

# Developmentally regulated expression of the transcriptional cofactors/histone acetyltransferases CBP and p300 during mouse embryogenesis

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**ABSTRACT** CBP (CREBBP/ CREB-binding protein) and p300 are related signal-dependent transcriptional cofactors and histone acetyltransferases. They are both implicated in tumorigenesis and mutations in the human *CBP* gene have been found in Rubinstein-Taybi syndrome (RTS), which is characterized by multiple developmental defects and mental retardation. Studies with *CBP* and *p300* mouse mutants indicate that both proteins are required for normal development, and that there is an essential gene dosage-sensitive role for these transcriptional cofactors in embryogenesis, cell differentiation and proliferation. Although it is generally believed that the expression of CBP and p300 is ubiquitous, we report here that they are developmentally regulated during mouse embryogenesis. In the developing CNS, CBP and p300 proteins were found throughout the newly formed neural plate, but their expression was later restricted to the dorsal parts of the developing neural tube. Later in neural development, CBP and p300 proteins could also be found in subsets of ventral neurons, including motor neurons and oligodendrocytes. During organogenesis, CBP and p300 proteins were expressed in specific cell types of the developing heart, vasculature, skin, lung and liver. Many of these tissues and organs are known to be affected in mutant mice lacking CBP and/or p300, and in RTS patients. Interestingly, while CBP and p300 proteins show extensive overlapping expression during mouse embryogenesis, we observed that their subcellular localization is developmentally regulated in several cell types. Taken together, our results suggest that there are common, as well as distinct, biochemical functions of CBP and p300 during mouse development.

**KEY WORDS:** *Rubinstein-Taybi syndrome, neural development, heart, hair follicle, Sonic hedgehog*

## Introduction

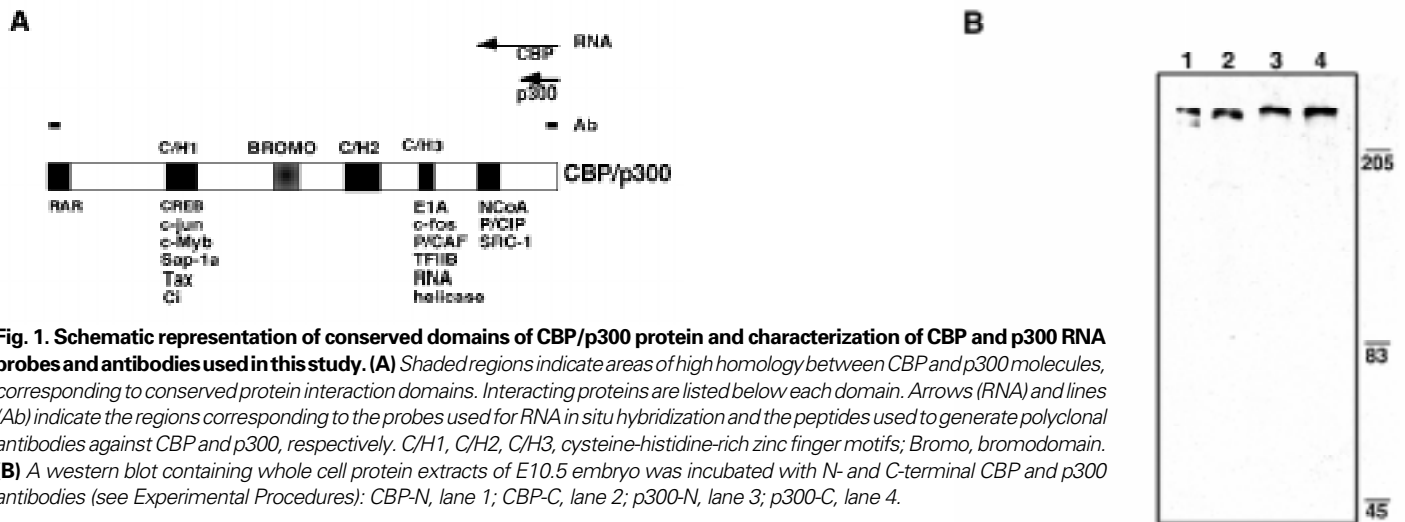
CBP and p300 are histone acetyltransferases, which presumably remodel chromatin structure, and function as cofactors in multiple signal-dependent transcriptional events (for review, see Giles *et al.*, 1998). CBP was originally isolated as a protein interacting with the cAMP-responsive transcription factor CREB (Chrivia *et al.*, 1993), and p300 was identified as one of the cellular proteins targeted by the adenovirus E1A protein (Eckner *et al.*, 1994). CBP and p300 are closely related proteins (over 60% identical at the amino-acid level), which interact directly with a variety of sequence-specific transcription factors (like CREB, JUN and p53), several components of the basal transcriptional machinery, other histone acetyltransferases (SRC1, ACTR and P/CAF), as well as viral oncoproteins (E1A, large T antigen and Tax). CBP and p300 are evolutionarily conserved as

their *Caenorhabditis elegans* and *Drosophila* homologs show striking structural similarity to their mammalian counterparts and perform similar functions as transcriptional cofactor and histone acetyltransferases (Akimaru *et al.*, 1997a; Shi and Mello, 1998).

CBP/p300 plays pivotal roles in embryonic development and is implicated in tumorigenesis. The role of CBP/p300 in development and cancer was first suggested by the observations that the human *CBP* gene was disrupted in a dominant genetic disorder, Rubinstein-Taybi Syndrome (RTS), which is characterized by craniofacial and limb defects, mental retardation as well as developmental anomalies of the eye, heart, kidney, lung, skin and

*Abbreviations used in this paper:* CREB, cAMP-response element binding protein; RA, retinoic acid; RTS, Rubinstein-Taybi syndrome.

