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CBP/p300 Functions as a Possible Transcriptional Coactivator of Ah Receptor Nuclear Translocator (Arnt)[1]

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A heterodimer of AhR (aryl hydrocarbon receptor) and Arnt (AhR nuclear translocator) conveys a transactivation signal of aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and 3-methylcholanthrene to the genes for a group of drug-metabolizing enzymes. This inducible expression of the genes is inhibited by adenovirus E1A, suggesting that CBP/p300 is somehow involved in the transactivation of the genes by the AhR and Arnt heterodimer. Yeast and mammalian two hybrid systems revealed that CBP/p300 interacted with the transactivation domain of Arnt, but not with that of AhR, *via* the CREB-binding domain. The pull down assay using GST-Arnt hybrid protein confirmed the interaction between Arnt and CBP/p300. Considering these results and that Arnt or Arnt2 functions as a common partner in the formation of transcriptional regulators with other bHLH/PAS proteins such as AhR, HLF, and HIF-1α, the possibility arises that CBP/p300 is extensively involved as a coactivator in the transactivation process by bHLH/PAS (a conserved sequence motif among Per, Arnt, and Sim) heterodimer transcription factors through interaction with Arnt or Arnt2.

Key words: coactivator, drug-inducible expression, heterodimer, transcriptional activation.

Transcriptional activation of eukaryotic genes in response to exogenous or endogenous signals is accomplished through multiple sequence-specific protein/DNA and protein/protein interactions. DNA-binding proteins that recognize appropriate DNA sequences in the promoter of a target gene can interact with other transcription factors to transmit transcription-enhancing effects to the basic transcription machinery, including TFIID and RNA polymerase II. Transcription of CYP1A1 (cytochrome P-450c) gene is induced by polycyclic aromatic hydrocarbons such as 3-methylcholanthrene and TCDD (*1, 2*). Analysis of CYP1A1 gene regulation by means of DNA transfer experiments revealed that a high level of inducible expression required at least two regulatory DNA elements, designated XRE (xenobiotic responsive element) and BTE (basic transcription element, a GC box sequence), besides the TATA box sequence (*3-7*). The regulatory factor bound to the XRE is a heterodimer complex consisting of AhR [aryl hydrocarbon receptor (*8, 9*)] and Arnt [(AhR nuclear translocator (*10*)] (*11-13*), while Sp1 is a regulatory factor acting on the BTE (*14*).

In cotransfection assays, the drug-inducible expression was repressed by adenovirus E1A (*15*). Analyses of functional domains of AhR and Arnt using chimeric proteins fused to GAL4 DNA binding domain revealed that AhR and Arnt carry the transactivation domains in their C-terminal halves (*16-19*) and that E1A markedly repressed the transactivation by GAL-Arnt chimeric protein, while that by GAL-AhR protein was relatively resistant to inhibition by E1A (*16*). Since no evidence for direct interaction between E1A and Arnt has been obtained in several trials, the transactivation activity of Arnt is considered to be mediated by a coactivator whose activity is hindered by E1A. Recently, various transcriptional cofactors, such as CBP (*20*), p300 (*21*), PC4 (*22*), Dr1 (*23*), N-CoR (*24*), SRC-1 (*25, 26*), and SMRT (*27*) have been identified and characterized. These cofactors, designated as coactivators or corepressors depending on their transcriptional properties, do not directly bind the DNA sequence, but mediate transcriptional effects, either stimulative or repressive, from the sequence-specific transcription factors to the general transcription machinery through interacting with their cognate DNA-binding proteins. Of these cofactors, CBP (CREB binding protein) was originally found to be a coactivator for CREB (cAMP-responsive element-binding

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Abbreviations: AhR, arylhydrocarbon receptor; Arnt, AhR nuclear translocator; BTE, basic transcription element; CAT, chloramphenicol acetyltransferase; CBP, CREB-binding protein; CYP, cytochrome P450; DTT, dithiothreitol; GST, glutathione S-transferase; HIF-1α, hypoxia inducible factor-1α; HLF, HIF-1α-like factor; HLH, helix-loop-helix; PAS, Per-Arnt-AhR-Sim; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; SRC-1, steroid hormone receptor coactivator; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic responsive element.

protein) (*20*) and is now known to be a common coactivator for numerous DNA-binding transcription factors including AP1 (*28, 29*), steroid hormone receptors (*26, 30*), MyoD (*31*), and Myb (*32, 33*), in addition to CREB. Upon activation as a result of phosphorylation at Ser 133 by protein kinase A, CREB binds to the CRE (cAMP responsive element) to recruit CBP to the promoter of the target genes and transmits the transactivation activity to the general transcription machinery *via* interaction with CBP (*20, 34*). p300 which was identified as an E1A-associated factor, has now been found closely to resemble CBP, both structurally and functionally (*35-37*). Since both CBP and p300 are known to be targets for a repressive effect of E1A, inhibition of the enhanced expression of CYP1A1 gene by E1A led us to investigate whether CBP/p300 mediates the transactivation of CYP1A1 gene by AhR/Arnt heterodimer.

