Cdx2 encodes for a homeodomain protein that is expressed in intestinal epithelial cells. The Cdx2 protein triggers intestinal differentiation in cell lines and is necessary for maintenance of the intestinal phenotype in mice. CBP (CREB-binding protein) is a transcriptional co-activator that interacts with many transcription factors and components of the basal transcriptional machinery. In this study, we demonstrate that CBP is markedly induced upon differentiation of the Caco-2 intestinal cell line and augments Cdx2-dependent transcriptional activity. Cdx2 interacts with the amino-terminal domain of CBP, and the two proteins coexist in vivo within the same nuclear protein complex. Moreover, expression of the CBP domain that interacts with Cdx2 acts as a dominant-negative inhibitor of transcriptional activation by Cdx2. These findings demonstrate a direct interaction between an intestinal homeobox protein and CBP and suggest that CBP participates in the network of transcriptional proteins that regulate intestinal differentiation.

In mammals, visceral endoderm gives rise to the gastrointestinal epithelium. Several caudal-type homeodomain proteins of intestinal epithelium are expressed in the differentiated phenotype of these em-

We hypothesized that co-factors that link DNA-binding proteins to the basal transcriptional apparatus may be involved in regulation of Cdx2-dependent transcription. CBP (CREB-binding protein) was first characterized as a co-factor for the CREB-binding protein that potentiates transcriptional activity (8). Since then, CBP, and its family member p300, have been shown to bind to interact with many transcription factors in a number of different families in addition to CBP, including helix-loop-helix proteins and nuclear receptors (9–11). CBP functions as an activator of complex transcriptional regulatory elements by enhancing the interaction between transcription factors and basal transcriptional complexes. In this study, we show that CBP interacts with Cdx2 and is markedly induced upon differentiation of the Caco-2 intestinal cell line and augments Cdx2-dependent transcriptional activity. Cdx2 interacts with the amino-terminal domain of CBP, and the two proteins coexist in vivo within the same nuclear protein complex. Moreover, expression of the CBP domain that interacts with Cdx2 acts as a dominant-negative inhibitor of transcriptional activation by Cdx2. These findings demonstrate a direct interaction between an intestinal homeobox protein and CBP and suggest that CBP participates in the network of transcriptional proteins that regulate intestinal differentiation.

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1. The abbreviations used are: SI, sucrase-isomaltase; CBP, CREB-binding protein; GST, glutathione S-transferase; PBS, phosphate-buffered saline; HA, hemagglutinin.

2. This work was supported by National Institutes of Health Grant RO1-DK46704 and the Molecular Biology Core of the Center for Molecular Studies in Digestive Diseases at the University of Pennsylvania (P30-DK50306) (to P. G. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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CBP and Cdx2 protein level during Caco-2 cell differentiation. The expression of CBP and Cdx2 proteins during Caco-2 cell differentiation were measured by immunoblotting (Experimental Procedures). Total cellular protein was extracted 2 days before confluency (lane 1), at confluency (lane 2), 3 days after confluency (lane 3), 7 days after confluency (lane 4), and 14 days after confluency (lane 5). Protein integrity was confirmed by expression of E-Cadherin.

luciferase assay kit (Promega Corp.). Each transfection was performed in duplicate and repeated at least 3 times. As a measure of transfection efficiency, 300 ng of pCMV-β-galactosidase were co-transfected in each experiment, and the results were reported as light units per unit of β-galactosidase.

In Vitro GST-Protein Interaction Assay—GST-protein interaction assays were performed as described elsewhere (19) with slight modifications. Briefly, bacterial expression of GST fusion proteins was induced in medium containing 0.1 μM isopropyl-1-thio-β-D-galactopyranoside for 3 h. The bacterial pellet was resuspended in 500 μl of PBS and sonicated to disrupt the bacteria. The bacterial remnants were removed by centrifugation, and the supernatant was incubated with 75 μl of GST beads (Amersham Pharmacia Biotech) on a rotating wheel for 30 min at 4 °C. The beads were washed 4 times with PBS and then with HND buffer (10 mg/ml bovine serum albumin, 20 mM Hepes, 50 mM NaCl, 0.1% Nonidet P-40, 5 mM dithiothreitol).

Proteins were labeled with [35S]methionine by coupled in vitro transcription and translation using the TNT reticulocyte lysate system (Promega) according to the manufacturer’s instructions. Proteins with bound fusion proteins were incubated with 1 mg of GST beads (Amersham Pharmacia Biotech) in 200 μl of PBS, 0.1% Nonidet P-40, 2 mM KCl, 800 μM KH2PO4, pH 7.4, in a 2% skim milk. Cdx2 was detected using rabbit anti-Cdx2 polyclonal antibody.2 CBP was detected using rabbit polyclonal A-22 antibody in combination with anti-mouse antibodies for E-Cadherin (Santa Cruz Biotechnology). E1A to affect Cdx2-dependent activation of the SI promoter in Caco-2 cells and abolished superactivation of the SI promoter when Cdx2 was overexpressed (Fig. 2A). Moreover, an E1A mutant that lacks the ability to interact with retinoblastoma protein but does bind to CBP (E1ACXdir) also inhibited Cdx2 activation of the promoter. In contrast, expression of a mutant form of E1A (E1AD2–36) that is unable to bind CBP, had no effect on SI promoter activation by Cdx2 (Fig. 2A). We next examined the ability of E1A to affect Cdx2-dependent activation of the SI promoter in

