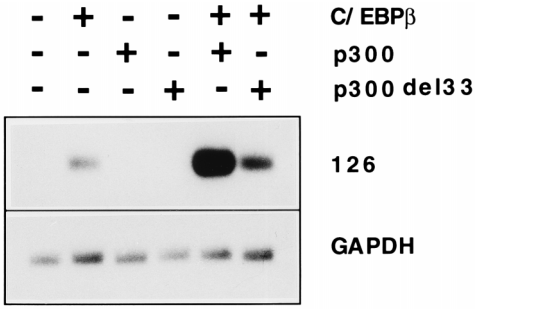

FIG. 4. Synergistic activation of the endogenous gene *126* by C/EBPβ and p300. Northern blot analysis of polyadenylated RNA from QT6 cells, transfected with combinations of expression vectors for C/EBPβ (0.5 μg), p300 (10 μg), and p300del33 (4 μg). The amount of expression vector for mutant p300 was reduced to compensate for the slightly higher amount of p300 protein produced from the mutant expression vector. Control transfections contained equivalent amounts of the appropriate empty expression vectors. The blot was hybridized to probes specific for the chicken gene *126* (top) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (bottom).

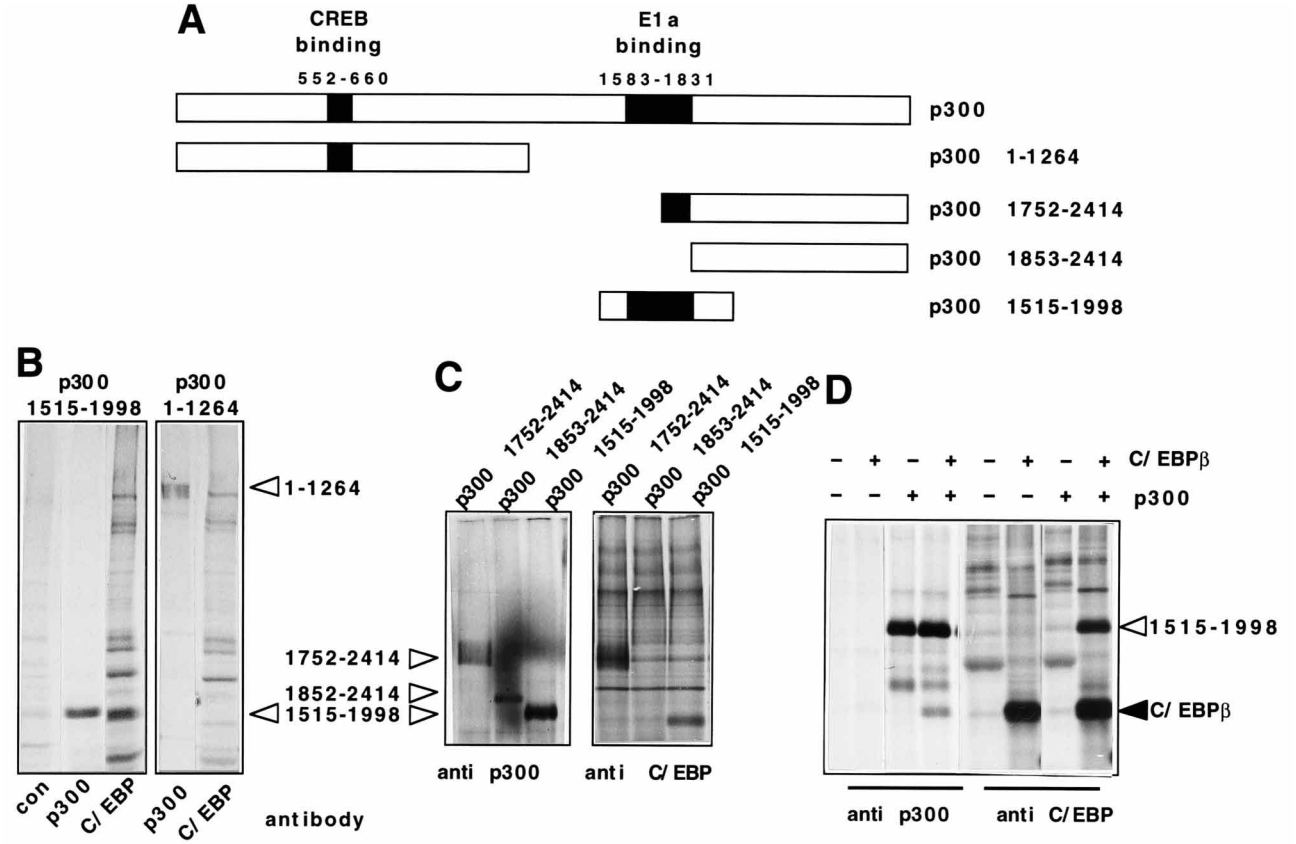

FIG. 5. C/EBPβ and p300 interact in vivo. (A) Schematic overview of p300 subfragments used for in vivo coprecipitation studies. The positions of CREB and E1A binding regions are marked by black boxes. (B and C) QT6 cells were transfected with expression vectors for full-length C/EBPβ (pCDNA3-CCR, 1 μg) and for different parts of p300 (p300/1-1264, 10 μg; all other constructs, 5 μg), as indicated at the top. Twenty-four hours after transfection, the cells were radiolabeled with [^{35}S] methionine and cell extracts were immunoprecipitated with antibodies specific for C/EBPβ or p300 or preimmune serum (con), as indicated. The immunoprecipitates were analyzed by SDS–10% PAGE and autoradiography. (D) QT6 cells were cotransfected with expression vectors for C/EBPβ (pCDNA3-CCR, 1 μg) and a subfragment of p300 encompassing amino acids 1500 to 1983 (5 μg), as indicated at the top. Twenty-four hours after transfection, cells were radiolabeled with [^{35}S]methionine and analyzed by immunoprecipitation with C/EBPβ- or p300-specific antisera, as described for panels B and C. The positions of the p300 polypeptides and of C/EBPβ are marked in panels B to D. Additional bands are due to nonspecific precipitation of proteins.

tain smaller portions of p300 showed that the C/EBPβ binding site maps to amino acids 1752 to 1859 of p300 (Fig. 6).

