FEBS Letters 510 (2002) 50–56 FEBS 25612

NGF-dependent and tissue-specific transcription of *vgf* is regulated by a CREB–p300 and bHLH factor interaction

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Received 12 November 2001; accepted 23 November 2001

First published online 4 December 2001

Edited by Julio Celis

Abstract Neurotrophins support neuronal survival, development, and plasticity through processes requiring gene expression. We studied how *vgf* target gene transcription is mediated by a critical promoter region containing E-box, CCAAT and cAMP response element (CRE) sites. The p300 acetylase was present in two distinct protein complexes bound to this region. One complex, containing HEB (ubiquitous basic helix–loop–helix (bHLH)), bound the promoter in non-neuronal cells and was involved in repressing *vgf* expression. Neurotrophin-dependent transcription was mediated by the second complex, specific for neuronal cells, which included CRE binding protein and MASH1 (neuro-specific bHLH), bound the CCAAT motif, and was target of neurotrophin signalling. The interaction, mediated by p300, of different transcription factors may add specificity to the neurotrophin response.

Key words: Neurotrophin; *Vgf*; Transcription; Basic helix–loop–helix; cAMP response element binding protein; p300

1. Introduction

Neurotrophins (nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), NT4/5 and NT6) control neuronal survival, differentiation and plasticity, and are involved in learning and memory [1–3]. They use two types of receptors: TrkA–C tyrosine kinases, specific for different neurotrophins, and $p75^{NTR}$, which is bound by all and lacks kinase activity. $p75^{NTR}$ signals survival or apoptosis via JNK, p53, nuclear factor-κB and other intermediates, whereas Trk family receptors promote differentiation and survival through Ras/mitogen-activated protein kinase (MAPK), PI3K/AKT, and PLC-γ signalling cascades [1,2]. cAMP response element binding proteins (CREBs) are critical mediators of neurotrophin-dependent gene expression and of transcription of 'memory genes' during LTP [4,5]. Neurotrophins cause sustained activation of MAPKs (ERK/MAPKs) through cooperation of Ras and cAMP-dependent protein kinase signalling pathways [6]. This switches on Rsk2, a kinase that phosphorylates CREB bound to the CRE [7,8]. Since CREB is ubiquitous and responds to a variety of different stimuli, other factors must be involved in conferring transcriptional specificity to neurotrophin signals. We addressed this question by studying *vgf* transcription, which is developmentally regulated, modulated by electrical activity, and selectively induced by NGF, BDNF and NT3 [9–11]. *Vgf* encodes a precursor protein that undergoes regulated secretion and cleavage into peptides, regulating the body energy balance [12]. Tissue specificity and neurotrophin responsiveness involve a tripartite promoter region of about 110 bp (from −180 to −70) that includes E-box (CAGGTG), CCAAT (T*CCAAT*CATTGGA) and CRE (CAT*TGACGTCA*ATG) consensus sequences [10,11]. The E-box was shown to have a dual role as repressor and weak enhancer in non-neuronal and neuronal cells, respectively [13], and to bind HEB, a member of the E protein family (HEB, E2A, E2-2) of ubiquitous basic helix–loop–helix (bHLH) transcription factors [14]. The *vgf* CRE, by itself, mediates the weak transcriptional response elicited by cAMP in PC12 cells, but it is not sufficient for the stronger activation by NGF, which requires the CCAAT site as well [10,11]. CREB and the CREB family proteins ATF1 and ATF2 were shown to bind the *vgf* CRE in in vitro binding experiments, whereas the CCAAT bound factors were not characterized [10]. By using as probe the whole 110 bp region, rather than short sequences containing a single protein binding site, we showed the presence of two protein complexes interacting with the *vgf* promoter in vitro. The C complex, neuro-specific, strongly binds the CCAAT sequence, while the E–CRE complex, ubiquitous, interacts with E-box and CRE sites [13].

In this work, we have investigated which factors are present in the two complexes and what is their function. The p300 acetylase was present in both complexes, which appeared to have opposite functions in transcriptional regulation. The E–CRE complex, which involved HEB, appeared to have a role in tissue-specific repression, whereas the C complex, which included CREB and the neuro-specific bHLH MASH1, appeared to mediate neurotrophin responsiveness.

