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A roof plate-dependent enhancer controls the expression of Homeodomain only protein in the developing cerebral cortex

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Abstract

The smallest known homeodomain protein, *Homeodomain only protein* (*Hop*), was identified and described here as a temporally and spatially restricted gene in the neurogenic regions of the developing murine CNS including the cerebral cortex. Furthermore, an evolutionarily conserved 418 base pair upstream *cis*-regulatory DNA sequence was found to confine the *Hop* expression to the CNS of transgenic mice, but not to the heart which is the second major *Hop* expressing organ (Chen, F., Kook, H., Milewski, R., Gitler, A.D., Lu, M.M., Li, J., Nazarian, R., Schnepp, R., Jen, K., Biben, C., Runke, G., Mackay, J.P., Novotny, J., Schwartz, R.J., Harvey, R.P., Mullins, M.C., Epstein, J.A., 2002. *Hop* is an unusual homeobox gene that modulates cardiac development. Cell 110, 713–723; Shin, C.H., Liu, Z.P., Passier, R., Zhang, C.L., Wang, D.Z., Harris, T.M., Yamagishi, H., Richardson, J.A., Childs, G., Olson, E.N., 2002. Modulation of cardiac growth and development by HOP, an unusual homeodomain protein. Cell 110, 725–735). The forebrain enhancer activity was successfully reproduced in vitro utilizing a combination of the electroporation and the organotypic brain culture method. Using this approach, the minimal requirement for the forebrain-specific enhancer sequence was delineated down to 200 base pairs. We further demonstrate that the *Hop* enhancer activity is inducible ectopically in a transgenic tissue by wild-type roof plate transplantation in vitro. Thus *Hop* is regulated in the forebrain by a so far unidentified paracrine signaling factor from the roof plate. Furthermore, the identified enhancer sequence provides an important tool for the targeted expression of transgenes in the medial cortex and the cortical hem.

Keywords: *Homeodomain only protein*; *Hop*; Enhancer; Cerebral cortex; Roof plate; Cortical hem; Transgene; Electroporation; Tissue transplantation; Brain slice culture

Introduction

The development of the mammalian cerebral cortex is associated with an intriguing process by which certain molecular expression boundaries are first established and then translated into morphologically distinct domains. The establishment of cortical area identities depends on the concerted action of transcription factors that form expression gradients in the cortical primordium. For example, the homeodomain transcription factors *Pax6* and *Emx2* show complementary expression gradients and mice lacking either of these genes develop displaced cortical area identities (Bishop et al., 2000, 2002; Muzio et al., 2002b).

Similar to the caudal neural tube whose patterning depends on signals from the roof plate and floor plate (for a review, see Helms and Johnson, 2003; Jessell, 2000) the cortical patterning depends on signaling centers that are situated at the margins of the cortical primordium and release paracrine signaling factors (reviewed in Grove and Fukuchi-Shimogori, 2003; O'Leary and Nakagawa, 2002). These signaling centers are the FGF expressing anterior neural ridge (Shimamura and Rubenstein, 1997), the WNT and BMP expressing cortical hem (Grove et al., 1998), and the EGF expressing anti-hem (Assimacopoulos et al., 2003). In addition, SHH from the prechordal mesoderm and the medial ganglionic eminence is suggested to pattern the ventral forebrain (Kohtz et al., 1998; Shimamura and Rubenstein, 1997). Loss of function and gain of function studies of many of these signaling molecules demonstrate

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their importance for patterning the cerebral cortex (Fukuchi-Shimogori and Grove, 2001; Galceran et al., 2000; Garel et al., 2003; Kohtz et al., 1998; Lee et al., 2000; Shimogori et al., 2004) in part mediated by their interactions with the *Pax6* and *Emx2* functions (Assimacopoulos et al., 2003; Fukuchi-Shimogori and Grove, 2003; Muzio et al., 2002a,b).

During the development three subdivisions become apparent in the medial cortex, namely the cortical hem as a transitory field adjacent to the archicortex, the choroid plexus and most medially the telencephalic roof. The cortical hem has been implicated in the establishment of medial structures, such as the hippocampus. Thus, *Gli3*-deficient *extra toes* (XT^J) mutants lack typical cortical hem markers (Grove et al., 1998) and fail to develop a hippocampus (Theil et al., 1999). Similar defects are seen in mice mutant for the cortical hem factor *Wnt3a* in which a rudimentary hippocampal primordium persists and does not develop into a mature structure (Lee et al., 2000). Finally, the hippocampus development is altered in mice that express a mutated form of the WNT downstream mediator *Lef1* (Lee et al., 2000).

The *Emx2* gene has been established as a downstream target of the cortical hem signaling. Thus, *Emx2* expression is absent in the cortical hem-deficient forebrain of the $Gli3^{XTJ/XTJ}$ mutant (Theil et al., 1999) and the forebrain-specific *Emx2* expression is mediated by a specific enhancer sequence which is regulated by the action of WNT and BMP factors (Theil et al., 2002). As another signaling center in the dorsal midline of the forebrain, the roof plate exerts a cortical patterning function already before the cortical hem appears. For example, the *Lhx2* expression gradient in the ventricular zone of the cerebral cortex has been shown to depend on roof plate derived BMP signaling (Monuki et al., 2001). Both *Emx2* and *Lhx2* show medial-high to lateral-low expression gradients in the cortical ventricular zone and depend on signaling from the medial signaling centers. It can be assumed also that the expressions of other genes forming similar gradients in the cerebral cortex are mediated by roof plate or cortical hem signaling.

In spite of such detailed information about some cortical patterning molecules (for a review, see Grove and Fukuchi-Shimogori, 2003), our understanding of corticogenesis is far from complete. It seems crucial to discover additional molecules that are expressed in the developing cortex and characterize their expression patterns. Besides the above-mentioned medial cortex-specific *Emx2* enhancer (Theil et al., 2002), only few other *cis*-regulatory elements, such as those of *NF-L* (Charron et al., 1995), *Sox2* (Zappone et al., 2000), *Otx2* (Kurokawa et al., 2004), *Ngn1* (Blader et al., 2004), and *Ngn2* (Scardigli et al., 2003), have been linked to gene expression in the developing cerebral cortex. Therefore, the characterization of other gene regulatory elements underlying regionalized gene expression, such as in the medial cerebral cortex, and linking them to the action of cortical signaling centers will provide us a better understanding of the gene regulation and patterning processes in the cerebral cortex.

In order to identify such genes, which are differentially expressed in various developing brain domains, we have performed an Affymetrix DNA Microarray expression analysis (manuscript in preparation). Here we describe the spatially and temporally regulated expression of *Homeodomain only protein* (*Hop*) in the developing cerebral cortex and other structures of the central nervous system. Until now the *Hop* gene has been only implicated with the development of the heart, where it is very strongly expressed (Chen et al., 2002; Shin et al., 2002). In the heart, *Hop* acts downstream of *Nkx2.5* inhibiting gene transcription by either direct interactions with the transcription factor SRF (serum response factor; Chen et al., 2002; Shin et al., 2002) or recruitment of histone deacetylase activity (Hamamori and Schneider, 2003). Neither the function of *Hop* nor its regulation in the cerebral cortex had been studied so far.

As described here, the *Hop* gene is expressed in the developing medial cortex and cortical hem as well as other structures of the central nervous system, such as the neural tube and cerebellum. Subsequently, we have identified a 418-bp *Hop* enhancer element that confined *lacZ* reporter expression specifically to all *Hop*-positive sites within the central nervous system of transgenic mice. Applying a novel method that involves electroporation and organotypic brain culture, we then established the minimal sequence requirements of the forebrain activity of the *Hop* enhancer being represented in a 200-bp sequence. The 200-bp forebrain enhancer of *Hop* does not contain functional transcription factor binding sites that have been shown to be involved in cortical gene regulation thus far. However, our transplantation experiments in vitro demonstrate a dependence of the activity of the *Hop* forebrain enhancer on roof plate signaling. The *Hop* forebrain enhancer can therefore be regarded as a novel sequence element activating transcription in the medial cerebral cortex downstream of roof plate signaling.

Materials and methods

Mouse strains

The outbred mouse strain NMRI is maintained in our laboratory. Inbred FVB female mice were obtained from Harlan Winkelmann. *Lef1*-deficient mice were obtained from the laboratory of Dr. R. Grosschedl (University of Munich) and genotyped as previously described in Galceran et al., 2000.

Sequence alignment and analysis

Murine and human genomic sequences were obtained from the CELERA database (Kerlavage et al., 2002) and

aligned with the CLUSTAL-W algorithm (Thompson et al., 1994). Potential transcription factor binding sites were searched in the TRANSFAC database (Wingender et al., 2000) utilizing the MatInspector program (Quandt et al., 1995).

Microarray hybridization

E16.5 NMRI brains were dissected in ice-cold PBS. Small portions from the rostral and caudal thirds of the medial cortex as well as the frontal, parietal, and occipital lateral cortex were dissected out and collected in RNA later (Ambion). Tissue from several embryos was pooled and total RNA was extracted utilizing the QIAGEN RNeasy system (Qiagen). RNA labeling, hybridization, and analysis of the MGU74v2 series of microarrays (Affymetrix) were performed as described in the manufacturers guidelines. A manuscript concerning our microarray expression analysis is in preparation.