The E1A binding domain of p300 acts as a dominant-negative inhibitor of C/EBPβ. To confirm the interaction between C/EBPβ and the E1A binding region of p300 by an independent experimental approach, we investigated whether overexpression of the minimal C/EBPβ binding region of p300 (amino acids 1752 to 1859) on its own inhibits C/EBPβ-mediated transactivation. To provide this region of p300 with a nuclear translocation signal, we fused it to the Gal4 DNA binding domain. We then assessed the ability of the fusion protein to act as a dominant-negative inhibitor of C/EBPβ-mediated transactivation. As illustrated in Fig. 7, expression of the Gal4-p300 fusion protein indeed inhibited transactivation by C/EBPβ. Control experiments showed that the decreased transactivation by C/EBPβ was not due to a reduction of the amount of C/EBPβ.

p300 interacts with amino-terminal sequences of C/EBPβ. As an alternative strategy to validate the interaction between C/EBPβ and the E1A binding region of p300, we performed two-hybrid-type experiments using Gal4-C/EBPβ and a VP16-p300 fusion protein which contains amino acids 1752 to 1998 of p300. As shown in Fig. 8A, the Gal4-C/EBPβ fusion protein had undetectable activity on its own; however, in the presence of p300-VP16 its activity was drastically increased. p300-VP16 had no effect on the promoter in the absence of a Gal4-C/EBPβ fusion protein. Furthermore, the VP16 transactivation domain by itself did not affect the activity of the Gal4-C/EBPβ fusion protein. Control Western blotting experiments, whose results are depicted in Fig. 8B, showed that the increased activity of Gal4-C/EBPβ in the presence of p300-VP16 was not due to an increase in the amount of the Gal4 or VP16 fusion proteins. This experiment supports the notion that C/EBPβ interacts with the E1A binding region of p300.

Successive deletion of sequences from the amino terminus of C/EBPβ caused a gradual decrease of the activity of the reporter gene in the presence of p300-VP16, indicating that the amino terminus of C/EBPβ is responsible for the interaction with p300. The amounts of the various Gal4-C/EBP fusion proteins used in this experiment were comparable, as determined by Western blotting (data not shown). The gradual loss of activity suggests that the sequences responsible for the interaction with p300 are not confined to a distinct region within C/EBPβ but rather that different parts of the amino terminus of C/EBPβ contribute to the interaction with p300.

