Neuron, Vol. 36, 597–610, November 14, 2002, Copyright ©2002 by Cell Press

An Essential Role for a MEK-C/EBP Pathway during Growth Factor-Regulated Cortical Neurogenesis

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Summary

Mammalian neurogenesis is determined by an interplay between intrinsic genetic mechanisms and extrinsic cues such as growth factors. Here we have defined a signaling cascade, a MEK-C/EBP pathway, that is essential for cortical progenitor cells to become postmitotic neurons. Inhibition of MEK or of the C/EBP family of transcription factors inhibits neurogenesis while expression of a C/EBPβ mutant that is a phosphorylation-mimic at a MEK-Rsk site enhances neurogenesis. C/EBP mediates this positive effect by direct transcriptional activation of neuron-specific genes such as Tα1 α-tubulin. Conversely, inhibition of C/EBP-dependent transcription enhances CNTF-mediated generation of astrocytes from the same progenitor cells. Thus, activation of a MEK-C/EBP pathway enhances neurogenesis and inhibits gliogenesis, thereby providing a mechanism whereby growth factors can selectively bias progenitors to become neurons during development.

Introduction

Development of the mammalian nervous system is determined by a complex interplay between intrinsic genetic mechanisms and extrinsic cues such as growth factors. Within the central nervous system, this interplay has perhaps been best studied in the developing cortex (Lillien, 1998; Shen et al., 1998). In vivo, cortical neurogenesis occurs during embryogenesis, while gliogenesis largely occurs postnatally. Remarkably, this same temporal sequence can be replicated with cultured embryonic cortical stem cells, with neurons generated first and glia second (Qian et al., 2000). Growth factors do, however, alter this sequence; CNTF prematurely induces astrocyte formation (Bonni et al., 1997; Nakashima et al., 1999), while FGF2 is necessary for neurogenesis (Raballo et al., 2000), and PDGF and the neurotrophins enhance generation of neurons in the presence of FGF2 (Williams et al., 1997; Park et al., 1999; Ghosh and Greenberg, 1995). In this regard, the intracellular mechanisms regulating astrocyte formation have been well studied (Bonni et al., 1997; Nakashima et al., 1999; Sun et al., 2001), but little is known about signaling pathways that promote cortical neurogenesis.

To address this issue, we have utilized embryonic cortical progenitor cells isolated at a time point when neurogenesis commences in vivo. These cycling, nestin-positive progenitors undergo the transition to postmitotic neurons when cultured in FGF2 (Slack et al., 1998; Gloster et al., 1999; Toma et al., 2000; Ghosh and Greenberg, 1995), an event that requires the pRb family (Slack et al., 1998), and that is regulated by the inhibitory HLH Id2 (Toma et al., 2000; Lasorella et al., 2000). Cortical precursor cells also require Mash1 and Neurogenin2 to bias them toward a neuronal rather than glial fate (Nieto et al., 2001). However, while growth factors such as PDGF enhance neurogenesis in these progenitors (Williams et al., 1997; Park et al., 1999), the signaling cascades linking growth factors to pRb and the HLHs are unknown.

In this regard, the C/EBPs are one family of transcription factors (Williams et al., 1991) known to couple growth factor signal transduction to cellular differentiation in numerous developing nonneural tissues (reviewed in Johnson and Williams, 1994; Lekstrom-Hines and Xanthopoulos, 1998). The C/EBP family is composed of basic leucine zipper DNA binding proteins (C/EBPs α, β, γ, δ, ϵ, and ζ) that recognize a common DNA binding sequence (Johnson and Williams, 1994), and that are expressed in virtually all tissues, including the developing and adult brain (Sterneck and Johnson, 1998; Sterneck et al., 1998). C/EBPs regulate differentiation by direct *trans*-activation and/or repression of gene expression, and proliferation via multiple mechanisms, including interactions with the pRb family (Chen et al., 1996).

Here, we have asked whether the C/EBPs might provide a link between growth factor-mediated neurogenesis and the transcription of early neuronal genes. These studies show that C/EBP activation regulates the generation of neurons versus astrocytes, thereby providing a mechanism whereby growth factors can selectively bias cortical progenitors to become neurons during development.

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Results

MEK Activity Is Important for Cortical Neurogenesis

To address the signaling mechanisms underlying cortical neurogenesis, we examined progenitors isolated from E12-E13 mouse cortex and plated in FGF2 (Toma et al., 2000; Slack et al., 1998; Gloster et al., 1999); these cells are dividing, nestin-positive progenitors, many of which exit the cell cycle and express the panneuronal markers βIII-tubulin, neuron-specific enolase (NSE), MAP-2, neurofilament-M, and NeuN, as well as a neuron-specific Tα1 α-tubulin promoter linked to a nuclear β-galactosidase reporter gene (Gloster et al., 1994, 1999).

We first confirmed, as previously documented (Williams et al., 1997; Bonni et al., 1997; Park et al., 1999), that PDGF enhanced neurogenesis and CNTF enhanced gliogenesis in these cultures. Progenitors were cultured from E13 line K6 Tα1:nlacZ transgenic mouse embryos (Gloster et al., 1994); this transgene is induced in virtually all postmitotic cortical neurons (Gloster et al., 1999). Cells were then treated for 5 days with FGF2 plus or minus 50 ng/ml PDGF and immunostained for NSE or for the β-galactosidase gene product of Tα1:nlacZ (Figure 1A). This analysis revealed that relative to FGF2 alone, FGF2 plus PDGF treatment enhanced the number of cells expressing NSE or Tα1:nlacZ by approximately 2.0-fold ($p < 0.05$ in two independent experiments). We then confirmed that CNTF promoted gliogenesis by treating cultures with FGF2 plus 50 ng/ml CNTF for 5 days and then immunostaining for the astrocyte-specific glial fibrillary acidic protein (GFAP). GFAP was not expressed in progenitors cultured in FGF2 alone over this timeframe, while FGF2 plus CNTF caused 8%–25% of the cells to express GFAP in four independent experiments (Figure 1B).

One pathway activated by the PDGF and FGF receptors and implicated in cellular differentiation is the MEK-ERK pathway (Davis, 1995). To examine a potential role for this pathway in neurogenesis, we first examined it biochemically, performing Western blots for phosphorylated, activated ERKs, direct downstream substrates of MEK. PhosphoERK was detectable in progenitors cultured in FGF2, and addition of 50 ng/ml PDGF for 10 min caused an increase in phosphoERK (Figure 1C). We then utilized a recombinant adenovirus expressing an HA-tagged, dominant-inhibitory form of MEK (DN-MEK) (U. Zirrgeibel et al., submitted) to ask whether MEK-ERK pathway activation was important for neurogenesis. We have previously used recombinant adenovirus to manipulate both cortical progenitor cells and neurons (Slack et al., 1998, 1996; Toma et al., 2000; Wartiovaara et al., 2002), and have demonstrated that these vectors do not have any effect on neurogenesis or on cell survival under conditions used here.

As a prelude to biological experiments, we confirmed by Western blot analysis that a DN-MEK protein of the appropriate size was expressed in infected progenitors (Figure 1D). We then confirmed that MEK activity was inhibited; cells were infected upon plating with 50 MOI of DN-MEK or GFP adenoviruses, and maintained 3 days in FGF2. Double-label immunocytochemistry for phosphoERK and for the HA-tag in DN-MEK revealed that 90% of control, GFP-positive cells expressed detectable phosphoERK, while only 14% of DN-MEK-positive cells did so.

Having confirmed that the DN-MEK adenovirus inhibited ERK activation, we then asked whether this affected cell survival. Cells were infected with 50 MOI of DN-MEK or GFP adenoviruses, were cultured for 3 or 5 days in FGF2, and then analyzed for apoptosis. Double-label analysis for the viral gene product and for TUNEL (Figure 1E) revealed a low level of apoptosis in control or GFP-infected cells, and showed that this was not affected by DN-MEK expression at 3 or 5 days. We then asked whether the MEK pathway was important for neurogenesis. Cells were infected with 50 MOI of DN-MEK adenovirus and were cultured for 3 days in FGF2 or for 5 days in FGF2 plus 50 ng/ml PDGF. As controls, sister cultures were infected with 50 MOI of adenoviruses expressing either GFP or the wild-type MEK protein. Double-label immunocytochemistry revealed that DN-MEK, but not wild-type MEK or GFP, inhibited induction of both the Tα1:nlacZ transgene and NSE (Figure 1F and 1G). Similar results were obtained in the presence of FGF2 plus PDGF (Figure 1H). These results were not due to adenoviral infection, since similar results were obtained when progenitors were transfected with the same HA-tagged DN-MEK construct; approximately 40% of GFP-expressing cells coexpressed the neuron-specific marker NeuN at 3 days, while only approximately 25% of DN-MEK-expressing cells did so ($p < 0.05$). Thus, MEK activity is essential for neurogenesis in the presence of FGF2 with or without PDGF.

The C/EBP Transcription Factor Family Is Essential for Cortical Neurogenesis

The C/EBP family is known to regulate cellular differentiation downstream of MEK in nonneural cells (Davis, 1995). To determine a potential role for C/EBPs during neurogenesis, we first asked whether they were expressed in cortical progenitors. RT-PCR analysis demonstrated that cortical progenitors expressed C/EBPs α, β, and δ (Figure 2A). We then asked whether C/EBPs had any role in cortical neurogenesis, taking advantage of a recombinant adenovirus expressing an acidic form of C/EBP (A-C/EBP) that strongly and selectively binds to all C/EBP family members and inhibits their binding to DNA (Greenwel et al., 2000; Krylov et al., 1997). To confirm the specificity of this A-C/EBP protein for members of the C/EBP family, and not for other leucine-zipper transcription factors, gel shift assays were performed. Incubation of a molar equivalent of purified A-C/EBP with C/EBPα completely inhibited the ability of C/EBPα homodimers to bind and shift a radiolabeled double-stranded DNA containing the cognate DNA binding site (Figure 2C). In contrast, even a 100-fold excess of A-C/EBP had no effect on the binding of CREB, vitellogenin binding protein (VBP), or a fos/junD heterodimer to their cognate binding sites (Figure 2C).