2. Materials and methods

2.1. Cell culture and transfections

Cells were cultured as previously described [11,13]. NIH3T3 cells

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Abbreviations: bHLH, basic helix–loop–helix; NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT3–6, neurotrophins 3–6; CRE, cAMP response element; CREB, CRE binding protein

PII: S0014-5793(01)03227-6

(3×10^5 cells/35 mm plate) and PC12 cells (3×10^6 cells) were transfected overnight by Lipofectamine (Gibco BRL). Transfections contained 0.5 μg reporter plasmid, the indicated amounts of expression vector DNAs and 0.2 μg pRSV-βgal as internal standard; vector DNA was added to keep the DNA amount constant. Following transfection, NIH3T3 cells were cultured for 24–30 h and PC12 cells were subcultured in two dishes, one of which was treated with 100 ng/ml NGF for 36 h. Cells were lysed and processed for CAT activity as described [13]. Reported CAT values, normalized to β-galactosidase activity of the standard, are relative to those observed with the reporter alone. Each value represents the average, and standard deviation of the mean, of at least three independent transfections.

For HEB detection, nuclear proteins were extracted from a 100 mm plate of pCSHEB-transfected NIH3T3 cells. Western blots were probed with anti-HEB (Santa Cruz, 1/100), followed by protein A–horseradish peroxidase and ECL detection (Amersham).

For differentiation analysis, PC12 cells (1.5×10^6 cells) were transfected with 1 μg pRSV-βgal and the indicated amounts of E1A dl922–947 DNA. After 24 h cells were resuspended in 1% serum, replated, stimulated with 100 ng/ml NGF, grown for 3–4 days, fixed (2% formaldehyde–0.2% glutaraldehyde in 0.1 M phosphate buffer) and stained for β-galactosidase with X-gal solution. Cells were counted as differentiated if the length of neurites was at least 1.5 times the cell body diameter.

2.2. Plasmid construction

−179*vgf*CAT was previously described [13]; p4EC*vgf*CAT was generated by polymerization and cloning of a −178 to −132 *vgf* promoter oligonucleotide into pLBKΔ44CAT *Xba*I–*Bgl*II sites [15]. To construct pCSHEB, the HEB sequence was PCR-amplified with oligonucleotides 5′-GAGAGAGGATCCATGAATCCCCAGCAACAACG-C-3′ and 5′-GCCTAGCTCGATACAATAAACGCCAT-3′ and inserted between pGEX-2TK *Bam*HI–*Sma*I sites. The GST-HEB sequence was then amplified with primers 5′-GAGAGAATCGATGTC-CCCTATACTAGGTTA-3′ and 5′-CCTACTGAGAATTCTGGAA-CTGGC-3′ and cloned in pCS (from D. Turner) *Cla*I–*Sna*BI sites. For GST-ΔbHEB construction, the sequence encoding HEB last 92 amino acids was amplified with 5′-GAACGCTTAGGATCCCGG-GATATTAA-3′ and 5′-CCTACTGAGAATTCTGGAACTGGC-3′ oligonucleotides and cloned into pGEX-2T *Bam*HI–*Eco*RI sites. Constructs were verified by sequencing. pRSVMASH1 was by D. Anderson, the E1A12S mutant dl922–947 by A. Felsani.

2.3. Electrophoretic mobility shift assays (EMSAs)

Gel shifts were performed with a −183 to −65 *vgf* probe, as described [13]. The CCAAT competitor oligonucleotide sequence is 5′-TACTGCGTCACATCAGGCCGGGAGCGACGCTTATCCTCCA-ATCATTG-3′ [13]. The following antibodies were added before the probe: αCBP [16], αHEB (Santa Cruz), αMASH1 [17], αCREB (New England Biolabs), αphospho-CREB (Upstate Biotechnology) rabbit antisera (1 μl each); AC238, Rw128 and Rw144 p300 monoclonals [18]; pre-immune sera as control. AC238 (0.5 μl) was used as ascite fluid, Rw128 and Rw144 (5 μl each) as culture supernatants. Rw144 does not recognize CBP under the diluted conditions it was employed; AC238 recognizes both p300 and CBP [18]. GST and GST-ΔbHEB proteins were affinity-purified on glutathione agarose beads and checked by SDS–PAGE.