In situ hybridization

NMRI whole embryos or isolated brains were fixed in ice-cold 4% PFA and processed for paraffin or cryosectioning following standard histology protocols. The 255-bp *Dra*III fragment of RIKEN clone *AK009007* was cloned blunt ended into the *Sma*I site of pBluescript II KS+ (Stratagene) and used to generate the *Hop*-specific RNA probes. DIG or radioactively labeled probes were transcribed with T3 RNA polymerase (for antisense) or T7 (for sense). In situ hybridization was performed as previously described (Moorman et al., 2001; Stoykova and Gruss, 1994).

Plasmid construction and mutagenesis

For transgenic analysis, several genomic DNA fragments were PCR amplified using PCR primers with introduced *Sal*I restriction sites and subsequently cloned into the *Sal*I site (pD1, pD3–pD9) or *Xho*I site (pD2) of the pTrap vector (Pfeffer et al., 2000). After subcloning into pTrap, all PCR produced fragments were sequenced using a beta-globin reverse primer (5′ AGCAATAGATGGCTCTGCCCTGAC). The primer pairs used for amplification were as follows (*Sal*I sites underlined, mutated bases in bold): pD1, pD2, forward 5′AAA-*GTCGAC*CAACTTTCAAAGGCTTTACGTTTC, reverse 5′AAAGTCGACGAAGAGTGGCTTAAGGGCTCTGC; pD3, forward 5′AAAGTCGACTAGATCTCTTTGACACACTC, reverse 5′AAAGTCGACAACAGCTGCCTGCTAGCAG; pD4, forward 5′AAAGTCGACTCCCTGGCTGCCTGCGGC, reverse 5′AAAGTCGACTAATAGCCTCCTGTTCTAAAAG; pD5, forward 5′AAAGTCGACTCCCTGGCTGCCTGCGGC, reverse 5′TTTGTCGACAAGGCAGTCAGCACAA-GTCGACAAGGCAGTCAGCACAAGGGTGGATTTC; pD6, forward 5′AAAGTCGACTCCCTGGCTGCCTGCGGC, reverse 5′AAAGTCGACAGGCATCTTAATTCCTGGCTG; pD7, forward 5′AAAGTCGACTGGTCTCCAAGACAGCTCC, reverse 5′AAAGTCGACTAATAGCCTCCTGTTCTAAAAG; pD8, forward 5′AAAGTCGACAGCCGCTAAGCGG-GTCGACAGCCGCTAAGCGGTAGTCAC, reverse 5′AAA-GTCGACTGCCTGCTAGCAGAAGTCAC; pD9, forward 5′AAAGTCGACTCCGCTTTTGTTGACAGAG, reverse 5′TTTGTCGACAAGGCAGTCAGCACAAGGGTGGATTTC; pD5ΔOct6, forward 5′AAAGTCGACTCCCTGGCTGCC-TGCGGC, reverse 5′TTTGTCGAC AAGGCAGTCAGCA-CAAGGGTGGATTTCACAGGCATCTT**GGA**TCCTG.

For the mutation of both putative TCF/Lef1 binding motifs in pD3ΔTCF two PCR reactions with the primer pairs 5′AAAGTCGACTCCCTGGCTGCCTGCGGC/ 5′CTTTCTTTTCGGCTT*G***A**T**A**GTTGGCCCTTGGC and 5′GCCAAGGGCCAAC**T**A**TC**AAGCCGAAAAGAAAG/ 5′AAAGTCGACTGCCTGCTAGCAGAAGTCAC were performed. The PCR products were cleaned up, annealed, and further used as templates for a subsequent PCR reaction with the forward mutated primer 5′AAAGTCGAC TCCCTGGCTGCCTGCGGCTTGCTTTCTATGGCAT**G** TT**ACT**TTAGAC and the reverse primer 5′AAAGTCGAC TGCCTGCTAGCAGAAGTCAC. The final product was cloned and sequence verified.

Generation and analysis of transgenic mice

Transgenic mice were generated by microinjection of fertilized eggs from FVB mice as described previously (Brink et al., 2001) and were identified by polymerase chain reaction using tail DNA. Expression of the transgene was analyzed by staining whole mouse embryos or sectioned brains for β-galactosidase activity following standard protocols (Gossler and Zachgo, 1993). Sections were counterstained using 0.1% neutral red.

Electroporation

Plasmid DNA for electroporation was prepared with the QIAGEN Plasmid Maxi Kit (Qiagen), 1× phenol-chloroform, and 1× chloroform extracted, precipitated, and dissolved in sterile PBS. 2–3 μl of a plasmid solution containing 700 ng/μl of a construct driving an EGFP sequence from a CMV promoter (CMV-EGFP; Spieler et al., 2004) and 400 ng/μl of a deletion construct of interest (pD) were microinjected into the lateral ventricle of isolated E14.5 brains (NMRI) using microcapillaries from Clark Electromedical Instruments. Injected brains were placed between 8 mm distant platinum electrodes and five rectangular 70-V pulses with 50 ms duration and 1 s intermission were applied using an Electro Square Porator ECM 830 (Btx Inc.).

Organotypic slice culture and roof plate transplantation

For brain slice culture, electroporated or transgenic brains were embedded in 2% low melting point agarose (Invitrogen) in EBSS (Gibco BRL) and vibratome sectioned

into 350 μm coronal slices with a manual vibratome (TSE Systems). Protocols for organotypic slice culture were modified from previous publications (Anderson et al., 1997) using 0.3 μm polycarbonate tissue culture inserts (Nunc) and Neurobasal A medium (Gibco BRL), supplemented with 1× B27 (Gibco BRL), 10% fetal calf serum (Pan Biotech GmbH), 50 μg/ml streptomycin (Gibco BRL), 50 U/ml penicillin (Gibco BRL), and 2 mM glutamine (Gibco BRL). Tissue culture was performed at standard conditions (5% [CO_2], 95% humidity, 37°C).

For transplantation experiments, E10.5 wild-type forebrains were dissected as illustrated in Fig. 4A. Dorsal midline tissue corresponding to the roof plate, or ventral forebrain tissue corresponding to the ganglionic eminence, were removed and placed in direct contact to the ventral and lateral pallial regions of organotypically cultured transgenic brain slices. Transplanted tissue was in part visualized by briefly dipping it in inert dyes, such as DiI or carmine red (Sigma). After 48 h, brain cultures were fixed for 30 min with 1% formaldehyde/0.2% glutaraldehyde/0.02% NP-40 in PBS and further processed for lacZ staining.

Image recording and processing

Images were recorded with a digital camera (Colerview 12) on a microscope (BX60, Olympus) or binocular (SZX12, Olympus). EGFP signals were visualized using an MGFP filter from Olympus. Images were processed and mounted using Photoshop 6.0 (Adobe).

Results

The Hop gene is expressed in the developing nervous system in a temporally and spatially restricted fashion

Using Affymetrix DNA microarrays for expression analysis we identified *Homeodomain only protein* (*Hop*) as a highly regionalized gene in the E16.5 cerebral cortex (manuscript for the microarray expression screen in preparation). *Hop*, whose cortical expression was briefly reported elsewhere (Funatsu et al., 2004), showed the highest hybridization signal in samples of the rostro-medial cortex, whereas the hybridization signals in samples of the frontal, parietal, occipital, and caudo-medial cortices were reduced by the fold changes of 2.6, 3.0, 3.7, and 1.9, respectively.

In situ hybridization experiments revealed that at E11.5 (embryonic day 11.5) *Hop* transcripts are present in the heart, in the ventricular zone (vz) of the spinal cord, and in a specific hindbrain region that corresponds to the prospective cochleo-vestibular nucleus. No expression was detectable in the developing forebrain at this stage (Figs. 1A and B). First forebrain expression was detectable at E12.5 in the most medial aspect of the cortex, the anlage of the archipallium. At E12.5 *Hop* expression persisted in the ventricular zone of the spinal cord, in the anlage of the cochleo-vestibular hindbrain nucleus, and in the heart (Figs. 1C and D). At E11.5 as well as at E12.5 the expression of *Hop* in the ventricular zone of the spinal cord was higher in the basal plate than in the alar plate and absent from the floor plate and roof plate (Figs. 1B and D). The forebrain expression level continued to remain low at E14.5 (Figs. 1E–G) but became higher and reached its peak at E16.5. At this stage the *Hop* expression formed an expression gradient in the cortical ventricular zone with high expression rostro-medially and low expression caudo-laterally (Figs. 1H–J). In addition, the cortical hem and a stream of cells leaving the dentate notch towards the developing dentate gyrus became *Hop* positive (Fig. 1I). Expression at E18.5 resembled that of E16.5 with more *Hop*-positive cells having reached the dentate gyrus (Figs. 1K–M). We further examined the postnatal expression at P6 and found that cells in the ependymal layer, the CA3 field of the hippocampus, and the entire dentate gyrus express *Hop* (Figs. 1N–P). Eventually little *Hop* message was detectable in the adult cerebral cortex (P21) where positive cells were solely found in the subgranular layer of the dentate gyrus. Due to their distribution pattern and sparseness, these cells resemble much the dentate gyrus stem cell population (Fig. 1Q). However, their identity remains at this time elusive. At P6 and P21 strong *Hop* expression was seen in the cerebellum in cells surrounding the Purkinje cells, thus most likely representing a subpopulation of the Bergmann glia cells (arrowheads Figs. 1P and R). An in situ hybridization using sense RNA probes on E16.5 cortex yielded no signal and served as a control (data not shown). Taken together, *Hop* expression is subject to a temporal and spatial regulation during vertebrate nervous system development.