We then used A-C/EBP to ask whether C/EBPs were essential for cortical neurogenesis; progenitors were isolated from Tα1:nlacZ mice, and infected with 50 MOI of adenoviruses expressing flag-tagged A-C/EBP or GFP. Western blot analysis confirmed that the infected cells expressed A-C/EBP (Figure 2B). We then measured

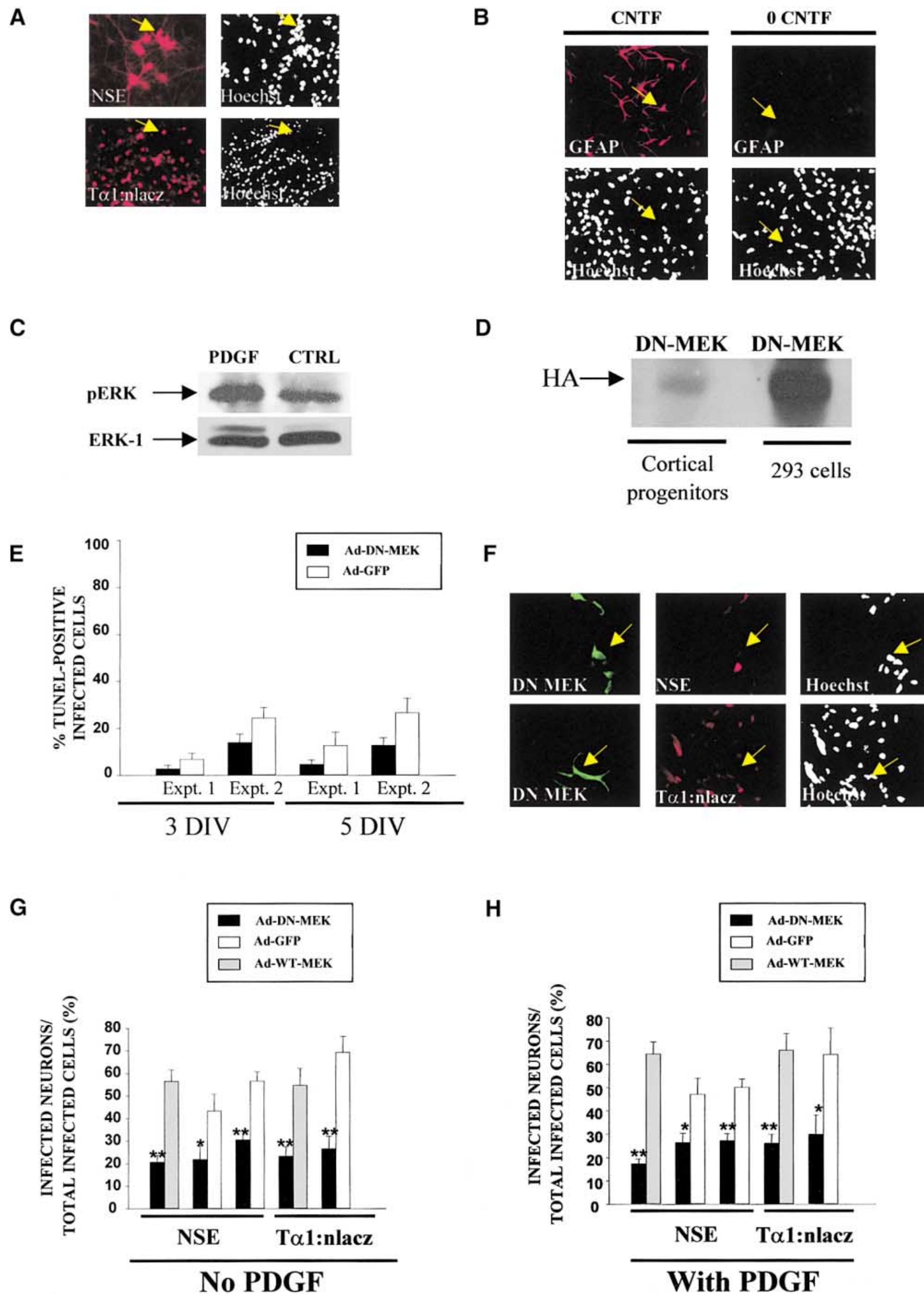


Figure 1. The MEK Pathway Is Important for the Generation of Neurons from Cortical Progenitor Cells

(A) Immunostaining for NSE (top two panels) and for the β-galactosidase product of Tα1:nlacZ (bottom two panels) in progenitors maintained in FGF2 and PDGF for 5 days. Each pair of panels represents the same field, with the left panel being the immunostaining and the right panel the Hoechst labeling to show all of the nuclei in the field.

(B) Immunostaining for GFAP in progenitors maintained for 5 days in FGF2 with (two left panels) or without (two right panels) 50 ng/ml CNTF.

(C) Western blot analysis for the phosphorylated, activated form of ERK (pERK) in progenitors maintained in FGF2 alone or stimulated for 10 min with 50 ng/ml PDGF. The bottom panel is a reprobe for total ERK-1 protein.

(D) Western blot analysis for the HA-tag in the DN-MEK protein, showing expression of a protein of the appropriate size in progenitors and 293 cells.

(E) Quantitation of the number of cells expressing either GFP or the HA-tag in the DN-MEK protein that were TUNEL positive at 3 and 5 days following adenovirus infection. Each pair of bars represents one experiment. No significant difference was observed between GFP- versus DN-MEK-positive cells ($p > 0.05$).

(F) Double-label immunocytochemistry for the HA-tag in DN-MEK and for NSE (top three panels) or the Tα1:nlacZ gene product (bottom three panels) in progenitors infected with the DN-MEK adenovirus for 3 days. In both cases, the left panels show HA-positive cells (DN MEK), the middle the neuron-specific marker (NSE or Tα1:nlacZ), and the right the Hoechst staining for the same field. Arrows indicate cells positive for DN-MEK, but not for neuronal proteins.

(G and H) Quantitation of data similar to that shown in (F), for cells infected with adenoviruses expressing DN-MEK, wild-type MEK (WT-MEK), or GFP and grown in FGF2 for 3 days (G) or FGF2 plus 50 ng/ml PDGF for 5 days (H). Each pair of bars derives from an independent experiment. *$p < 0.05$; **$p < 0.005$.

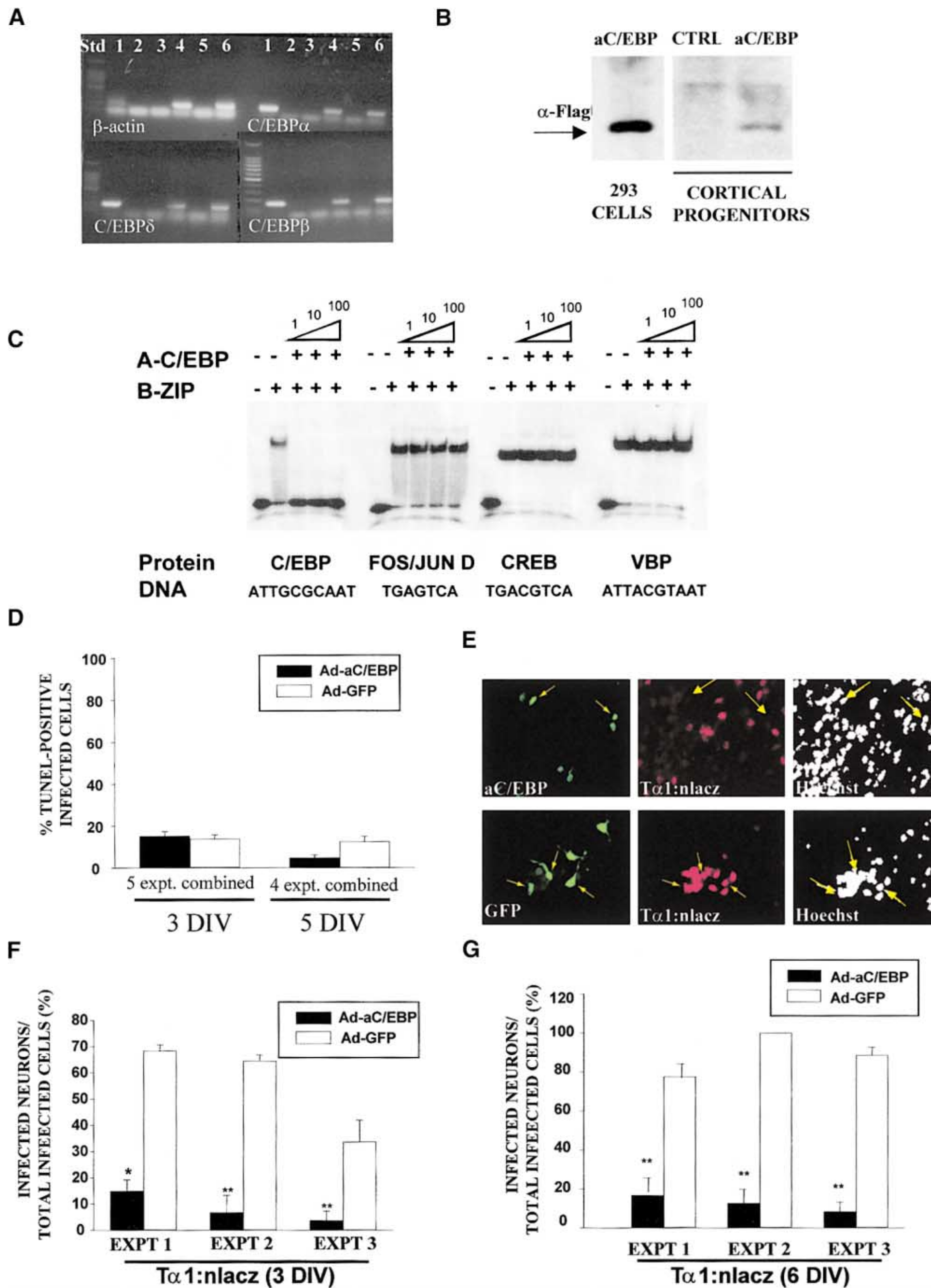


Figure 2. Transcriptional Induction of the Ta1 α-Tubulin Promoter Is Inhibited in Cortical Progenitors Expressing A-C/EBP, which Inhibits *trans*-Activation via All C/EBP Family Members