Interaction of p300 with endogenous C/EBP. To investigate whether endogenous p300 and C/EBP interact, we performed coimmunoprecipitation experiments using extract prepared from [^{35}S]methionine-labeled HeLa cells. We used this human cell line because antibodies recognizing chicken p300 are pres-

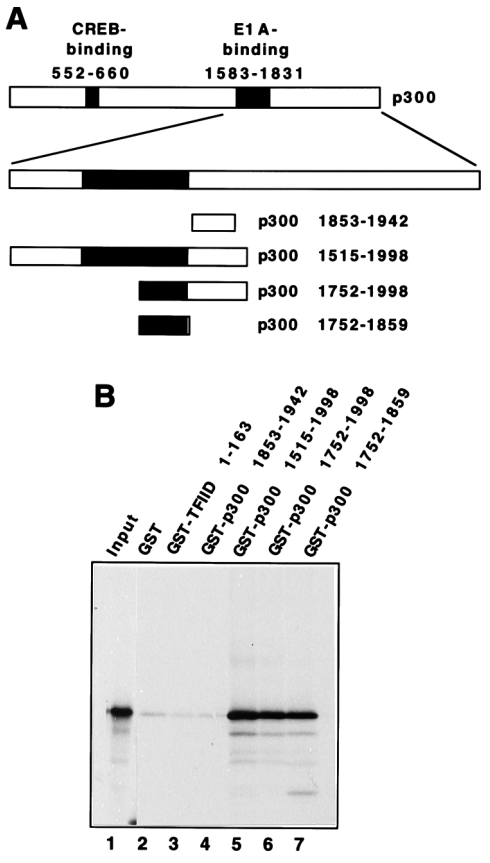

FIG. 6. C/EBPβ and p300 interact in vitro. (A) Schematic illustration of GST-p300 fusion proteins. The CREB and E1A binding regions are highlighted by black boxes. (B) Bacterially expressed GST fusion proteins were bound to glutathione-agarose, incubated with in vitro-translated, radiolabeled C/EBPβ, and subjected to the GST pull-down assay, as described in Materials and Methods. Input (lane 1) and bound proteins (lanes 2 to 7) were analyzed by SDS–10% PAGE and autoradiography.

ently not available. Immunoprecipitation was carried out in two steps. Equal amounts of HeLa cell extract were first immunoprecipitated with p300- or C/EBPβ-specific antibodies or preimmune serum. Immunoprecipitates were then dissolved and reprecipitated with p300-specific antibodies to reduce the background of nonspecific proteins. As shown in lane 1 of Fig. 9, the p300 antibody recognized two protein species (labeled with arrowheads) which presumably correspond to full-length p300 and a proteolytic degradation product of p300. C/EBPβ-specific antiserum (lane 3) but not preimmune serum (lane 2) coprecipitated a small amount of full-length p300 and of the p300 degradation product. This experiment therefore suggests that endogenous p300 and C/EBPβ interact in HeLa cells.

p300 stimulates the synergy between Myb and C/EBPβ. The chicken *mim-1* gene is a physiological target gene for Myb and C/EBP which is activated synergistically by Myb and C/EBP family members (8, 40). The synergistic activation of the *mim-1* gene is mediated by a composite promoter element consisting of a pair of binding sites for Myb and C/EBP (34). Recent evidence has suggested that CBP is a coactivator for Myb and that Myb binds to the CREB binding site of CBP (14, 42). The data presented here show that C/EBP, in contrast to Myb, interacts with the E1A binding region of p300. Thus, it is conceivable that p300 simultaneously interacts with Myb and C/EBP at the *mim-1* promoter, with two different protein domains. We were therefore interested to examine whether p300 affects the synergy between Myb and C/EBP.

The experiment whose results are shown in Fig. 10A illustrates the effect of p300 on the activation of the *mim-1* promoter by v-Myb and C/EBPβ. In the absence of p300, only weak synergy between Myb and C/EBP was observed, due to the low amounts of expression vectors transfected. p300 significantly stimulated the activity of both factors acting individually. Interestingly, the synergistic activation of the promoter observed when both factors were present together was stimulated to a greater extent than the activation mediated by each factor acting on its own (6.1- and 8.0-fold versus 17.3-fold). This observation suggested that p300 increases the synergy of v-Myb and C/EBPβ.

