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TTF-1/NKX2.1 up-regulates the *in vivo* transcription of nestin

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ABSTRACT *TTF-1/NKX2.1*, also known as T/EBP, is a homeodomain-containing gene involved in the organogenesis of the thyroid gland, lung and ventral forebrain. We have already reported that in 3T3 cells, *TTF-1/NKX2.1* up-regulates the transcription of nestin, an intermediate filament protein expressed in multipotent neuroepithelial cells, by direct DNA-binding to a HRE/CRE-like site (NestBS) within a CNS-specific enhancer. Here, we demonstrate that *TTF-1/NKX2.*1 is co-expressed with *nestin* in the embryonal forebrain. We also performed a transgenic mouse embryo analysis in which NestBS was replaced by the canonical *TTF-1/NKX2.1* consensus DNA-binding site (as identified in many thyroid- and lung-specific genes and very divergent from NestBS) or a random mutation. We observed β-galactosidase expression in forebrain regions where *TTF-1/NKX2.1* is expressed in wild-type embryos, and -to a minor extent- in rostralmost telencephalic regions and thalamus, whereas no β-galactosidase expression was detected in forebrains of embryos bearing the random mutation. These data show that *TTF-1/NKX2.1* regulates the transcription of the *nestin* gene *in vivo* through the NestBS site, suggesting that nestin might be at least one of the effectors of *TTF-1/NKX2.*1 during forebrain development. Finally, we have shown that the transactivating effect of TTF-1/NKX2.1 on the CNS-specific enhancer is unaffected by Retinoic Acid Receptor-α.

KEY WORDS: *CNS development, hypothalamus, medial ganglionic eminence, transcriptional regulation*

Introduction

TTF-1/NKX2.1, also known as T/EBP, is a homeodomain-containing transcription factor belonging to the NKX gene family required for the tissue specific-expression of several thyroid- and lung-specific genes (Guazzi *et al.,* 1990; Missero *et al.,* 1998; Whitsett *et al.,* 1998). *TTF-1/NKX2.1* is also expressed in restricted areas of the developing forebrain, namely in the diencephalon (i.e., in the hypothalamus) and in the telencephalon (i.e., in the medial ganglionic eminence, preoptic area, and anterior endopeduncular area) (Lazzaro *et al.,* 1991; Price *et al.,* 1992; Sussel *et al.,* 1999). In addition, *TTF-1/NKX2.1* is one of the genes involved in the early organization of the vertebrate rostral brain, playing a role in the early patterning of the developing head in longitudinal and transverse domains (Rubenstein *et al.,* 1998; Shimamura *et al.,* 1995; Wilson and Rubenstein, 2000). A subset of precursor cells expressing *TTF-1/NKX2.1* migrates tangentially from the medial ganglionic eminence of the ventral telencephalon to the developing striatum and to the developing cortical plate (Marìn and Rubenstein, 2001; Anderson *et al.,* 2001). *TTF-1/NKX2.1* expression in the adult persists in the hypothalamus and in the subfornical organ, a circumventricular organ of the third ventricle (Lee *et al.,* 2001; Son *et al.,* 2003). *TTF-1/NKX2.1* homozygous mutant mice were born dead and lacked completely the thyroid gland and the lung parenchyma, the entire pituitary was missing, and extensive defects were found in the

Abbreviations used in this paper: β-Gal, β-galactosidase; bp, base-pairs; d.p.c, days-post-coitum; CNS, central nervous system; HRE/CRE, hormone receptor element/cAMP-responsive element; IF, immunofluorescence; RAR, retinoic acid receptor; PBS, phosphate-buffered-saline solution; TTF-1, thyroid transcription factor-1; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

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ventral region of the forebrain (Kimura *et al.,* 1996). It has also been reported that in *TTF-1/NKX2.1* knockout mice a ventral-to-dorsal transformation of the pallidum primordium into a striatal-like anlage takes place (Sussel *et al.,* 1999).