In the present paper, we show that Arnt actually interacts with CBP/p300 in its C-terminal activation domain by using yeast and mammalian two hybrid systems. In cell extracts of 293T cells transfected with GST-tagged Arnt or AhR, and CBP expression plasmids, CBP was coprecipitated with GST-Arnt, but not GST-AhR on glutathione Sepharose beads. Experiments using a variety of deletion mutants of these factors revealed that CBP/p300 bound to the transcriptional activation domain of Arnt *via* their CREB-binding domains. Since Arnt2 (*38*) was also found to interact with CBP/p300 in the activation domains common to Arnt and since either Arnt or Arnt2 functions as a common partner with other bHLH/PAS (a conserved sequence motif among Per, Arnt, and Sim) transcription factors such as HIF-1α (*39*) and HLF (*40*), it is reasonable to conclude that CBP/p300 is a common coactivator for a group of bHLH/PAS transcription factors.

EXPERIMENTAL PROCEDURES

Expression Plasmids of Arnt and CBP in Yeast Cells—Expression plasmids for the yeast two hybrid system encoding the GAL4 DNA-binding domain (GBT9) and GAL4 activation domain (GAD424) were kindly provided by Dr. S. Fields (State University of New York at Stony Brook). Expression plasmids of GADArnt(1-789), GADArnt2 and GADAhR were described previously (*38*). GADArnt(1-617) was made by inserting an *Xba*I linker into the *Pst*I site of GADArnt(1-789) to create a termination codon. GADArnt(1-214), GADArnt(116-513), and GADArnt (597-789) plasmids were generated by inserting the 650 bp *Nco*I/*Apa*LI, 1,200 bp *Asp*718 and 680 bp *Nae*I/*Xho*I fragments from BKSArnt (*11*) into appropriate sites of GAD424, respectively.

GBT-CBP(1-1891) plasmid was produced by inserting the 5.7 kbp *Sma*I fragment of pRc/RSVCBP (a kind gift from Dr. R.H. Goodman, Oregon Health Sciences University, Oregon) into the blunt-ended *Bam*HI site of GBT9. GBT-CBP(1-452) plasmid was produced by inserting the 1,380 bp *Eco*RI fragment of GBT-CBP(1-1891) into the *Eco*RI site of GBT9. GBT-CBP(452-721) and GBT-CBP-(721-1679) plasmids were generated by inserting the blunt-ended 1,380, 800, and 2,900 bp *Eco*RI fragments of pRc/RSV CBP into the blunt-ended *Eco*RI and *Bam*HI sites of GBT9, respectively. GBT-CBP(1678-2441) plasmid was made by inserting the 2,900 bp *Eco*RI fragment of CBP into the *Eco*RI site of GBT9.

Expression Plasmids in Mammalian Cells—pBosArnt was made by inserting the 2.5 kbp *Nco*I/*Xho*I fragment from pBKS-Arnt (*11*) into the blunt-ended *Xba*I of pEFBos (a kind gift from Dr. S. Nagata, Osaka University, Osaka) (*41*). pBosCBP and pBosp300 were made by inserting the 7.3 kbp *Bam*HI fragment from pRc/RSV CBP and the 7.2 kbp *Not*I/*Hin*dIII fragment from pCMVβ p300-CHA (a kind gift from Dr. D.M. Livingston, Dana-Farber Cancer Institute, Boston), into the blunt-ended *Xba*I of pEFBos, respectively. pBosGST was generated as follows. The glutathione S-transferase-coding fragment of pGEX2T (Pharmacia) was amplified by the PCR method using the primers GST-N (5′-ccaccatgtcccctatactaggtt-3′) and GT-3′ (5′-ctatctatctatgaattcccggggatccacgcggaaccag-3′). pBosGST-Arnt and pBosGST-AhR were constructed by inserting the 2.5 kbp *Nco*I/*Xho*I fragment from pBKS-Arnt and the 2.5 kbp *Xho*I/*Sph*I fragment from pBKS-AhR into the blunt-ended *Bam*HI site of pBosGST, respectively. For the mammalian two hybrid system, the expression vectors [pBosGBT, pBosGBT-CBP(452-721), pBosGAD, pBosGADArnt(1-798), pBosGADArnt(1-612), pBosGADAhR, and pBosGBTArnt] were constructed by inserting the *Hin*dIII fragments from respective yeast expression vectors described above into the blunt-ended *Xba*I site of pEFBos.

All constructs were confirmed by sequencing.