3. Results

3.1. Role of the C complex in NGF-dependent transcription

Phospho-CREB is a critical mediator of neurotrophin-dependent transcription, and is rapidly detected following NGF treatment of PC12 cells [5,7,8]. To clarify which of the two complexes bound to *vgf* promoter was involved in neurotrophin responsiveness, we studied by gel shift how CREB or phospho-CREB antibodies affected DNA binding in extracts of PC12 cells treated with NGF for different time periods. The CREB antibody recognizes both phosphorylated and non-phosphorylated form of CREB, whereas the phospho-CREB antibody is specific for phosphorylation on serine 133. Surprisingly, the CREB antibody inhibited C complex binding in all samples including the NGF untreated control. The phospho-CREB antibody had an impact on this complex that was stronger with increasing NGF incubation time (Fig. 1). This shows that CREB interacts with the *vgf* promoter within the

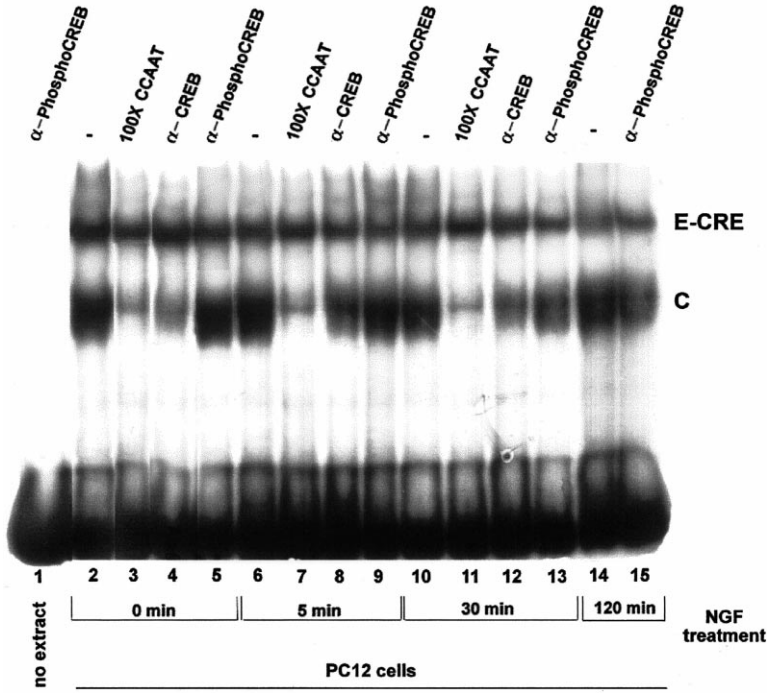

Fig. 1. NGF-induced CREB phosphorylation in the C complex. EMSA on nuclear extracts of PC12 cells treated with NGF (0–120 min). Binding reactions contained extract and *vgf* promoter probe alone (lanes 2, 6, 10, 14) or also included CCAAT competitor oligonucleotide (100-fold excess; lanes 3, 7, 11), anti-CREB (lanes 4, 8, 12) or anti-phospho-CREB serum (lanes 5, 9, 13, 15); lane 1 has probe and anti-phospho-CREB, but no extract.

CCAAT bound complex, pointing to this complex as the mediator of NGF-dependent transcription. On the contrary, the E–CRE complex was unaffected, indicating that CREB was either not present or inaccessible to the antibodies; possibly a different CREB family protein is involved. These data, obtained with a complex promoter sequence, add information to results obtained with a single binding site, such as CRE. The *vgf* CRE, alone, binds perfectly well CREB [10], while this transcription factor appeared to be preferentially associated to the CCAAT, in the context of the *vgf* promoter.

3.2. Tissue specificity: MASH1

Due to its tissue specificity, we expected that the promoter was bound by neuro-specific proteins in PC12 cells. Tissue-specific bHLH factors, functioning in cell differentiation as heterodimers with E proteins, represented natural candidates, since the HEB E protein was shown to bind the *vgf* promoter E-box [13,14]. On the other hand, the E-box bound complex is not tissue-specific, while the CCAAT bound complex is [13]. To find out whether HLH proteins were present, we utilized GST-ΔbHEB, a chimera between GST and the HLH dimerization domain of HEB, as a DNA binding inhibitor in gel shift assays with PC12 cell extracts. GST-ΔbHEB has the potential to dimerize with a variety of bHLH proteins [15]; DNA binding of the dimers is prevented since GST-ΔbHEB lacks the basic region and two basic regions are required for DNA binding [14]. As a matter of fact, it was able to inhibit in vitro binding of purified HEB to the E-box (not shown). GST-ΔbHEB affected the HEB containing E–CRE complex, as expected (Fig. 2). Surprisingly, and more efficiently, it inhibited binding of the C complex (Fig. 2A, lanes 2–5), suggesting that a protein capable of interacting with the HLH domain of HEB was involved. Such a protein was identified as the neuro-specific bHLH MASH1, which is present in PC12 cells, heterodimerizes with HEB, and has a role in neuronal differentiation [14,17,19]. A MASH1 antiserum (Fig. 2B) prevented binding of the C complex, which was unaffected by control antiserum or by antibodies against a number of transcription factors (not shown).