(A) RT-PCR analysis of C/EBP family members α, β, and δ in cortical progenitor cells. As a control, cells were also analyzed for β-actin mRNA. Std is the molecular weight standard, lane 1 is mouse genomic DNA as a positive control (the primers are located within the same exon), lane 2 is water, lanes 3 and 5 are progenitor cell RNA with no RT, and lanes 4 and 6 are two different preparations of progenitor cell mRNA.

(B) Western blot analysis for the flag-tagged (α-Flag) A-C/EBP protein in lysates of infected progenitors or 293 cells.

(C) Specificity of A-C/EBP for C/EBPs and not other leucine-zipper transcription factors. The purified transcription factors (B-ZIPs) C/EBPα, CREB, VBP, and the fos/junD heterodimer were mixed with purified A-C/EBP at concentrations ranging from equimolar to a 100-fold molar excess, prior to incubation with a labeled, double-stranded oligonucleotide encoding their cognate DNA binding sequence (denoted in the figure).

(D) Quantitation of TUNEL-positive cells expressing either flag-tagged A-C/EBP or GFP at 3 or 5 days (DIV) following adenoviral infection. Results are the mean ± SE of five (3 DIV) or four (5 DIV) individual experiments.

(E) Double-label immunocytochemistry of progenitors for adenovirally driven expression of flag-tagged A-C/EBP (aC/EBP) or GFP (both in green) and the nuclear β-galactosidase reporter gene (in red) driven from the Tα1:nlacZ transgene. Right panels are Hoechst staining for the same fields. Note that many GFP-positive but not A-C/EBP-positive cells express the transgene (arrows).

(F and G) Quantitation of data similar to that shown in (E) in progenitors infected with A-C/EBP (Ad-aC/EBP) or GFP (Ad-GFP) adenoviruses for 3 (F) or 6 days (G), and then analyzed for expression of Tα1:nlacZ. In total, 14 individual experiments gave similar results. Results indicate mean ± SE. *$p < 0.05$, **$p < 0.005$.

cell survival at 3 and 5 days following infection. Double-label analysis for the viral gene product and TUNEL (Figure 2D) revealed that A-C/EBP expression had no effect on survival relative to controls. Moreover, approximately the same number of cells were A-C/EBP positive at 3 and 6 days postinfection (approximately 20%), indicating that A-C/EBP expressing cells did not die during this time period. Finally, Hoechst staining of similar cultures at 6 days postinfection demonstrated that approximately 8% and 11% of GFP- versus A-C/EBP-infected cells displayed the fragmented nuclear morphology typical of apoptosis, a difference that was not statistically significant ($p = 0.171$, results from four experiments). Thus, inhibition of the C/EBPs had no effect on cell survival at any time point during these experiments.

We next asked whether C/EBPs were essential for progenitors to transcribe the neuronal Tα1 α-tubulin promoter. Double-label immunocytochemistry of cells infected for 3 days with A-C/EBP or GFP adenoviruses revealed that only 3%–14% of A-CEBP-infected cells expressed β-galactosidase from the Tα1:nlacZ promoter, relative to 33%–68% of the control, GFP-positive cells (Figures 2E and 2F). To determine whether A-C/EBP was inhibiting as opposed to delaying induction of the transgene, we performed similar experiments at 6 days postinfection. Even at this later time point, only 8%–16% of A-C/EBP-positive cells expressed the transgene versus 78%–100% of GFP-positive cells (Figure 2G).

To determine whether the C/EBPs might play a more general role in regulating induction of the neuronal phenotype, we performed similar experiments examining NSE. Immunocytochemistry of cells cultured with FGF2 for 3 days revealed that 60% or more of the cells transduced with GFP or β-galactosidase adenoviruses expressed NSE, while only 0%–7% of cells expressing A-C/EBP did so (Figure 3A). A similar decrease was observed in cells cultured for 3 days in FGF2 plus PDGF (Figure 3B). Experiments performed at a later time point with FGF2 plus PDGF confirmed that, as seen with Tα1:nlacZ, this inhibition of NSE expression persisted for at least 5 days (Figure 3C).

To confirm the specificity of these findings, we transfected progenitors with a construct encoding C/EBPβ lacking the *trans*-activation domain; this truncated protein binds to C/EBP family members and inhibits transcriptional activation (Baer and Johnson, 2000). We first ensured that we could transfect cycling progenitors; immunocytochemical analysis for Ki67, a protein expressed in dividing cells, revealed that approximately 70% of cells transfected with a GFP expression construct were actively dividing (Figure 3D). We then transfected progenitors with DN-C/EBP; double-label analysis at 3 days showed that DN-C/EBP significantly inhibited the induction of NSE relative to GFP-transfected cells (Figure 3E). Thus, C/EBP transcriptional activity is essential for induction of neuronal gene expression as progenitors become neurons.

Phosphorylation of C/EBP on the Rsk Site Promotes Neurogenesis

Activation of MEK leads to phosphorylation of C/EBPs and enhanced transcriptional activity. One kinase involved in this pathway is Rsk, which is activated by MEK/ERK and which directly phosphorylates mouse C/EBPβ on Thr217 (Buck et al., 1999). To determine whether Rsk might be one way that MEK "talks to" the C/EBPs, we performed Western blots for phosphorylated Rsk1. This analysis revealed that progenitors expressed Rsk1 and that it was activated by FGF2 (Figure 3F). To test the hypothesis that MEK might promote neurogenesis via an Rsk1-C/EBP pathway, we then utilized a C/EBPβ mutant in which Thr217 was replaced by glutamate (Glu-217), thereby acting as a phosphorylation mimic at the Rsk site.

For these experiments, we first showed that progenitors were reliably cotransfected using independent plasmids expressing GFP and red fluorescent protein (RFP) (data not shown). We then cotransfected progenitor cells shortly after plating with the C/EBPβ Glu-217 mutant and GFP, or, as controls, with wild-type C/EBPβ and GFP or GFP alone. Two days later, we performed immunocytochemistry for NSE, NeuN, or βIII-tubulin. This analysis (Figure 3G) revealed that approximately 30%–40% of cells transfected with either GFP or wild-type C/EBPβ expressed one of these neuronal genes, while 50%–60% of cells expressing the C/EBPβ Glu-217 mutant were positive for neuronal markers (Figure 3H). Thus, overexpression of a C/EBPβ mutant that is a phosphorylation mimic at the Rsk site, but not C/EBPβ alone, enhances neurogenesis.

The C/EBP Family Robustly *trans*-Activates the Tα1 α-Tubulin Promoter by Binding to Sites Conserved in the Fish α1-Tubulin Gene

Data presented here indicate that phosphorylation of the C/EBPs in response to activation of the MEK pathway leads to transcription of neuron-specific genes, and hence, induction of a neuronal phenotype. As one potential direct transcriptional target of the C/EBPs, we focused upon the promoter for the Tα1 α-tubulin gene; both the endogenous Tα1 α-tubulin gene and its isolated promoter are induced as soon as cortical progenitors become postmitotic neurons (Miller et al., 1987; Gloster et al., 1994, 1999), and we show here that transcription of this promoter in transgenic cells is inhibited by A-C/EBP (Figures 2F and 2G). To define sequences within the Tα1 promoter that might represent functional C/EBP binding sites important for neuronal gene expression, we compared it to the goldfish α1-tubulin gene that, like the rodent promoter, is neuron-specific and reinduced during neuronal regeneration (Hieber et al., 1998). This comparison revealed three sequence motifs of 6–11 nucleotides that were almost identical in sequence in the two promoters, and that were in similar locations within the first −200 nucleotides (Figure 4A). Two of these motifs, those located at −71 and −120, are at the core of putative C/EBP family binding sites (Figure 4A).