We have shown before that an interaction between v-Myb and C/EBPβ, mediated by the DNA binding domains of both proteins, is necessary for both factors to synergize (34). The finding that p300 stimulates the synergy between Myb and C/EBP prompted us to also examine whether the presence of p300 abrogates the requirement for direct interaction between v-Myb and C/EBPβ. To address this issue, we employed a hybrid protein (referred to as CJ) which consists of the amino terminus of C/EBPβ and the DNA binding domain of c-Jun; as

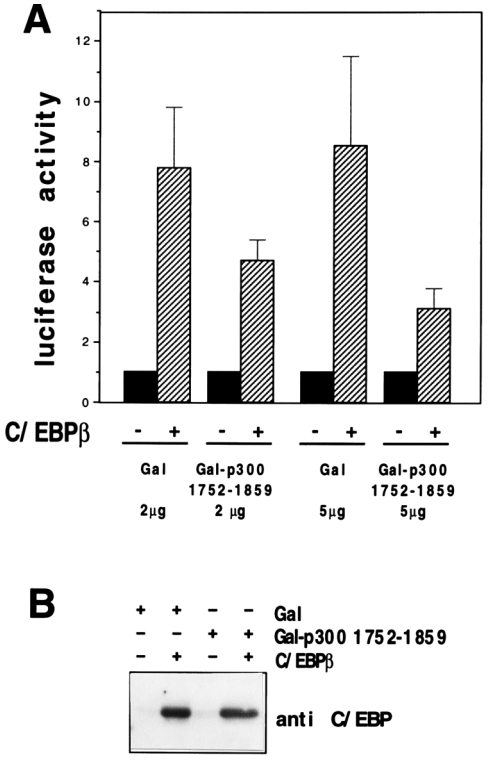

FIG. 7. The E1A binding domain of p300 acts as a dominant-negative inhibitor of C/EBPβ. (A) QT6 cells were transfected with the *mim-1* reporter gene p-240Luc (3 μg), the β-galactosidase plasmid pCMVβ (0.2 μg), and the C/EBPβ expression vector pCDNA3-CCR (1 μg, hatched bars) or the same amount of empty expression vector (black bars). Additionally, transfections included different amounts of expression vector encoding only the Gal4 DNA binding domain (pCMV-Gal-1) or a Gal4-p300 fusion protein containing amino acids 1737 to 1844 of p300 (pCMV-Gal-SN1G/2), as indicated at the bottom. The columns show the average luciferase activity, normalized with respect to the β-galactosidase activity. Thin lines show standard deviations. (B) Total cellular protein of cells transfected with different combinations of expression vectors (same amounts as in the right half of panel A) was analyzed by Western blotting with antibodies specific for chicken C/EBPβ. The activity of the cotransfected pCMVβ plasmid was measured to ensure that transfection efficiencies of different plates were similar. Only the part of the gel containing C/EBPβ is shown.