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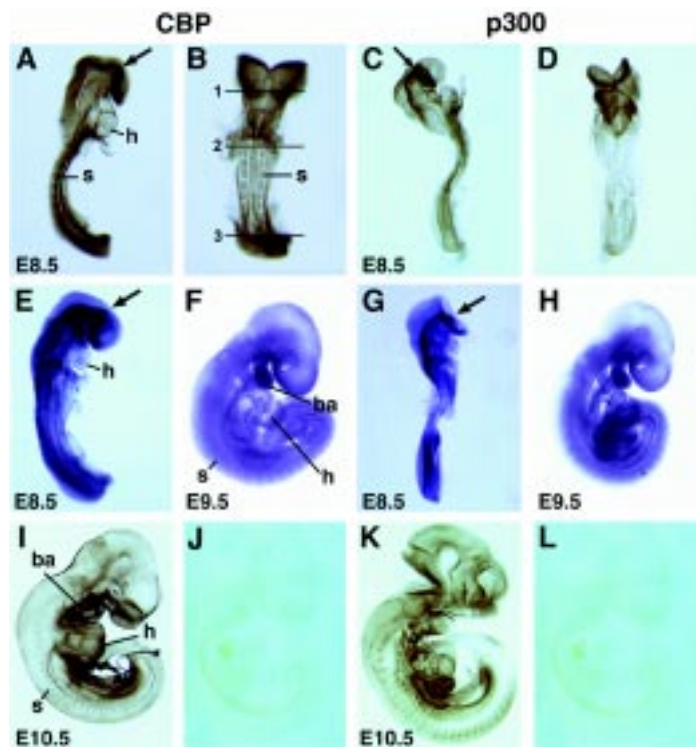
**Fig. 1. Schematic representation of conserved domains of CBP/p300 protein and characterization of CBP and p300 RNA probes and antibodies used in this study.** (A) Shaded regions indicate areas of high homology between CBP and p300 molecules, corresponding to conserved protein interaction domains. Interacting proteins are listed below each domain. Arrows (RNA) and lines (Ab) indicate the regions corresponding to the probes used for RNA *in situ* hybridization and the peptides used to generate polyclonal antibodies against CBP and p300, respectively. C/H1, C/H2, C/H3, cysteine-histidine-rich zinc finger motifs; Bromo, bromodomain. (B) A western blot containing whole cell protein extracts of E10.5 embryo was incubated with N- and C-terminal CBP and p300 antibodies (see Experimental Procedures): CBP-N, lane 1; CBP-C, lane 2; p300-N, lane 3; p300-C, lane 4.

testes (see review by Rubinstein, 1990; Petrij *et al.*, 1995). RTS patients also have an increased predisposition to cancer (Miller and Rubinstein, 1995). Somatic inactivation of both p300 alleles has been observed in gastric and colon cancers, supporting the hypothesis that CBP/p300 function as tumor suppressors (Muraoka *et al.*, 1996). CBP and p300 are also involved in somatic translocations associated with various types of hematological malignancies (see Giles *et al.*, 1998). In contrast to the tumor suppressor function of wild type CBP and p300, it has been postulated that the CBP and p300 fusion proteins generated by these chromosomal translocations act as dominant oncoproteins affecting cell cycle control and/or cell differentiation.

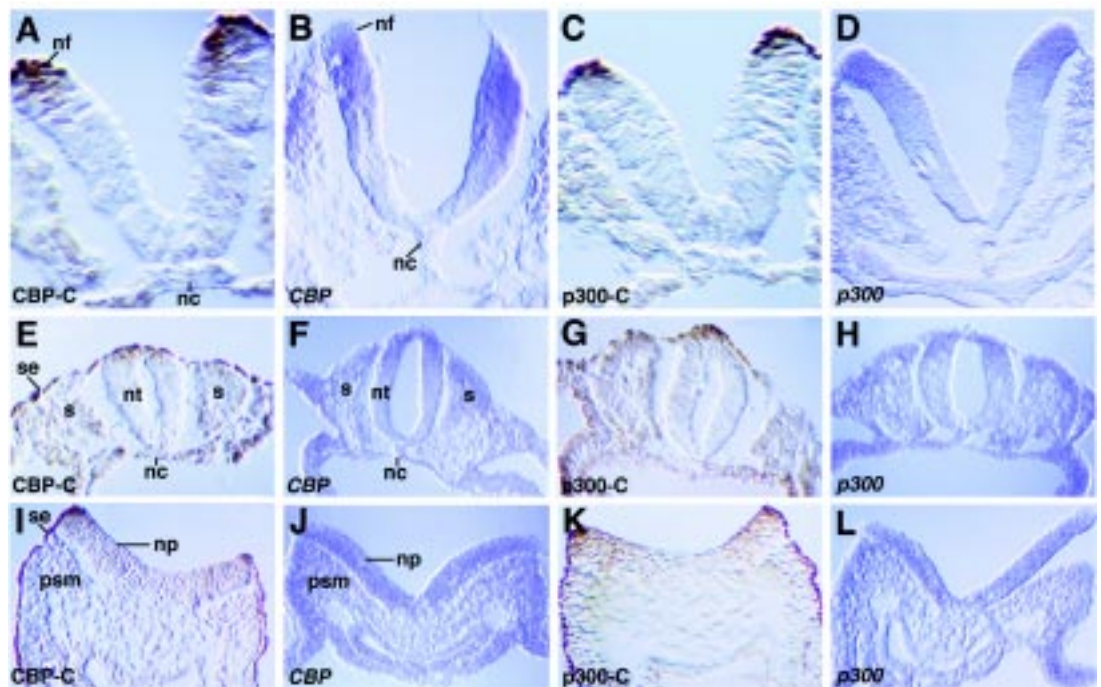
Recent targeted mutagenesis studies in mice have demonstrated that mammalian development is extremely sensitive to the *Cbp* and *p300* gene dosage. Consistent with the notion that RTS is caused by the inactivation of one copy of *CBP*, mice heterozygous for a targeted mutation of *Cbp* have been shown to exhibit skeletal abnormalities, some of which resemble RTS (Tanaka *et al.*, 1997). Analysis of *p300* mutant mice indicated that the gene dosage of *p300* is also important for development (Yao *et al.*, 1998). All *p300*<sup>-/-</sup> embryos die at or before E11.5 with severe CNS and heart abnormalities, and some *p300*<sup>+/-</sup> mice die *in utero* with neural tube closure defects. Strikingly, *Cbp*<sup>+/-</sup>, *p300*<sup>+/-</sup> mouse embryos display an exencephalic phenotype similar to *p300*<sup>-/-</sup> and *Cbp*<sup>-/-</sup> mouse embryos. These observations suggest that CBP and p300 can perform similar biochemical functions during embryonic development.

