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Neuralization of the *Xenopus* Embryo by Inhibition of p300/ CREB-Binding Protein Function

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p300/ CREB-binding protein (CBP) is a transcriptional coactivator for a plethora of transcription factors and plays critical roles in signal transduction pathways. We report that the inhibition of p300/CBP function in the *Xenopus* embryo abolishes non-neural tissue formation and, strikingly, initiates neural induction and primary neurogenesis in the entire embryo. The observed neuralization is achieved in the absence of anterior or posterior gene expression, suggesting that neural fate activation and anterior patterning may represent distinct molecular events. We further demonstrate that the neuralizing and anteriorizing activities of chordin and noggin are separable properties of these neural inducers. This study reveals that all embryonic cells possess intrinsic neuralizing capability and that p300/ CBP function is essential for embryonic germ layer formation and neural fate suppression during vertebrate embryogenesis.

Key words: p300; CBP (CREB-binding protein); neural induction; neuralization; transcription suppression; germ layer formation; embryogenesis; Xenopus

Early vertebrate development, including neural development, has been investigated extensively in *Xenopus laevis* and has been found to require coordinated spatial and temporal regulation of distinct signaling pathways (for review, see Kessler and Melton, 1994; Harland and Gerhart, 1997; Heasman, 1997). The formation of non-neural germ layers requires the activation of fibroblast growth factor (FGF; for mesoderm), activin/Vg1 (for mesoderm and endoderm), and bone morphogenetic protein (BMP; for epidermis) signal transduction pathways (Amaya et al., 1991; Hemmati-Brivanlou and Melton, 1992; Sasai et al., 1995; Wilson and Hemmati-Brivanlou, 1995; Henry et al., 1996; Chang et al., 1997; Joseph and Melton, 1998). Neural formation is induced by neuralizing signals, such as noggin and chordin, from the dorsal mesoendoderm, the Spemann organizer. These signals are believed to function primarily as antagonists for BMP signaling (Sasai and De Robertis, 1997; Wilson and Hemmati-Brivanlou, 1997). The nuclear events underlying neural versus non-neural developmental pathways are not well understood.

p300 and related CBP (*C*REB-*b*inding *p*rotein) have emerged as central integrators in signal transduction and transcriptional activation (Eckner, 1996; Janknecht and Hunter, 1996; Goldman et al., 1997; Shikama et al., 1997). p300 was discovered as a cellular protein targeted by the adenovirus early region 1A (E1A) oncoprotein (Yee and Branton, 1985; Harlow et al., 1986; Eckner et al., 1994), whereas CBP was identified as a transcriptional coactivator for the *c*AMP-*r*esponsive *e*lement *b*inding protein, CREB (Chrivia et al., 1993). p300 and CBP share similar amino acid sequences and biological properties and commonly are described by the generic designation of p300/CBP (Goldman et al., 1997; Shikama et al., 1997). In the mammalian cell culture system p300/CBP not only associates with a plethora of DNA-binding transcription activators but also complexes with the basal transcription machinery and possesses intrinsic and associated histone acetyltransferase activity important for chromatin remodeling (for review, see Eckner, 1996; Janknecht and Hunter, 1996; Goldman et al., 1997; Shikama et al., 1997; Wade and Wolffe, 1997). Although p300/CBP plays a central and global role in signaling integration and transcription activation in cell culture models, p300/CBP function in embryonic development, in particular in vertebrate development, has not been characterized fully. The *Drosophila* CBP is necessary for the *dorsal*, *hedgehog*, and *decapentaplegic* signaling pathways (Akimaru et al., 1997a,b; Waltzer and Bienz, 1999) and antagonizes *wingless* signaling (Waltzer and Bienz, 1998). In the nematode *Caenorhabditis elegans*, interference of CBP function blocks mesodermal and endodermal differentiation (Shi and Mello, 1998). Mice homozygous mutant for either p300 or *cbp*, and mice double heterozygous for p300 and *cbp*, exhibit similar pleiotropic developmental defects and die during embryonic day 9.5–11.5 (Yao et al., 1998). However, common and gene dosage-dependent functions of p300 and CBP (Yao et al., 1998) have precluded the revelation of their role in early vertebrate development.

We report that in *Xenopus* embryos p300/CBP function is essential for embryonic germ layer formation and the specification of neural versus non-neural developmental pathways. In the absence of p300/CBP function the entire embryo adopts neural fate. Our results also suggest that neural fate activation and anterior patterning may represent distinct molecular events.

MATERIALS AND METHODS

cDNA constructs and synthetic RNA. cDNAs for 12S E1A, 12S E1A–RG2, and 12S E1A–DL120–140 (Kannabiran et al., 1993; Wang et al., 1993) were subcloned into the pCS2$^+$ vector (Turner and Weintraub,

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1994). p300N was generated by deleting the coding sequence 3′ to the unique *Stu*I site in the human p300 cDNA and was subcloned into pCS2$^+$. Thus p300N protein contains amino acids from residue one to 1691, lacking the C-terminal third of the protein. p300(dl10) (Lee et al., 1996) was cloned into pCS2$^+$. Capped RNAs were transcribed *in vitro* from linearized plasmids with SP6 RNA polymerase according to the manufacturer's recommended procedure (Ambion, Austin, TX).