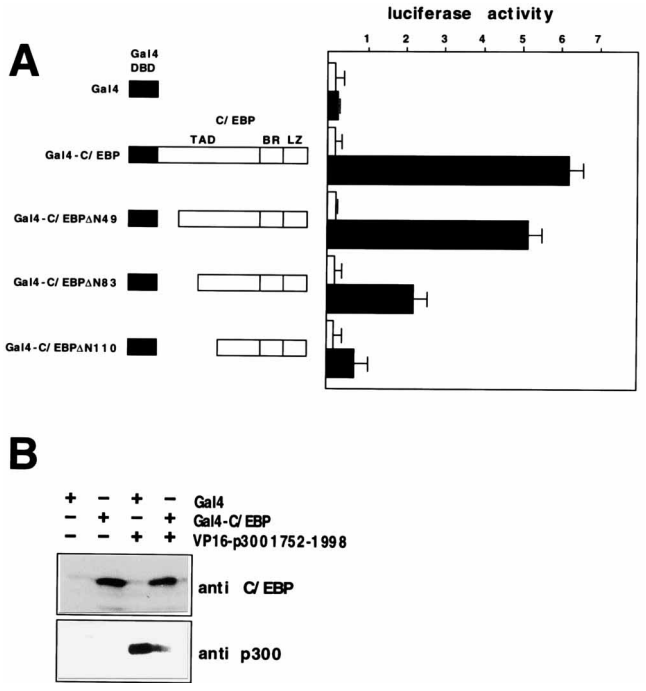

FIG. 8. p300 interacts with amino-terminal sequences of C/EBPβ. (A) QT6 cells were transfected with the Gal4-responsive reporter plasmid pG5E4-38Luc (3 μg), the β-galactosidase plasmid pCMVβ (0.2 μg), and the expression vectors for the Gal4-C/EBPβ fusion proteins shown schematically on the left (0.02 μg each). Western blotting experiments confirmed that the different Gal4-C/EBPβ fusion proteins were produced in similar amounts in the transfected cells (data not shown). In addition, an expression vector for a hybrid protein consisting of the VP16 transactivation domain fused to p300 amino acids 1737 to 1983 (pCDNA3-SN1G/3-VP16, 2 μg) was included (black bars). As control (white bars), an expression vector encoding only the VP16 transactivation domain was used (pCDNA3-Vp16, 2 μg). The columns on the right show the average luciferase activity, normalized with respect to the cotransfected β-galactosidase plasmid. Thin lines show standard deviations. (B) QT6 cells were transfected with the indicated expression vectors (same amounts as in panel A) and analyzed by Western blotting with C/EBPβ- or p300-specific antibodies (top and bottom, respectively).

shown before (34), the CJ protein does not interact with v-Myb and fails to synergize with v-Myb on a modified *mim-1* reporter gene (referred to as pDel130/33TRE-Luc) in which the relevant C/EBP binding site had been mutated to a Jun binding site. Figure 10B confirms our previous observation that v-Myb and the CJ protein do not synergize in the absence of p300, although each of them activates the reporter gene. However, in the presence of p300, but not the del33 mutant of p300, strong synergy between v-Myb and the CJ protein was observed (Fig. 10B). This experiment shows that in the presence of p300 direct interaction between Myb and C/EBP is not strictly required for synergistic activation by both factors. More important, this experiment directly demonstrates that p300 increases the synergy between Myb and C/EBP.

DISCUSSION

p300 is a coactivator for C/EBPβ. The communication between transcription factors and the basal transcriptional machinery is one of the key steps in the regulation of eukaryotic gene expression. Transcriptional coactivators such as p300 and CBP connect, via protein-protein interactions, transcription factors to the transcriptional machinery and thus are considered important elements of gene regulation networks. p300 and CBP appear to be particularly versatile coactivators because they interact with several different transactivators.

A number of observations described here establish a link between p300 and the C/EBP family. Firstly, we have found that transactivation mediated by C/EBPβ and C/EBPα (data not shown) is strongly inhibited by the adenovirus E1A protein. Analysis of the effects of specific E1A mutants confirmed that the inhibitory effect of E1A is dependent on its p300 binding activity. In accordance with this notion, we found that the E1A-mediated inhibition of C/EBP activity is reversed by ectopic expression of p300. The E1A-induced inhibition of C/EBPβ, therefore, seems not to be due to interference of E1A with the stimulatory effect of Rb on C/EBPβ described recently (11). Secondly, using an artificial C/EBP-responsive reporter gene we have shown that ectopic expression of p300 increases the transactivation potential of C/EBPβ and C/EBPα. Thirdly, several in vivo and in vitro assays show that C/EBPβ and p300 interact with each other. Lastly, a subfragment of p300 containing the C/EBPβ binding site acts as a dominant-negative inhibitor for C/EBPβ-mediated transactivation. Together, these findings strongly suggest that p300 is a bona fide coactivator for C/EBPβ and, presumably, for other members of the C/EBP family.

p300 potentiates the activation of an endogenous C/EBP-responsive gene by C/EBPβ. The most compelling evidence for a role of p300 in C/EBPβ-mediated gene activation stems from the analysis of the effect of p300 on the expression of an endogenous, chromatin-embedded C/EBP-responsive gene. This gene is referred to as *126* and was originally identified in a screen of genes differentially expressed in avian myeloblastosis virus-transformed versus E26-transformed myelomonocytic cells (39). Expression of *126* mRNA is increased by C/EBPβ (reference 27 and this study), indicating that *126* is a C/EBP-regulated gene. Our analysis shows that p300 potentiates the activation of the *126* gene by C/EBPβ but that p300 has no effect on the expression of the gene in the absence of C/EBPβ. These observations indicate that the observed stimulation of C/EBPβ-mediated transactivation by p300 is not an artifact caused by the use of artificial reporter genes. As far as we are aware, our observations provide the first evidence for coactivation of a natural, chromatin-embedded gene by p300.