Since in *TTF-1/NKX2.1* K.O. mice the organogenesis of the ventral forebrain is profoundly perturbed, elucidating the mechanisms by which *TTF-1/NKX2.1* is able to play its fundamental role in the development of the forebrain might be important to achieve a better comprehension of brain organogenesis. In order to better elucidate the function of *TTF-1/NKX2.1* in the developing forebrain, we performed an analysis of brain-specific *TTF-1/NKX2.1* target genes, and we found that *TTF-1/NKX2.1* ectopic expression in fibroblastic cells strongly up-regulates the endogenous transcription of the neuroepithelial cell marker *nestin*, by direct DNA-binding to an already characterized enhancer capable of recapitulate the CNS-specific expression of the nestin gene in transgenic mice (Lonigro *et al.,* 2001; Zimmerman *et al.,* 1994). *TTF-1/NKX2.1* bound to the CNS-specific enhancer through a NestBS site, already known by transgenic mice analysis to be essential for proper *nestin* expression in the telencephalic portions of the forebrain from 12.5 d.p.c onwards (Josephson *et al.,* 1998). In this study, we have exploited the role of *TTF-1/NKX2.1* in the *in vivo* regulation of the *nestin* gene transcription analyzing transgenic mouse embryos in which the NestBS site was replaced by a random mutation or by the canonical *TTF-1/NKX2.1* consensus DNA-binding site, previously identified in many thyroid- and lung-specific regulatory DNA sequences (Damante *et al.,* 1994; Bohinski *et al.,* 1994). In this way, if *TTF-1/NKX2.1* is indeed the protein binding to the *nestin* gene *in vivo*, presumably it would be still able to recognize a mutation bearing the canonical *TTF-1/NKX2.1* DNA-binding site, but not a random mutation. Moreover, since it has been reported that Retinoic Acid Receptor (RAR)–α was able to bind *in vitro* to the NestBS sequence (Lothian *et al.,* 1999) and the surfactant protein-B promoter–stimulation by retinoic acid was dependent on juxtaposed *RAR-*α and *TTF-1/NKX2.1* sites (Naltner *et al.,* 2000), we tested the hypothesis that an interplay occurs between these two transcription factors in the context of the *nestin* enhancer.

Results

TTF-1/NKX2.1 *and* nestin *are co-expressed in murine embryonal brain*

As a first step, in order to elucidate if *TTF-1/NKX2.1* plays a role in the regulation of the *nestin* gene *in vivo*, we assessed if *TTF-1/NKX2.1* and *nestin* were actually co-expressed in the murine developing forebrain. To this aim, we performed a double immunohistochemistry staining for TTF-1/NKX2.1 and nestin protein on forebrain sections from wild type 14 d.p.c. mouse. As shown in Fig. 1A, we found that *TTF-1/NKX2.1* and *nestin* were co-expressed, respectively, in the nucleus and in the cytoplasm of the majority of the cells within the domain of expression of *TTF-1/NKX2.1*. In order to quantify this expression, we also performed immunofluorescence (IF) assays on cells dissociated from whole E14 mouse brains with antibodies against *TTF-1/NKX2.1* and *nestin*, and the nuclei were visualized by DAPI staining. Again, a clear nuclear signal for *TTF-1/NKX2.1* was observed, while the anti-nestin antibody showed a cytoplasmic signal (Fig. 1B). However, cells positive for *TTF-1/NKX2.1* were often found in clusters, suggesting that they derived either from rapidly dividing cells in the overnight cell culture or from dissociation of tightly linked embryonal cells. Therefore, we performed a flow cytometric analysis (i.e., without any previous plating step) on freshly dissociated cells from murine E14 brains using the same antibodies against *TTF-1/NKX2.1* and *nestin* used in IF assays: we found that about 18% of the total cells were positive for *TTF-1/NKX2.1*, 65% for *nestin* and 15% were double positive. Remarkably, ~ 83% of the *TTF-1/NKX2.1*-expressing cells were also positive for *nestin* (Fig. 1C).

TTF-1/NKX2.1 *is able to recognize a mutagenized* NestBS *site when replaced by a DNA-binding site bearing its core recognition sequence 5'-CAAG-3', and not by a random sequence, in co-transfection assay*

We have previously shown that *TTF-1/NKX2.1* in 3T3 cells is able to transactivate a CNS-specific enhancer of the *nestin*