Embryo manipulation, histology, in situ *hybridization, and explants.* Eggs were obtained from female *Xenopus laevis*, and embryos were raised in 0.1× MMR buffer (100 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM HEPES, 1 mM EDTA, pH 7.8). Embryos were staged according to Nieuwkoop and Faber (1994). Injections of RNAs into embryos were done in 3% Ficoll/0.1× MMR buffer. All injections were done at two-cell stage (for vegetal injections) or four-cell stage (for dorsal or ventral injection), with two blastomeres injected with an equal amount of RNA; the exception was ultraviolet (UV) rescue experiments in which only one animal cell was injected at the 16-cell stage. Dorsal blastomeres at the four-cell stage were recognized by their lighter pigmentation. Fixation, sectioning, and staining of embryos were performed as described (Christian and Moon, 1993). Whole-mount *in situ* hybridization was performed according to Harland (1991), using *in vitro* synthesized antisense probes labeled with digoxygenin–UTP. Explants were performed as described (Henry et al., 1996) and were done in at least two independent experiments. Briefly, for animal pole explants the embryos were injected at two-cell stage in the animal pole. Caps were dissected at stage 8 and cultured until the specified stages. For hormone treatment, basic fibroblast growth factor (bFGF; Life Technologies, Gaithersburg, MD) was used at a final concentration of 50 ng/ml. Activin (from the National Hormone and Pituitary Program, a part of the National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD) was used at a final concentration of 5 ng/ml for mesoderm induction and 50 ng/ml for endoderm induction. For ventral marginal explants the embryos were injected at four-cell stage in the ventral equatorial region. Ventral explants were dissected from stage 10 embryos when the dorsal lip was easily recognizable. For vegetal pole explants the embryos were injected at two-cell stage in the vegetal pole. Explants were dissected from stage 9 embryos and cultured until the specified stages. For UV rescue experiments the embryos were UV-irradiated within 25 min of fertilization in a UV cross-linker (model CL-1000 of Ultra-Violet Products, Cambridge, UK), with the energy meter level at 120 mJ. β-Gal lineage tracing was done as described (Chitnis et al., 1995).

Immunoblot of E1A. RNA (1 ng) was injected into the animal pole of each embryo at two-cell stage. Stage 9 embryonic extracts were made by homogenizing 10 embryos in 400 μl of cold NP-40 buffer as described (He et al., 1995). In all, 30 μl of each extract (0.7 embryo equivalent) was run on 12% SDS-PAGE and transferred to a 0.2 μm nitrocellulose filter (Schleicher & Schuell, Keene, NH). The filter was blocked with 2% BSA in TBST (10 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween 20). The monoclonal antibody specific for E1A was used at 1:40 in 5% BSA plus 3% milk in TBST for overnight incubation at 4°C. Bound antibodies were visualized with peroxidase-conjugated secondary antibody (used at 1:10,000; Calbiochem, La Jolla, CA) and the ECL detection system (Amersham, Arlington Heights, IL).

Reverse transcription-PCR (RT-PCR). Semiquantitative RT-PCR was performed within linear ranges as described (Wilson and Melton, 1994). Some of the PCR primers were from the *Xenopus* home page (for neurogenin, krox20, otx2, Xlhbox6, neuroD, *n*-tubulin, xif3), De Robertis Lab home page (for globin, cytokeratin, N-CAM), and the following references: EF-1α and Xwnt-8 (LaBonne and Whitman, 1994), mix-1 (Wilson and Melton, 1994), *siamois* (Zeng et al., 1997), chordin (Fagotto et al., 1997), Xsox-17α (Hudson et al., 1997), Mixer (Henry and Melton, 1998), msx1 (Suzuki et al., 1997b), endodermin (Sasai et al., 1996), *goosecoid* (Hemmati-Brivanlou et al., 1994), Xbra (Gupta and Mayer, 1998), and Xnr3 (Darras et al., 1997). The rest of the primers were designed for this paper and are as follows, with the size of each PCR product and cycle numbers indicated in parentheses. These PCRs were performed at 94, 72, and 55°C with 1 min for each temperature segment, using *Taq* polymerase from Life Technologies. *zic-r1* (284 bp, 20): 5′-ttg aag gct gtg aca gac-3′ (u), 5′-atg tac tgc tga gga gct-3′ (d); *zic2* (311 bp, 20): 5′-ccc aga act taa aca acg gc-3′ (u), 5′-cag gtg aat gaa ggg tac gt-3′ (d); *zic3* (291 bp, 25): 5′-caa cag tga gga acc ttc ca-3′ (u), 5′-ggg ctt tgt tag tct gta gc-3′ (d); *sox2* (315 bp, 20): 5′-acc gct atg atg tca gtg-3′ (u), 5′-ctg agg cac tct gat agt-3′ (d); *sox3* (229 bp, 20): 5′-atg ggc tca gtg gtg aaa tc-3′ (u), 5′-ata tgt gag tga gag gta cc-3′ (d); *soxD* (272 bp, 18): 5′-ctg act tga gaa tct agg gg-3′ (u), 5′-gtc aca gtg tgt gcg aca aa-3′ (d); *Xhl* (298 bp, 25): 5′-cga gag gat gtg gaa aga ag-3′ (u), 5′-agg gga ggc gtg tat tta ag-3′ (d); *livertine* (257 bp, 25): the upper primer is the same as for Xhl, 5′-gtc cta ttg tca tcg tac cc-3′ (d); *gli1* (204 bp, 25): 5′-aag ctt cct cac act tga cc-3′ (u), 5′-gct ctg cgc cat aga taa tc-3′ (d); *Xiro2* (336 bp, 22): 5′-cta cag gcc cac aaa cta tg-3′ (u), 5′-cat tgt ttg cag agg gtc ac-3′ (d); *Xiro3* (230 bp, 22): 5′-caa cgg agg tca caa gat ca-3′ (u), 5′-aac cat acg aac tca gct gc-3′ (d); *etr-1* (270 bp, 22): 5′-ccc agt tgg atc ctc tac aa-3′ (u), 5′-cgc ggt cta caa aca ctt tg-3′ (d); and *pv1* (268 bp, 25): 5′-ggc ccc tca gaa ttc ata ca-3′ (u), 5′-gtt caa atg tac tga gcc cc-3′ (d). All PCRs were done at least twice, and consistent results were obtained.

RESULTS

Induction of non-neural tissues requires p300/CBP

To study p300/CBP in early *Xenopus* embryogenesis, we took advantage of the highly specific inhibitory effect of the adenovirus E1A protein toward p300/CBP (for review, see Goldman et al., 1997; Shikama et al., 1997). Because E1A also binds to and inhibits the function of the Rb family of tumor suppressor proteins (for review, see Nevins, 1992; Moran, 1993), we further used two E1A mutants, i.e., E1A–RG2 (referred to as RG2) and E1A–DL120–140 (referred to as DL120–140). RG2 contains a single amino acid substitution (arginine to glycine) at the second residue position, thereby having a significantly compromised ability to bind and inhibit p300/CBP (Wang et al., 1993). DL120–140 harbors a deletion of the Rb binding domain and does not bind Rb but binds to and inhibits p300/CBP comparable to the wild-type E1A (Kannabiran et al., 1993). We also generated a truncated p300, referred to as p300N, that lacks the C-terminal third of the protein containing binding sites for coactivator proteins (Goldman et al., 1997; Shikama et al., 1997). We reasoned that p300N is likely to function in a dominant-negative manner to inhibit wild-type p300/CBP function.