C/EBPβ interacts with the E1A binding region of p300. We have demonstrated that C/EBPβ and p300 interact in vivo and

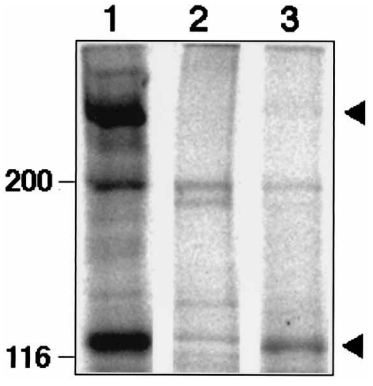

FIG. 9. Coimmunoprecipitation of endogenous p300 and C/EBP. HeLa cells were labeled for 4 h with 500 μCi of [^{35}S]methionine per ml. Equal amounts of extract (prepared from these cells as described in Materials and Methods) were immunoprecipitated first with the p300-specific monoclonal antibody AC238 (lane 1), with C/EBPβ-specific rabbit antiserum (lane 3), or with preimmune serum (lane 2). Immunoprecipitates were dissolved by boiling them in a small volume of sample buffer, diluted in lysis buffer, reprecipitated with the AC238 antibody, and finally analyzed by SDS-PAGE. The numbers to the left indicate the positions of molecular mass markers (in kilodaltons). The positions of full-length p300 and a proteolytic degradation product are marked by arrowheads.

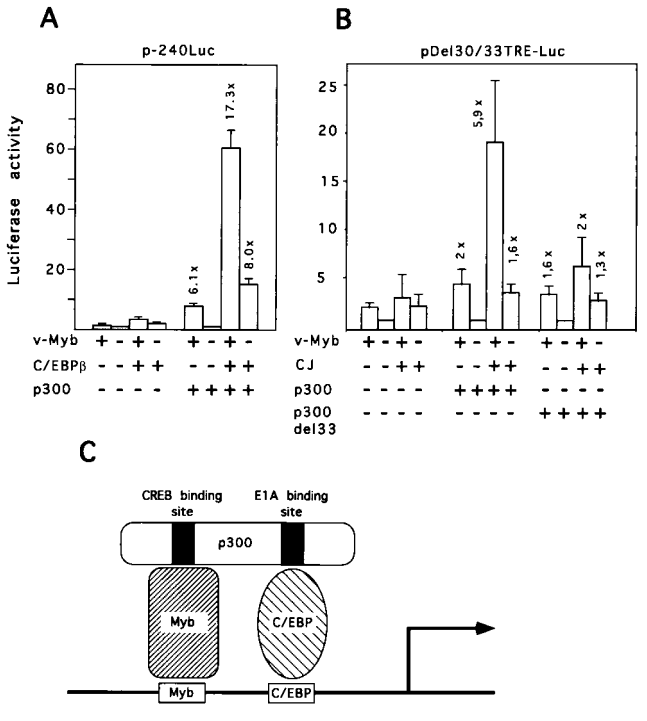

FIG. 10. p300 synergizes with v-Myb and C/EBPβ at the *mim-1* promoter. (A) QT6 cells were transfected with the *mim-1* reporter gene p-240Luc (3 μg); the β-galactosidase plasmid pCMVβ (0.2 μg); and expression vectors for C/EBPβ (pCRNC-CCR, 0.1 μg), v-Myb (pVM134, 0.3 μg), and p300 (pCMV-p300CHA, 5 μg), as indicated at the bottom. Control transfections (marked with minus signs) contained equivalent amounts of the appropriate empty expression vectors. The columns show the average luciferase activity, normalized with respect to the cotransfected β-galactosidase plasmid. The activity of the reporter gene in the absence of v-Myb and C/EBPβ was designated as 1. Thin lines show standard deviations. The numbers on top of the columns indicate the stimulation of the luciferase activity by p300. (B) QT6 cells were transfected with the reporter gene pDel30/33TRE-Luc (3 μg); the β-galactosidase plasmid pCMVβ (0.2 μg); and expression vectors for a hybrid protein consisting of the DNA binding domain of c-Jun and the amino terminus of C/EBPβ (pCDNA3-CJ, 1 μg), v-Myb (pVM134, 0.5 μg), p300 (pCMV-p300CHA, 5 μg), and p300del33 (pCMV-p300del33CHA, 2 μg), as indicated at the bottom. Control transfections (marked with minus signs) contained equivalent amounts of the appropriate empty expression vectors. The cells were analyzed as described for panel A. (C) Model illustrating the interaction of p300 with Myb and C/EBP.

in vitro and have mapped the amino acid sequences responsible for this interaction to the C-terminal part of p300. The minimal C/EBPβ binding region of p300 corresponds to amino acids 1752 to 1859 of p300. Although the sequences required for E1A and C/EBPβ binding are located in the same region of p300, they are not identical. This is evident from the fact that a deletion mutant of p300, del30, which no longer binds to E1A, still interacts with and stimulates C/EBPβ.