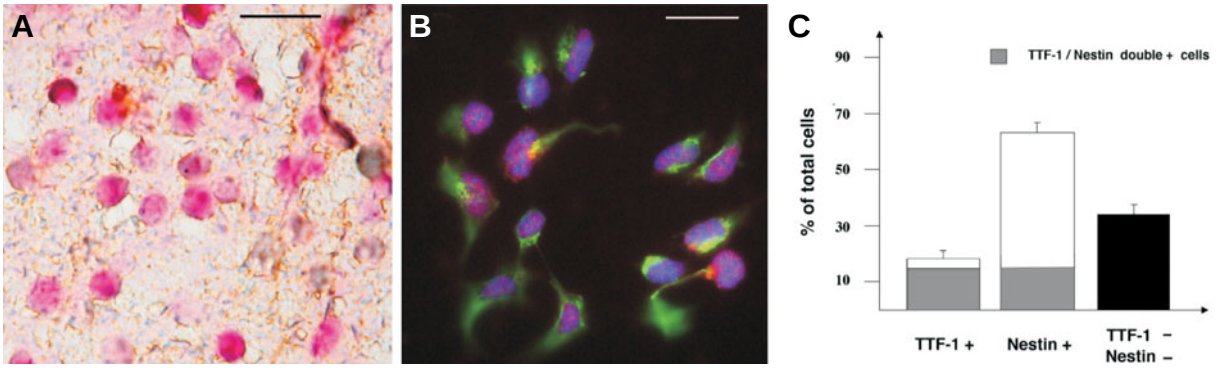

Fig. 1. TTF-1/NKX2.1 and nestin are co-expressed in murine E13 forebrains. (A) *Double immunohistochemistry on E13 mouse forebrain section with an anti-TTF-1/NKX2.1 (red, nuclear) and an anti-nestin (brown, cytoplasmic) antibody.* **(B)** *Immunofluorescence assay on cells dissociated from E14 forebrains: TTF-1/NKX2.1 protein is detected by TRITC-conjugated secondary antibody in the nucleus (red), nestin protein by FITC-conjugated secondary antibody in the cytoplasm (green) and DAPI stains the nuclei (blue). Scale bar, 10* μ*m.* **(C)** *Flow cytometric analysis on freshly dissociated primary neuronal E14 cells double-stained with antibodies against TTF-1/NKX2.1 and nestin shows that ~ 83% of the TTF-1/NKX2.1-positive cells display also nestin staining.*

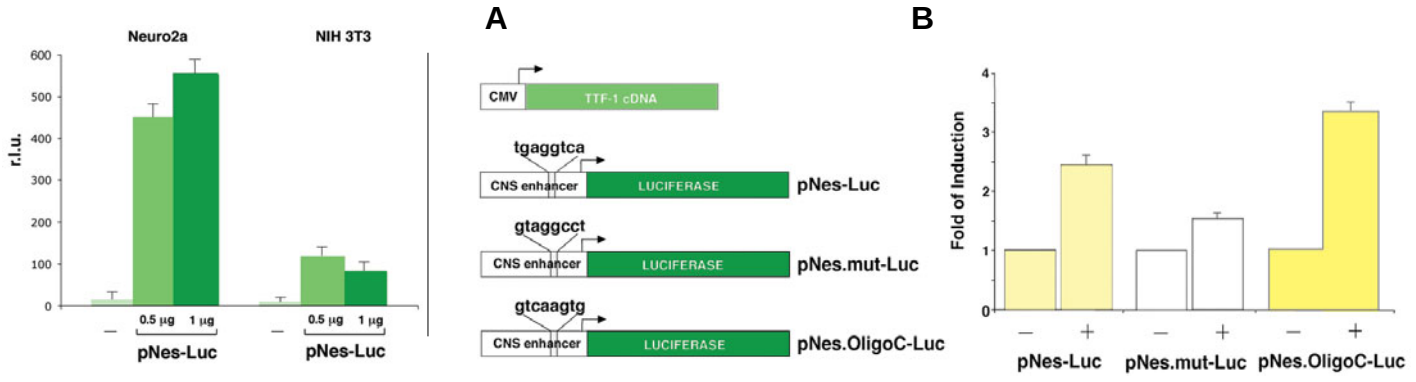

Fig. 2 (Left). The CNS-specific enhancer of the *nestin* gene imparts tissue-specificity to the luciferase reporter gene in cell culture. *The reporter construct* pNes-Luc, *containing the CNS-specific enhancer of the* nestin *gene, showed a 4.5-fold increase in basal activity when transfected in neural (Neuro2A) vs. non-neural (NIH3T3) cells. r.l.u., relative luminescence units.*

Fig. 3 (Right). TTF-1/NKX2.1 protein recognizes its canonical DNA-binding site in the context of the CNS-specific enhancer. (A) *The expression vector* pCMV-TTF1 *encoding for* TTF-1/NKX2.1 *(top) and the reporter constructs pNes.Luc, pNes.mut-Luc and pNes.OligoC-Luc bearing a wild type* NestBS, *a random mutation and a mutation containing the canonical DNA-binding site of* TTF-1/NKX2.1, *respectively.* **(B)** *Co-transfection assays in 3T3 cells of the plasmids described in (A). Luciferase activity expressed as fold of induction over the basal transcriptional activity (arbitrarily considered equal to 1) of each reporter construct. -/+, without and with the pCMV-TTF1 vector, respectively.*

gene through direct DNA binding to a NestBS site similar to HRE/CRE, bearing a 5'-TGAGGTCA-3' core sequence (Lonigro *et al.,* 2001). We then compared the basal transcriptional activity of this enhancer in two different cell lines of neural (Neuro2A) or non-neural (NIH3T3) origin by transfection of the previously described luciferase reporter construct pNes-Luc, bearing the CNS-specific enhancer (Lonigro *et al.,* 2001). We found that the basal activity of the enhancer was more efficient (4.5-fold higher) in the neural rather than in the non-neural cells, and that it was able to impart a tissue-specific expression to the luciferase reporter gene in cell culture (Fig. 2).