Yeast Two Hybrid System—Transformation of *Saccharomyces cerevisiae* SFY526 and measurement of β-galactosidase activity of the transformants were performed as described previously (*38*), with some modifications, *i.e.*, the transformants were cultured in SD medium [yeast nitrogen base without amino acids (Gibco), 2% glucose] for both leucine and tryptophan requirements.

Cell Culture and Transfection—293T cells (human embryonic kidney cell line), were maintained in DMEM supplemented with 10% FCS. The cells were transfected with appropriate expression plasmids by the calcium-phosphate method as described by Gorman *et al.* (*42*) and the expressed CAT activity was determined as described (*43*). The CAT assays were repeated at least three times in independent transfection experiments and the results were normalized with respect to β-galactosidase activity expressed from pEFBosLacZ plasmid used as a standard (a kind gift from Mr. J. Mimura, Tohoku University, Sendai). pG5E1bCAT was a kind gift from Dr. M. Green (Harvard University, Cambridge).

In Vivo Binding Assay—Expression plasmids of GST-Arnt, GST-AhR, CBP, and p300 were transfected into 293T cells by the calcium-phosphate method. After incubation for 36 h, cells were washed with ice-cold phosphate-buffered saline, followed by resuspension in buffer D [20 mM Hepes (pH 7.9), 420 mM NaCl, 0.2 mM EDTA, 20% glycerol, 1 mM DTT, 1 mM PMSF, 2 mg/ml Aprotinin, 10 mM NaF]. The cells were subjected three times to freeze and thaw treatments, and centrifuged at 10,000$\times g$ for 15 min at 4°C. The NaCl concentration of the supernatants was adjusted to 100 mM with a buffer (20 mM Hepes, 5 mM $MgCl_2$, 10 mM NaF, 1 mM PMSF, 1 mM DTT, 1.3 mg/ml BSA) and the supernatants were incubated for 2 h at 4°C with gentle rocking and then incubated for another 3 h at 4°C after addition of glutathione-Sepharose beads (Pharmacia). The samples were washed four times with RIPA

buffer [10 mM Tris (pH 7.5), 100 mM NaCl, 2 mM EDTA, 0.01% NP40], then subjected to 6% SDS-PAGE and immuno-blotting analyses (*43*), and visualized with anti-CBP (Santa Cruz) or anti-human p300 CT antibody (Upstate Biotechnology).

RESULTS

In Vivo Binding of CBP to the Transactivation Domain of Arnt—We previously demonstrated that the adenovirus E1A repressed the drug-inducible expression driven by CYP1A1 gene promoter in transient transfection assays using Hepa-I cells (*15*). Here, we showed that the expression of E1A 12S repressed the drug-inducible expression of CYP1A1 gene in a dose-dependent manner, but not the basal expression (Fig. 1). Since E1A was not found to interact directly with the AhR/Arnt heterodimer (data not shown), it was speculated that the transactivation of the AhR/Arnt transcription factor was mediated by an E1A-sensitive mechanism. This speculation lead us to investigate whether CBP/p300 interacts with AhR and/or Arnt by using the yeast two hybrid system based on the *S. cerevisiae* strain SFY526 carrying the LacZ reporter gene with binding sites for GAL4 (UAS). Several parts of the CBP cDNA were fused to the GAL4 DNA-binding domain (DBD) to express hybrid proteins as bait (Fig. 2), and

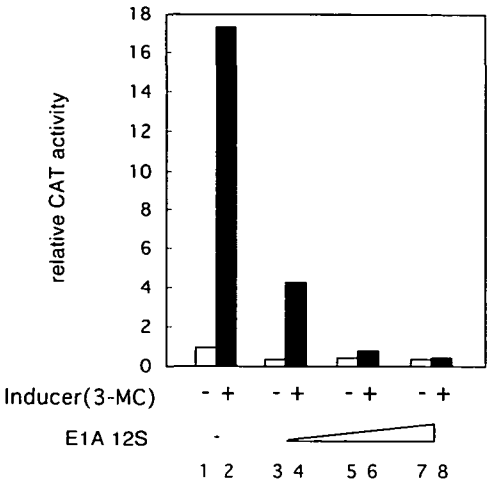

Fig. 1. **Inhibition of the drug-inducible expression of CYP1A1 gene by coexpression of adenovirus E1A.** Adenovirus 12S E1a (10 ng, 100 ng, and 1 μg) (*15*) was transfected into Hepa-I cells along with the reporter pMC6.3K (2 μg) (*5*). The cells were cultured for 40 h in the presence or absence of 3-MC (1 μM) and then lysed as described (*43*). The CAT activities in the cell extracts were measured three times in independent transfection experiments as described under "EXPERIMENTAL PROCEDURES." The CAT activities were expressed relative to the standard activity in lane 1.