Injection of RNA for E1A, DL120–140, or p300N into the dorsal equatorial region, which is fated to become dorsal mesoderm, resulted in severe defects in axial development, whereas embryos injected with RNA for RG2, which does not bind p300/CBP, developed normally (Fig. 1*A*,*B*, Table 1). Histological examination demonstrated a lack of dorsal axial structures such as the notochord and muscle in these affected embryos (Fig. 1*B*). At the molecular level the expression of an early pan-mesodermal marker, Xbra (a T-box transcription factor; Smith et al., 1991), was inhibited completely on the injected side (dorsal or ventral) by E1A, DL120–140, or p300N, but not by RG2 (Fig. 1*C*). On the contrary, neither E1A nor p300N affected the endogenous dorsal-specific expression of Xnr3 (a member of the TGF-β superfamily; Smith et al., 1995) or ectopic Xnr3 expression induced at the ventral side by the overexpression of β-catenin (Fig. 1*D*). These results suggested that the inhibition of p300/CBP function specifically disrupted mesoderm induction without affecting Wnt/β-catenin signaling, which directly activates Xnr3 expression (McKendry et al., 1997).

When RNA for E1A, DL120–140, or p300N was injected into the vegetal pole region that is fated to become endoderm, the injected embryos also exhibited anterior truncations and a lack of A/P axis (Table 2). This phenotype was reminiscent of that generated by reagents that block the endodermal development required for head and mesoderm induction (Hudson et al., 1997; Henry and Melton, 1998; Joseph and Melton, 1998). Embryos injected vegetally with RG2 RNA developed normally (Table 2). Indeed, the expression of two early endodermal-specific markers, *sox-17α* (an HMG box transcription factor; Hudson et al., 1997) and Mixer/mix-3 (a homeodomain transcription factor; Henry

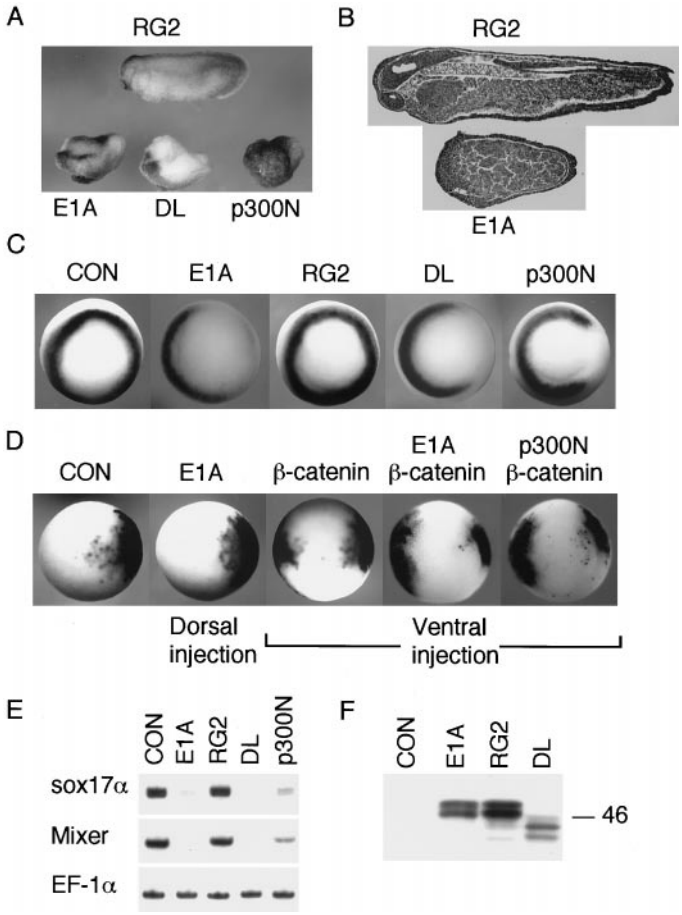

Figure 1. Inhibition of p300/CBP by E1A, E1A–RG2, and p300N interferes with mesoderm and endoderm development. *A*, Phenotypes of embryos injected dorsally at four-cell stage with E1A, RG2, DL120–140 (*DL*), and p300N RNAs, shown at stage 25. E1A, DL120–140, and p300N-injected embryos cleaved and developed normally up through the late blastula stage (stage 9) but began to exhibit severe gastrulation abnormality from the early gastrula stage (stage 10.5). A failure of blastopore closure was observed in many of the injected embryos (stage 13). Most of injected embryos displayed a lack of any axial development at stage 25. The RG2-injected embryo is phenotypically identical to normal controls. RNA injected per embryo: 0.1 ng for E1A, RG2, and DL120–140; 2 ng for p300N. Note that because p300N mRNA and protein sizes are each approximately seven times of those for E1A, the efficiency of synthesis and the local concentration of p300N protein are likely to be much smaller than those of E1A in the embryo if the same amount of RNA were to be injected. *B,* Sagittal sections of a RG2 (*top*) and an E1A-injected embryo (*bottom*) at stage 25. Note that the RG2-injected embryo exhibits normal development, whereas the E1A-injected one lacks any axial tissues, such as the notochord and muscles. Axial tissues are absent in all sections (data not shown). *C*, Inhibition of Xbra expression examined at stage 10.5 by *in situ* hybridization. Note that the control (*CON*) and RG2-injected embryo exhibit a uniform circumferential expression pattern, whereas expression is abolished on the injected side (either dorsal or ventral) in E1A, DL120–140, or p300N-injected embryos. Dorsal is to the *right*. RNA injected per embryo: 20 pg for E1A, RG2, and DL120–140; 500 pg for p300N. *D*, Lack of inhibition of Xnr3 expression by *in situ* hybridization analysis, examined at stage 10.5. Note that the endogenous Xnr3 expression is not blocked by dorsally injected E1A, and an ectopic induction of Xnr3 by ventrally injected β-catenin (0.5 ng RNA per embryo) is not inhibited by coinjected E1A or p300N. Ectopic activation of *goosecoid* expression by β-catenin was inhibited significantly by E1A, as expected (data not shown), because *goosecoid* expression depends on mesoderm-inducing signals (Watabe et al., 1995). Dorsal is to the *right*. RNA amount injected is the same as in *C*. *E*, Inhibition of Xsox-17α and Mixer expression assayed by quantitative RT-PCR. In this and all of the following RT-PCR results, EF-1α is used as a control for the template amount. The RNA amount injected is the same as in *A*. *F*, Immunoblot of stage 9 embryo extracts to examine protein levels of E1A, E1A–RG2 (*RG2*), and E1A–DL120–140 (*DL*) from injected synthetic RNAs. *CON*, Uninjected control extract.