We then focused primarily on the identification and characterization of the regulatory sequence elements underlying the highly regionalized expression of *Hop* in the nervous system, in particular the cerebral cortex.

The Hop gene expression in the nervous system is regulated by a specific enhancer sequence

Indispensable genes and gene regulatory sequences are highly conserved during evolution. A DNA sequence comparison between the mouse and the human *Hop* loci revealed the existence of a 418-base pairs (bp) element (*Hop* E1) located about 7 kb upstream of the first exon and containing 340 nucleotide identities between the two genomes (Figs. 2A and B). Within the examined sequences no other conserved element of similar quality was found. Database search revealed no transcripts containing the E1 sequence, thus indicating that E1 is likely to be a conserved regulatory element.

To test this idea, we generated transgenic mice with a 706-bp genomic fragment encompassing the 418-bp *Hop*

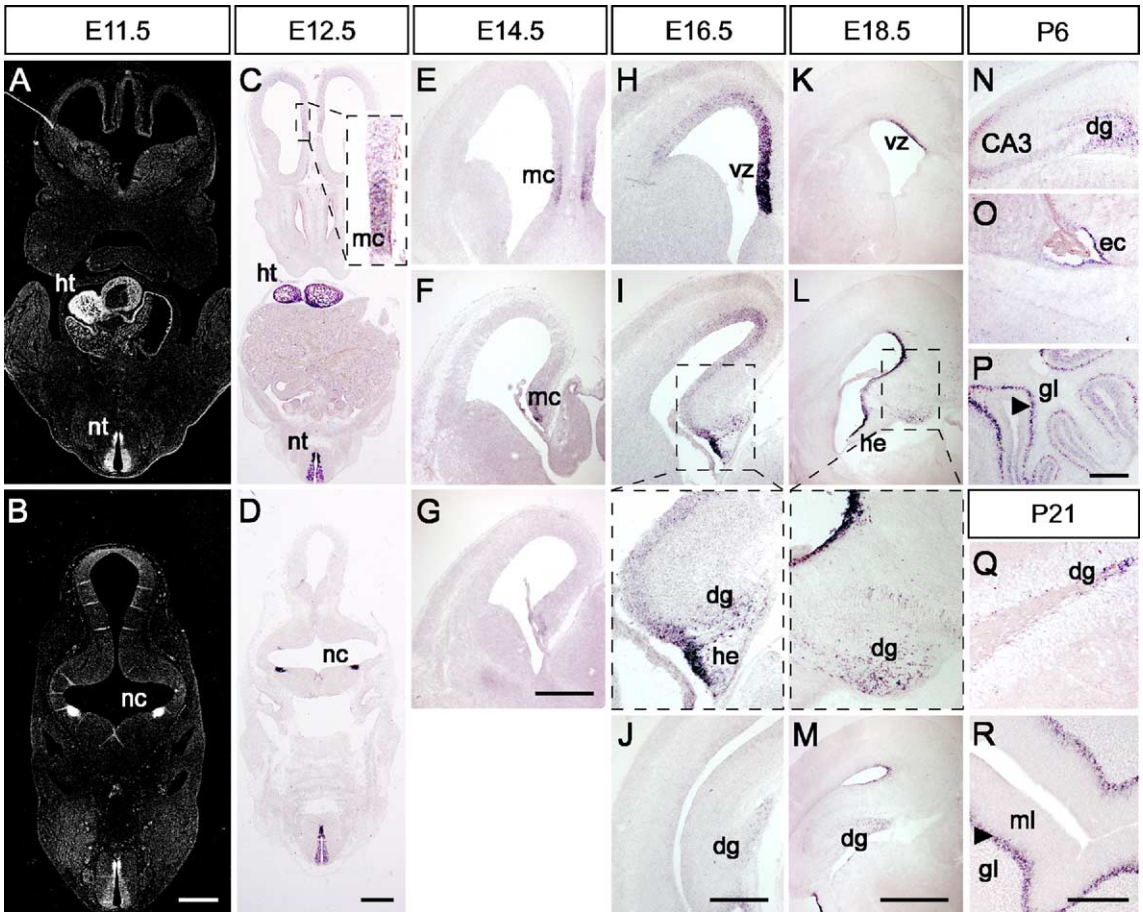

Fig. 1. *Hop* gene expression in the developing mouse embryo as detected by in situ hybridization. At E11.5 (A, B) and E12.5 (C, D) *Hop* expression was detected in the heart (ht) and neural tube (nt) as well as in the anlage of the nucleus cochleo-vestibularis of the hindbrain (nc). From E12.5 onwards *Hop* transcripts became apparent in the medial cortex (mc, C) where they persisted at E14.5 (mc, E–G). Later on, at E16.5 (H–J) and E18.5 (K–M), strong *Hop* expression was seen in the cortical ventricular zone (vz) forming a medial-high to lateral-low gradient. At these stages *Hop* transcripts were further seen in the cortical hem (he) and developing dentate gyrus (dg). At P6 *Hop* expression was detected in the entire dentate gyrus (dg, N), the CA3 field of the hippocampus (CA3, N), the ependymal cell layer (ec, O), and in the cerebellum in cells that most likely represent a subpopulation of the Bergmann glia cells (arrowhead in P). In the adult brain *Hop* expression persisted in these cells (arrowhead in R) and in the subgranular layer of the dentate gyrus (dg, Q). Gl, granular layer; ml, molecular layer. Scale bars are equal to 500 μm (A, B; C, D; H–J; K–M), 400 μm (E–G), and 250 μm (N–P; Q, R).

E1 element linked to a beta globin minimal promoter-*lacZ* reporter gene cassette (construct pD1; Fig. 6A). Analyses of these mice showed that indeed the 706-bp fragment was able to mimic the *lacZ* reporter activity in the *Hop* expressing regions of the embryonic brain and the spinal cord (compare Figs. 1 and 3). Such *lacZ* activity patterns were reproduced in three out of five independent transgenic mouse lines. When we compared the *lacZ* patterns among these positive mouse lines some variable ectopic reporter activity was observed, most likely due to different integration sites among the independent transgenic lines. No reproducibly specific reporter activity was detected outside the nervous system. Especially the heart, the major *Hop* expressing organ, was devoid of reporter activity. We concluded that the E1 element (plus the flanking sequences present in the construct) serves as a nervous-system-specific enhancer for the *Hop* expression. The kinetics and expression of the *lacZ* reporter activity in one founder line are shown in Fig. 3.

At E12.5 the specific beta galactosidase reporter activity was detected in the medial cerebral cortex, the anlage of the cochleo-vestibular nucleus of the hindbrain, and the ventricular zone of the spinal cord (Figs. 3I–K). The *lacZ* staining in the ventricular zone of the spinal cord was strong in the most dorsal part of the alar plate and reduced in the entire basal plate, the floor plate and the roof plate were devoid of reporter activity (Fig. 3K). In the E16.5 cortex, *lacZ* was detectable in the ventricular zone of the rostro-medial cortex (Fig. 3L), the cortical hem, and cells migrating from the dentate notch towards the developing dentate gyrus (Fig. 3M). Thus, the E1 enhancer-driven reporter activity strongly correlated with the *Hop* expression in the developing nervous system. Merely some divergence was noticed in the most lateral ventricular zone of the E16.5 cortex where no *lacZ* activity (Fig. 3L), but slight *Hop* expression was seen by in situ hybridization (Fig. 1H). The presence of other *Hop* enhancer sequences, which might exist and extend the expression further laterally, cannot be