3.3. Presence of p300 in two vgf promoter complexes

CBP and p300 are highly related proteins that interact with sequence-specific transcription factors to promote assembly of transcription initiation complexes. They are able to bind CREB as well as ubiquitous or tissue-specific bHLH proteins [18,20]. In order to assess the presence of p300/CBP, we investigated whether antibodies specific for these coactivators affected complex formation in gel shift assays with a *vgf* promoter probe and PC12 (Fig. 3A) or NIH3T3 (Fig. 3B) cell nuclear extracts. Fig. 3A (lanes 2–4) illustrates that the lower mobility component of the C complex doublet was affected by all p300 antibodies, suggesting that p300 participates in the CCAAT bound complex. The ubiquitous E–CRE complex as well included p300, since it was affected by the p300 monoclonal antibodies Rw144 and AC238 in both cell lines; the CBP antiserum had no effect (Fig. 3A,B). Similarly to the p300/bHLH complex in myogenic C2C12 cells [18], the E–CRE complex was not supershifted by the Rw128 monoclonal antibody, likely because the p300 epitope is not exposed.

3.4. Role of HEB, MASH1 and p300 in vgf transcription

HEB binds an E-box involved in transcriptional inhibition in non-neuronal cells [13]. To determine whether it had a repression function on *vgf*, NIH3T3 fibroblasts were transfected with the −179*vgf*CAT reporter (Fig. 4A) and increasing amounts of the HEB expression vector. Although *vgf* is transcriptionally silent in such cells, a transfected *vgf* promoter has some transcriptionally activity, due to its high copy number [11]. *Vgf* transcription was inhibited by HEB in a dose-dependent manner (Fig. 4B). To show that the E-box was implicated in repression, the expression vector was cotransfected with 4EC*vgf*CAT (Fig. 4A), harboring four repetitions