and Melton, 1998; Mead et al., 1998), was inhibited in embryos injected with RNA for E1A, DL120–140, or p300N, but not with RG2 RNA (Fig. 1*E*). Thus, endodermal development depends on p300/CBP function as well. Because E1A, RG2, and DL120–140 proteins were expressed at comparable levels in injected embryos (Fig. 1*F*), the lack of RG2 effect on embryogenesis is most likely attributable to its inability to interfere with p300/CBP function.

To investigate how p300/CBP function is required for germ layer formation, we performed induction assays in animal pole explants. The isolated explant, which forms epidermis by the endogenous BMP signaling, can be induced to form both mesoderm and endoderm by exogenous activin or mesoderm by FGF (for review, see Kessler and Melton, 1994; Harland and Gerhart, 1997; Heasman, 1997). As shown in Figure 2, E1A, DL120–140, or p300N blocked the induction of mesodermal markers, such as Xbra and Xwnt-8, by FGF and activin (Fig. 2*A*) and the induction of dorsal mesodermal markers such as *goosecoid* (a homeobox gene; Cho et al., 1991) and chordin by activin (Fig. 2*B*). The activin induction of early endoderm markers, such as *sox-17α*, Mixer, and a late endoderm marker endodermin (Sasai et al., 1996), also was inhibited (Fig. 2*B*). In addition, morphological elongation induced by either activin or FGF in animal explants was blocked (data not shown). RG2 affected neither mesodermal and endodermal gene induction nor elongation by activin or FGF (Fig. 2*A,B*; data not shown).

We further found that BMP-4 signaling generated by a constitutively activated ALK2 (a BMP-4 receptor; Suzuki et al., 1997a) in animal explants was prevented by E1A, DL120–140, or p300N, but not by RG2 (Fig. 2*C*). This was demonstrated by the inhibition of the induction of *msx-1* and *pv1*, which are homeobox genes induced by BMP-4 (Ault et al., 1997; Suzuki et al., 1997b). Hence, p300/CBP function is required for BMP signaling, which is critical for epidermal formation. Consistent with the studies in the whole embryo, Wnt or β-catenin induction of Xnr3 and *siamois* (a homeobox gene directly activated by β-catenin; Brannon et al., 1997) was not affected to any significant degree by E1A or p300N (Fig. 2*D*).

Although similar inhibitory activities of E1A, DL120–140, and p300N, together with the lack of the inhibitory activity of RG2, strongly suggested the specific inhibition of p300/CBP function, we further addressed the specificity issue by examining whether the wild-type p300 function can rescue the effect of E1A and p300N. We used p300(dl10) to restore p300 function in the embryo, because for unknown reasons we were unable to obtain the full-length p300 protein via injection of the wild-type p300 RNA (data not shown). p300(dl10) harbors an internal deletion of the E1A-binding domain (residue 1679–1812; Lee et al., 1996). Similar to other analogous E1A-binding domain deletions of p300, p300(dl10) retains the full activity of wild-type p300 but is refractory to E1A inhibition (Eckner et al., 1994; Lee et al., 1996). As shown in Figures 2*E* and 3, the inhibition of mesodermal, endodermal, or epidermal gene expression by E1A or p300N was rescued completely by coinjection with p300(dl10). Taken together, our data suggest that p300/CBP function is required for the induction of non-neural germ layers.

Table 1. Dorsal injection

RNA-injected	Control (%)	E1A (%)	RG2 (%)	DL (%)	p300N (%)
Phenotype					
No axial structures	0 (0)	117 (75)	0 (0)	59 (80)	84 (53)
Partial axial defects	0 (0)	39 (25)	8 (11)	14 (20)	75 (47)
Normal embryos	60 (100)	0 (0)	65 (89)	0 (0)	0 (0)
Total	60	156	73	73	159

Axial deficiency in embryos injected with E1A, RG2, DL, and p300N. RNA amount injected per embryo is as in Figure 1*A*. Partial axial defects are defined as lacking parts or entire head structures but retaining the posterior body axis. Note that partial axial defects in 11% RG2-injected embryos were much milder than those of E1A, DL, or p300N-injected ones.

Table 2. Vegetal injection

RNA-injected	Control (%)	E1A (%)	RG2 (%)	DL (%)	p300N (%)
Phenotype					
No axial structures	0 (0)	36 (66)	0 (0)	51 (70)	36 (54)
Partial axial defects	0 (0)	18 (34)	0 (0)	21 (30)	31 (46)
Normal embryos	60 (100)	0 (0)	77 (100)	0 (0)	0 (0)
Total	60	54	77	72	67

Axial deficiency in embryos injected with E1A, RG2, DL, and p300N. RNA amount injected per embryo is as in Figure 1*A*. Partial axial defects are defined as lacking parts or entire head structures but retaining the posterior body axis.