Besides E1A, several proteins have been shown to interact with the C-terminal region of p300 or of the closely related CBP, such as c-Fos (5), TFIIB (28), YY1 (30), MyoD (53), $p160^{SRC-1}$ (24), P/CAF (51), p53 (4, 18, 31), and $pp90^{rsk}$ (38). This domain of p300-CBP might therefore provide a means for cross-talk between these proteins and could serve as an integration point for different transcription factors. It will be interesting to precisely delineate the sequence requirements for interaction with each of these factors and to determine whether different factors interacting with the C-terminal domain of p300 can affect each other's function, either positively or negatively, by stimulating or inhibiting the other's interaction with p300.

We have mapped the amino acid sequences of C/EBPβ involved in the interaction with p300 to the N-terminal half of the protein. This region of C/EBPβ contains several amino acid sequence stretches that are conserved between different members of the C/EBP family and might provide the basis for the interaction of p300 with other members of this family, such as C/EBPα. In addition, this region harbors the transactivation domain of C/EBPβ (16, 27). The gradual loss of the p300-C/EBPβ interaction detected in a two-hybrid-type experiment (Fig. 8) makes it unlikely that there is one distinct binding site for p300 but suggests rather that different parts of the amino terminus of C/EBPβ contribute to the interaction with p300.

p300 stimulates the synergy between Myb and C/EBP. Transcription of the *mim-1* gene is restricted to the myelomonocytic lineage of the hematopoietic system (41, 45) and depends on the cooperation of Myb and C/EBP transcription factors (8, 40). The synergy between Myb and C/EBPβ has been shown to involve an interaction of both proteins which is mediated by their DNA binding domains (34). Interestingly, expression of Myb and C/EBP is sufficient to activate the endogenous *mim-1* gene in heterologous cell types, such as fibroblasts or erythroblasts (8, 40). This finding has implicated Myb and C/EBP in a molecular switch which controls the expression of *mim-1* and probably of other genes during myelomonocytic differentiation (8, 37, 40).

Myb has been shown to interact with the CREB binding site of p300/CBP (13, 38), suggesting that p300 and CBP are integral components of the Myb-C/EBP switch. Since Myb and C/EBPβ bind to different subdomains of p300/CBP, it is conceivable that p300 and CBP interact with Myb and C/EBP at the same time and thereby stimulate the synergy between these factors. Our results show that p300 indeed increases the synergy between Myb and C/EBPβ. This was particularly evident in experiments in which a modified version of C/EBPβ containing the DNA binding domain of c-Jun was used. As shown previously (34), this chimeric protein is unable to synergize with v-Myb in the absence of p300. However, in the presence of p300 expression the C/EBPβ-Jun hybrid protein efficiently synergizes with Myb. It therefore appears that the requirement for direct interaction between Myb and C/EBPβ, which we have described before (34), can be overcome if sufficient amounts of p300 are provided. More important, these findings suggest a novel function for p300: in addition to its role in linking certain transcription factors to the basal transcriptional machinery, p300, and presumably CBP, also facilitates the communication and cooperation between different transcription factors.

Our observations confirm and extend the results of Oelgeschläger et al. (42), who have shown that CBP stimulates the synergy between Myb and C/EBP. Although direct evidence for an interaction of CBP and C/EBP is missing so far, p300 and CBP appear to display very similar activities towards Myb and C/EBP. Since cooperation between Myb and C/EBP is crucial for the cell-type-specific expression of *mim-1* and presumably other genes during myelomonocytic differentiation, these observations also imply that p300 and CBP are involved in activating specific genes during the differentiation of myelomonocytic cells.

ACKNOWLEDGMENTS

We thank T. Kouzarides, H. Land, E. Harlow, D. Livingston, S. Ness, and J. Nevins for plasmids; R. Eckner for advice and the gift of the p300-specific antibody AC238; and L. Lay for preparing the photographs.

This work was supported by a grant (SFB364/A3) from the Deutsche Forschungsgemeinschaft and the Zentrum für Klinische Forschung (ZKF) of the University of Freiburg.

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