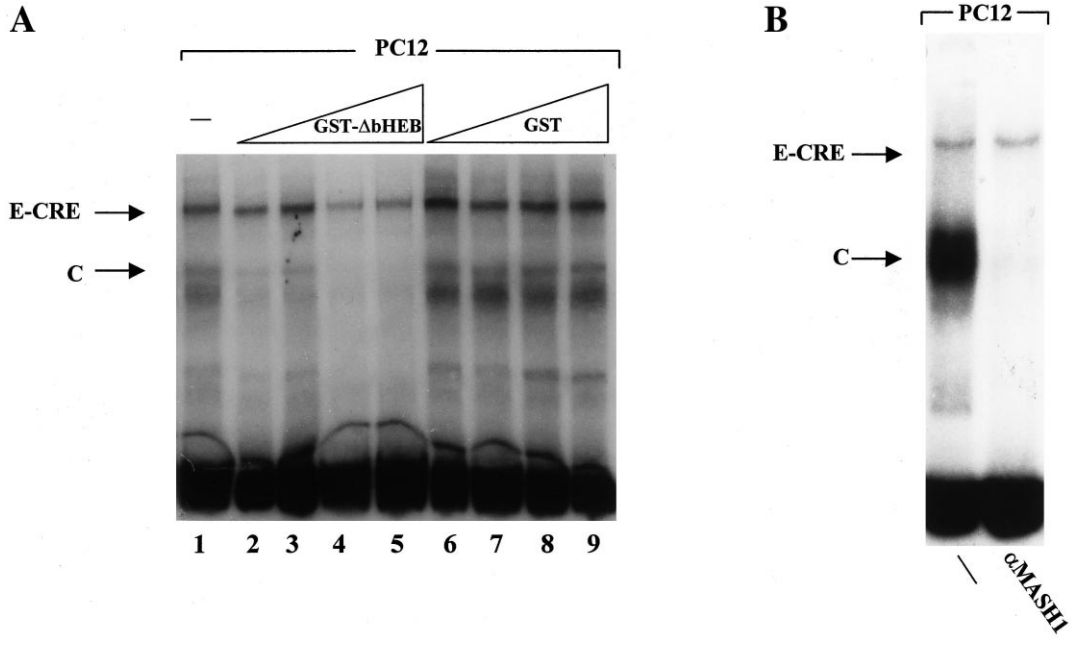

Fig. 2. MASH1 is required for C complex formation. A: GST-ΔbHEB inhibited C complex DNA binding. Gel shift assays in the presence of increasing amounts (100–500 ng) of GST-ΔbHEB (lanes 2–5) or GST protein (lanes 6–9). E–CRE complex and C complex are indicated. B: MASH1 antibodies prevent C complex formation.

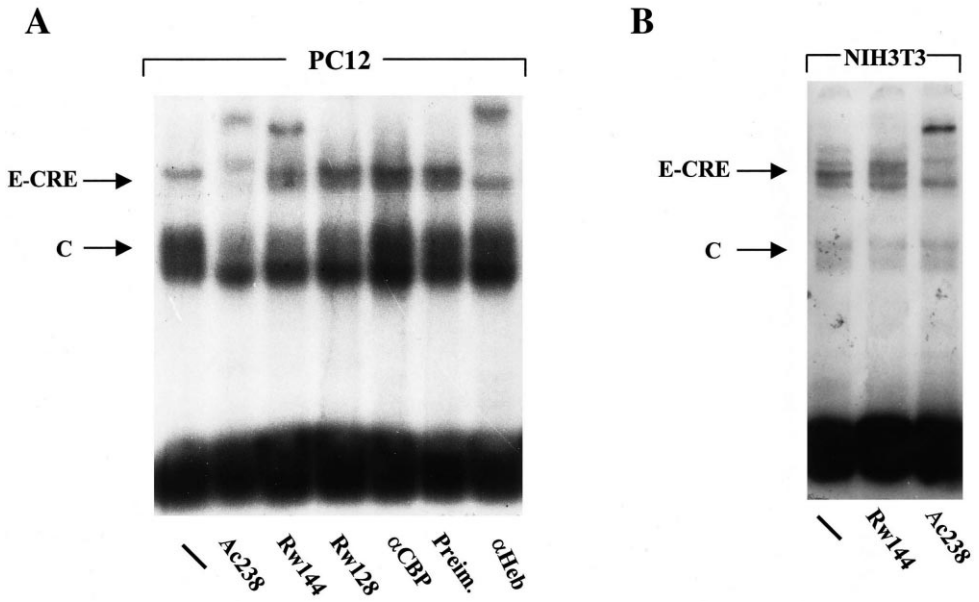

Fig. 3. p300 binds the *vgf* promoter. EMSA on PC12 (A) or NIH3T3 (B) cell nuclear extracts. E–CRE and C complexes are indicated by arrows. The first lane in each panel shows the pattern with extract alone; for the other lanes, the indicated antibodies were added. The E–CRE complex was supershifted by the two p300 monoclonals AC238 and Rw144, and by the HEB serum. The C complex was affected by all p300 antibodies (AC238, Rw144 and Rw128).

of E-box and CCAAT, upstream of an alkaline phosphatase minimal promoter. Transcription was still inhibited (Fig. 4B). The E-box weakly promotes *vgf* transcription in PC12 cells [13]. Consistently with this observation, *vgf* transcription was weakly up-regulated by HEB overexpression in this cell line (Fig. 5A). Activation by HEB was more evident following NGF treatment, suggesting that NGF turns on a HEB cofactor such as, for instance, MASH1, up-regulated by NGF and able to heterodimerize with HEB [17,19]. MASH1 was involved in the complex bound to the CCAAT site (Fig. 2B),