Inhibition of p300/CBP function initiates neuralization and neurogenesis without anteroposterior patterning

Because p300/CBP plays a critical role in chromatin remodeling and transcription activation, we next asked whether p300/CBP is required for neural development and neural-specific gene activation. Strikingly, in the absence of any neuralizing signals, E1A, DL120–140, or p300N, but not RG2, induced early neural-specific genes in animal explants. Most prominently, all members of the *zic* family of zinc-finger transcription factors, *zic-r1*/*opl*, *zic2* and *zic3* as well as *sox3*, an HMG-box transcription factor, were strongly induced (Fig. 3). The *zic* genes and the *sox3* gene are among the earliest markers known to be expressed specifically in the neuroectoderm region and induced by endogenous neuralizing signals (Nakata et al., 1997; Penzel et al., 1997; Brewster et al., 1998; Kuo et al., 1998; Mizuseki et al., 1998a). Importantly, *zic-r1* and *zic3* are sufficient, both *in vivo* and *in vitro*, to activate the entire neural developmental cascades, including anterior patterning, primary neurogenesis, and neural crest formation (Nakata et al., 1997; Kuo et al., 1998; Mizuseki et al., 1998a), whereas *zic2* is required for the regulation of primary neurogenesis and neural crest differentiation (Brewster et al., 1998). We also observed a strong induction of a proneural gene neurogenin (Xngnr-1), a bHLH transcription factor that initiates and regulates primary neurogenesis (Ma et al., 1996). Some other early pan-neural markers, such as Xhl and livertine, which encode related growth factors potentially involved in neural morphogenesis (Aberger et al., 1996; Ruiz i Altaba and Thery, 1996), also were induced. When compared with neural induction by neural inducers, levels of neural gene induction by E1A or p300N were similar to those by chordin or noggin (Fig. 3*A*). Furthermore, E1A plus chordin did not exhibit additive or synergistic effects on neural gene induction (Fig. 3*A*). The observed neural induction was at the expense of epidermis, as indicated by the downregulation of cytokeratin, an epidermal marker (Fig. 3*A*), and was direct because it occurred in the absence of any mesoderm, which was abolished in the absence of p300/CBP function (see Fig. 2*A,B*). Induction of *zic-r1*, *zic2*, *zic3*, and *sox3* by E1A or p300N was fully suppressed by p300(dl10) (Fig. 3*B*), further indicating that E1A and p300N induced neural gene expression by an inhibition of p300/CBP function. Taken together, these results suggested that the inhibition of p300/CBP function initiated neural induction in animal explants.

Interestingly, whereas chordin, noggin, and other known neural-inducing signals lead to neural fate determination and a concomitant anterior patterning of the neural tissue, neither E1A, DL120–140 nor p300N induced anterior or posterior markers, such as otx2 and Xif3 (forebrain), En2 (midbrain), krox20 (hindbrain), and HoxB9 (spinal cord) (Fig. 3*A*; data not shown). In fact, E1A completely blocked the induction of otx2 and Xif3 by chordin/noggin (Fig. 3*A*). These results suggested that the neuralizing and anteriorizing activities of chordin and noggin are two distinct properties: neural fate determination induced by chordin or noggin is mimicked by suppressing p300/CBP, whereas anterior patterning by chordin or noggin requires p300/CBP function. We note that the inhibition of p300/CBP did not lead to the expression of neural cell adhesion molecule (N-CAM), a pan-neural marker (Fig. 3*A*); in fact, E1A also inhibited chordin/noggin induction of N-CAM (Fig. 3*A*). Although N-CAM has been used widely as a neural marker, its expression during neural induction, detectable at approximately stage 12–13 (Kintner and Melton, 1987) (data not shown), lags significantly behind the expression of *zic* and *sox* genes that are detectable at approximately stage 8–9 (Nakata et al., 1997; Penzel et al., 1997; Kuo et al., 1998; Mizuseki et al., 1998a). Thus, the inhibition of p300/CBP function appears to initiate the early phase of neuralization.

Inhibition of p300/CBP rescues early neural development in ventralized embryos

To investigate whether blocking p300/CBP function is sufficient to initiate *de novo* neural development in the whole embryo, we examined the ability of E1A and p300N to rescue neural development in UV-treated embryos. UV treatment of the one-cell

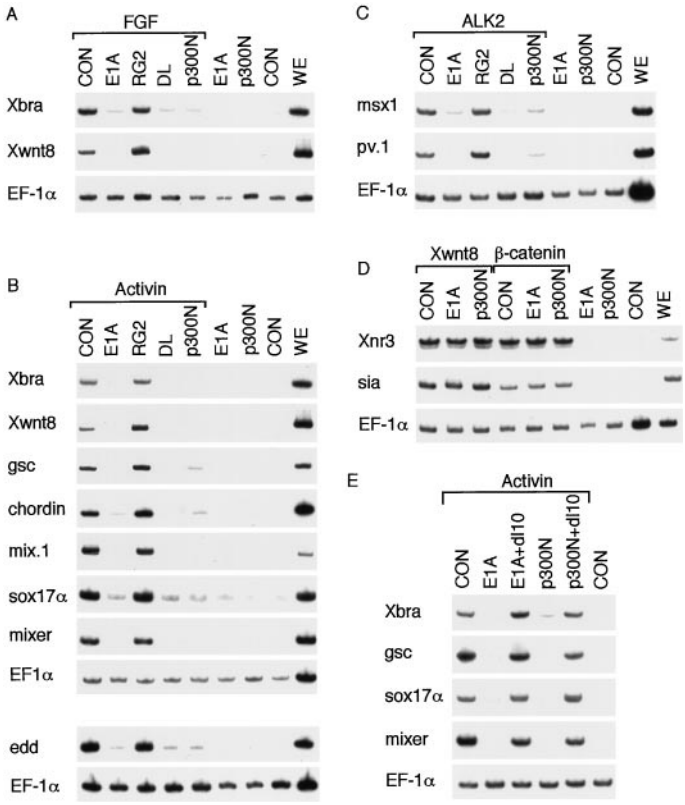

Figure 2. Inhibition of mesoderm, endoderm, and epidermis formation in animal pole explants. *A,* Inhibition of mesoderm markers induced by FGF examined at stage 10.5. *WE* is the positive control for RT-PCR, using the cDNA from whole embryos at the equivalent stage. RNA injected per embryo: 0.1 ng for E1A, RG, and DL; 3 ng for p300N. bFGF concentration is 50 ng/ml. *B*, Inhibition of mesoderm and endoderm markers induced by activin examined at stage 10.5. *mix1* is a homeobox gene expressed in both mesoderm and endoderm. Endodermin induction by activin was examined at stage 35. The RNA amount that was injected is the same as in *A*. Activin concentration: 5 ng/ml (for mesoderm induction) and 50 ng/ml (for endoderm induction). *C*, Inhibition of early BMP-4 target genes induced by an activated ALK2, a BMP-4 receptor. RNA injected per embryo: 0.05 ng for E1A, RG, and DL; 3 ng for p300N; 0.2 ng for ALK2. *D*, A lack of inhibition of Xwnt-8 or β-catenin signaling. RNA injected per embryo is the same as in *A*, except 10 pg for Xwnt-8 and 0.1 ng for β-catenin. *E*, E1A or p300N inhibition of mesodermal and endodermal gene expression was rescued by p300(dl10), which has wild-type p300 function but is refractory to E1A inhibition (Eckner et al., 1994; Lee et al., 1996). RNA injected per embryo is the same as in *A*, except 1 ng for p300(dl10).