To ask whether these conserved elements were functional C/EBP sites, we performed heterologous cotransfection experiments. Initially, we utilized a minimal 176 nucleotide Tα1 α-tubulin promoter that contains all three conserved sites (Figure 4A) and that is sufficient to target neuronal gene expression in transgenic mice (P.H., A.S., A.P., and F.D.M., unpublished data). Expression plasmids encoding C/EBP α, β, or δ were cotransfected

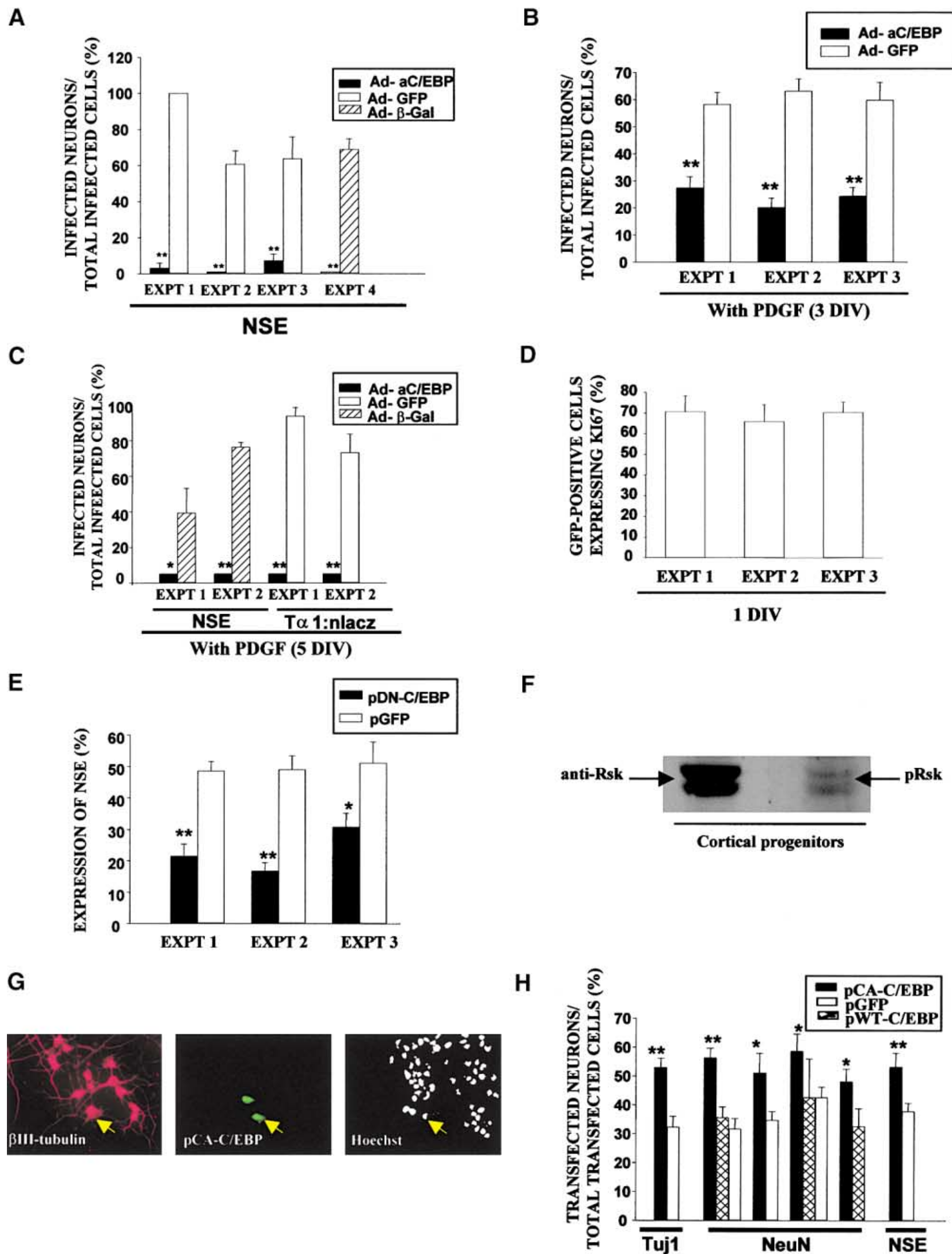


Figure 3. Inhibition of the C/EBP Family Prevents Cortical Progenitors from Becoming Neurons, while a C/EBP Mutant that Is a Phosphorylation Mimic at the Rsk Site Promotes Neurogenesis

(A) Quantitation of double-label immunocytochemistry of progenitors infected with A-C/EBP (Ad-aC/EBP), GFP (Ad-GFP), or β-galactosidase (Ad-β-Gal) adenoviruses and analyzed for NSE. 11 individual experiments gave similar results. Results represent mean ± SE. **$p < 0.005$.

(B and C) Quantitative double-label analysis similar to that shown in (A), except that the cells were cultured for 3 (B) or 5 days (C) in the presence of FGF2 plus PDGF.

(D) Quantitation of the number of GFP-positive cells expressing Ki67 1 day following transfection. Results derive from three experiments.

(E) Quantitation of immunocytochemistry of progenitors transfected with DN-C/EBPβ (pDN-C/EBP) or GFP (pGFP) expression plasmids and then analyzed for expression of NSE. **$p < 0.005$.

(F) Western blot analysis for the phosphorylated, activated form of Rsk (pRsk) or total Rsk (anti-Rsk) in progenitors cultured 1 day in FGF2.

(G) Immunocytochemical analysis for neuron-specific βIII-tubulin in cells cotransfected with the C/EBP Glu-217 mutant (CA-C/EBP) and GFP. The right panel is Hoechst staining of the same field, and the arrow indicates a double-labeled neuron.

(H) Quantitation of data similar to that shown in (G); progenitors were cotransfected for 2 days with the C/EBP Glu-217 mutant plus GFP (pCA-C/EBP), wild-type C/EBP plus GFP (pWT-C/EBP), or GFP alone (pGFP). For βIII-tubulin, results from three experiments were combined, while each set of bars for NeuN and NSE derive from independent experiments.

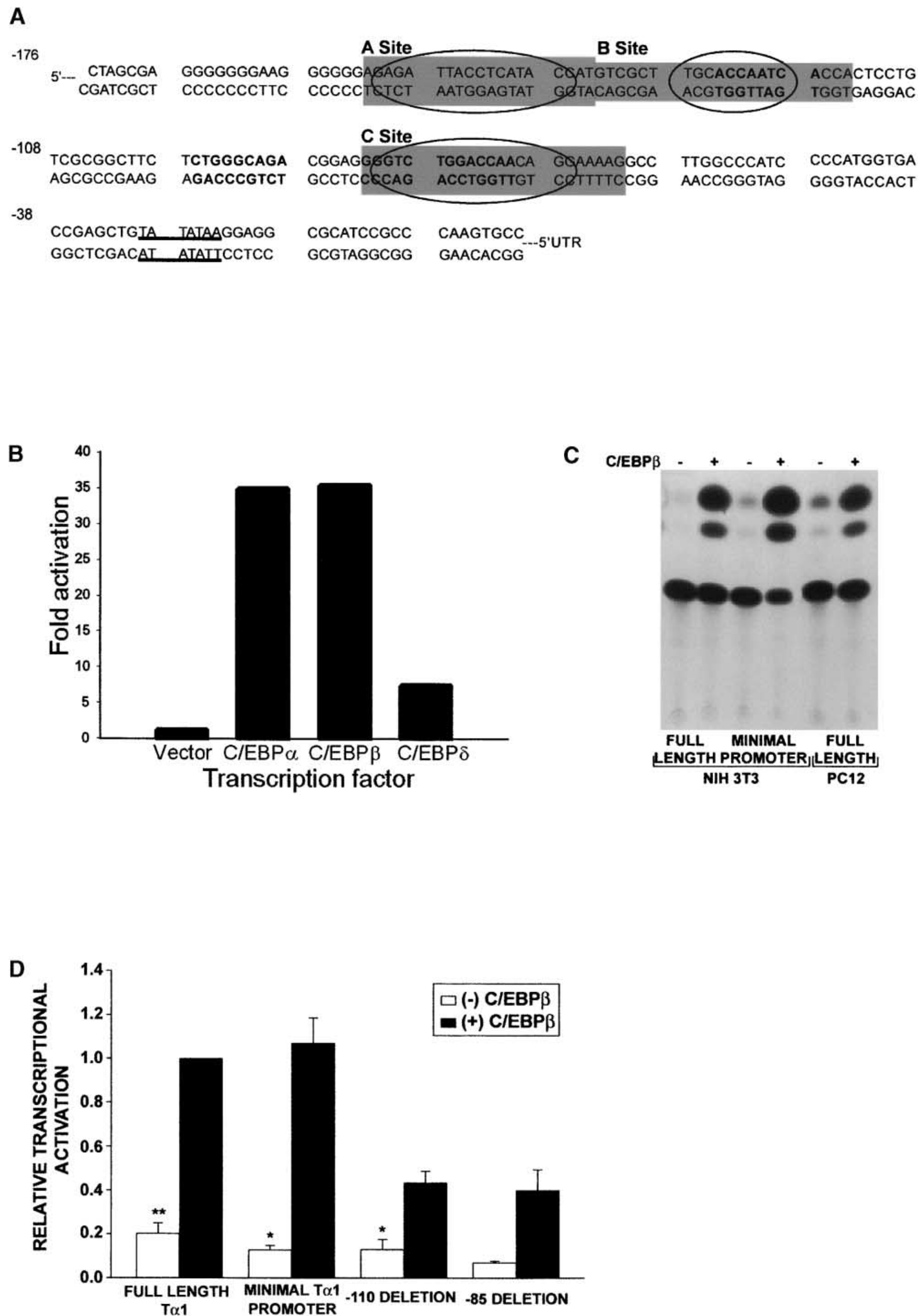


Figure 4. The C/EBP Family *trans*-Activates the Tα1 α-Tubulin Promoter, as Determined Using Heterologous Cotransfections

(A) Sequence of a minimal 176 nucleotide Tα1 α-tubulin promoter showing sequence motifs conserved in sequence and location in the goldfish α1-tubulin promoter (bold letters), the putative C/EBP binding sites (circles), and the oligonucleotides used for the gel shift assays (boxes, denoted A Site, B Site, and C Site). The underlined sequence is the TATAA box.