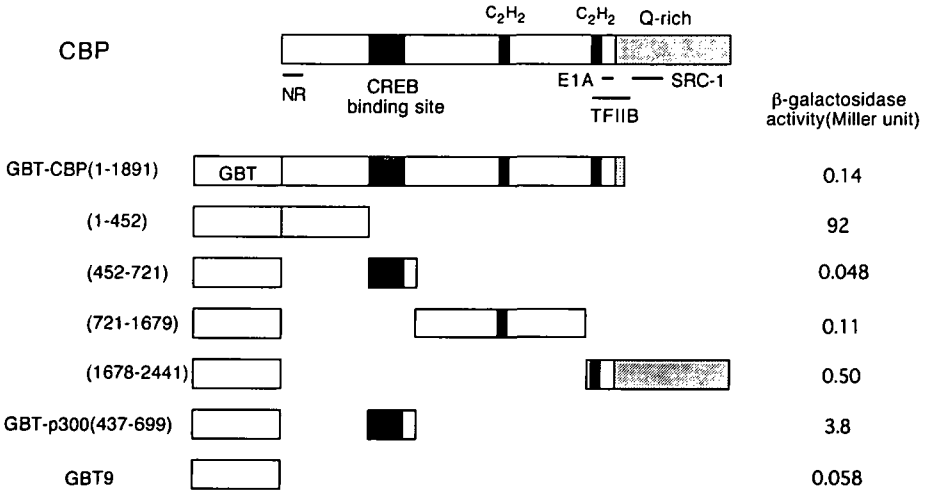

Fig. 2. **Schematic representation of several domains of CBP fused to the GAL4 DNA-binding domain (GBT) using the yeast two hybrid system.** β-Galactosidase activities of the yeasts transformed with GAD424 and several GBT-CBP plasmids are shown on the right.

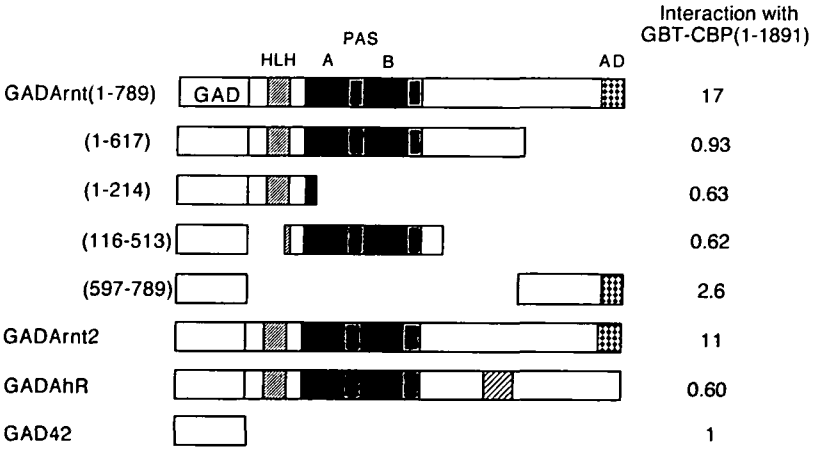

Fig. 3. **Interaction between CBP/p300 and Arnt assayed by the yeast two hybrid system.** Schematic representation of several domains of Arnt, Arnt2, and AhR fused to the GAL4 transactivation domain (GAD). β-Galactosidase activities of the yeasts transformed with GBT-CBP(1-1891) and several GAD fusion plasmids are shown on the right.

cDNAs for several functional domains of Arnt and AhR were fused to the GAL4 transactivation domain (AD) for construction of prey plasmids (Fig. 3). In the control experiments using GAD424 plasmid which encodes the GAL4 AD and GBT-CBP(1-452) or GBT-CBP(1678-2441), the β-galactosidase activities were increased, in contrast to the case of GAD424 and GBT9, which encodes only the GAL4 binding domain (Fig. 2). In accordance with the result for GBT-CBP(1678-2441), Goodman *et al.* reported that CBP had a transactivation domain in the C-terminus which was considered to interact with TFIIB (*34*). However, our results in the yeast two hybrid system revealed that CBP contained an additional transactivation domain in the N-terminus which was also reported to be necessary to interact with the steroid hormone receptor superfamily (*26*).

As shown in Fig. 3 and Table I, remarkable enhancement of β-galactosidase activity was observed when the yeast was transformed with GADArnt(1-789) and GBT-CBP(1-1891) (17-fold), indicating interaction between Arnt and CBP. Arnt2, a neural-specific homologue of Arnt in mouse embryos (*38*), was also found to interact with CBP, while AhR, a partner molecule of Arnt in the inducible expression of drug-metabolizing enzyme genes, showed little effect on the β-galactosidase expression, even in the presence of 3-MC as a ligand for AhR (data not shown). It is interesting to note that Arnt2 possesses an activation domain with close sequence similarity to Arnt at the C-terminus (*38*). We localized the interaction domains of Arnt and CBP by introducing a series of deletion mutations into CBP and Arnt. Arnt contains the characteristic structural motifs of bHLH and PAS domains necessary for the DNA-binding and dimerization activities with itself and other bHLH/PAS proteins, and these domains apparently did not contribute to the interaction with CBP. On the other hand, deletion of the transactivation domain of Arnt from GADArnt(1-789) [GADArnt(1-617)] markedly reduced the β-galactosidase activity to the background level, while a GADArnt(597-789) fusion protein which carries the transactivation domain exhibited a weakly but significantly enhanced β-galactosidase expression, indicating interaction with GBT-CBP(1-1891). These results suggested that CBP mediated transactivation effects of Arnt through their mutual interaction.