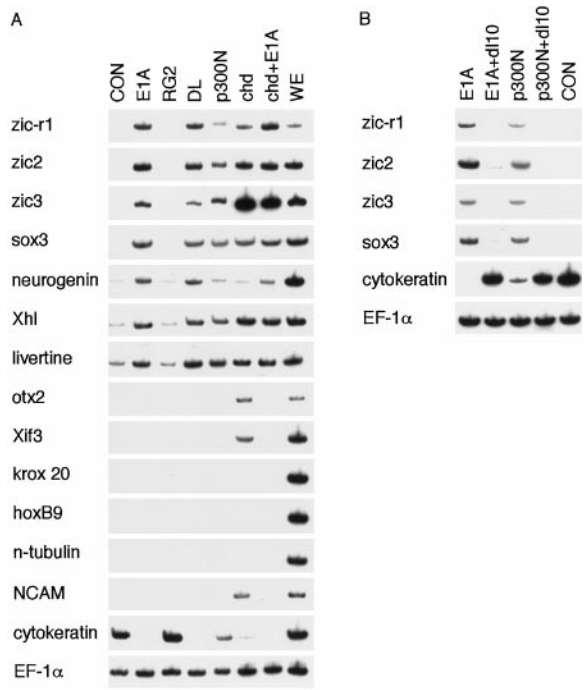

Figure 3. Neuralization without anteroposterior patterning in animal pole explants. *A,* Induction of neural markers was examined at stage 20 by RT-PCR. Note the concomitant suppression of cytokeratin, an epidermal marker. RNA injected per embryo: 5 pg for E1A, RG, and DL; 3 ng for p300N; 1 ng for chordin (*chd*). Note that neurogenin was not induced by chordin at this stage, as expected from published results (see Discussion; Lamb et al., 1993; Papalopulu and Kintner, 1996). Similar results were obtained when noggin was used in place of chordin (data not shown). *B*, E1A or p300N induction of neural markers and suppression of cytokeratin could be rescued by p300(dl10), coinjected at 1 ng of RNA per embryo.

stage embryo abolishes the formation of the Spemann organizer and thus the production of neuralizing signals, thereby leading to ventralized embryos lacking any neural tissue (Gerhart et al., 1989). Normal development, including neural development in such UV-treated embryos, can be rescued by either chordin or noggin (Smith and Harland, 1992; Sasai et al., 1994, 1995). As shown in Figure 4, *A* and *B*, E1A as well as p300N, but not RG2, rescued early neural development. This rescue was comparable to that achieved by chordin, as demonstrated by the induction of *zic-r1*, *zic-2*, *sox3*, and neurogenin, as well as of many other early neural-specific transcription factors such as *sox2* and *soxD* (HMG-box; Mizuseki et al., 1998a,b), gli1 (zinc finger; Lee et al., 1997), Xash3 (bHLH; Turner and Weintraub, 1994), and Xiro2 and Xiro3 (homeobox; Bellefroid et al., 1998; Gomez-Skarmeta et al., 1998) (Fig. 4*B*). Also rescued was the expression of etr-1 (a pan-neural marker) and XCG (a cement gland marker) (Fig. 4*B*). Note that the rescue of early neural development by E1A or p300N was in the absence of any axial development (data not shown), because p300 function is required for dorsal mesoderm formation (see Figs. 1, 2).

UV-treated embryos rescued by chordin exhibited progressive neural development, including anteroposterior patterning, neural crest formation, and neuronal differentiation, as manifested by the expression of otx2, xif3, krox20, and slug (a neural crest marker; Mayor et al., 1995) as well as *n*-tubulin (a neuronal differentiation marker; Chitnis et al., 1995) (Fig. 4*B*). However, UV-treated embryos injected with E1A or p300N did not express any of these regional, crest, or differentiation markers (Fig. 4*B*), consistent with results from the animal pole explant assay. Therefore, the inhibition of p300/CBP function *in vivo* initiated neural fate determination and neurogenesis, but further neural development was disrupted before any anteroposterior patterning and progression of neuronal differentiation.

Inhibition of p300/CBP neuralizes mesoderm and endoderm

The inhibition of p300/CBP function blocked epidermal differentiation and promoted neuralization in prospective ectoderm (animal pole explants) from which neural tissue normally derives (see Figs. 3, 4). Because the inhibition of p300/CBP function also blocked mesoderm and endoderm formation *in vivo* (see Fig. 1),