Although it is well established that CBP and p300 are involved in transcription, cancer and development, very little is known about their expression during development. In this study, we report the analysis of CBP and p300 expression during mouse embryogenesis by using RNA *in situ* hybridization and immunohistochemistry. In contrary to the general belief that CBP and p300 expression is ubiquitous, their expression was shown to be regulated during mouse embryogenesis in a stage- and tissue-specific manner. Consistent with the notion that CBP and p300 can perform similar biochemical functions during development, they were co-expressed during mouse embryogenesis. CBP and p300 proteins were found to be highly expressed in many tissues that are known to be affected in *Cbp* and *p300* mutant mice as well as RTS patients.

Intriguingly, the subcellular distribution of CBP and p300 were also developmentally regulated in some cell types. Our results provide evidence suggesting that CBP and p300 can perform common as well as distinct biochemical functions during mouse development.



**Fig. 2. Analysis of CBP and p300 expression in E8.5-E10.5 mouse embryos by whole-mount immunohistochemistry and RNA *in situ* hybridization.** E8.5-E10.5 embryos were incubated with anti-peptide polyclonal CBP (A,B,I,J) and p300 (C,D,K,L) antibodies. The specificity of the antibodies was tested by preincubating CBP-N (J) and p300-N (L) antibodies with synthetic peptide before application to the embryos. E8.5-E9.5 embryos were examined by RNA *in situ* hybridization using *Cbp*- (E,F) and *p300*- (G,H) specific antisense RNA probes. Arrows indicate the strong RNA and protein expression in the developing cranial neural folds. The levels of sections in Figure 3 are indicated in Figure 2B. ba, first branchial arch; h, heart; s, somites.



**Fig. 3. Protein and mRNA distribution of CBP and p300 during neural patterning.**

Sections through the foregut (A-D), heart (E-H) and tail (I-L) levels of E8.5 mouse embryo were used for section immunohistochemistry (A,C,E,G,I,K) and RNA *in situ* hybridization (B,D,F,H,J,L). The levels of sections are indicated in Figure 2B (level 1 corresponds to Figures 3A-D, level 2 to 3E-H and level 3 to 3I-L). Frozen sections were incubated with CBP-C (A,E,I) and p300-C (C,G,K) antibodies. nc, notochord; nf, neural folds; np, neural plate; nt, neural tube; psm, presomitic mesoderm; s, somites.

## Results

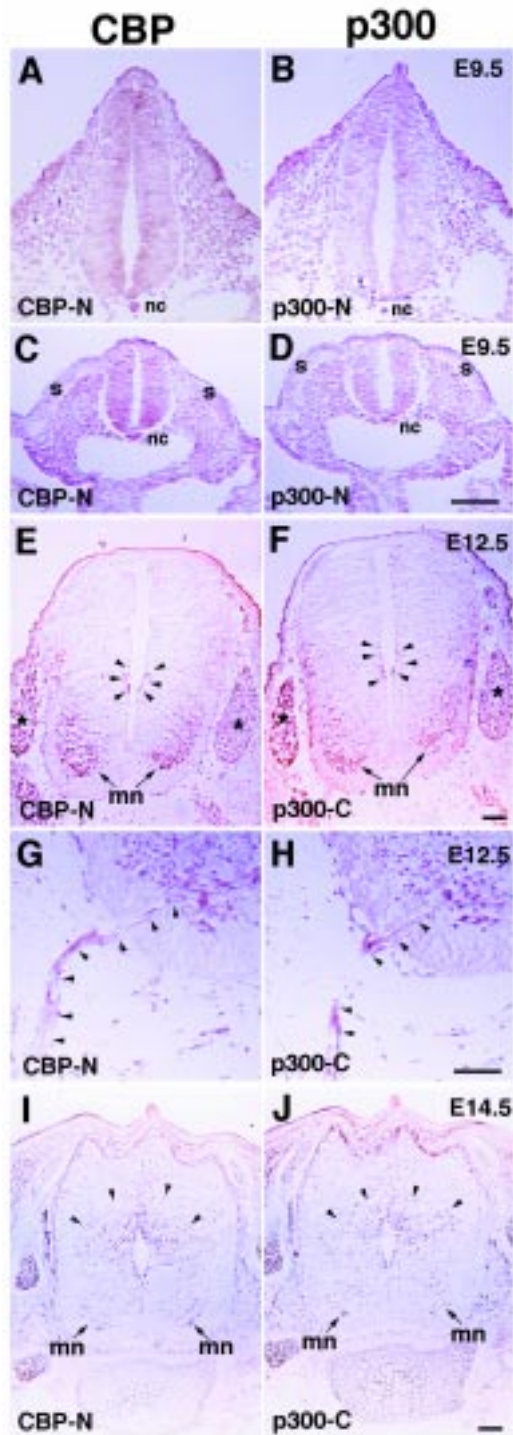
To study the developmental expression of *Cbp* and *p300* during mouse embryogenesis, we performed RNA *in situ* hybridization as well as immunohistochemistry on whole-mount embryos (E7.5-E10.5) and embryo sections (E10.5-E18.5). Two CBP-specific and two p300-specific antibodies (see Fig. 1 and Materials and Methods for details) were used in this study. The specificity of the antibodies was confirmed by western blotting (Fig. 1). In general, protein distribution is concordant with RNA distribution except that protein expression appears to be more restricted. The specificity of immunostaining was confirmed by preincubation of the antibodies with specific peptide competitors (see Fig. 2I-L). By both assays, *Cbp* and *p300* expression could be detected as early as E7.5 (the earliest stage we have examined). While their expression was found to be quite ubiquitous in E7.5-E9.5 mouse embryos (Fig. 2A-H), there was a developmental stage- and tissue-specific restriction of *Cbp* and *p300* expression during organogenesis. Furthermore, although *Cbp* and *p300* displayed mostly overlapping expression throughout mouse embryogenesis, immunohistochemical studies revealed some clear difference in both the cellular and subcellular distribution of the proteins. In the following sections, we will present their expression patterns with special emphasis to RTS and the phenotypes observed in *Cbp* and *p300* mutant mice.