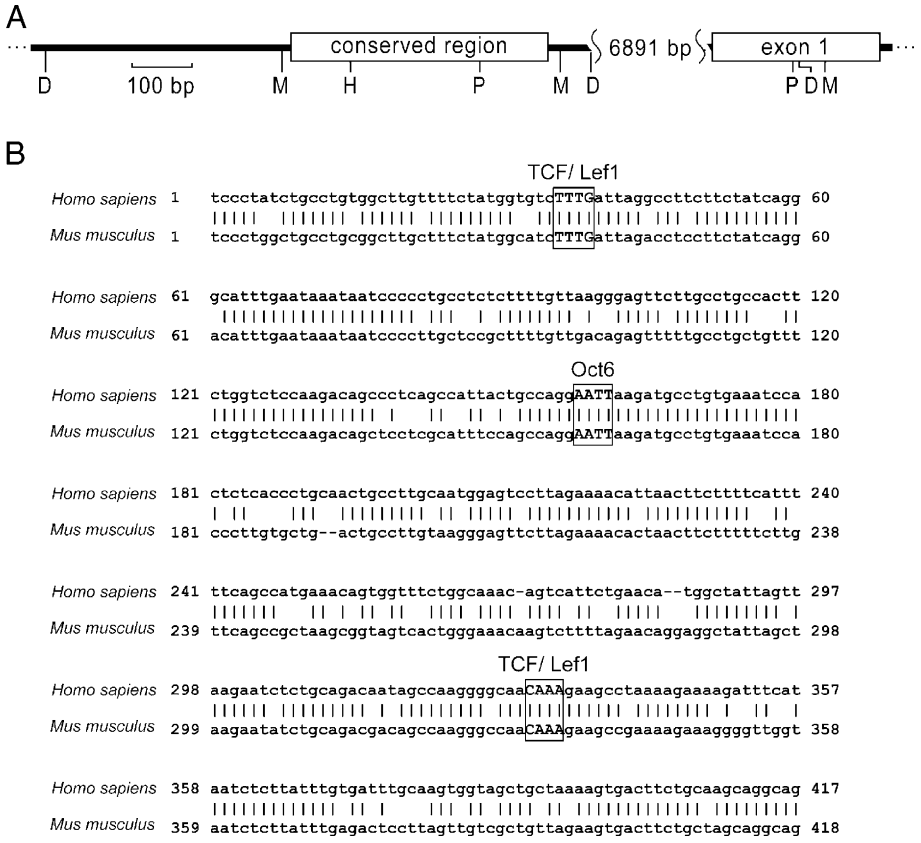

Fig. 2. Location and sequence of the evolutionarily conserved *Hop* E1 upstream regulatory element. A conserved 418-bp sequence element is situated approximately 7 kb upstream of the first exon (A) and reveals a high degree of sequence conservation between the murine and human genomes (B). The *Hop* E1 element contains putative transcription factor binding sites (core bases capitalized), amongst them two TCF/Lef1 binding motifs and one Oct6 binding motif. Restriction enzyme sites shown in panel A are as follows: D, *Dra*II; H, *Hin*cII; M, *Mbo*I; P, *Pst*I.

excluded at this time. Nevertheless, the identified E1 element seems to account for most of the *Hop*-specific expression in the cortex, hindbrain, and spinal cord; but not other organs, such as heart.

We further examined the temporal expression pattern of the *Hop* E1 transgene in three transgenic mouse lines. The endogenous *Hop* gene expression and the transgene expression correlated well in the neural tube, where they first appeared at E11.5 (Chen et al., 2002; Shin et al., 2002; this publication). In the dorsal forebrain, the *Hop* expression started at E12.5 (Fig. 1C), while enhancer-driven *lacZ* was detectable already as early as E10.5 (Fig. 3D). One reason might be that the low expression level at earlier stages could not be detected due to the lower sensitivity of the in situ hybridization as compared to the transgenic *lacZ* activity. Alternatively, additional negative regulatory sequences might be responsible for an early repression of the *Hop* transcription.

Ectopic roof plate transplants are able to induce Hop enhancer activity

In the medial forebrain the roof plate and the cortical hem are strong sources of paracrine signaling molecules, such as members of the BMP and WNT families (Furuta et al., 1997; Grove et al., 1998). As mentioned above, the medial-high to lateral-low expression gradients of *Emx2* and *Lhx2* in the cerebral cortex have been suggested to depend on the signaling from these medial signaling centers (Bulchand et al., 2001; Theil et al., 2002). To assess whether also the medial-high to lateral-low gradient of *Hop* expression and enhancer activity depends on paracrine signaling from the medial signaling centers, we performed a series of transplantation experiments in vitro. In these experiments forebrains from E11.5 to E14.5 pD1-transgenic embryos were put in organotypic slice culture (acceptor). Transplants were isolated from the dorsal midline of E10.5 wild-type forebrains (donor), thus representing the roof plate, and transplanted into the lateral forebrain regions of acceptor tissue that are normally devoid of *Hop* expression and enhancer activity (Fig. 4A). Whereas endogenous enhancer activity was always present in the medial cortex of the acceptor tissue, successful transplants yielded additional ectopic enhancer activity in the lateral forebrain (arrowheads in Fig. 4B). However, *Hop* enhancer activity could be induced only when the recipient brains were taken from stages E11.5 (Fig. 4B) or E12.5, but not older (data not shown). From this observation we concluded that roof plate signals are sufficient to induce *Hop* enhancer activity in the forebrain and that the

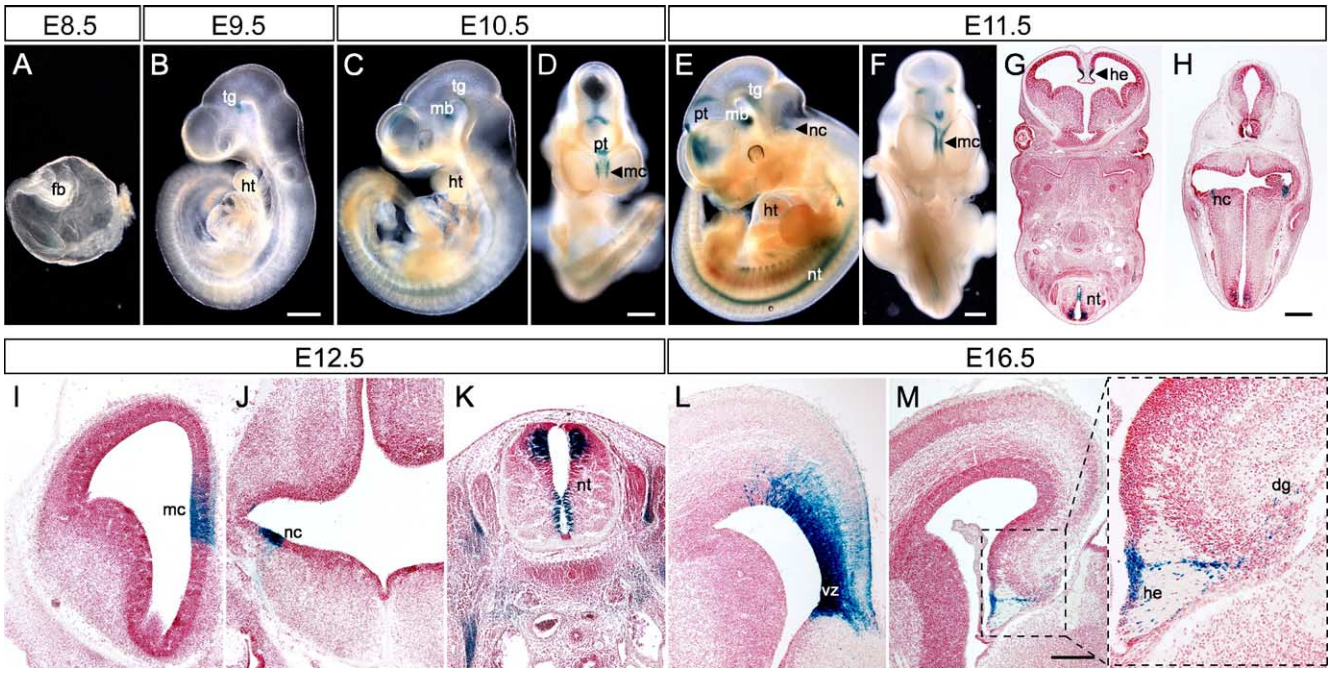

Fig. 3. The *Hop* E1 element is capable of driving the *lacZ* expression from a minimal beta globin promoter in vivo. At E11.5 (E–H), E12.5 (I–K), and E16.5 (L, M) *lacZ* activity was detected in regions that showed strong *Hop* expression (also compare to Figs. 1E–M), such as the ventricular zone of the neural tube (nt; E, G, K), the medial cerebral cortex (mc; D, F, I), and the cortical hem (he; G, M), the anlage of the nucleus cochleo-vestibularis of the hindbrain (nc; E, H, J), the ventricular zone of the rostro-medial cortex (vz; L), and the developing dentate gyrus (dg; M). Ectopic *lacZ* was present in the mesencephalic tegmentum through several stages (tg; B, C, E), in the pretectum (pt; D, E), the anlage of the mammillary bodies (mb; C, E), as well as in the ribs (E). At E8.5 no *lacZ* activity was present in the developing central nervous system including the forebrain (fb; A). The heart (ht; B, C, E) was devoid of *lacZ* activity at all stages. Scale bars are equal to 500 μm (A, B; C, D; E, F; G, H) and 250 μm (I–M).