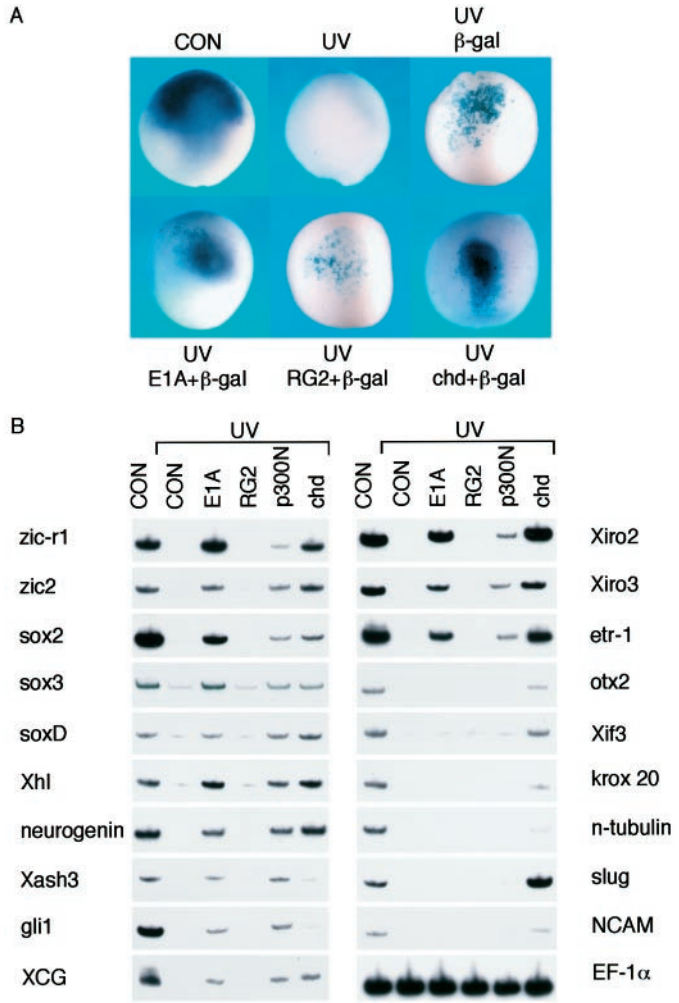

Figure 4. Rescue of early neural development in UV-ventralized embryos. RNA injected per embryo: 20 pg for E1A and RG, 3 ng for p300N, and 150 pg for chordin. *In situ* hybridization and RT-PCR were performed on embryos at stage 14. *A*, Rescue of *zic-r1* expression by E1A and chordin, but not by RG2 or β-galactosidase (used as a lineage-tracing marker). Note that the expression of *zic-r1* overlaps with X-gal staining in E1A or chordin (*chd*)-injected embryos. *B*, Rescue of neural markers examined by RT-PCR.

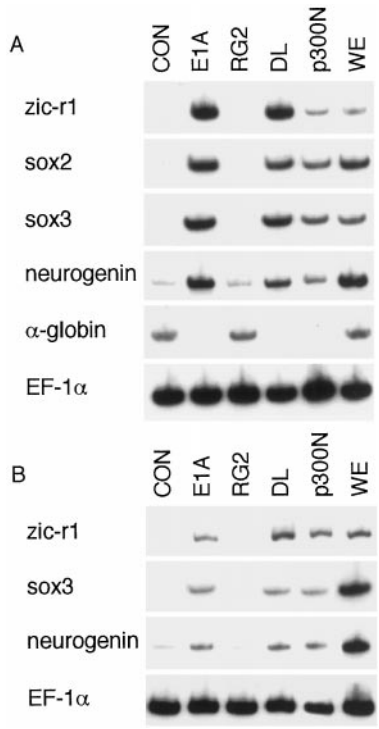

Figure 5. Neural gene induction in ventral marginal explants (prospective ventral mesoderm) and vegetal pole explants (prospective endoderm). RNA injected per embryo: 20 pg for E1A, RG, and DL; 3 ng for p300N. *A,* Induction of neural markers and suppression of a mesoderm marker in ventral marginal explants examined at stage 20 by RT-PCR. α-Globin is a hematopoietic (ventral mesoderm) marker. *B*, Induction of neural markers in vegetal explants examined at stage 20 by RT-PCR. Note the concomitant suppression of endoderm markers (see Fig. 1*E*).

we asked whether such inhibition neuralizes prospective mesoderm and endoderm as well. In ventral marginal explants that are fated to become ventral mesoderm such as hematopoietic cells, the E1A, DL120–140, or p300N, but not RG2, completely suppressed globin gene expression while activating *zic-r1*, *sox2, sox3*, and neurogenin expression (Fig. 5*A*). Similarly, in vegetal pole explants that are fated to become endoderm, the E1A, DL120–140, or p300N, but not RG2, activated *zic-r1, sox3*, and neurogenin expression (Fig. 5*B*) while suppressing *sox-17α* and Mixer expression (see Fig. 1*E*). These results suggest that the inhibition of p300/CBP function initiates neuralization in the entire embryo.

DISCUSSION

We demonstrate that the inhibition of p300/CBP function abolishes the formation of non-neural tissues while concomitantly initiates neural induction and primary neurogenesis in the entire *Xenopus* embryo, thereby revealing that all embryonic cells possess intrinsic neuralizing capability. We also show that neuralization can occur in the absence of any regional patterning, suggesting that neural fate determination and anterior patterning may represent distinct molecular events. We further provide the first evidence that the neuralizing and anteriorizing activities of chordin and noggin are distinguishable properties of these neural inducers. Our study places p300/CBP at essential crossroads for neural versus non-neural specification pathways during early vertebrate embryogenesis and reveals multiple key functions of p300/CBP in distinct aspects of neural development.

We used E1A, E1A mutant derivatives, and a dominant-negative p300N to inhibit endogenous p300/CBP function in the embryo. The specificity of this inhibition is demonstrated independently by the following: (1) E1A–RG2, an E1A mutant (with a single amino-acid substitution) that cannot bind effectively to p300/CBP, does not perturb normal development; (2) E1A–DL120–140, an E1A mutant with the Rb-binding domain deleted, behaves the same as wild-type E1A in all assays that were performed; (3) p300N, a truncated mutant p300, behaves the same as wild-type E1A and E1A–DL120–140 in all assays that were performed; (4) p300(dl10), a p300 allele that has the wild-type p300 function and is resistant to E1A inhibition, rescues the effect of E1A and p300N on all of the neural or non-neural molecular

markers that were examined. These results are most consistent with the interpretation that the observed phenotypic effect by E1A or p300N is attributable to the inhibition of p300/CBP function.

p300/CBP function in non-neural versus neural determination

p300/CBP function is essential for the induction of all non-neural germ layers. Such a requirement most likely is derived from the key roles p300/CBP plays in FGF, activin/Vg-1, and BMP signal transduction pathways. Our results highlight p300/CBP, for which the transcripts are present in the early embryo as maternal mRNAs (Fujii et al., 1998) (data not shown), as a signal integrator during early vertebrate embryogenesis, as has been observed in variety of signaling pathways, including TGF-β signaling in the mammalian cell culture system (for review, see Goldman et al., 1997; Shikama et al., 1997) (Feng et al., 1998; Janknecht et al., 1998; Pouponnot et al., 1998; Shen et al., 1998; Topper et al., 1998; Nakashima et al., 1999); and during invertebrate embryogenesis (Akimaru et al., 1997a,b; Shi and Mello, 1998; Waltzer and Bienz, 1999). *Drosophila* CBP recently was reported to have an inhibitory role for *wingless* signaling (Waltzer and Bienz, 1998). We did not observe any activation of Wnt downstream target genes by E1A or p300N in the *Xenopus* embryo (see Figs. 1*D*, 2*D*). We speculate that this may be attributable to the presence of Groucho proteins that repress Wnt signaling in *Drosophila*, *Xenopus* embryos, and mammalian cells (Cavallo et al., 1998; Roose et al., 1998).