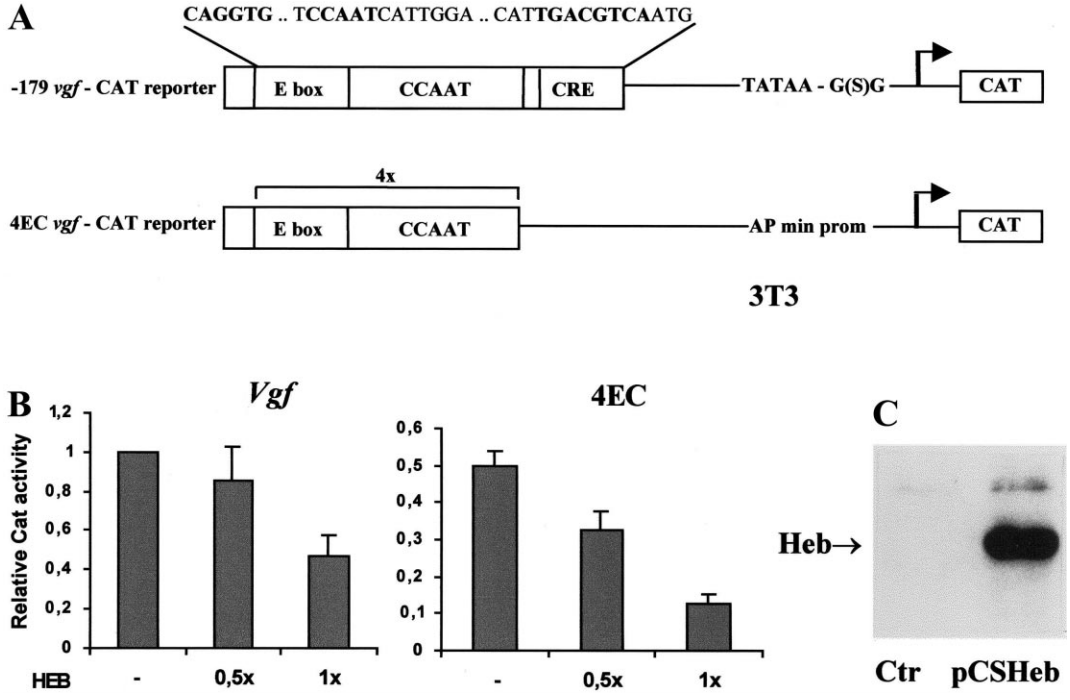

Fig. 4. *Vgf* transcription is repressed by HEB in fibroblasts. A: Organization of −179*vgf*CAT and 4EC*vgf*CAT reporter plasmids. The nucleotide sequences are relative to E-box, CCAAT and CRE sites. B: CAT assays of NIH3T3 cells transfected with −179*vgf* or 4EC*vgf* CAT reporters and either empty vector (–) or pCSHEB. The relative amounts (0.5×; 1×) of HEB expression vector compared to the reporter are indicated. Unlike *vgf* transcription, RSV promoter driven transcription (pRSVCAT plasmid) was not repressed by HEB (not shown). C: HEB expression in control or pCSHEB-transfected NIH3T3 cells (Western blot).

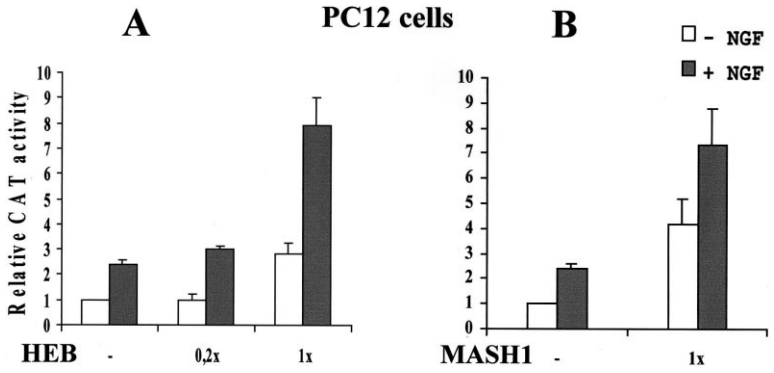

Fig. 5. *Vgf* transcription is activated by HEB and MASH1 in PC12 cells. CAT assays of PC12 cells transfected with −179*vgf*CAT and either empty vector (–) or increasing amounts of HEB (A) and MASH1 (B) expression vectors, in the presence or absence of NGF. The relative amounts of expression vectors compared to reporter plasmid are shown.

necessary for activation by NGF [10]. Accordingly, its overexpression activated *vgf* transcription in PC12 cells (Fig. 5B); it is unclear whether MASH1/HEB heterodimers have a role.