Then we examined the interaction surface of CBP with GADArnt(1-789) by constructing several expression plasmids for GBT-CBP derivatives. GBT-CBP(452-721), which encodes the CREB-binding domain of CBP remarkably enhanced the β-galactosidase expression in association with GADArnt(1-789). The CREB-binding domain of p300 was also involved in interaction with GADArnt(1-789) as shown in Table I. These results showed that CBP/p300

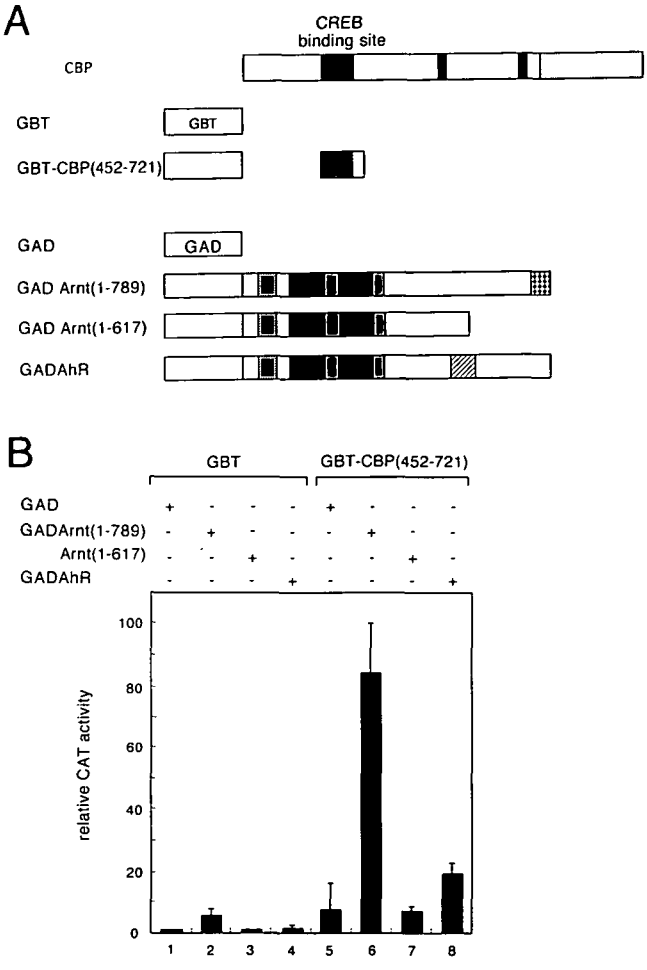

Fig. 4. **Interaction between CBP and Arnt in mammalian two hybrid system.** (A) Schematic representation of GBT-CBP(452-721), GADArnt hybrids and GADAhR. (B) pBosGBT-CBP(452-721) (2 μg) and pBosGADArnt(1-789) or pBosGADArnt(1-617) or GADAhR (2 μg) were cotransfected into 293T cells along with the reporter pG5E1bCAT (3 μg) and pEFBosLacZ (50 ng) which was used as an internal control for efficiency of transfection. The cells were cultured for 40 h after transfection of these plasmid DNAs and the CAT activities were measured as described in "EXPERIMENTAL PROCEDURES."

TABLE I. **Interaction of Arnt and Arnt2 with CBP/p300 in the yeast two hybrid system.** Yeast strain SFY526 containing LacZ under the control of the GAL1 promoter was transformed with the plasmids indicated in the table, and β-galactosidase activities were determined as described in "EXPERIMENTAL PROCEDURES." β-Galactosidase activities of transformants with the combination of GAD424 as prey and the GBT fusion plasmids described on the left side of the table as bait were arbitrarily taken as standard for a series of experiments. The β-galactosidase activities of a series of various parts of Arnt, Arnt2, and AhR as prey with each of the baits were normalized relative to the standards. "−," not determined.