The inhibition of p300/CBP function leads to the initiation of neural induction, as demonstrated by the activation of a large array of neural-specific genes, most of which are transcription factors essential for early neural development. These genes, including *zic* genes, *sox* genes, the Xiro family, and the neurogenin family, initiate and regulate overlapping but distinct aspects of neural development, including neural fate determination and primary neurogenesis (for review, see Sasai, 1998). Such an outcome was not fully anticipated, because p300/CBP plays a major role in transcriptional activation, as exemplified by numerous studies in mammalian cell models and by its requirement in all non-neural tissue formation in the *Xenopus* embryo. These results suggest that transcriptional activation mechanisms that govern neural versus non-neural cell fate determination are fundamentally different. Thus, whereas non-neural gene expression relies on p300/CBP-dependent transcription activation, neural-specific gene activation during neural fate determination may, at least in part, depend on transcription de-repression.

Neural gene induction by the inhibition of p300/CBP function is comparable to that achieved by the endogenous neural inducers chordin and noggin. It has been shown that neural-inducing signals in *Xenopus* prevent epidermal formation in the ectoderm by blocking BMP signaling, thereby permitting ectoderm cells to adopt the "default" neural fate (for review, see Hemmati-Brivanlou and Melton, 1997; Sasai and De Robertis, 1997; Wilson and Hemmati-Brivanlou, 1997). However, such a "default" mechanism for vertebrate neural induction has received some challenge by the observation that the BMP/chordin antagonism is not sufficient for neural induction in the chick embryo (Streit et al., 1998). Our finding, that the inhibition of endogenous p300/CBP function results in neuralization of all embryonic germ layers, provides a striking demonstration that neural fate determination in the *Xenopus* embryo is a suppressed developmental pathway of all embryonic cells and is inhibited by a transcription apparatus involving p300/CBP. In light of p300/CBP function in histone acetylation and chromatin remodeling, it is conceivable that the neural fate suppression occurs via chromatin modification. It wasshown recently that viral and cellular mechanisms exist that permit specific modulation of the histone acetyltransferase (HAT) activity of p300/CBP by E1A and a tissue-specific transcription factor (Chakravarti et al., 1999; Hamamori et al., 1999). These findings raise an intriguing question of whether p300/CBP HAT activity is modulated differentially in neural versus non-neural tissues during germ layer formation.

Neuralization and anterior patterning induced by chordin/noggin may represent distinct events

During *Xenopus* neural induction, anterior patterning occurs concomitantly with neural fate determination (Waddington, 1940; Yamada, 1950; Nieuwkoop, 1952). Because all known neural inducers seem to induce anterior neural tissue directly and because other types of signals such as FGF, Wnt-3A, or retinoic acid can transform the anterior neural tissue into posterior ones (for review, see Sasai and De Robertis, 1997; Wilson and Hemmati-Brivanlou, 1997), the two-step or activation–transformation model of neural induction (for review, see Nieuwkoop et al., 1985; Saxen, 1989) has been widely accepted. According to this model the first signal, a neuralizing signal from the organizer, directly induces the anterior neural tissue (forebrain), and a second caudalizing signal transforms the anterior neural tissue into progressively more posterior ones. We showed that, although the inhibition of p300/CBP function activates neural fate determination as chordin or noggin does, this does not lead to the expression of any anterior or posterior markers. On the contrary, anterior patterning by chordin/noggin is blocked in the absence of p300/CBP function. These results provide a unique example of neural tissue being induced in the absence of any regional gene expression in the *Xenopus* embryo. Uncoupling between neuralization and regionalization also was observed during neural induction in chick embryos (Streit et al., 1997). These results suggest that neural fate determination and anterior patterning may represent two distinct molecular events and that neuralizing and anteriorizing activities of chordin and noggin are separable properties of these neural inducers. Thus, the neuralizing activity of chordin and noggin is independent of p300/CBP function and, in fact, is mimicked by the inhibition of p300/CBP function, whereas the anteriorizing activity of chordin and noggin is p300/CBP-dependent.

In *Xenopus* embryos the first wave of neuronal differentiation occurs during primary neurogenesis when groups of uncommitted neural plate cells exit the cell cycle and differentiate into motor neurons, sensory neurons, and interneurons (Hartenstein, 1989). This differentiation process appears prefigured and initiated by the proneural gene neurogenin (Ma et al., 1996). How the specific temporal and spatial pattern of neurogenin expression is initiated is mainly unknown, although many transcription factors, including Zic, Sox, Xiro, and XBF proteins, can, directly or indirectly, affect neurogenin expression (Nakata et al., 1997; Bellefroid et al., 1998; Bourguignon et al., 1998; Brewster et al., 1998; Gomez-Skarmeta et al., 1998; Kuo et al., 1998; Mariani and Harland, 1998; Mizuseki et al., 1998a,b). Because neurogenin expression can be activated in all embryonic germ layers on the inhibition

of endogenous p300/CBP function, we suggest that all embryonic cells possess the intrinsic capability to initiate neurogenin expression and neuronal differentiation and that this intrinsic capability is suppressed via transcriptional suppression involving p300/CBP.

p300/CBP: A neural fate suppresser in both invertebrates and vertebrates

In fruit fly *Drosophila* and nematode *C. elegans*, CBP function is essential for mesodermal and endodermal formation (Akimaru et al., 1997a; Shi and Mello, 1998; Waltzer and Bienz, 1999). In addition, the *cbp* (RNAi) mutant nematode exhibits an increased number of cells that express certain neural markers, although the underlying mechanism remains to be elucidated (Shi and Mello, 1998). These results, together with the data presented here, suggest that an essential requirement of p300/CBP function in non-neural germ layer specification and in the suppression of neural fate determination is conserved evolutionarily from invertebrates to vertebrates.

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