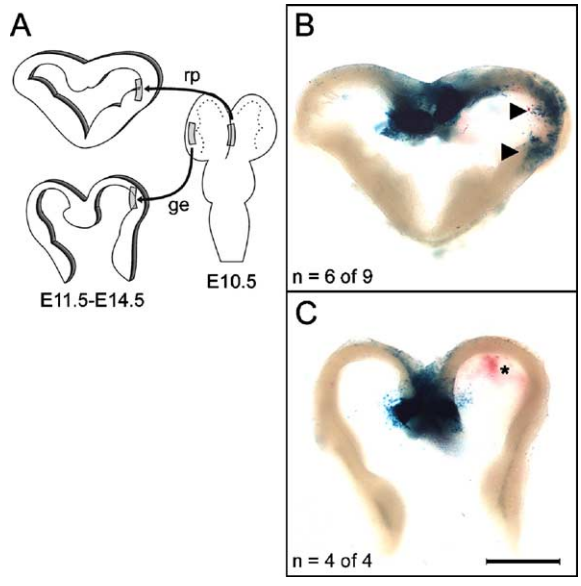

Fig. 4. Strategy and results of the in vitro roof plate transplantation experiments. Either roof plate (rp) or ganglionic eminence (ge) tissues were taken from wild-type E10.5 embryonic brains and transplanted into the ventral pallium of E11.5 pD1-transgenic brains (A). After 2 days in slice culture the recipient forebrains developed ectopic enhancer activity in the lateral and ventral pallial regions overlaying the roof plate transplants (arrowheads, B). No enhancer induction was seen after transplantation of E10.5 ganglionic eminences (asterisk, C) into the lateral E11.5 forebrain. The transplanted tissues were visualized with karmin red (B, C). In all transgenic brains the endogenous enhancer activity was present in the medial cortex and cortical hem (B, C). Scale bars are equal to 500 μm (B–C).

competence to respond to these signals is lost in forebrains older than E12.5. Control transplants taken from wild-type E10.5 ganglionic eminences were incapable of inducing enhancer activity (asterisk in Fig. 4C).

An electroporation-based system for the delineation of the Hop-specific enhancer sequence in brain slice cultures

To further delineate the sequence requirements of E1 to act as an enhancer we adapted an in vitro system combining electroporation-mediated plasmid transfer and organotypic brain culture. Utilizing this approach we aimed at a high throughput screening of deletion constructs to identify the minimal sequence requirement for the *Hop* E1 activity.

For electroporation, a plasmid containing solution was injected into the lateral ventricle of E14.5 wild-type brains. Thereafter the brains were placed in drops of PBS centered between electrodes (Fig. 5A). Five 70-V pulses were applied. The electroporated brains were vibratome sectioned and put in culture. After 24 h the slice cultures were fixed and stained for *lacZ* activity. To monitor the exact sites of electroporation a CMV-EGFP (CMV promoter driven enhanced green fluorescent protein sequence) plasmid was coelectroporated with the deletion construct of interest.

We first determined whether the enhancer activity pattern seen in vivo (in transgenic mice) is reproducible through

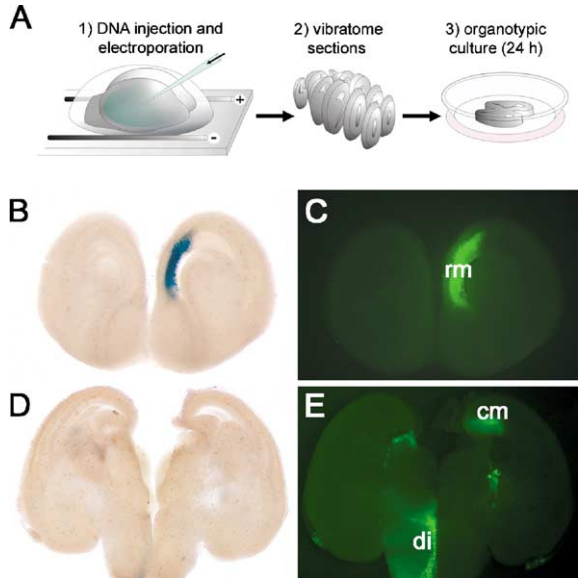

Fig. 5. Electroporation and organotypic slice culture strategy. The procedure of electroporation-mediated gene transfer involves the injection of a DNA solution into the lateral ventricle of E14.5 brains (A, 1), followed by vibratome sectioning (A, 2) and 24 h of organotypic slice culture (A, 3). CMV-EGFP, a plasmid expressing the EGFP cDNA from the CMV promoter, and pD1, a plasmid containing the *Hop* E1 element and a beta globin minimal promoter-*lacZ* cassette, were coelectroporated. EGFP-positive cells were typically seen in the rostro-medial (rm, C) and caudo-medial (cm, E) as well as in the diencephalic (di, E) ventricular zone. Among the targeted regions only the rostro-medial cortex (B), but not the caudo-medial cortex or the diencephalic region (D) developed E1-driven *lacZ* activity, thus reflecting the regional specificity of the enhancer activity.

electroporation in vitro (in brain slice cultures). Therefore a plasmid mixture of CMV-EGFP and pD1 was electroporated. When the anode was placed towards the uninjected hemisphere (Fig. 5A), a typical pattern of EGFP-positive cells was seen in the medial ventricular zone (Fig. 5C). Due to some leakage of plasmid solution through the interventricular foramen, EGFP-positive cells were also present in the diencephalic ventricular zone (Fig. 5E). Among the EGFP-positive electroporated brain regions, only the rostro-medial cortex, but not the caudo-medial cortex or the diencephalic ventricular zone developed *lacZ* activity (Figs. 5B and D). The in vivo regional specificity of E1 activity along the rostro-caudal axis and the telencephalic–diencephalic subdivision was therefore well preserved and reproducible in vitro.

We then proceeded to perform further deletion analyses of the *Hop* E1 enhancer fragment by the electroporation and organotypic culture method. To begin with, we electroporated two pD1 plasmid versions containing the enhancer in sense and antisense orientation as well as pD2 containing the 706-bp enhancer fragment located at the 3′-end of the *LacZ* cassette (Fig. 6A). In all these experiments full reporter activity was observed (Figs. 7A–C, A′–C′). *Hop* E1 therefore fulfils all criteria of a classical enhancer, namely independence of position and orientation from the promoter (Banerji et al., 1981; Fromm and Berg, 1983).

We attempted next to delineate the minimal sequence requirements of the *Hop* enhancer activity in the medial pallium. pD3, another deletion construct, contained only the 418-bp element conserved between the human and the mouse genome (Fig. 6A). With pD3 full reporter activity was observed (Figs. 7D and D′). Of the several putative transcription factor binding sites predicted by MatInspector (Quandt et al., 1995), two TCF/Lef motifs in pD3 attracted our attention. The Lef1 transcription factor is strongly expressed in the ventricular zone of the medial cortex (Galceran et al., 2000) and might therefore account for the *Hop* enhancer activity. However, a deletion construct containing the 418 bp conserved E1 element with point mutations in both TCF/Lef motifs (pD3ΔTCF; Figs. 6A and B) showed reporter activity that was identical to that of the non-mutated plasmid (compare Figs. 7D, D′, E, and E′).

Further deletion analyses allowed us to establish a 200-bp minimal enhancer sequence (pD5; Fig. 6A) yielding strong reporter activity (Figs. 7G and G′). The 200-bp minimal enhancer could not be further deleted at its 5′- or 3′-ends without severely reducing or abolishing its activity (pD6 and pD9; Figs. 6 and 7I, I′, L, L′). A putative Oct6 binding motif was also detected in the 200-bp fragment, which might be essential for its enhancer activity. Mutating this binding motif (pD5ΔOct6; Figs. 6A and B) however did not abolish reporter activity (Figs. 7H and H′), thus indicating its dispensability. Moreover two 180-bp subfragments from the center or the 3′ regions of E1 (pD7 and pD8; Fig. 6A) did not result in any enhancer activity (Figs. 7J, J′, K, and K′), thus corroborating the fact that the construct pD5 harbors the minimal enhancer element. For the sake of reproducibility and comparability among all experiments, the electroporation, culture, and staining conditions were kept constant and the CMV-EGFP plasmid was included as an internal control.

Among the TCF/Lef transcription factors, *Lef1* shows the strongest expression in the medial cortex (Galceran et al., 2000). However, as mentioned above, our deletion analysis suggested that the putative TCF/Lef binding sites in the *Hop* enhancer are dispensable and that the cortical *Hop* expression might therefore not depend on the *Lef1* transcription factor. Further support for this assumption was gained from the analysis of the *Hop* expression in the cerebral cortex of *Lef1*-deficient brains. As expected, the expression of *Hop* was neither absent nor reduced in the *Lef1*-deficient cortex when compared to wild type (data not shown).