Results and Discussion

Cdx2 is an important transcription factor for activating the SI promoter in intestinal cell lines (2). Caco-2 cells, derived from a human colonic adenocarcinoma, spontaneously differentiate following confluence in culture, including a marked increase in the expression of SI mRNA and protein (6, 20, 21). Therefore, we examined the expression of Cdx2 protein during Caco-2 cell differentiation to determine whether an increase in this transcription factor may be involved in SI induction. The amount of Cdx2 protein was not different in pre- and postconfluent cells in comparison to E-Cadherin, a protein that is unchanged during Caco-2 differentiation (22) (Fig. 1). Because

2 D. Silberg and P. G. Traber, unpublished results.
In previous studies, we have shown that Cdx2 is able to activate transcription from an enhancer upstream of a minimal thymidine kinase promoter (pTK-SIF1(+4)-luc) (18). As with the SI promoter, Cdx2-induced transactivation of this construct was inhibited by wild-type E1A, but not by the E1A mutant that is unable to bind to CBP (Fig. 2C). These modifications in luciferase activity were directly related to the presence of the SIF1 elements, because no luciferase activity was unchanged when transfections were done using pTK-luc (Fig. 2C). These results suggest that CBP may be directly involved in the ability of Cdx2 to activate gene transcription.

The ability of CBP to directly augment transactivation by Cdx2 was examined by co-transfection of expression vectors for Cdx2 and CBP with the Cdx2-responsive reporter plasmid pTK-SIF1(+4) in Caco-2 (Fig. 3A). CBP overexpression in Caco-2 cells resulted in a dose-dependent increase in Cdx2-dependent transactivation with a maximum of a 3-fold increase over that with Cdx2 alone (Fig. 3A). The induction of transactivation by CBP was greater in the presence of higher levels of Cdx2, suggesting that Cdx2 may be limiting in Caco-2 cells. Addition of the wild-type E1A expression vector resulted in loss of reporter gene expression whereas E1AΔ(Δ2–36) had no effect on the ability of CBP to augment Cdx2 transactivation (Fig. 3A). Transient overexpression of CBP or Cdx2 in Caco-2 cells did not change the endogenous expression of Cdx2 and CBP, respectively (Fig. 3B). Furthermore, transient overexpression of E1A or E1AΔ(Δ2–36) in Caco-2 cells did not interfere with Cdx2 and CBP expression (Fig. 3B). These results demonstrate that in Caco-2 cells, CBP is able to increase Cdx2 transactivation activity when SIF1 elements are located immediately upstream of a heterologous promoter.

In previous studies, we have shown that Cdx2 is able to activate transcription when its DNA binding elements are placed in an enhancer position on heterologous promoters (18). These studies further showed that the cellular context is important for Cdx2 to activate transcription from an enhancer.
context, because Caco-2 cells supported Cdx2-dependent enhancer activation, whereas NIH-3T3 cells did not. These results led to the conclusion that there may be cell-specific adaptor proteins that are important for Cdx2 function on certain transcriptional elements (18). We found that CBP was not able to augment Cdx2 transactivation when Cdx binding elements were placed in an enhancer context (data not shown). Thus, consistent with the widespread expression of CBP, it does not appear to be responsible for the previously observed cell-specific function of Cdx2 on enhancer elements.

The mechanism by which CBP augments Cdx2 transactivation activity could be via direct protein-protein interaction, by common interaction with a third protein, or by indirect effects on other components of the transcriptional machinery. To determine whether there is direct interaction between Cdx2 and CBP, in vitro protein interaction assays were performed. The bacterially expressed fusion protein GST-CBP(1–450) was able to interact with in vitro labeled Cdx2 (Fig. 4A). In contrast, there was very weak interaction with GST-CBP(451–682) and no interaction with GST-CBP(1000–1500). These data raised the question of whether the homeodomain of Cdx2 interacted with GST-CBP(1–450), similar to a recently described POU-homeodomain protein Pit-1 (26). A peptide containing the homeodomain of Cdx2 and excluding the majority of the amino- and carboxyl-terminal domains was found to bind specifically to GST-CBP(1–450) (Fig. 4B).

We next examined whether CBP and Cdx2 interact within the cell. Co-immunoprecipitation was performed to determine whether Cdx2 and CBP coexist within the same protein complex. Protein extracts of Caco-2 cells transfected with expression vectors for Flag-CBP and/or Flag-Cdx2 were subjected to immuno- precipitation followed by immunoblot analysis. Results of these experiments showed that Cdx2 was immunoprecipitated with Flag-CBP and that CBP immunoprecipitated with Flag-Cdx2 (Fig. 4C). Antibodies were unable to precipitate Flag-Cdx2 and anti-CBP antibodies were unable to precipitate CBP or CBP associated in an immunoprecipitable protein complex in Caco-2 cells.

The interaction of Cdx2 with CBP provides an avenue to test the functional implications beyond the subfamily of POU-homeodomain proteins that are important for Cdx2 function on certain promoters and thus may influence intestinal differentiation and development. Taken together, these results demonstrate that the amino-terminal domain of CBP is able to interact with Cdx2 and that this interaction results in increased Cdx2-dependent transactivation. Recently, it has been shown that CBP interacts with the POU-homeodomain protein Pit-1 and that this interaction is functionally important for transcriptional activation (26). Our findings represent the second description of an interaction between homeodomain proteins and CBP and extend the regulatory implications beyond the subfamily of POU-homeodomain proteins. Cdx2 has been shown to be a critical component of the transcriptional machinery of intestinal epithelial cells and, as a result, plays a critical role in intestinal development and differentiation. Based on these studies, we hypothesize that CBP may modulate the function of Cdx2 on complex promoters and thus may influence intestinal differentiation and development.

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