(B) Relative activation of the Tα1 α-tubulin minimal promoter, determined in NIH 3T3 cells by cotransfecting the reporter plasmid Tα1 minimal promoter:CAT with saturating amounts of expression plasmids encoding C/EBP α, β, or δ. Fold activation indicates the relative level of acetylated chloramphenicol produced when the reporter plasmid was cotransfected with the C/EBP-expressing plasmid versus the empty expression vector. Values were determined from saturation curves using varying amounts of expression construct with a fixed amount of reporter plasmid. In all cases, the values are those obtained using the same amount (4 μg) of reporter plasmid.

(C) Representative CAT assay of NIH 3T3 and PC12 cells cotransfected with C/EBPβ, and either the 1.1 kb (full-length) or 176-nucleotide (minimal promoter) reporter constructs.

(D) Cotransfection assays to map the sites necessary for C/EBP-mediated *trans*-activation of the minimal Tα1 promoter. Relative transcriptional activation indicates the level of β-galactosidase activity detected in NIH 3T3 cells cotransfected with plasmids expressing the same nlacZ reporter gene from various Tα1 promoter deletion constructs with or without the C/EBPβ expression plasmid. Results indicate the mean ± SEM from four separate experiments. Full-length Tα1 indicates the 1.1 kb promoter, minimal Tα1 promoter indicates the 176 nucleotide promoter fragment, and −110 and −85 deletion refers to promoter fragments truncated 5′ to those nucleotides. *$p < 0.05$; **$p < 0.005$.

with a reporter construct comprised of this minimal Tα1 promoter linked to chloramphenicol acetyl-transferase (CAT). All results were normalized to a cotransfected CMV-lacZ construct. These experiments revealed that all three C/EBP family members robustly *trans*-activated the minimal Tα1 promoter (Figure 4B). Saturation experiments with a fixed amount of Tα1 reporter construct and varying amounts of the C/EBP expression constructs revealed that C/EBPs α and β induced 35-fold increases in CAT activity, while C/EBP δ induced an approximately 10-fold increase (Figure 4B). To determine whether additional C/EBP sites were upstream of −176, we performed similar studies in NIH 3T3 cells and in the PC12 neuronal cell line using the previously described 1.1 kb Tα1 promoter (Gloster et al., 1994, 1999). CAT assays revealed that both the minimal and 1.1 kb promoters were *trans*-activated to a similar degree by C/EBPβ in NIH 3T3 cells (Figure 4C). A similar degree of *trans*-activation was observed in PC12 cells (Figure 4C) and in cos cells (data not shown).

To more precisely define the C/EBP sites responsible for this robust *trans*-activation, we generated deletion mutants of a minimal Tα1 promoter:nlacZ construct. One construct included sequences to −110, thereby eliminating the first conserved sequence motif and an additional potential C/EBP binding site (A and B sites; see Figure 4A). A second construct included sequences to −85, and eliminated the second conserved sequence, but left intact the third conserved motif (C site; see Figure 4A). Cotransfection assays in NIH 3T3 cells revealed that C/EBPβ led to a robust increase in β-galactosidase activity as driven either by the 1.1 kb or 176 nucleotide Tα1 promoters (Figure 4D). The magnitude of this increase was similar for both promoter constructs but was lower than with the CAT assays, potentially due to differences in stability of the two reporter proteins. Deletion of the sequences from −110 to −176 resulted in a consistent decrease in C/EBP-mediated *trans*-activation from 10- to 3-fold (Figure 4D). Further deletion of the promoter to −85 had no further effect, indicating (i) that site(s) essential for C/EBP *trans*-activation are located between −176 and −110, and downstream of −85 (Figure 4A), and (ii) that the conserved motif between −85 and −110 is not important for C/EBP *trans*-activation.

To further confirm that the conserved sequence elements were C/EBP binding sites, we performed gel shift assays. Oligonucleotides were generated to a consensus C/EBP site (Williams et al., 1995), as well as to sites A, B, and C in the minimal promoter (Figure 4A), and were used in gel shifts with liver nuclear extracts, a rich, well-characterized source of C/EBP activity. The consensus C/EBP oligonucleotide formed one major complex when incubated with liver nuclear extract (Figure 5A). A complex of similar size was observed with extracts from cortical progenitor cells (Figure 5B), from embryonic brain (Figure 5E), or from cortical cells isolated from the telencephalon at time points ranging from E12 to E18 (data not shown), consistent with the expression of C/EBPs in developing cortical progenitors and neurons (Figure 2). This complex formation was inhibited by competition with a 125-fold excess of cold consensus oligonucleotide, as well as by a similar excess of oligonucleotides corresponding to sites A, B, or C from the Tα1 promoter (Figures 5A, 5B, and 5E). Importantly, complex formation was not inhibited by a similar excess of a site C oligonucleotide (C^{MUT}) mutated at the core of the consensus C/EBP site (Figure 5A). Finally, inclusion of an anti-C/EBPβ antibody in gel supershift assays led to the appearance of a larger molecular weight complex (Figures 5A, 5B, and 5E), confirming that the complexes assayed here contained C/EBPs, and demonstrating that functional C/EBPβ is expressed in developing cortical progenitors and neurons.

Oligonucleotides to sites A, B, or C from the Tα1 promoter all formed similar sized complexes when incubated with liver (Figures 5A and 5C), developing brain (Figure 5D), or cortical progenitor cell nuclear extracts (Figure 5E). Complex formation with the three Tα1 promoter oligonucleotides was inhibited by inclusion of an excess of either the same oligonucleotide or of the consensus C/EBP site oligonucleotide (Figures 5A–5E). For the site C oligonucleotide, a site C oligonucleotide mutated outside the putative C/EBP binding site inhibited complex formation, while an oligonucleotide mutated within the core did not (Figure 5C). For all three oligonucleotides (sites A, B, and C), supershift assays with anti-C/EBPβ confirmed the presence of C/EBPβ in the complexes (Figures 5C–5E; data not shown).

To conclusively demonstrate that sites A, B, and C were responsible for the robust *trans*-activation of the Tα1 promoter by members of the C/EBP family, we mutated these sites within the context of the minimal promoter. Specifically, site A was mutated from AGAGATT ACCTCATACCAT to AGAGA*CC*ACCTC*G*TACCAT, site B from GTCGCTTGCACCAATCACCA to GTCGC*CC*GC ACC*G*ATCACCA, and site C from GGGTCTGGACCAA CAG to GGGGTCTGGA*GGGG*CAGGAAAAG. Initially, as a control, we made oligonucleotides containing the same mutations, and performed gel shift assays. These experiments revealed that the C/EBP binding complexes formed with the oligonucleotides for sites A, B, or C could not be competed out with the mutated oligonucleotides for those same sites (Figure 6A). We then generated the same mutations within the context of the Tα1 minimal promoter driving the CAT reporter gene, and performed CAT assays on NIH 3T3 cells heterologously cotransfected with C/EBPβ plus the mutated versus wild-type minimal promoter (Figure 6B). All data were normalized to a cotransfected CMV-lacZ construct. These assays revealed that, while the mutations had little or no effect on the basal level of transcription from the minimal promoter, the robust C/EBP-mediated transcription was greatly decreased (Figure 6B). Thus, these three sites are in fact bona fide C/EBP binding sites, and they are essential for the robust, C/EBP-mediated transcriptional activation of the Tα1 promoter.

Inhibition of C/EBPs Enhances CNTF-Mediated Gliogenesis

Together, these data support the hypothesis that MEK activation leads to Rsk-mediated phosphorylation of C/EBPs, and that C/EBPs then promote neurogenesis from cortical progenitor cells by directly *trans*-activating neuronal genes such as Tα1 α-tubulin. To determine the fate of cortical progenitors expressing A-C/EBP, we first asked whether they were subverted to an astrocytic