As overexpression of p300, a very abundant protein, might not be very informative, its role in *vgf* transcription was analyzed by inhibition with a mutant E1A adenoviral protein. The wild-type E1A protein interacts with both p300 and the pocket (pRb family) proteins, interfering with gene expression [21,22]. To specifically target p300 we resorted to dl922–947, a mutant deleted in CR2, the pocket protein interaction region. E1A mutant transfection inhibited *vgf* transcription in PC12 cells, in agreement with the observation that p300 is present in the C complex, involved in transcriptional control (Fig. 6A). E1A interacting proteins, most likely p300, are known to be required for differentiation in response to NGF [21]: we used the dl922–947 mutant to more specifically assay for a role of p300. Following transfection with the mutant and a β-gal expression plasmid, PC12 cells were induced to differentiate with NGF. A dose-dependent inhibition of neurite outgrowth was caused by the E1A mutant in transfected cells, identified by β-gal staining, showing that p300 is needed for differentiation (Fig. 6B).

4. Discussion

Vgf neural specificity and neurotrophin responsiveness were shown to be linked to two promoter bound complexes, E–CRE and C. Transcription in non-neuronal cells was inhibited by a component of the E–CRE complex, HEB, while neurotrophin signalling targeted the CCAAT bound complex, which included CREB. The p300 histone acetyl transferase [23–25] was required for *vgf* transcription and, as previously suggested [21], for neurite extension in response to NGF. The presence of this coactivator in two complexes with opposite roles in *vgf* transcription might be explained if its functions were differentially modulated by MASH1 and HEB, bHLH proteins present in C and E–CRE complex, respectively. This is conceivable, since p300 activity in other systems was shown to be stimulated by the tissue-specific bHLH MyoD, and to be suppressed by another bHLH, Twist [14,18,25]. Moreover, a transcriptional repression domain was found to be present within p300 [26]. A negative regulation of p300 by HEB, although not directly proven, would be compatible with the findings that HEB and p300 concurred to the E–CRE complex and that HEB repressed the promoter in fibroblasts (Fig. 4). It would also explain why cAMP is unable to stimulate *vgf* transcription in non-neuronal cells [27], CRE binding factors requiring the coactivator [7,24]. A repressive role of HEB is consistent with the observations that HEB binds the CAGGTG E-box, which restrains *vgf* expression in non-neuronal cells, that E-boxes with the same sequence inhibit GATA-3 gene promoter in non-T-cells, and that $p75^{NTR}$ gene transcription is inhibited by HEB [13,28,29]. Since HEB is present at high levels in the developing nervous sys-

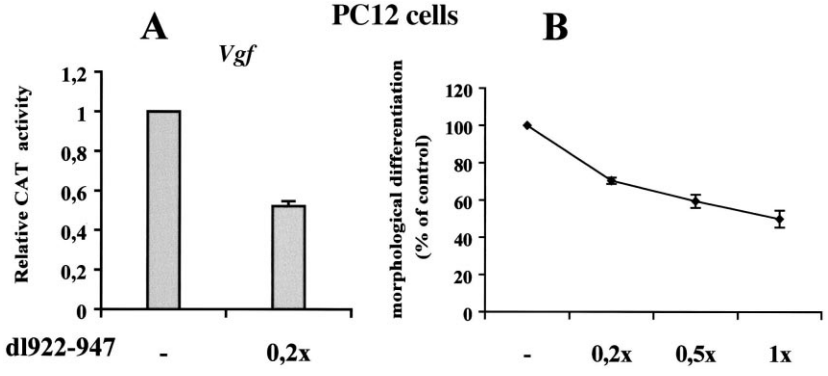

Fig. 6. *Vgf* transcription (A) and neurite outgrowth (B) are inhibited by an E1A mutant protein that selectively binds p300. A: CAT activities of PC12 cells transfected with −179*vgf*CAT (0.5 μg) and E1A12S mutant dl922–947 expression vector (0.1 μg), and treated with NGF. B: Inhibition of neurite outgrowth in PC12 cells transfected with pRSV-βgal (1 μg) and increasing amounts (0.2, 0.5 and 1 μg) of E1A dl922–947 expression vector.