### Neural development

Mutant mice lacking either *Cbp* or *p300* function have recently been shown to exhibit a severe neural tube closure defect and, strikingly, *Cbp;p300* double heterozygous mutant embryos also develop a similar phenotype (Yao *et al.*, 1998). These observations indicate that both CBP and p300 are essential for normal development of the CNS and their gene dosage is critical. During neural development and differentiation, we found that CBP and

p300 display a dynamic expression pattern. At E8.5, both *Cbp* and *p300* transcripts were broadly distributed throughout the developing neural tube (Fig. 2E,G). In the caudal part of the embryos, cells at all mediolateral positions of the newly formed neural plate expressed *Cbp* and *p300* transcripts (Fig. 3J,L). At more rostral levels where the neural tube had begun to fold, they were not expressed medially (not shown) and their transcripts were further restricted to the dorsal part of the neural fold/tube in the head and trunk regions, respectively (Fig. 3B,D,F,H). Immunohistochemical studies revealed a similar and even more profound accumulation of CBP and p300 proteins to the dorsal regions of the developing CNS (Fig. 3A,C,E,G). This progressive dorsal expression of CBP and p300 in the developing neural tube is reminiscent of the expression of PAX3/7 and MSX1/2 which are up-regulated by both the dorsalizing BMP/TGF $\beta$  signal and inhibited by the ventralizing Shh signal during neural patterning (Liem *et al.*, 1995; Ericson *et al.*, 1996).

Later in neural development, we observed a change in CBP and p300 expression (Fig. 4). In the developing hindbrain of E9.5 mouse embryos, high levels of CBP and p300 proteins were found in the ventricular epithelium (Fig. 4A,B). At this stage, CBP and p300 were detected throughout the neural tube in the tail region which is relatively less differentiated (Fig. 4C,D). In the spinal cord of E12.5 mouse embryos, CBP and p300 were highly expressed in two symmetrical groups of cells in the ventral ventricular epithelium dorsal to the floor plate, regions comprising the precursors of oligodendrocytes (Fig. 4E,F). In addition, CBP and p300 were also expressed in two bilaterally symmetrical columns of cells in the ventral spinal cord adjacent to the floor plate, the region that comprises motor neurons (Fig. 4E,F). Consistent with the notion that these CBP/p300-positive cells are oligodendrocyte precursors and motor neurons, CBP and p300 expression was found at E14.5 in scattered cells which appear like oligodendrocytes (see Richardson *et al.*, 1997) and in the motor neuron region



**Fig. 4. Expression of CBP (A,C,E,G,I) and p300 (B,D,F,H,J) proteins in E9.5-E14.5 spinal cord.** At E9.5, CBP and p300 were found at higher levels in the ventricular zone of the neural tube at the foregut level (A,B) whereas, CBP and p300 proteins were expressed throughout the neural tube in the tail region (C,D). At E12.5, CBP (E) and p300 (F) proteins were found in the motor neuron area (indicated by arrows), some cells in the ventricular zone (arrowheads) and in the dorsal root ganglia (asterisks). Their expression was also detected in the axons of the motor neurons (G,H). At E14.5, CBP (I) and p300 (J) proteins were found in scattered oligodendrocyte-like cells (indicated by arrowheads) in addition to the expression in the motor neuron area (arrows). mn, motor neuron; nc, notochord; s, somites. Bar, 100  $\mu$ m.

(Fig. 4I,J). In parallel experiments, we confirmed that CBP/p300-positive cells in the motor neuron region overlap with Isl1/2-positive motor neurons (data not shown). Furthermore, CBP and p300 were also found in axons projected from ventral neurons suggesting that these latter groups of CBP/p300-positive cells are motor neurons (Fig. 4G,H). In the PNS, high levels of CBP and p300 proteins were found in the dorsal root ganglia and sympathetic ganglia (Fig. 4E,F,I,J; data not shown).

#### Vertebral and limb development

Heterozygous *Cbp* mutant mice exhibit limb and vertebral abnormalities similar to those observed in patients with RTS which carry *CBP* mutations (see Tanaka *et al.*, 1997). *In vitro* experiments have also shown that CBP and p300 are involved in muscle-specific gene expression (Eckner *et al.*, 1996; Puri *et al.*, 1997; Sartorelli *et al.*, 1997). Consistent with a role in limb and somite development, CBP and p300 proteins were found in the developing limbs, notochord and somites (Figs. 2A-H, 3E-H and 5A-D; data not shown). Later in development, they were expressed in somite derivatives such as muscle (data not shown) and cartilage (Fig. 5E,F). These data support the hypothesis that CBP and p300 play a role in the differentiation of muscle and cartilage cells.