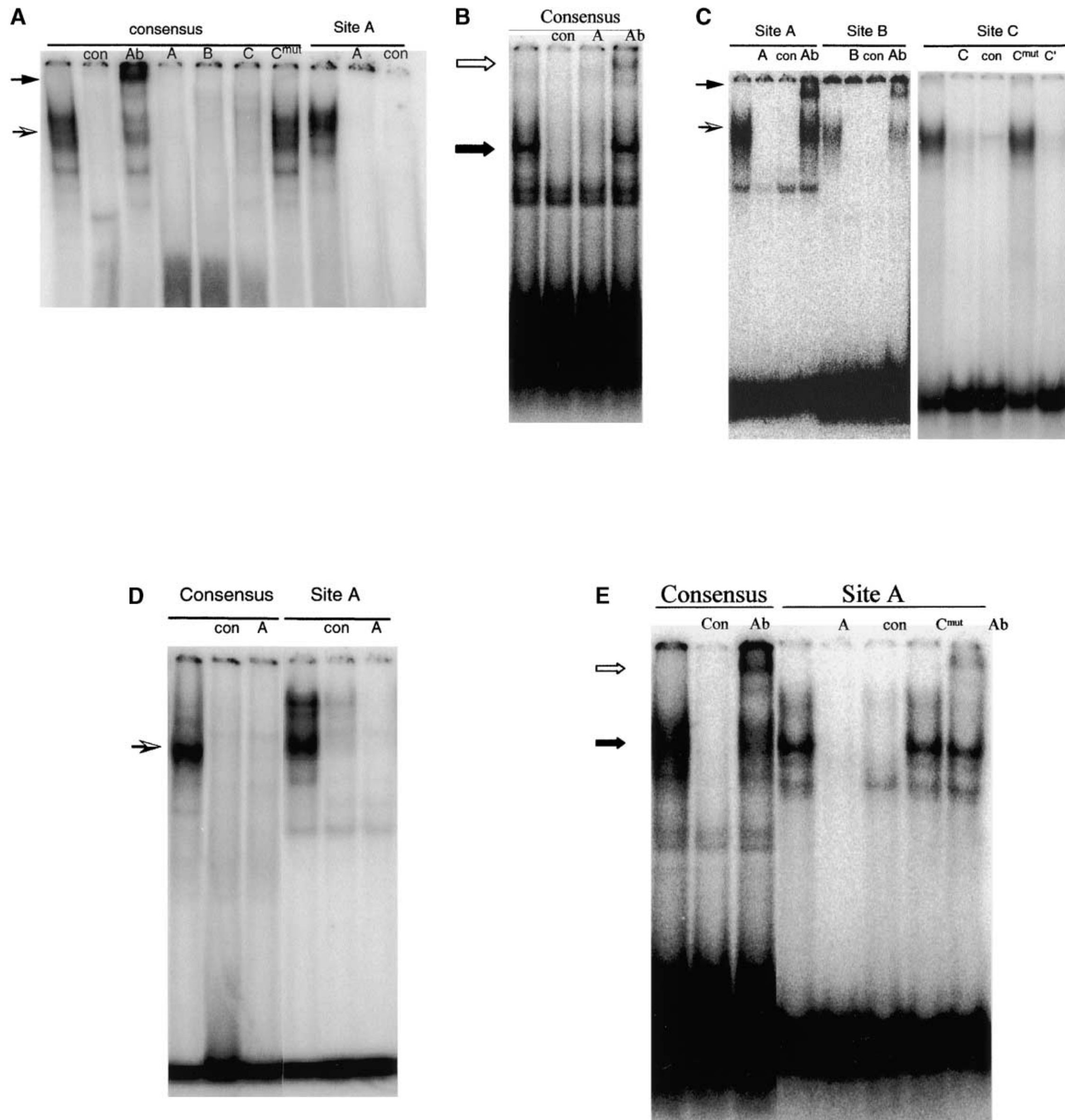


Figure 5. Gel Shift Assays for the Three Consensus C/EBP Sites in the Tα1 Promoter in Nuclear Extracts from Liver, Developing Brain, and Cortical Progenitor Cells

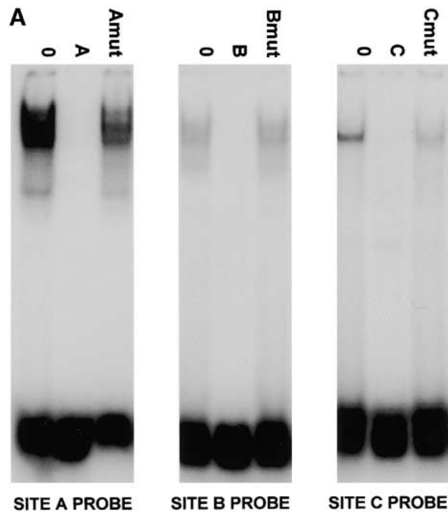
(A) Liver nuclear extracts were incubated with radiolabeled oligonucleotides corresponding to a consensus C/EBP binding site (TCGACTCCCT GATTGCGCAATAGGCTCC) (Williams et al., 1995), or the A site from the Tα1 promoter (see Figure 4A), plus or minus a 125-fold excess of either the consensus oligonucleotides (con) or of oligonucleotides corresponding to sites A, B, or C from the Tα1 promoter (see Figure 4A). As a control, competitions were performed with a C site oligonucleotide mutated within the consensus C/EBP binding motif (C^{mut}). A major complex of similar size (open arrow) was formed with both radiolabeled oligonucleotides. The consensus oligonucleotide was also incubated with anti-C/EBPβ (Santa Cruz) (lane marked Ab), which induced a supershift to a higher molecular weight (black arrow).

(B) Nuclear extracts from cortical progenitors were incubated with the radiolabeled consensus C/EBP oligonucleotide. Competitions were performed with a 125-fold excess of oligonucleotides corresponding to the consensus sequence (con) or to site A (A). A larger band appeared when the complex was incubated with an anti-C/EBPβ antibody (Ab; open arrow).

(C) Liver nuclear extracts were incubated with radiolabeled oligonucleotides corresponding to the A, B, or C sites from the Tα1 promoter, plus or minus a 125-fold excess of either the consensus oligonucleotide (con) or oligonucleotides corresponding to the A, B, or C sites. As a control, competitions were performed with a C site oligonucleotide mutated within the putative C/EBP binding sequence (C^{mut}) or outside this sequence (C′). The open arrow indicates the major shifted complex. Oligonucleotides to sites A and B were also incubated with anti-C/EBPβ (Ab), which caused a supershift (black arrow).

(D) Nuclear extracts from the E18 brain were incubated with radiolabeled oligonucleotides corresponding to the consensus C/EBP binding site or the A site from the Tα1 promoter with or without a 160-fold excess of the same cold oligonucleotides. The major complex is similar for both oligonucleotides, but an additional complex is formed with the A site oligonucleotide.

(E) Nuclear extracts from cortical progenitor cells (for the Site A oligonucleotide) or from liver (for the consensus oligonucleotide) were incubated with oligonucleotides corresponding to the consensus sequence or Site A. Competitions were performed with oligonucleotides to the consensus or to the A site, and supershifts were performed using the antibody to C/EBPβ (Ab). The black arrow denotes the major complex, while the open arrow denotes the supershifted complex.



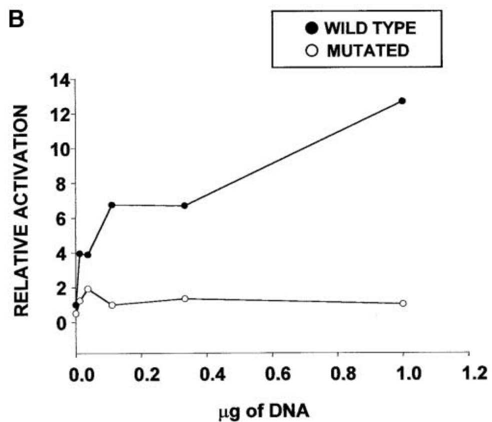


Figure 6. Sites A, B, and C Are Essential for C/EBPs to *trans*-Activate the Tα1 α-Tubulin Promoter

(A) Gel shift assays with liver nuclear extracts and radiolabeled, double-stranded oligonucleotides corresponding to sites A, B, or C, and then competed with a 450-fold excess of either the wild-type or mutated (Amut, Bmut, and Cmut) oligonucleotides for the same sites.

(B) Relative levels of activation of the wild-type versus mutant Tα1 α-tubulin minimal promoter, as determined in NIH 3T3 cells by cotransfecting a fixed amount of the reporter CAT plasmids with increasing amounts of the C/EBPβ expression plasmid. All data were normalized to a cotransfected CMV:lacZ control plasmid. Relative activity indicates the relative level of acetylated chloramphenicol produced when the reporter plasmid was cotransfected with the C/EBP-expressing plasmid versus cotransfection with the empty expression vector.

fate (as occurs with CNTF treatment), or whether they remained undifferentiated. To distinguish these two possibilities, at 3–4 days postplating, we performed double-label analysis for flag, to detect A-C/EBP-expressing cells, and GFAP or nestin, markers for astrocytes and neural progenitor cells, respectively. This analysis revealed that, in 5 separate experiments, the large majority of A-C/EBP-positive cells coexpressed nestin (Figure 7A), while in 11 separate experiments, none of the A-C/EBP-positive cells expressed GFAP, suggesting that they remain as undifferentiated progenitor cells.

We then asked whether inhibition of C/EBP affected the gliogenesis that occurs when CNTF is added to the cultures in the presence of FGF2. Progenitors were infected with A-C/EBP, β-galactosidase, or GFP adenoviruses and then exposed to FGF2 plus 50 ng/ml CNTF. Immunostaining 5 days later revealed that while 10%–30% of control, GFP- or β-galactosidase-expressing cells coexpressed GFAP, 70%–90% of the A-C/EBP-positive cells expressed this glial marker (Figure 7C). Thus, inhibition of C/EBP activity biases cortical progenitor cells to become astrocytes in response to CNTF, suggesting that C/EBP transcriptional activity not only promotes neurogenesis, but also actively inhibits gliogenesis in cortical progenitor cells.

Discussion

Data presented here support four major conclusions. First, these studies demonstrate that the activities of both MEK and the C/EBP family are necessary for cortical progenitors to become neurons in the presence of FGF2 and/or PDGF. Second, our data with the C/EBPβ Rsk-site phosphorylation mimic suggests that a MEK-Rsk pathway directly promotes the generation of neurons from cortical progenitors via phosphorylation of C/EBPs. Third, experiments with the Tα1 α-tubulin promoter indicate that the C/EBPs promote the progenitor-to-neuron transition by direct transcription of neuron-specific genes. Finally, our studies examining glial differentiation suggest that activated C/EBPs not only promote neurogenesis, but also inhibit growth-factor-mediated gliogenesis. Together, these data support a model where growth factors that enhance the generation of neurons do so via activation of a MEK-Rsk-C/EBP signaling cascade that leads directly to transcription of early, neuron-specific genes, while at the same time inhibiting the cells from responding to growth factors that promote gliogenesis, such as CNTF.