		GAD424	Arnt(1-789)	Arnt(1-617)	Arnt(1-214)	Arnt(116-513)	Arnt(597-789)	Arnt2	AhR
GBT9		1	1.2	1	0.72	0.88	0.43	1	−0.42
GBT-CBP	(1-1891)	1	17	0.93	0.63	0.62	2.6	11	0.6
	(1-452)	1	1.5	−	−	−	−	−	−
	(452-721)	1	48	0.75	0.5	0.65	1.6	41	1
	(721-1679)	1	2.2	−	−	−	−	−	−
	(1678-2441)	1	2	−	−	−	−	−	−
GBT-p300	(437-699)	1	24	−	−	−	−	−	−

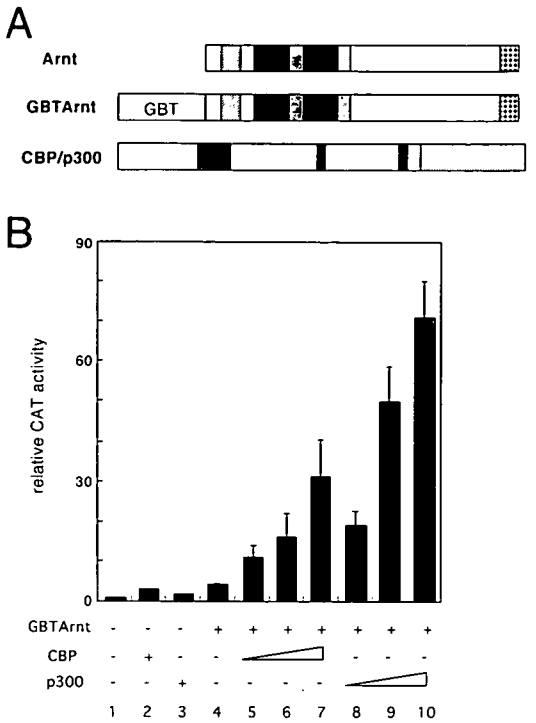

Fig. 5. **Interaction between CBP/p300 and Arnt in mammalian two hybrid system.** (A) Schematic representation of GBTArnt and CBP/p300. (B) pBosGBTArnt (100 ng) and pBosCBP/p300 (0.2, 0.6, and 2 μg) were cotransfected into 293T cells along with the reporter pG5E1bCAT (3 μg) and pEFBosLacZ (50 ng) which was used as internal control for efficiency of transfection. Incubation of the cells transfected with these plasmids and subsequent determination of the CAT activities were as described in the legend to Fig. 4.

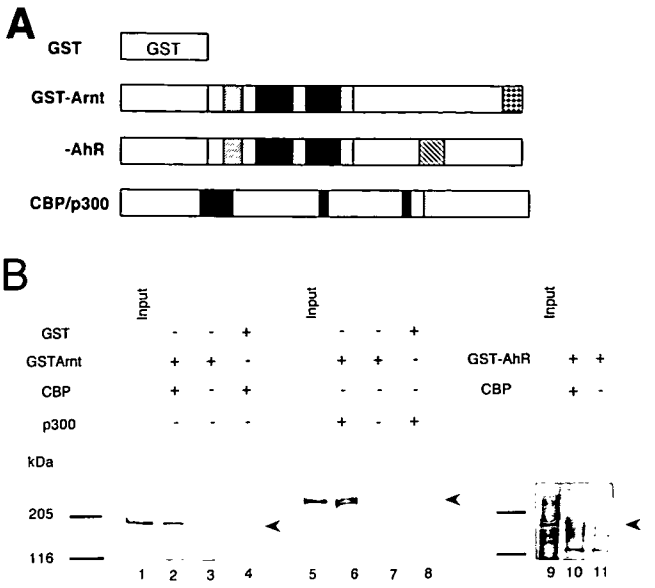

Fig. 6. ***In vivo* interaction between CBP/p300 and Arnt by pull down assay.** (A) Schematic representation of GSTArnt, GST-AhR, and CBP/p300. (B) Human embryonic kidney 293T cells were transfected with the expression plasmids of GST-Arnt or GST-AhR and CBP/p300 (5 μg), and cultured for 40 h. The cell extracts were prepared and incubated with glutathione beads for 2 h. The bound proteins were eluted from the glutathione beads with the sample buffer by boiling and the eluates were subjected to 6% SDS-PAGE. After their translocation to a membrane filter by blotting, the proteins were visualized by the immuno-staining method with anti-CBP or anti-p300 antibody. "Input" indicates whole cell extracts loaded in lanes 1, 5, and 9. Positions of molecular mass markers are shown on the left.

interacted with Arnt *via* the CREB-binding domain.

Interaction between CBP and Arnt in Mammalian Two Hybrid System—Specific interactions between the CBP fragment and Arnt were investigated by using a mammalian two hybrid system. cDNAs from yeast expression plasmids of GBT-CBP(452-721), GADArnt(1-789), GADArnt(1-617), and GADAhR were subcloned into mammalian expression vectors and the resultant expression plasmids (Fig. 4A) were cotransfected into a human embryonic kidney cell line, 293T cells, along with the reporter plasmid pG5E1bCAT with the GAL4 binding sequence in the promoter. The interaction between Arnt and CBP was assessed by measuring the CAT activity expressed from the reporter plasmid. When BosGBT and several GADArnt derivatives or GADAhR were transfected into the cells, the expressed CAT activities were around the background level (Fig. 4B, lanes 1-4). Transfection of GADArnt(1-789) expression plasmid along with BosGBT-CBP(452-721) markedly enhanced the CAT activity (lane 6, 16-fold, compare lanes 2 and 6), while deletion of the Arnt transactivation domain [GADArnt(1-617)] abolished most of the enhanced CAT expression (lane 7). GADAhR expression plasmid also showed only a slight enhancement of the CAT expression (lane 8).