#### Heart development

Heart development is perturbed in mouse embryos lacking p300 (Yao *et al.*, 1998). In p300 mutant hearts, the ventricular chamber exhibited reduced trabeculation and the expression of cardiac muscle-specific genes was significantly reduced. *Cbp* and p300 expression could be detected in the developing heart as early as E8.5 (Fig. 2A,C,E,G). They continued to be highly expressed in the heart at E9.5 and E10.5 (Fig. 2F,H,I,K). At E12.5, CBP and p300 were expressed in the trabeculated walls of the ventricles (Fig. 5G,H,I,J) as well as in the walls of the atria (data not shown). At E14.5, CBP and p300 expression in the heart was no longer detectable by immunohistochemistry (data not shown). It is worth noting that one third of RTS patients suffer from congenital or other heart defects (Rubinstein, 1990; Stevens and Bhakta, 1995) and CBP and p300 are involved in the expression of cell type-specific gene expression in cardiac myocytes (Bishopric *et al.*, 1997; Hasegawa *et al.*, 1997). Together, these observations suggest that both CBP and p300 have a critical function in heart development.

#### Skin development

During skin development, we found that CBP and p300 displayed a biphasic expression. At E8.5, high levels of CBP and p300 proteins could be detected in the surface ectoderm (Fig. 3E,G,I,K). Later at E12.5, their expression was only found in the peridermal layer of the surface ectoderm (Fig. 6A,B). After the peridermal layer shed off, CBP and p300 protein expression was not detectable in the surface ectoderm until E16.5 (Fig. 6C-F). At E16.5, their expression could be found throughout the surface ectoderm in both the intermediate and basal layers of the epidermis (Fig. 6E,F). Later at E18.5, they were expressed in the basal cell layer and also in some cells of the suprabasal layer of the epidermis (Fig. 6G-J). Epidermal derivatives such as hair and whisker also expressed CBP and p300 (Figure 6G,H,K,L and data not shown). CBP and p300 proteins were highly expressed in the developing hair shaft, including the germinal matrix and the inner

and outer root sheaths, which are derived from the epidermis. Interestingly, RTS patients are known to have high incidence of skin defects such as keloids and hypertrophic scars as well neoplasms (Hendrix and Greer, 1996). Based on our results, we hypothesize that CBP and p300 may play multiple roles during skin and hair development.

#### Development of other organs

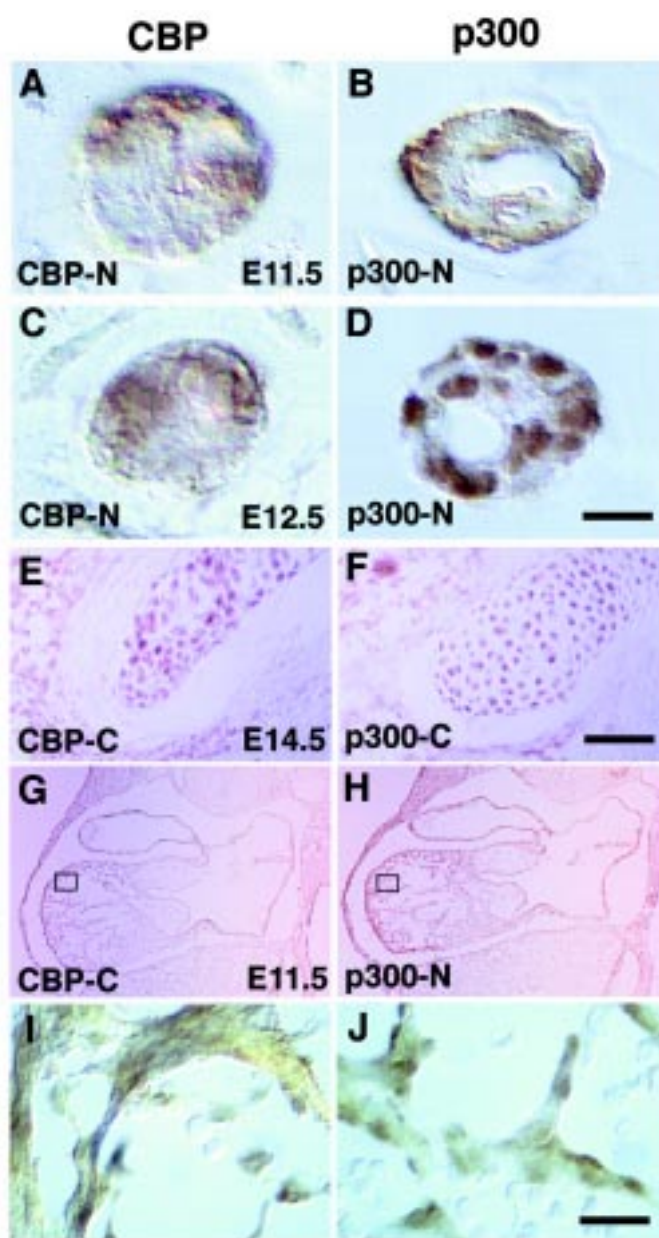
RTS is associated with a high incidence of malformations and diseases of the eye, lung and kidney (Rubinstein, 1990). Consistent with these findings, CBP and p300 were expressed during eye, lung and kidney development. At E18.5, CBP and p300 proteins were found in both neural (retina) and non-neural (lens) components of the eye (Fig. 7A,C). In the lens, high level of expression could be found in the anterior cuboid epithelium (Fig. 7B,D). Both CBP and p300 were expressed in the developing foregut endoderm which gives rise to the lung. At E12.5, they were strongly expressed in the epithelium of the oral cavity as well as the rest of the foregut (Fig. 7E,F and data not shown). At E14.5, both CBP and p300 proteins could be found in the epithelium of the esophagus (Fig. 7G-H), whereas CBP was detected only in the lung epithelium and p300 protein was detected in some mesenchymal cells adjacent to the lung epithelium (Fig. 7I-J). In the developing kidney, CBP and p300 proteins were found in some mesenchymal cells of the mature glomeruli (data not shown). Furthermore, their expression could also be found in the endothelial cells in the blood vessel (Fig. 7K,L) and some scattered cells in the liver (Fig. 7M,N). These liver cells may be precursors of B lymphocytes since CBP and p300 have been implicated in the transcriptional control of B lymphocyte gene expression (Eckner *et al.*, 1996). In summary, the restricted expression of CBP and p300 in these cell types is consistent with their suggested functions.