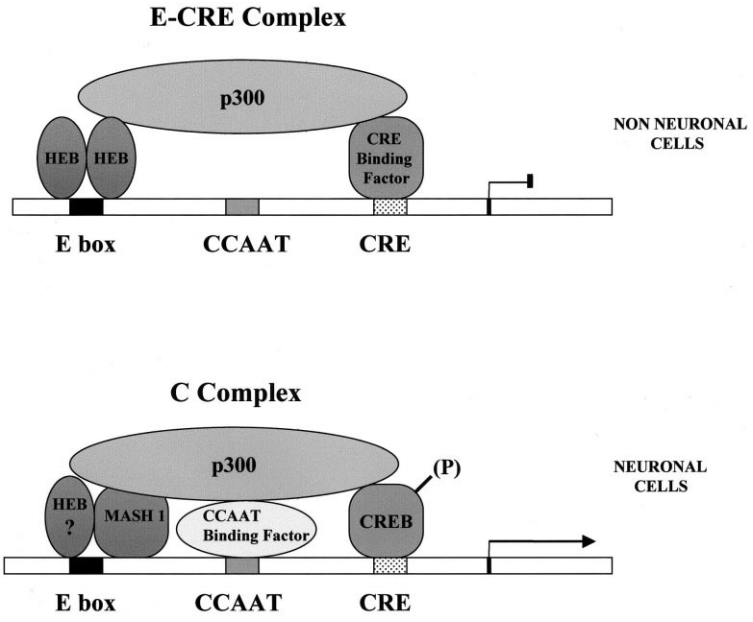

Fig. 7. Model for *vgf* promoter regulation. Different complexes bind the *vgf* promoter in non-neuronal and neuronal cells, respectively. The E–CRE complex includes HEB, CREB like factors and p300; the neuro-specific C complex includes p300, MASH1, CREB and a CCAAT binding factor. We propose that bHLH proteins recruit transcriptional adaptors–chromatin remodelling complexes and modulate their activity. In non-neuronal cells p300 activity is inhibited by HEB, resulting in *vgf* transcriptional repression, whereas p300 inhibition is relieved by the neuro-specific bHLH MASH1 in neuronal cells. Incoming signals from neurotrophin receptors, inducing modifications of CREB and possibly other proteins in the C complex, allow p300 activity and gene transcription.

tem, before overt differentiation [19,30], it might function to repress neuro-specific genes during early stages of development. The *vgf* promoter is no longer silent in neuronal cells, where it is ready to be activated by neurotrophins. This is reflected by the replacement of an inhibitory E–CRE complex with an activating C complex, which involves CREB, p300 and MASH1. According to our model, the neuro-specific bHLH facilitates chromatin modifications by p300, making the promoter competent for activation by neurotrophin signalling, which impinges on CREB (Fig. 7). It may appear surprising that a sequence different from their canonical DNA targets is so relevant for *vgf* transcriptional activation by CREB and MASH1. It should be considered that we are dealing with a large complex, to which they could be recruited via protein–protein interactions. As a matter of fact it was already reported that both CREB and HLH transcription factors are able to regulate cell type-specific gene transcription in a manner that may not require direct binding to their preferred DNA recognition sequence [14,31].

None of the proteins that were shown to be involved in the C complex specifically binds the CCAAT sequence, indicating that the complex must include at least another factor that specifically recognizes this site. A candidate is the NF-Y heteromeric protein, the major CCAAT recognizing factor in vertebrate cells, which was shown to interact with p300 [32].

In conclusion, the neurotrophin-dependent expression of *vgf* appears to be achieved by the synergy of different factors (bHLHs, CREB, p300 and others) present in a large complex that binds the promoter and is target of NGF signalling. The interaction of different proteins on a target gene promoter can function to confer specificity to neurotrophin signalling, via the interaction between CREB and a neuro-specific bHLH protein, and to integrate different cues that bear upon distinct transcription factors. It remains to be investigated whether the bHLH–p300–CREB interaction is peculiar to *vgf* or it represents a more general mechanism for gene regulation by neurotrophins and for conferring neural specificity to CREB-mediated transcription.

Acknowledgements: We acknowledge support from Telethon and AIRC (to S.N.). We are grateful to N. Rizzo for technical assistance and D. Mercanti for supplying NGF. We thank D. Anderson, R. Eckner, A. Felsani, D. Livingston, P. Sassone Corsi, T. Kadesch for providing plasmids and antibodies. G.M. and M.P. were supported by Telethon, B.I. by AIRC, L.S. and S.G. by C.N.R.

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