To examine further this association between Arnt and CBP in the mammalian two hybrid system, we cotransfected expression plasmids of Arnt fused to the GAL4 binding domain (GBTArnt) and CBP/p300 into 293T cells (Fig. 5, A and B). The activated CAT expression induced by transfection of GBTArnt (Fig. 5B, lane 4) was further enhanced by coexpression of CBP or p300 in a dose-dependent manner (Fig. 5B, lanes 5-10). Since E1A is known to be expressed in 293T cells, transactivation activities of endogenous CBP/p300 are thought to be repressed to some extent. Thus, the transfected expression plasmid of CBP/p300 produced a sufficient amount of CBP/p300 to overcome this repression by E1A and to enhance further the activated CAT expression by GBTArnt. These results confirmed that CBP/p300 interacted with Arnt in mammalian cells as well as yeast cells.

Interaction between CBP or p300 and Arnt in Cells—To substantiate further the interaction between CBP/p300 and Arnt, we investigated by means of pull down experiments whether CBP/p300 was associated with Arnt in cells cotransfected with the expression plasmids of Arnt and CBP/p300. In this assay, Arnt was GST-tagged on the N-terminus (Fig. 6A, GST-Arnt). The cell extracts were prepared from 293T cells cotransfected with CBP/p300 and GST-Arnt expression vectors and incubated with glutathione-Sepharose beads. The proteins adsorbed on the Sepharose beads were eluted in the sample buffer and subjected to SDS-PAGE. The proteins bound to the GST-Arnt were detected by the immuno-blot method using either anti-CBP or anti-p300 antibody. As shown in Fig. 6B, CBP was adsorbed on the glutathione Sepharose beads only when CBP was coexpressed with GST-Arnt in the 293T cells (lane 2), while it was not detected in the Sepharose

beads when expressed together with the truncated GST (lane 4). The same result was obtained with the experiment using p300 expression plasmid (lane 6). These results showed that both CBP and p300 interacted with Arnt *in vivo*. The size of CBP expressed in 293T cells appeared to be smaller than that expected for the full length CBP, but is consistent with the value reported for *in vitro*-synthesized CBP (*30*). Similar experiments were carried out by using GST-tagged AhR. CBP was barely found associated with GST-tagged AhR (lane 10), in agreement with the result of yeast two hybrid experiments.

DISCUSSION

By using two hybrid systems in yeast and mammalian cultured cells, we revealed that Arnt, but not AhR of the AhR/Arnt heterodimer, interacted with CBP/p300 in the C-terminal region containing its assigned activation domain. The CREB-binding site of CBP/p300 was found to be involved in the interaction with Arnt. This interaction was confirmed by a pull down experiment using an expression plasmid of GST-Arnt fusion protein. Arnt2 has its activation domain at the C-terminus with a close sequence similarity to that of Arnt (*38*), and was also found to interact with CBP/p300. Arnt and Arnt2 proteins constitute a family of sequence-specific transcription factors with characteristic structural motifs of basic helix-loop-helix and PAS together with AhR, mSim1 (*44*), mSim2 (*45*), dSim, dTrh (*46, 47*), HIF-1α, and HLF. These members of the family form heterodimers to function as sequence-specific transcription regulators which recognize their own enhancer DNA elements. Since Arnt and Arnt2 are known to be common partners in the heterodimer formation of the bHLH/PAS proteins, it is possible that CBP/p300 functions as a coactivator common to all the functional combinations of bHLH/PAS transcription factors in association with Arnt or Arnt2. During the preparation of this paper, Arany *et al.* reported that p300 also binds HIF-1α under hypoxic conditions, inducing the expression of genes encoding certain glycolytic enzymes, erythropoietin, and VEGF (*48*). The binding site of p300 to HIF-1α was found to be the first cysteine/histidine-rich region, C/H1, different from that to Arnt or Arnt2 which is the CREB-binding site, although the interactive site on HIF-1α remains to be identified. These observations suggest that the two chains of the HIF-1α/Arnt heterodimer interact independently with the two sites of CBP/p300. It has been reported that Arnt and AhR have their own transactivation domains in the C-terminal halves (*16-19*). The activity of the activation domain of Arnt is sensitive to the inhibition of E1A, while that of AhR is relatively insensitive to E1A (*16*). If the two activation domains of the AhR/Arnt heterodimer function independently, the pathway of the transactivation signal of AhR to the basic transcriptional machinery remains unknown. Based on deletion experiments with AhR and Arnt, Whitlock *et al.* claimed that the activation domain of AhR contributed mainly to the transcriptional activation due to the AhR/Arnt heterodimer in drug-inducible gene expression, because little transactivation activity was observed with a heterodimer of Arnt and truncated AhR which lacks the C-terminal activation domain (amino acid 424 to 805) (*49*). However, their suggestion may be incorrect, because (1) a closer look at the results of their deletion experiments showed that deletion of the Arnt activation domain substantially reduced (about 50%) the transactivation activity of the AhR/Arnt heterodimer (*18*), (2) further deletion from amino acid 424 to 82 in the truncated AhR restored the transactivation activity of Arnt in the heterodimer of AhR/Arnt, indicating that the sequence from amino acid 82 to 424 of AhR possesses an inhibitory effect on the transactivation activity of Arnt in the AhR/Arnt heterodimer (unpublished observation), and (3) a modified AhR with replacement of the Arnt activation domain (a.a. 597 to 789) for that of AhR (a.a. 564 to 805) formed a heterodimer with Arnt to show a transactivation activity on the XRE enhancer as high as or even higher than that of the AhR/Arnt heterodimer (unpublished observation).