#### Subcellular localization of CBP and p300

Previous studies have indicated that CBP and p300 proteins are mostly restricted to the nucleus of the cultured cells (Yaciuk and Moran, 1991; Chrivia *et al.*, 1993; Eckner *et al.*, 1994; Puri *et al.*, 1997). Our studies here revealed that the subcellular localization of CBP and p300 are developmentally regulated during mouse embryogenesis. For example, in the developing notochord, while CBP protein was both nuclear and cytoplasmic (Fig. 5A,C), the subcellular localization of p300 proteins shifted from being uniformly distributed in the cells at E11.5 to nuclear at E12.5 as confirmed by DAPI-staining (Fig. 5B,D and data not shown). p300 was also found to be localized mostly in the cytoplasm in the basal cells of the epidermis (Fig. 6J). In many cell types, p300 protein was found to be nuclear (see Figs. 5D,F, 6B and 7D,F,J,L). On the other hand, CBP was either nuclear (see Figs. 5E and 7B) or both nuclear and cytoplasmic (Fig. 5A,C, 6A and 7E,I,K). These observations suggest that although CBP and p300 show extensive overlapping expression during mouse embryogenesis, they might serve different functions in various cell types depending on their subcellular distribution.

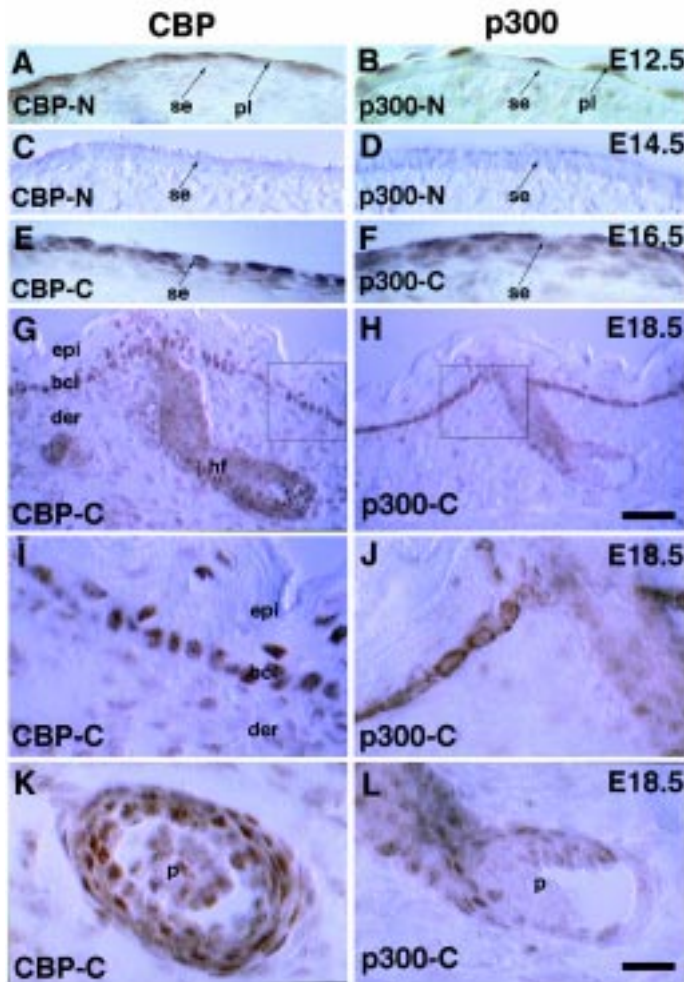
#### Discussion

This study establishes that CBP and p300 expression is developmentally regulated during mouse embryogenesis. Since CBP and p300 proteins can be found in all tissue culture cells



**Fig. 5. Expression of CBP and p300 proteins in the developing notochord, bone and heart.** Immunostaining of CBP and p300 proteins in E11.5 (A,B) and E12.5 (C,D) notochord, E14.5 rib cartilage (E,F) and E11.5 heart (G-J). Bar, 25  $\mu$ m in A-D; 100  $\mu$ m in E-F; 50  $\mu$ m in I-J.

examined, it is generally thought that their expression is ubiquitous. By using RNA *in situ* hybridization, Yao *et al.* (1998) recently reported that p300 mRNA is widely expressed at E14.5 and E16.5 during mouse embryogenesis. However, our immunohistochemical analysis demonstrates that p300 protein expression is developmentally regulated in a stage- and tissue-specific manner during mouse embryogenesis. Analysis of p300 mutants suggest that p300 is essential for cellular proliferation both *in vivo* and *in vitro*; embryos lacking p300 are significantly smaller than their wild-type counterparts and p300<sup>-/-</sup> mouse embryonic fibroblasts stop dividing after three to four cell generations in culture (Yao *et*



**Fig. 6. Expression of CBP and p300 proteins during skin development.**

At E12.5, CBP (A) and p300 (B) proteins were detected in the peridermal layer of the surface ectoderm. At E14.5, CBP and p300 protein expression was not found in the ectoderm after the periderm had shed off (C,D). CBP and p300 proteins were detected again throughout the surface ectoderm at E16.5 (E,F). At E18.5, CBP (G,I,K) and p300 (H,J,L) were highly expressed in the basal cell layer as well as the developing hair follicle. Higher magnification of the boxed areas (see G,H) illustrates that CBP protein was mainly nuclear in the basal cell layer (I) while p300 protein was both cytoplasmic and nuclear (J). During hair development, CBP and p300 proteins were expressed in the dermal papilla cells (K,L). bcl, basal cell layer; der, dermis; epi, epidermis; hf, hair follicle; p, dermal papilla; pl, periderm; se, surface ectoderm. Bar, 50  $\mu$ m in A-H; 25  $\mu$ m in I-L.

al., 1998). In fact, this observation might explain why ubiquitous expression of p300 is found in tissue culture cells.