Discussion

Hop is expressed in the neurogenic regions of the embryonic CNS

Here we identified the *Homeodomain only protein* (*Hop*) as a gene with spatially and temporally regulated expression

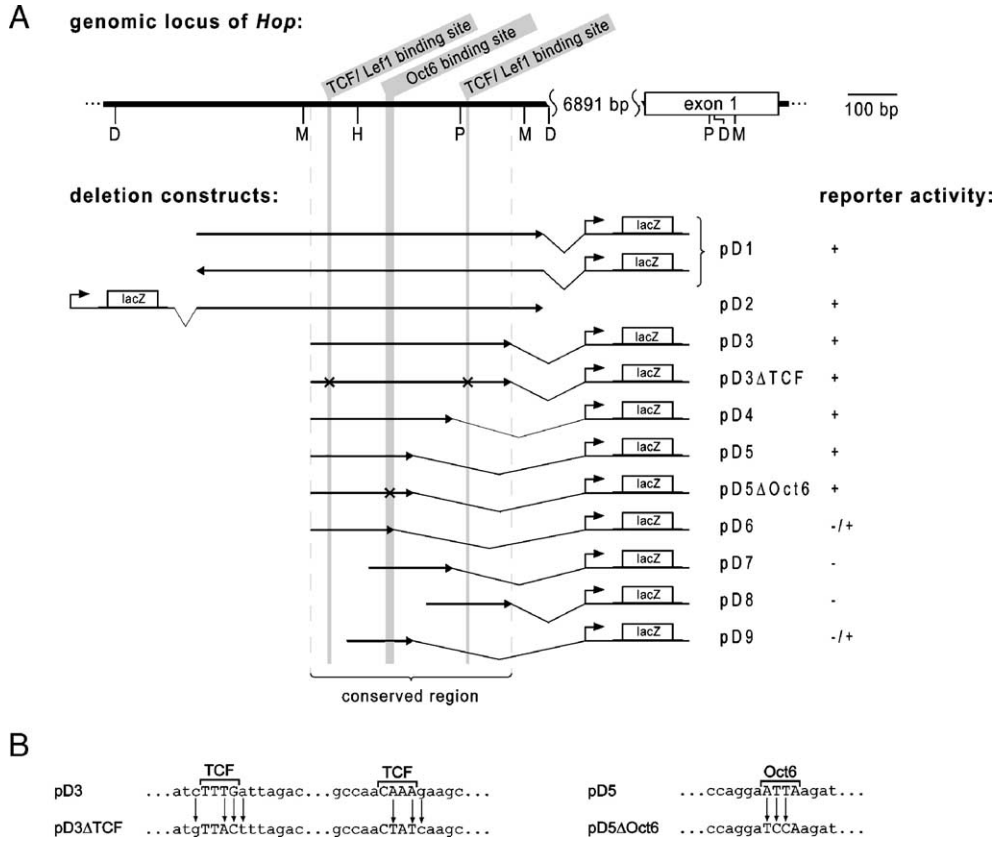

Fig. 6. Deletion mapping of the *Hop* E1 enhancer element. For deletion analysis several sub-fragments of E1 were linked to a beta globin minimal promoter-*lacZ* reporter cassette and tested for their enhancer activity in the medial cortex utilizing electroporation in organotypic slice culture (A). Full enhancer activity was thereby detected with constructs containing the E1 sequence independent of its position and orientation (A: pD1, pD2, 706 bp; pD3, 418 bp). pD4 (295 bp) or pD5 (200 bp) also yielded comparable enhancer activity (A). Further deletions (A: pD6, pD9) almost completely abolished the enhancer activity. Other sub-fragments of the PD3 construct were without enhancer activity (A: pD7, pD8). The mutation of putative TCF/Lef1 and Oct6 binding sites within the E1 sequence had no effect on its enhancer activity (A: pD3ΔTCF, pD5ΔOct6). The introduced point mutations are shown in panel B. For comparison see also Fig. 7.

in the developing central nervous system. An abundant *Hop* expression at sites of neurogenesis, such as the ventricular zone of the spinal cord and the cortex, the subgranular layer of the dentate gyrus, and the ependymal cell layer, suggests *Hop* as a putative regulator of proliferation and/or differentiation. Interestingly a similar function has been shown for *Hop* in cardiac development (Chen et al., 2002; Shin et al., 2002).

Hop lacks DNA binding activity and therefore does not directly regulate transcription. During cardiogenesis it acts as an antagonist of the transcription factor SRF (Chen et al., 2002; Shin et al., 2002). A similar mechanism of *Hop* activity in neurogenesis seems to be unlikely because we failed to detect any SRF protein in the ventricular zone of the E13.5 and E16.5 medial cortex using immunohistochemistry (data not shown). The applied rabbit anti-SRF polyclonal antibody (Santa Cruz) was capable to detect SRF protein in the heart, which thereby served as a control.

In addition to a putative function in neurogenesis, *Hop* might be associated with the establishment and/or maintenance of the dentate gyrus cells as they are *Hop*-positive during all phases of their development such as birth at the dentate notch, migration towards the dentate gyrus, and its colonization.

The spatially and temporally restricted expression of *Hop* in diverse tissues like brain and heart suggested that separate *cis*-regulatory elements control the activation and subsequent maintenance of *Hop* expression in different organs.

A novel enhancer confines expression to Hop-positive structures of the CNS

A regulatory sequence element located in the first intron of the *Hop* gene locus and capable of driving *Hop* expression solely and specifically in the heart has been described (Chen et al., 2002). Transcriptional activation from this cardiac *Hop* enhancer was mediated through the action of the Nkx2.5 transcription factor. However, this cardiac sequence element does not account for the strong *Hop* expression in the nervous system. By genomic DNA sequence comparison of the *Hop* locus we first identified a sequence element (*Hop* E1 element) with high conservation between the murine and the human genomes. Transgenic mouse lines were established with

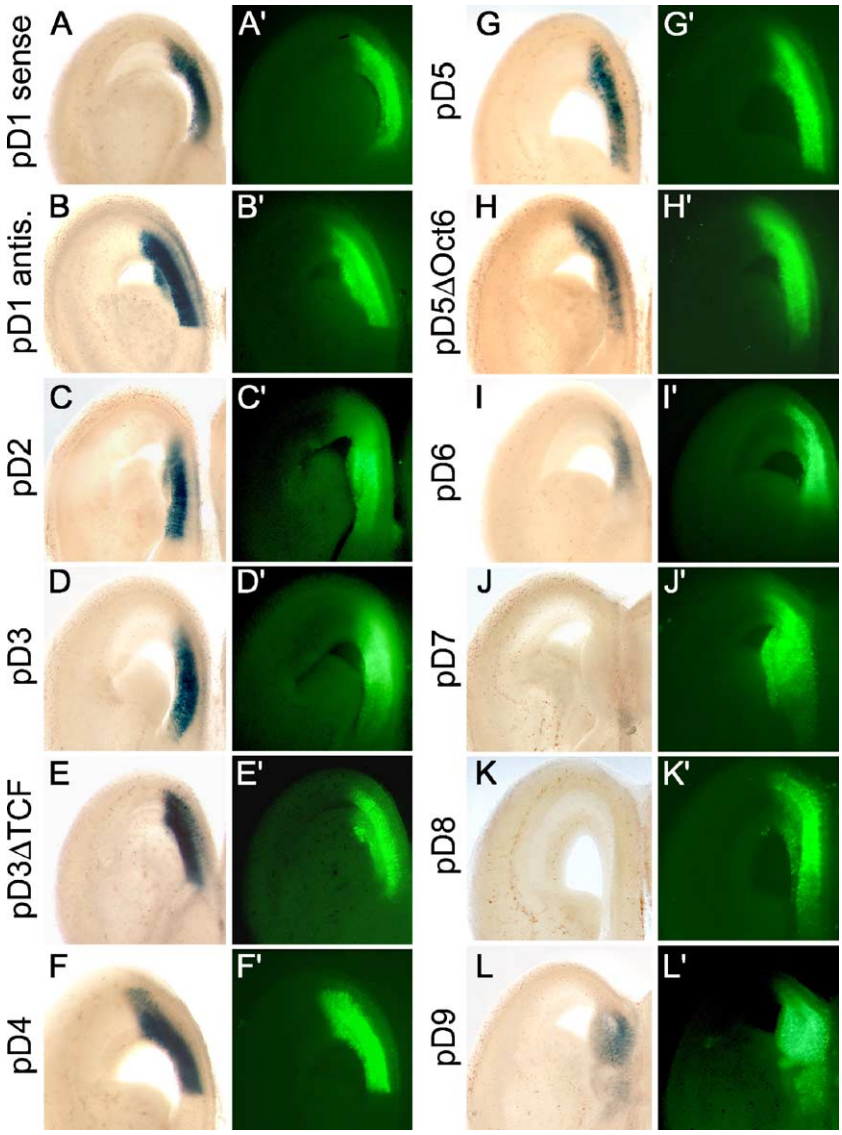

Fig. 7. Mapping of the *Hop* E1 enhancer activity. *lacZ* staining indicates enhancer activity detected after electroporation of various E1 deletion constructs (see Fig. 6A) into E14.5 brains and 24 h organotypic slice culture (A–L). The corresponding targeted regions are marked through the fluorescence of the coelectroporated CMV-EGFP plasmid (A′–L′). Strongly reduced or completely absent enhancer activity was detected with deletion constructs lacking the 200-bp E1 minimal enhancer region (pD6–pD9; I–L, I′–L′).

a 706-bp fragment encompassing the 418-bp element driving a minimal promoter-*LacZ* cassette. This novel DNA element was able to act as an enhancer and direct the *lacZ* reporter activity specifically to sites of the embryonic CNS that strongly correlate with sites of *Hop* expression, namely the ventricular zone of the neural tube, the anlage of the nucleus cochleo-vestibularis of the hindbrain, the forebrain roof plate and cortical hem, the rostro-medial cortical ventricular zone, and cells migrating from the dentate notch towards the dentate gyrus. E1-mediated transcription was completely absent from the developing heart at all investigated stages. Taken together, *Hop* expression in the developing heart and CNS is regulated through mutually exclusive *cis*-regulatory elements.