Previous work has clearly demonstrated that intrinsic genetic mechanisms play an essential role in regulating the generation of neurons from progenitor cells, but that these intrinsic programs are heavily influenced by environmental determinants such as growth factors (Shen et al., 1998; Lillien, 1998). One example of such a growth factor is PDGF which, when added to cultured cortical progenitors, enhances neurogenesis in a transcription-dependent fashion, and inhibits CNTF-mediated gliogenesis (Williams et al., 1997; Park et al., 1999). A second example is CNTF, which will prematurely induce the formation of astrocytes by cortical progenitor cells (Park et al., 1999). In this regard, a key question is how these growth factors signal to direct neural precursors to become neurons versus glial cells. This question has been answered for CNTF- and BMP-mediated astrocyte formation, which is at least partially due to activation of JAK-STAT (Bonni et al., 1997) and Smad-dependent (Nakashima et al., 1999) pathways, with synergistic interactions between the two pathways mediated via the tran-

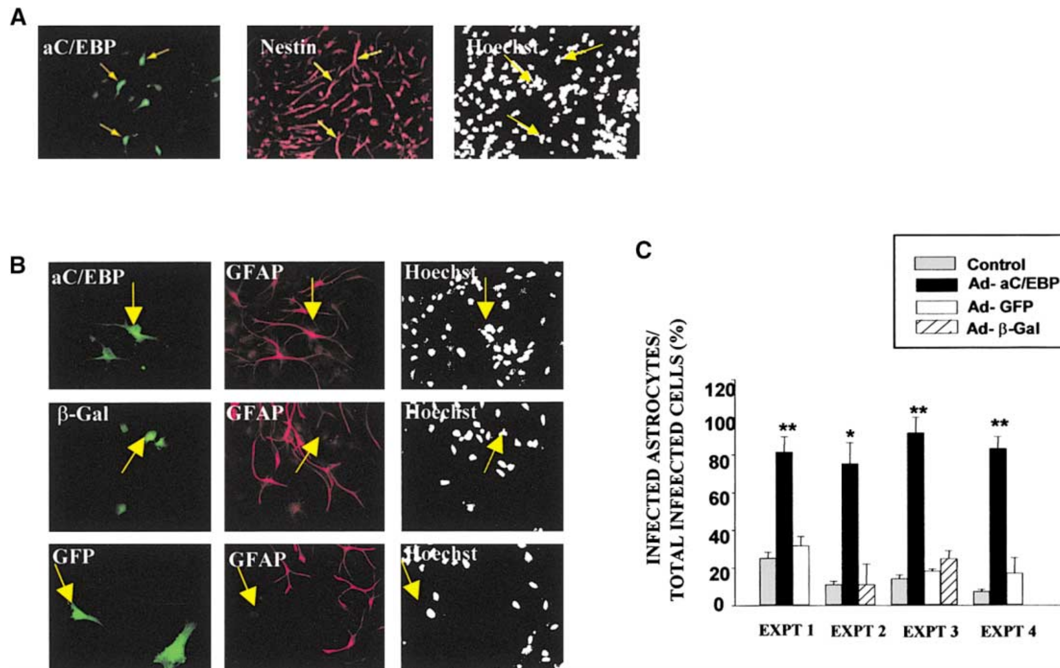


Figure 7. Inhibition of C/EBP Promotes Gliogenesis

(A) Immunocytochemistry for the flag tag in A-C/EBP and for the neural progenitor cell marker nestin showing that cells expressing aC/EBP express nestin (arrows). Similar results were obtained in five separate experiments.

(B) Immunostaining of progenitors transduced with A-C/EBP (top three panels), β-galactosidase (middle three panels), or GFP (bottom three panels) adenoviruses, and treated with FGF2 plus CNTF for 5 days. The left panels are immunostaining for the appropriate viral protein, the center for GFAP, and the right are Hoechst staining of the same fields. Arrows indicate the same cells in all three panels.

(C) Quantitation of data similar to that shown in (B), where progenitors were infected with A-C/EBP (Ad-aC/EBP), GFP (Ad-GFP), or β-galactosidase (Ad-B-gal) adenoviruses versus uninfected controls. Each group of bars derives from a different, independent experiment, and results are mean ± SE. $*p < 0.05$; $**p < 0.005$.

scriptional coactivators p300/CBP (Nakashima et al., 1999). However, the signaling pathways utilized by factors such as PDGF to promote the generation of neurons are largely unexplored. Thus, while a number of studies indicate that bHLH transcription factors such as Mash1 and Neurogenins1 and 2, as well as their negative regulators, the Ids, are key intrinsic determinants of neurogenesis from cortical precursors (Sun et al., 2001; Nieto et al., 2001; Toma et al., 2000), the work presented here identifies a novel, extrinsic, growth factor-mediated signaling cascade that promotes the generation of neurons. The importance of this pathway in vivo during embryogenesis is currently the subject of investigation.

Precedent for the C/EBP family of transcription factors acting to regulate cellular differentiation in response to growth factors derives from studies on a number of developing systems, most notably hematopoiesis and adipogenesis (Johnson and Williams, 1994; Lekstrom-Hines and Xanthopoulos, 1998). In these systems, various members of the C/EBP family act in concert with cell-type-specific determination signals to promote or repress transcription of genes essential for terminal differentiation. By analogy, we propose that in cortical progenitors, the C/EBPs act not as "determination" factors in the same sense as bHLHs like the neurogenins, but as differentiation factors responsible for initiating the transcription of early neuronal genes in response to growth factor cues. In this model, progenitor cells would be partially biased to become neurons as a consequence of the repertoire of HLHs that they express, and growth factor-mediated activation of the C/EBPs would provide the final "push" to trigger expression of a neuronal phenotype via transcription of genes such as Tα1 α-tubulin. Thus, expression of the appropriate bHLHs would be a requisite for the neurogenic actions of the C/EBPs; such necessary cooperativity would allow the C/EBPs to function as growth factor-regulated differentiation signals in a variety of different cell types. In this sense, the C/EBPs would act neither as classic differentiation or fate signals, but would instead act, with regard to their proneurogenic activities, as necessary cofactors for fate-biasing molecules such as the bHLHs and, in the case of their antigliogenic action, as negative modifiers of cues such as CNTF that directly promote an astrocytic fate.

How could the C/EBPs collaborate with the neurogenic bHLHs to generate a postmitotic neuron? One potential link between these two families of transcription factors are the transcriptional coactivators p300/CBP (Goodman and Smolik, 2000). Interestingly, p300/CBP bind to neurogenic bHLHs such as Neurogenin1 (Sun et al., 2001) as well as to C/EBPβ (Mink et al., 1997). Thus, p300/CBP may provide a key link between activated C/EBPs and neurogenic bHLHs during neurogenesis. Are the C/EBPs expressed at an appropriate time and place to cooperate with neurogenic bHLHs in such a fashion? The RT-PCR data presented here indicate that the mRNAs for C/EBPs α, β, and δ are all expressed in

cultured cortical progenitors, while the gel shift data confirms that functional C/EBPs, including C/EBPβ, are expressed in cultured cortical progenitors, in the embryonic brain, and in cells from the embryonic telencephalon from E12 to E18, likely both progenitors and neurons. Since neurogenic bHLHs are expressed in cortical progenitors both in vivo and in culture (Nieto et al., 2001; F.B.H. and F.D.M., unpublished data), then such interactions may well occur.

Do the C/EBPs also play a role in regulating terminal mitosis as progenitors become neurons? Although our data do not directly address this issue, the C/EBPs have been implicated in growth arrest in nonneural cells, an effect they mediate at least partially via the pRb family (Chen et al., 1996), which is essential for cortical progenitors to become neurons (Slack et al, 1996, 1998). Moreover, interactions between pRb and C/EBPβ are thought to enhance transcription of cell-type-specific genes during differentiation of adipocytes (Chen et al., 1996). Thus, interactions between the C/EBPs and the pRb family could provide an integral link between terminal mitosis and induction of the neuronal phenotype in cortical progenitor cells.

Data presented here indicate that one-way growth factors activate the C/EBP family during neurogenesis is via MEK-ERK-Rsk, a major, well-characterized kinase pathway downstream of tyrosine kinase receptors (Davis, 1995). In this regard, both FGF2 and PDGF activate C/EBPs in osteoblasts (Wadleigh and Herschman, 1999), and a MEK-ERK-Rsk-C/EBPβ pathway has recently been implicated in the control of hepatocyte proliferation in response to TGFα (Buck et al., 1999). However, while data presented here indicate that phosphorylation of C/EBPβ at the Rsk site is sufficient to enhance neurogenesis, C/EBPβ can also be directly phosphorylated by ERK itself (Nakajima et al., 1993; Williams et al., 1995). Thus, tyrosine kinase receptors may signal to activate the C/EBPs via several parallel signaling cascades, one of which is the MEK-ERK-Rsk pathway.

One of the surprising findings reported here is that activation of the C/EBPs not only promoted neurogenesis, but also inhibited gliogenesis in response to CNTF. Interestingly, previous work on PDGF during cortical neurogenesis would predict such a result; PDGF enhanced the generation of neurons from cortical progenitors at the same time that it inhibited CNTF-mediated astrocyte formation (Park et al., 1999), effects that could both be explained by the MEK-Rsk- C/EBP pathway defined here. Such a dual action may turn out to be the rule rather than the exception, since recent work with the bHLHs indicate that they too bias progenitors to become neurons at the same time that they inhibit them from becoming glial cells (Tomita et al., 2000; Nieto et al., 2001; Sun et al., 2001). For bHLHs such as Neurogenin1, these two effects are apparently mediated via different mechanisms, with the proneurogenic effects being transcriptionally mediated and the antigliogenic effects resulting from Neurogenin1-mediated sequestration of p300/CBP (Sun et al., 2001). For the C/EBPs, it is clear that at least some of the proneurogenic effects are transcriptionally mediated, but the mechanisms underlying the antigliogenic effects remain to be determined.