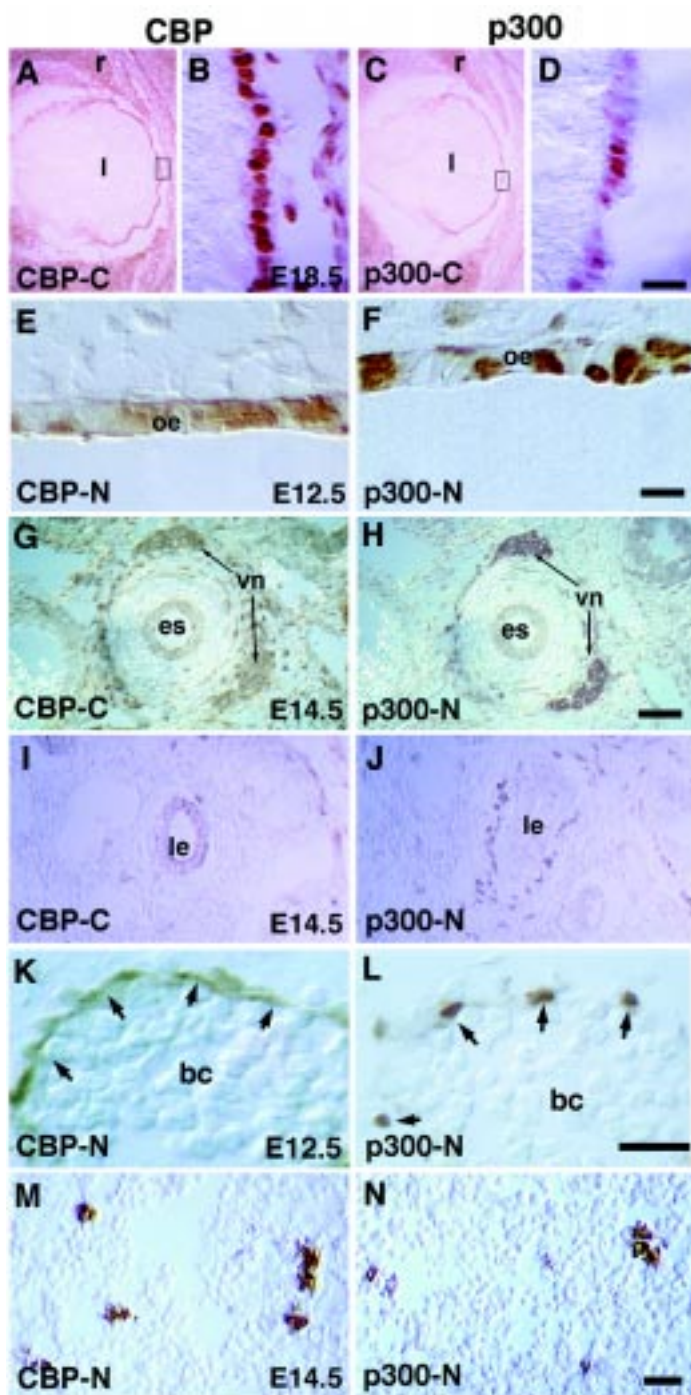
During mouse embryogenesis, CBP and p300 display almost identical expression patterns. The only difference we could identify in our study was in the developing lung, where CBP protein was found in the epithelium and p300 protein was detected in the mesenchyme (Fig. 7I,J). Most intriguingly, we observe a developmental stage-specific change in the subcellular distribution of CBP and p300 proteins. Although CBP and p300 are mostly restricted to the nucleus in cultured cells (Yaciuk and Moran, 1991; Chrivia et al., 1993; Eckner et al., 1994; Puri et al., 1997),

our results indicate that the intracellular distribution of CBP and p300 can be controlled during mouse embryogenesis. Since these transcriptional cofactors/histone acetyltransferases are thought to act both in the cytoplasm acetylating proteins and in the nucleus modifying chromatin structure, their subcellular localization might correlate with the cell- and stage-specific function of the protein during mouse embryogenesis.

Developmental anomalies observed in RTS patients as well as in *Cbp* and *p300* mutant mice indicate that the gene dosage of both CBP and p300 is essential for normal embryogenesis. One explanation is that a critical level of CBP/p300 proteins is important for normal development. The observation that *Cbp*<sup>+/-</sup>;*p300*<sup>+/-</sup> mouse embryos display a mutant phenotype similar to *p300*<sup>-/-</sup> and *Cbp*<sup>-/-</sup> embryos strongly supports the notion that CBP and p300 serve redundant functions during embryogenesis. It is also possible that CBP and p300 might possess distinct developmental functions due to differences in their function and/or expression. Supporting the latter explanation, our results indicate that there are indeed subtle differences between tissue-specific and intracellular expression patterns of CBP and p300 during mouse embryogenesis. Several recent observations also indicate that not all CBP and p300 functions are identical. First, embryonic fibroblasts from *p300*<sup>-/-</sup> mice were defective in retinoic acid (RA)-dependent but not cAMP-dependent transcription (Yao et al., 1998). Second, by using a ribozyme approach, Kawasaki et al. (1998) showed that p300, but not CBP, is required for RA-induced differentiation of F9 cells. Interestingly, the same approach demonstrated that expression of both CBP and p300 is required for RA-induced apoptosis and cell cycle exit indicating that various RA-dependent processes can have different requirements for CBP and p300. Third, activation of NF- $\kappa$ B and ATF/CREB pathways by the human T-cell leukemia virus type 1 Tax oncoprotein was shown to be mediated by distinct interactions with CBP and p300 (Bex et al., 1998). This study also demonstrated that endogenous CBP and p300 are localized in different nuclear compartments in a variety of cultured cells. Based on these observations, it is clear that there is also subtle functional difference between CBP and p300 proteins.