Hop E1 enhancer activity can be reproduced in vitro by a novel approach involving electroporation and organotypic slice culture

In the past electroporation-mediated gene transfer has been proven to be a versatile tool to study aspects of embryonic development (for a review, see Blader et al., 2004). Thereby electroporation was either performed in utero (Bai et al., 2003; Fukuchi-Shimogori and Grove, 2001; Tabata and Nakajima, 2001) or in vitro followed by whole embryo culture (Osumi and Inoue, 2001) or organotypic culture (Alifragis et al., 2004; Stühmer et al., 2002). Here we developed a modified variant of the electroporation and organotypic culture approach. Utilizing this technique we were able to reproduce the forebrain activity of the *Hop*

E1 element (as seen in transgenic mice) in a region-specific manner. Whereas the rostro-medial ventricular zone showed strong enhancer activity in vivo (Fig. 3L) and after electroporation in vitro (Fig. 5B), the caudo-medial ventricular zone of the cortex (Fig. 3M) and the diencephalic ventricular zone lacked enhancer activity in vivo as well as after electroporation in vitro (Fig. 5D). The all over good preservation of region specificity allowed us to perform the further deletion analyses of the forebrain E1 activity in vitro.

The electroporation and organotypic culture-based method presented here has the potential to complement the transgenic-based models of enhancer study. Salient features of this approach are its complete independence from genome integration site effects as they are seen in transgenic models and its high throughput capacity.

Hop expression in the forebrain requires a 200-bp minimal enhancer sequence

Applying the electroporation and organotypic culture method we could demonstrate that the *Hop* E1 element fulfils all criteria of being a classical enhancer element, such as independence of orientation and location from the promoter (Banerji et al., 1981; Fromm and Berg, 1983). Moreover, we could define the minimal requirements of *Hop* E1 enhancer activity in the forebrain being represented in a 200-bp sequence at the 5′-end of the conserved region. Interestingly, the 3′ half of the conserved region was dispensable for the forebrain-specific enhancer activity. Due to its strong activity the 200-bp *Hop* enhancer seems to carry all sequence motifs necessary for transcription activation in the medial forebrain. Although *Lef1* is strongly expressed in the *Hop*-positive rostro-medial cortex, mutations in two putative TCF/Lef binding sites did not prove these motifs to be functional for *Hop* expression. A normal expression of *Hop* in the *Lef1*-deficient cerebral cortex (data not shown) additionally emphasizes that the cortical *Hop* expression is independent of this transcription factor. The lack of effect after deletion of the corresponding putative binding sites can be explained as follows. These 4-base pair "sites" are not in any case optimal canonical sites for those factors though they are indeed the cores thereof. But these sites are so short that in a sequence of the total length shown here, their occurrence on a random basis is statistically not unlikely. Due to the lack of other known and functional transcription factor binding sites in the 200-bp minimal enhancer, it represents a novel element conferring gene expression to the medial cortical ventricular zone with yet unknown transcription factors binding to it.

Forebrain activity of the Hop E1 enhancer is inducible ectopically through roof plate transplants in vitro

The cortical medial-high to lateral-low expression gradients of *Emx2* and *Lhx2* are assumed to depend on diffusible signals from the medial signaling centers, the roof plate, and cortical hem (Bulchand et al., 2001; Monuki et al., 2001; Simeone et al., 1992). Here we provide experimental data suggesting that also the cortical expression of *Hop* depends on a medial signaling center, namely the roof plate. E10.5 wild-type roof plates that were brought into direct contact with transgenic brain in vitro, induced *Hop* E1 activity at sites usually devoid of enhancer activity, namely the lateral and ventral pallium (Fig. 4). We found that this inductive capacity is specific for roof plate tissue since transplantation experiments with tissue from the ganglionic eminences did not yield ectopic enhancer activity.

Although the transplanted roof plate tissues were always placed underneath the transgenic brain sections, the induced enhancer activity developed throughout its entire thickness of 350 μm. We suggest that this action at a distance is due to paracrine factors secreted by the transplanted roof plate tissue. Indeed, it is an accepted model that the forebrain roof plate acts as a source of diffusible activity, including BMP factors (Furuta et al., 1997). However, a direct dependence of the *Hop* enhancer activity on the BMP-mediated pathway cannot be suggested, since functional binding sites for its downstream mediators of the SMAD family were absent.

The competence of transgenic forebrain tissue, to respond to roof plate signals by up regulating the *Hop* enhancer activity, changed over time. The induction was high in E11.5 brains but strongly reduced or absent in older brains (data not shown). This disappearance of competence correlated chronologically with the onset of *Hop* expression in the medial cortex at E12.5. The induction of *Hop* E1 activity in the forebrain therefore seems to depend on roof plate signals, while its maintenance becomes roof plate independent from E12.5 onwards. However, both aspects, induction and maintenance of *Hop* expression, are apparently mediated by the same enhancer sequence.

In general, cortical gene expression is assumed to become an intrinsic feature of certain regions of the cerebral cortex at early stages of development. For instance, the expression of marker genes, such as *SCIP*, *KA1*, and *Steel*, has been shown to become intrinsic to the hippocampal anlage and independent from roof plate/cortical hem signaling at stages earlier than E12.5 (Tole and Grove, 2001). Similar findings apply to the marker transgene *H-2Z1* becoming an intrinsic feature of the prospective somatosensory area as early as E11.5 (Gitton et al., 1999). Furthermore, the commitment of regional identity in the cerebral cortex around the time of first neuronal production is consistent with results from transplantation experiments in the rat cerebral cortex (Barbe and Levitt, 1991) and chick optic tectum (Itasaki et al., 1991).

In conclusion, the *Homeodomain only protein*, *Hop*, is expressed and regulated in the developing murine forebrain by a 200-bp-specific *cis*-regulatory sequence. One or more so far unidentified paracrine signaling factor(s) from the roof plate can turn on the *Hop* enhancer-mediated transcriptional activity. The identified enhancer sequence

provides an important tool for the targeted expression of transgenes in the medial cortex and the cortical hem.

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References

Alifragis, P., Liapi, A., Parnavelas, J.G., 2004. Lhx6 regulates the migration of cortical interneurons from the ventral telencephalon but does not specify their GABA phenotype. J. Neurosci. 24, 5643–5648.

Anderson, S.A., Qiu, M., Bulfone, A., Eisenstat, D.D., Meneses, J., Pedersen, R., Rubenstein, J.L., 1997. Mutations of the homeobox genes Dlx-1 and Dlx-2 disrupt the striatal subventricular zone and differentiation of late born striatal neurons. Neuron 19, 27–37.

Assimacopoulos, S., Grove, E.A., Ragsdale, C.W., 2003. Identification of a Pax6-dependent epidermal growth factor family signaling source at the lateral edge of the embryonic cerebral cortex. J. Neurosci. 23, 6399–6403.

Bai, J., Ramos, R.L., Ackman, J.B., Thomas, A.M., Lee, R.V., LoTurco, J.J., 2003. RNAi reveals doublecortin is required for radial migration in rat neocortex. Nat. Neurosci. 6, 1277–1283.

Banerji, J., Rusconi, S., Schaffner, W., 1981. Expression of a beta-globin gene is enhanced by remote SV40 DNA sequences. Cell 27, 299–308.

Barbe, M.F., Levitt, P., 1991. The early commitment of fetal neurons to the limbic cortex. J. Neurosci. 11, 519–533.

Bishop, K.M., Goudreau, G., O'Leary, D.D., 2000. Regulation of area identity in the mammalian neocortex by Emx2 and Pax6. Science 288, 344–349.

Bishop, K.M., Rubenstein, J.L., O'Leary, D.D., 2002. Distinct actions of Emx1, Emx2, and Pax6 in regulating the specification of areas in the developing neocortex. J. Neurosci. 22, 7627–7638.

Blader, P., Lam, C.S., Rastegar, S., Scardigli, R., Nicod, J.C., Simplicio, N., Plessy, C., Fischer, N., Schuurmans, C., Guillemot, F., Strahle, U., 2004. Conserved and acquired features of neurogenin1 regulation. Development 131, 5627–5637.

Brink, C., Chowdhury, K., Gruss, P., 2001. Pax4 regulatory elements mediate beta cell specific expression in the pancreas. Mech. Dev. 100, 37–43.

Bulchand, S., Grove, E.A., Porter, F.D., Tole, S., 2001. LIM-homeodomain gene Lhx2 regulates the formation of the cortical hem. Mech. Dev. 100, 165–175.

Charron, G., Guy, L.G., Bazinet, M., Julien, J.P., 1995. Multiple neuron-specific enhancers in the gene coding for the human neurofilament light chain. J. Biol. Chem. 270, 30604–30610.

Chen, F., Kook, H., Milewski, R., Gitler, A.D., Lu, M.M., Li, J., Nazarian, R., Schnepp, R., Jen, K., Biben, C., Runke, G., Mackay, J.P., Novotny, J., Schwartz, R.J., Harvey, R.P., Mullins, M.C., Epstein, J.A., 2002. *Hop* is an unusual homeobox gene that modulates cardiac development. Cell 110, 713–723.