As shown in Fig. 1, E1A repressed the inducible expression of CYP1A1 to the basal level. This suggests that E1A inhibits not only the activity of Arnt *via* CBP/p300 but also other unknown transcriptional mechanisms including a transactivation by AhR. It was reported that E1A inhibits the activity of SWI/SNF (*50*), which plays a transcriptional role for reorganizing the chromatin structure. Modification of the chromatin structure around the XRE sequences in CYP1A1 promoter in response to the inducer was demonstrated by *in vivo* footprinting analyses (*51*).

Although we revealed an interaction of CBP/p300 with Arnt in *in vivo* binding assay by expressing these factors in 293T cells, we failed to detect this interaction in an *in vitro* binding assay using *in vitro* synthesized Arnt and bacterially expressed GST-CBP fusion protein under our experimental conditions (data not shown). These observations could be explained by either of the following two possibilities. Firstly, direct interaction between CBP/p300 and Arnt which may be too weak to be detected by the *in vitro* binding assay, but could be mediated or strengthened by other factor(s) missing in the system. It has recently been reported that CBP formed a complex with SRC-1(p160) to enhance synergistically steroid hormone receptor-dependent transcription (*52*). Interestingly, SRC-1 is a member of the bHLH/PAS family like Arnt and AhR. The bHLH and PAS motifs are known to function as protein/protein interaction interfaces. It would be interesting to investigate whether SRC-1 is involved in the transactivation pathway of the Arnt/AhR heterodimer. Secondly, post-translational modification of Arnt or phosphorylation may be required for the interaction between Arnt and CBP/p300, as reported for the interaction between CREB and CBP/p300. Since Arnt is believed to be phosphorylated to interact with AhR (*12*), we determined the phosphorylation site of Arnt by metabolic labeling of Arnt transiently expressed in 293T cells with ^{32}P-phosphate. While a major phosphorylation site of Arnt was a serine in the bHLH domain, the transactivation domain of Arnt was not significantly phosphorylated (data not shown). Replacement of the serine residue in the bHLH with alanine had little effect on the transactivation activity of AhR/Arnt heterodimer. Although these results suggested that phosphorylation of the major site is not involved in the transactivation activity of AhR/Arnt heterodimer, it remains to be seen whether a minor phosphorylation site plays a crucial role in the interaction between Arnt and CBP/p300.

It has been reported that the AhR/Arnt heterodimer and Sp1 synergistically enhance the inducible expression of the

CYP1A1 gene in response to inducers (*14*). This synergistic effect was found to result from direct interaction between the two transcription factors. When either the AhR/Arnt heterodimer or Sp1 binds its cognate DNA element, the DNA binding activity of the second factor was facilitated. The same situation has been recently reported in the expression of cholesterol-regulated genes such as LDL gene (*53*). The synergistic transcriptional activation of the genes by SREBP and Sp1 requires interaction between CBP and SREBP (*54*). It would be interesting to clarify whether CBP is commonly involved in the cooperative interaction between sequence-specific transcription factors, and if so, how.

Given the growing number of sequence-specific transcription factors whose effects are mediated by a coactivator complex containing CBP/p300 and SRC-1 (*55*), a pool of CBP/p300 coactivator is considered to function as a nuclear integrator of various signal transduction pathways (*26*). Some of the transcription factors are reported to compete with one another for binding with CBP/p300, resulting in antagonistic effects with respect to one another. The mechanisms of the antiestrogenic and other diverse toxicological effects exerted by TCDD should be reconsidered in this light (*56-60*).

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