Since CBP and p300 function in multiple signaling pathways in cultured cells, it is plausible to speculate that anomalies observed in RTS patients as well as in *Cbp* and *p300* mutant mice are caused by defects in multiple developmental signaling pathways. In *Drosophila*, CBP has been shown to be a transcriptional co-activator of the NF- $\kappa$ B homolog dorsal, which is involved in the activation of *twist*, and the *Gli* homolog *Cubitus interruptus*, which is required for hedgehog signaling (Akimaru et al., 1997a,b). Consistent with the above speculation, *p300*<sup>-/-</sup>, *Cbp*<sup>-/-</sup> and *Cbp*<sup>+/-</sup>;*p300*<sup>+/-</sup> mouse embryos exhibit an exencephalic phenotype strikingly similar to those observed in *twist* and *Gli3* mutant mouse embryos (Hui and Joyner, 1993; Chen and Behringer, 1995). While the molecular mechanism(s) underlying these neural tube defects remains unclear, further analysis of these mutant phenotypes should be highly informative for understanding the roles of CBP and p300 in these developmental signaling pathways.

In conclusion, our data show that the expression of CBP and p300 is developmentally regulated during mouse embryogenesis. There is a subtle difference between CBP and p300 protein expression at both the cellular and subcellular levels. These observations shed new light to the function of CBP and p300 during development and provide a molecular basis for understanding the



**Fig. 7. Expression of CBP and p300 proteins in the developing eye (A-D), foregut endoderm (E-J), endothelial cells (K,L) and liver (M,N).** Both CBP (A,B) and p300 (C,D) proteins were detected in the neural retina as well as the lens of the developing eye. In the lens, CBP and p300 were highly expressed in the anterior cuboid epithelium (B,D). In the developing foregut, CBP (E,G,I) and p300 (F,H) proteins were mostly expressed in the epithelium. High level of p300 protein was also detected in some mesenchymal cells adjacent to the lung epithelium (J). CBP and p300 protein expression was found in the endothelial cells (K,L) and some scattered liver cells (M,N). bc, blood cells; es, esophagus; l, lens; le, lung epithelium; oe, oral epithelium; r, retina; vn, vagus nerve. Bar, 25  $\mu$ m in B,D,F; 50  $\mu$ m in G,H,K-N.

anomalies observed in RTS patients as well as in *Cbp* and *p300* mutant mice.

## Materials and Methods

### Mouse embryo

CD1 mice were purchased from Charles Rivers (Wilmington, MA). Animals were mated overnight and females examined for a vaginal plug the following morning. Noon of the day of evidence for a vaginal plug was considered E0.5. 4-8 embryos were analyzed in each immunohistochemical and *in situ* hybridization experiment, and the experiments were repeated at least three times.

### Probes and *in situ* hybridization analysis

The plasmids used for the generation of antisense RNA probes were CMV-mCBP-HA-1 and CMV $\beta$ -p300 (Chrivia *et al.*, 1993; Eckner *et al.*, 1994). For *Cbp*, a 1.5 kb probe corresponding to 5,742 nt to 7,325 nt of the cDNA, was used. For *p300*, a 0.7 kb probe corresponding to 8,328 nt to 9,046 nt of the cDNA, was used. Specificity of hybridization was controlled using sense probes at E9.5 (data not shown). Whole-mount *in situ* hybridization was carried out essentially as described (Ding *et al.*, 1998) except that embryos (E8.5-E10.5) and limb buds (E11.5-E13.5) were permeabilized with 20  $\mu$ g/ml (embryos) or 10  $\mu$ g/ml (limbs) Proteinase K. After *in situ* hybridization, the embryos were fixed with 4% paraformaldehyde in PBS, incubated in 30% sucrose in PBS and mounted in OCT. Frozen sections were cut at 20  $\mu$ m.

### Antibodies and immunohistochemistry

Whole-mount (E8.5-E10.5) and section (E8.5-E18.5) immunohistochemistry was performed using polyclonal antibodies generated against the amino- and carboxyl-terminal peptides of CBP (CBP-N=A22 (cat# sc-369); CBP-C=C20 (cat# sc-583); Santa Cruz) and of p300 (p300-N=N15 (cat# sc-584); p300-C=C-20 (cat# 585); Santa Cruz). By western blot analysis, CBP-N and CBP-C antibodies were shown to be specific for CBP, and p300-N and p300-C antibodies were specific for p300 (Fig. 1).

Whole-mount immunohistochemistry was carried out essentially as described (Dent *et al.*, 1989). For section immunohistochemistry, embryos were fixed in 1% formaldehyde, 0.2% glutaraldehyde, 0.02% NP-40 in PBS for a duration of overnight to 7 days depending on the stage of the embryo. After fixation, embryos were incubated in 30% sucrose in PBS until the embryos sank to the bottom of the tube. Embryos were mounted in OCT and stored in -80°C. Frozen sections were cut 10-15  $\mu$ m thick. Endogenous peroxidases were inactivated by incubating the slides in 0.6% H<sub>2</sub>O<sub>2</sub> in methanol for 40 min. The unspecific protein binding sites on the sections were preblocked with blocking solution (1% blocking reagent from Boehringer Mannheim, 3% BSA, 20 mM MgCl<sub>2</sub>, 0.3% Tween-20 in PBS). CBP and p300 antibodies were diluted 1:200-1:400 in blocking solution and centrifuged at 13,000 rpm for 10 min. Slides were incubated with primary antibodies for 2 h at room temperature or overnight at 4°C and with secondary avidin-conjugated antibody (1:200 dilution) for one hour in a humidified chamber. Avidin and biotin reagents (ABC Santa Cruz kit or Vectastain Elite kit) were diluted 1:100 and incubated on the slides for 45 min. Color reaction was generated in developing reaction (0.1 M Tris pH7.5, 0.5 mg/ml DAB, 0.03% H<sub>2</sub>O<sub>2</sub>) for 2-20 min. The reaction was stopped by rinsing the slides in distilled water. Slides were then dehydrated and mounted in Permount.

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