Fromm, M., Berg, P., 1983. Transcription in vivo from SV40 early promoter deletion mutants without repression by large T antigen. J. Mol. Appl. Genet. 2, 127–135.

Fukuchi-Shimogori, T., Grove, E.A., 2001. Neocortex patterning by the secreted signaling molecule FGF8. Science 294, 1071–1074.

Fukuchi-Shimogori, T., Grove, E.A., 2003. Emx2 patterns the neocortex by regulating FGF positional signaling. Nat. Neurosci. 6, 825–831.

Funatsu, N., Inoue, T., Nakamura, S., 2004. Gene expression analysis of the late embryonic mouse cerebral cortex using DNA microarray: identification of several region- and layer-specific genes. Cereb. Cortex 14, 1031–1044.

Furuta, Y., Piston, D.W., Hogan, B.L., 1997. Bone morphogenetic proteins (BMPs) as regulators of dorsal forebrain development. Development 124, 2203–2212.

Galceran, J., Miyashita-Lin, E.M., Devaney, E., Rubenstein, J.L., Grosschedl, R., 2000. Hippocampus development and generation of dentate gyrus granule cells is regulated by LEF1. Development 127, 469–482.

Garel, S., Huffman, K.J., Rubenstein, J.L., 2003. Molecular regionalization of the neocortex is disrupted in Fgf8 hypomorphic mutants. Development 130, 1903–1914.

Gitton, Y., Cohen-Tannoudji, M., Wassef, M., 1999. Specification of somatosensory area identity in cortical explants. J. Neurosci. 19, 4889–4898.

Gossler, A., Zachgo, J., 1993. Gene and enhancer trap screen in ES cell chimeras. Gene Targeting: A Practical Approach. Oxford University Press, Oxford, pp. 181–225.

Grove, E.A., Fukuchi-Shimogori, T., 2003. Generating the cerebral cortical area map. Annu. Rev. Neurosci. 26, 355–380.

Grove, E.A., Tole, S., Limon, J., Yip, L., Ragsdale, C.W., 1998. The hem of the embryonic cerebral cortex is defined by the expression of multiple Wnt genes and is compromised in Gli3-deficient mice. Development 125, 2315–2325.

Hamamori, Y., Schneider, M.D., 2003. HATs off to Hop: recruitment of a class I histone deacetylase incriminates a novel transcriptional pathway that opposes cardiac hypertrophy. J. Clin. Invest. 112, 824–826.

Helms, A.W., Johnson, J.E., 2003. Specification of dorsal spinal cord interneurons. Curr. Opin. Neurobiol. 13, 42–49.

Itasaki, N., Ichijo, H., Hama, C., Matsuno, T., Nakamura, H., 1991. Establishment of rostrocaudal polarity in tectal primordium: engrailed expression and subsequent tectal polarity. Development 113, 1133–1144.

Jessell, T.M., 2000. Neuronal specification in the spinal cord: inductive signals and transcriptional codes. Nat. Rev., Genet. 1, 20–29.

Kerlavage, A., Bonazzi, V., di Tommaso, M., Lawrence, C., Li, P., Mayberry, F., Mural, R., Nodell, M., Yandell, M., Zhang, J., Thomas, P., 2002. The celera discovery system. Nucleic Acids Res. 30, 129–136.

Kohtz, J.D., Baker, D.P., Corte, G., Fishell, G., 1998. Regionalization within the mammalian telencephalon is mediated by changes in responsiveness to Sonic Hedgehog. Development 125, 5079–5089.

Kurokawa, D., Takasaki, N., Kiyonari, H., Nakayama, R., Kimura-Yoshida, C., Matsuo, I., Aizawa, S., 2004. Regulation of Otx2 expression and its functions in mouse epiblast and anterior neuroectoderm. Development 131, 3307–3317.

Lee, S.M., Tole, S., Grove, E., McMahon, A.P., 2000. A local Wnt-3a signal is required for development of the mammalian hippocampus. Development 127, 457–467.

Monuki, E.S., Porter, F.D., Walsh, C.A., 2001. Patterning of the dorsal telencephalon and cerebral cortex by a roof plate-Lhx2 pathway. Neuron 32, 591–604.

Moorman, A.F., Houweling, A.C., de Boer, P.A., Christoffels, V.M., 2001. Sensitive nonradioactive detection of mRNA in tissue sections: novel application of the whole-mount in situ hybridization protocol. J. Histochem. Cytochem. 49, 1–8.

Muzio, L., DiBenedetto, B., Stoykova, A., Boncinelli, E., Gruss, P., Mallamaci, A., 2002a. Conversion of cerebral cortex into basal ganglia in Emx2(−/−) Pax6(Sey/Sey) double-mutant mice. Nat. Neurosci. 5, 737–745.

Muzio, L., DiBenedetto, B., Stoykova, A., Boncinelli, E., Gruss, P., Mallamaci, A., 2002b. Emx2 and Pax6 control regionalization of the pre-neurogenic cortical primordium. J. Neurosci. 19, 877–885.

O'Leary, D.D., Nakagawa, Y., 2002. Patterning centers, regulatory genes and extrinsic mechanisms controlling arealization of the neocortex. Curr. Opin. Neurobiol. 12, 14–25.

Osumi, N., Inoue, T., 2001. Gene transfer into cultured mammalian embryos by electroporation. Methods 24, 35–42.

Pfeffer, P.L., Bouchard, M., Busslinger, M., 2000. Pax2 and homeodomain proteins cooperatively regulate a 435 bp enhancer of the mouse Pax5 gene at the midbrain-hindbrain boundary. Development 127, 1017–1028.

Quandt, K., Frech, K., Karas, H., Wingender, E., Werner, T., 1995. MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. Nucleic Acids Res. 23, 4878–4884.

Scardigli, R., Baumer, N., Gruss, P., Guillemot, F., Le Roux, I., 2003. Direct and concentration-dependent regulation of the proneural gene Neurogenin2 by Pax6. Development 130, 3269–3281.

Shimamura, K., Rubenstein, J.L., 1997. Inductive interactions direct early regionalization of the mouse forebrain. Development 124, 2709–2718.

Shimogori, T., Banuchi, V., Ng, H.Y., Strauss, J.B., Grove, E.A., 2004. Embryonic signaling centers expressing BMP, WNT and FGF proteins interact to pattern the cerebral cortex. Development 131, 5639–5647.

Shin, C.H., Liu, Z.P., Passier, R., Zhang, C.L., Wang, D.Z., Harris, T.M., Yamagishi, H., Richardson, J.A., Childs, G., Olson, E.N., 2002. Modulation of cardiac growth and development by HOP, an unusual homeodomain protein. Cell 110, 725–735.

Simeone, A., Gulisano, M., Acampora, D., Stornaiuolo, A., Rambaldi, M., Boncinelli, E., 1992. Two vertebrate homeobox genes related to the Drosophila empty spiracles gene are expressed in the embryonic cerebral cortex. EMBO J. 11, 2541–2550.

Spieler, D., Baumer, N., Stebler, J., Koprunner, M., Reichman-Fried, M., Teichmann, U., Raz, E., Kessel, M., Wittler, L., 2004. Involvement of Pax6 and Otx2 in the forebrain-specific regulation of the vertebrate homeobox gene ANF/Hesx1. Dev. Biol. 269, 567–579.

Stoykova, A., Gruss, P., 1994. Roles of Pax-genes in developing and adult brain as suggested by expression patterns. J. Neurosci. 14, 1395–1412.

Stühmer, T., Anderson, S.A., Ekker, M., Rubenstein, J.L., 2002. Ectopic expression of the Dlx genes induces glutamic acid decarboxylase and Dlx expression. Development 129, 245–252.

Tabata, H., Nakajima, K., 2001. Efficient in utero gene transfer system to the developing mouse brain using electroporation: visualization of neuronal migration in the developing cortex. Neuroscience 103, 865–872.

Theil, T., Alvarez-Bolado, G., Walter, A., Ruther, U., 1999. Gli3 is required for Emx gene expression during dorsal telencephalon development. Development 126, 3561–3571.

Theil, T., Aydin, S., Koch, S., Grotewold, L., Ruther, U., 2002. Wnt and Bmp signalling cooperatively regulate graded Emx2 expression in the dorsal telencephalon. Development 129, 3045–3054.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Tole, S., Grove, E.A., 2001. Detailed field pattern is intrinsic to the embryonic mouse hippocampus early in neurogenesis. J. Neurosci. 21, 1580–1589.

Wingender, E., Chen, X., Hehl, R., Karas, H., Liebich, I., Matys, V., Meinhardt, T., Pruss, M., Reuter, I., Schacherer, F., 2000. TRANSFAC: an integrated system for gene expression regulation. Nucleic Acids Res. 28, 316–319.

Zappone, M.V., Galli, R., Catena, R., Meani, N., De Biasi, S., Mattei, E., Tiveron, C., Vescovi, A.L., Lovell-Badge, R., Ottolenghi, S., Nicolis, S.K., 2000. Sox2 regulatory sequences direct expression of a (beta)-geo transgene to telencephalic neural stem cells and precursors of the mouse embryo, revealing regionalization of gene expression in CNS stem cells. Development 127, 2367–2382.