Together, these studies identify a novel growth factor-regulated signaling cascade, a MEK-C/EBP pathway that promotes the genesis of neurons from neural precursors. We propose that this pathway provides an intracellular mechanism for integrating and coupling diverse growth factor stimuli to the intrinsic cellular machinery, thereby ensuring that neurons are generated in appropriate numbers at the appropriate time during development.

Experimental Procedures

Cortical Cultures

Cortical progenitor cells were cultured from E12-E13 mouse embryos using protocols previously described (Slack et al., 1998; Gloster et al., 1999; Toma et al., 2000). Cell density was 100,000 cells per well. For adenovirus infection, progenitor cells were infected at the time of plating, and 24 hr later half the media was changed. Cells were fed every 2 days. For experiments with PDGF or CNTF, cells were plated first in FGF2 for 12 hr, and then PDGF or CNTF plus FGF2 was added to the media for an additional 2.5 or 4.5 days.

Recombinant Adenoviruses

The recombinant adenoviruses expressing β-galactosidase and GFP are made in the Ad5 backbone and have been previously described (Slack et al., 1996; Toma et al., 2000). The flag-tagged A-C/EBP construct has been previously described (Krylov et al., 1997; Greenwel et al., 2000) and was recombined into a replication-deficient adenovirus backbone deleted in E1 and E3 (Ad-aC/EBP). Wild-type or DN-MEK constructs (kindly provided by Natalie G. Ahn, University of Colorado) were cloned into the replication-deficient Ad5 backbone, which is deleted in E1 and E3 (U. Zirrgeibel et al., submitted). All adenoviruses drive expression from the CMV promoter and were purified and titered as previously described (Slack et al., 1996; Toma et al., 2000).

Immunocytochemistry and TUNEL

Immunocytochemistry and TUNEL were performed essentially as described (Wartiovaara et al., 2002; Toma et al., 2000). The following primary antibodies were used: polyclonal antibodies to β-galactosidase (ICN Biomedicals), NSE (Polysciences Inc.), nestin (Pharmingen), neurofilament-M (Chemicon), GFAP (DAKO), and monoclonal antibodies to the flag tag (Sigma), the HA-tag (Boehringer), βIII-tubulin (RDI), NeuN (Chemicon), anti-phosphoERK (Promega), or Ki67 (Pharmingen). All secondary antibodies were obtained from Jackson Immunoresearch Laboratories Inc. (West Grove, PA).

For quantitation, three to five random images of each treatment (per experiment) were captured and processed. Digital image acquisition and analysis was performed with the Northern Eclipse software (Empix Inc) using a Sony XC-75CE CCD video camera.

Western Blot Analysis

For biochemistry, cortical progenitor cells were cultured in 60 cm dishes, lysed, and analyzed as described previously (Toma et al., 2000). Blots were probed with anti-flag, anti-HA, anti-phosphoERK, anti-phosphoRsk (Santa Cruz), and anti-Rsk (Santa Cruz).

RT-PCR Analysis

Total RNA from cortical progenitor cells was isolated with Triazol as described by the manufacturer (GIBCO-BRL). cDNA was prepared from this RNA using random hexamer primers and M-MuLV reverse transcriptase as described by the manufacturer (MBI-Fermentas). The sequence of the primers used for the PCR reaction were: β-actin, 5′-TGGAGAAGAGCTATGAGCTGCCTG-3′ and 5′-GTGCCACCAGACAGCACTGTGTTG-3′; C/EBPβ, 5′-GCGCGAGCGCAACAACATC-5′ and 5′-TGCTTGAACAAGTTCCGCAG-3′; C/EBPα, 5′-AAGGCCAAGAAGTCGGTGGA-3′ and 5′-CAGTTCACGGCTCAGCTGTT-3′; and C/EBPδ, 5′-ACAGTCCGAGAAAAGGGCGC-3′ and 5′-CCAGGTCCCGGGTGAGCT-3′.

Transfection of Cortical Progenitor Cells

Cortical progenitor cells were plated at a density of 1×10^5 cells/well of a 4-well chamber slide (Nalgene-Nunc) in 700 μl of medium

plus FGF2. A total of 1 μg of DNA and 1 μl of lipofectamine (GIBCO-BRL) were added to 100 μl of OPTI-MEM (GIBCO-BRL) medium, which after mixing, was added to the plated cells overnight. The wild-type, dominant-negative, and Rsk-site phosphorylation mutant C/EBPβs were expressed in pCDNA3.1, containing the CMV promoter. GFP was expressed from pEGFP-N1 (Clontech). Wild-type and DN-MEK were expressed from the CMV promoter in the same Ad5-based plasmids used to generate the recombinant adenovirus.

Heterologous Cotransfection Assays

Plasmids used for the cotransfections included pMEX driving expression of C/EBPα, β, or δ (Williams et al., 1991), pSV2CAT, and pUC19 containing the various Tα1 α-tubulin promoter constructs driving expression of CAT or β-galactosidase. The β-galactosidase expression constructs were made by deleting a previously described Tα1:nlacZ transgene (Gloster et al., 1994) by excising sequences upstream of −176, −110, or −85 and leaving the remainder of the transgene. The point mutations in the Tα1 minimal promoter were made using site-directed mutagenesis. All constructs were confirmed by DNA sequencing. For cotransfections, cells were grown to 60%–80% confluence and were transfected with 1–4 μg of the reporter plasmid, up to 4 μg of plasmid expressing a C/EBP family member, and 1–4 μg of pSV2CAT or CMV:β-galactosidase plasmid for normalization. For saturation studies, varying amounts of the C/EBP expression plasmid were added, along with a carrier plasmid to ensure that the same amount of DNA was utilized at each point. Cells were transfected with Superfect (Qiagen) and harvested and lysed at 48 hr. β-galactosidase activity was measured using ONPG (Promega) as per the manufacturer's instructions. CAT assays were performed using BODIPY 1-deoxyCAM substrate (Stratagene) as per manufacturer's instructions.

Electrophoretic Mobility Shift Assays

The oligonucleotides used in the EMSAs are as follows (5′ to 3′, sense strand): consensus C/EBP, TCGACTCCCTGATTGCGCAAT AGGCTCC (Williams et al., 1995); site A, GAGAGATTACCTCATAC CAT; mutant site A, GAGAGA*CC*ACCTC*G*TACCAT site B, GTCGCTT GCACCAATCACCA; mutant site B, GTCGC*CCG*CACC*G*ATCACCA; site C, GGGGTCTGGACCAACAGGAAAAGG; oligonucleotide C[MUT] (site C with the putative C/EBP binding site mutated), GGGGT CTGGA*GGGG*CAGGAAAAGG; and oligonucleotide C′ (site C mutated outside of the putative C/EBP binding site) GGGGTCTGGAC CAACAG*CCCC*AGG. In all cases, complementary oligonucleotides were also synthesized in the opposite orientation. 3 μg of the two complementary oligonucleotides for each site were 5′ end labeled with 32P-ATP and T4 polynucleotide kinase (Promega), and the labeled oligonucleotides purified by gel filtration prior to annealing.

Nuclear extracts were made as previously described (Husain et al., 1996) with minor modifications. EMSAs were performed essentially as described (Lichtsteiner et al., 1987). 20–40 μg of liver or brain nuclear extract was incubated with 1.2 ng of annealed, 32P-labeled double-stranded oligonucleotide for 1 hr at 5°C–8°C in a 25 μl reaction volume containing 25 mM HEPES, 60 mM Kcl, 7.5% glycerol, 0.1 mM EDTA, 0.75 mM DTT, 5 mM $MgCl_2$, pH 7.9, and 1 μg of poly-dIdC. Samples were analyzed on 5% acrylamide, 0.4 M glycine gels (pH 8.5) and analyzed with a phosphoimager. Competition assays contained 150 or 200 ng of cold double-stranded oligonucleotides. Supershifts were performed using one of two different antibodies specific to C/EBPβ (Santa Cruz SC-150X, 8 μg; Geneka, as per manufacturers instructions). For competitions and supershifts, the cold oligonucleotide or antibody were preincubated with the nuclear extract at 5°C–8°C for 30 min prior to addition of labeled oligonucleotide. EMSAs were also performed using Geneka's Nushift C/EBPβ supershift kit, with similar results. Gel shift assays with purified leucine-zipper transcription factors and A-C/EBP were performed essentially as previously described (Moitra et al., 1998).

Acknowledgments

We would like to thank D. Kaplan and S. Stifani for discussions and advice, K. Wartiovaara and F. Said for amplifying and purifying adenoviruses, S. Morris for advice regarding transfection of progenitors, and the members of the Miller and Kaplan laboratories for advice, discussions, and technical support. This work was supported by grants from the Canadian Institutes of Health Research to F.D.M. F.D.M. is a CIHR Senior Scientist and a Killam Scholar, and F.B.H. is supported by the CIHR, FRSQ, and a Tomlinson Foundation Studentship. X.M.Y. was supported by a fellowship from the Canadian NeuroSciences Network during the course of this work.

Received: October 30, 2001
Revised: September 13, 2002

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