In transgenic mice bearing the core recognition sequence of NestBS mutagenized to 5'-GTAGGCCT-3', the β-gal expression was shown to be virtually absent in the telencephalon in comparison with the rest of the CNS, suggesting that this element was critical for proper *nestin* telencephalic expression *in vivo* from 12.5 to at least 14.5 d.p.c of mouse development (Josephson *et al.,* 1998). Therefore, we replaced the wild-type site within the NestBS sequence of the pNes-Luc construct with two different mutations in order to obtain two mutagenized constructs: the first (pNes.mut-Luc) bearing a random sequence identical to the DNA sequence used by Josephson *et al.* (1998) for the generation of transgenic mice described above (5'-GTAGGCCT-3'), the second (pNes.OligoC-Luc) bearing the DNA-binding site of *TTF-1/NKX2.1* (5'-GTCAAGTG-3') found within the rat thyroglobulin promoter (OligoC, from Civitareale *et al.,* 1989). The latter mutagenized pNes.OligoC-Luc construct contained the canonical 5'-CAAG-3' consensus core sequence of the *TTF-1/NKX2.1* protein, as identified in many thyroid- and lung-specific genes (Fig. 3A). Noticeable, only 4 out of 8 base pairs of the wild-type NestBS site were substituted in the pNes.mut-Luc construct, whereas 8 out of 8 bp were replaced in pNes-OligoC-Luc. We then evaluated whether the mutagenized constructs were able to differentially influence the extent of the transactivation mediated by the *TTF-1/NKX2.1* protein. Since, to our knowledge, a cell line of neural origin expressing *TTF-1/NKX2.1* was not available, we co-transfected the reporter constructs (pNes.mut-Luc, pNes.OligoC-Luc, and the wild-type pNes-Luc) with or without an expression vector encoding for *TTF-1/NKX2.1* (pCMV-TTF1) in 3T3 cells (Fig. 3A). We found that the wild-type pNes-Luc reporter construct showed a ~2.5-fold increase in transcriptional activity when co-transfected with pCMV-TTF1 (as reported in Lonigro *et al.,* 2001), whereas the pNes.mut-Luc failed to show a clear increase, and the pNes.OligoC-Luc showed a ~3.4-fold increase, suggesting that the random mutation negatively affects the transactivation mediated by *TTF-1/NKX2.1*, while the mutation containing the *TTF-1/NKX2.1* canonical DNA-binding site does not (Fig. 3B). Therefore, these data suggest that *TTF-1/NKX2.1* protein is still able to

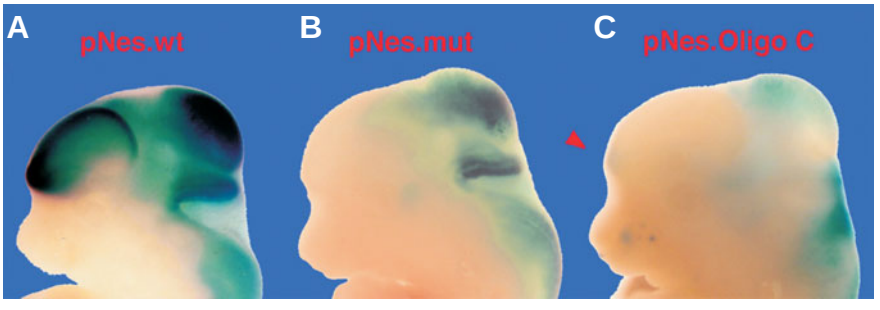

Fig. 4. Whole-mount β-galactosidase staining of 13 d.p.c. transgenic mouse embryos *bearing the pNes.LacZ* **(A)**, *pNes.mut-LacZ* **(B)**, *and pNes.OligoC-LacZ* **(C)** *constructs. An arrowhead indicates a faint expression in the rostralmost telencephalic regions observed only in pNes.OligoC-LacZ-bearing embryos. An ectopic expression in the whisker pad primordium in also visible. The difference in staining intensity between the two embryos is an artifact.*

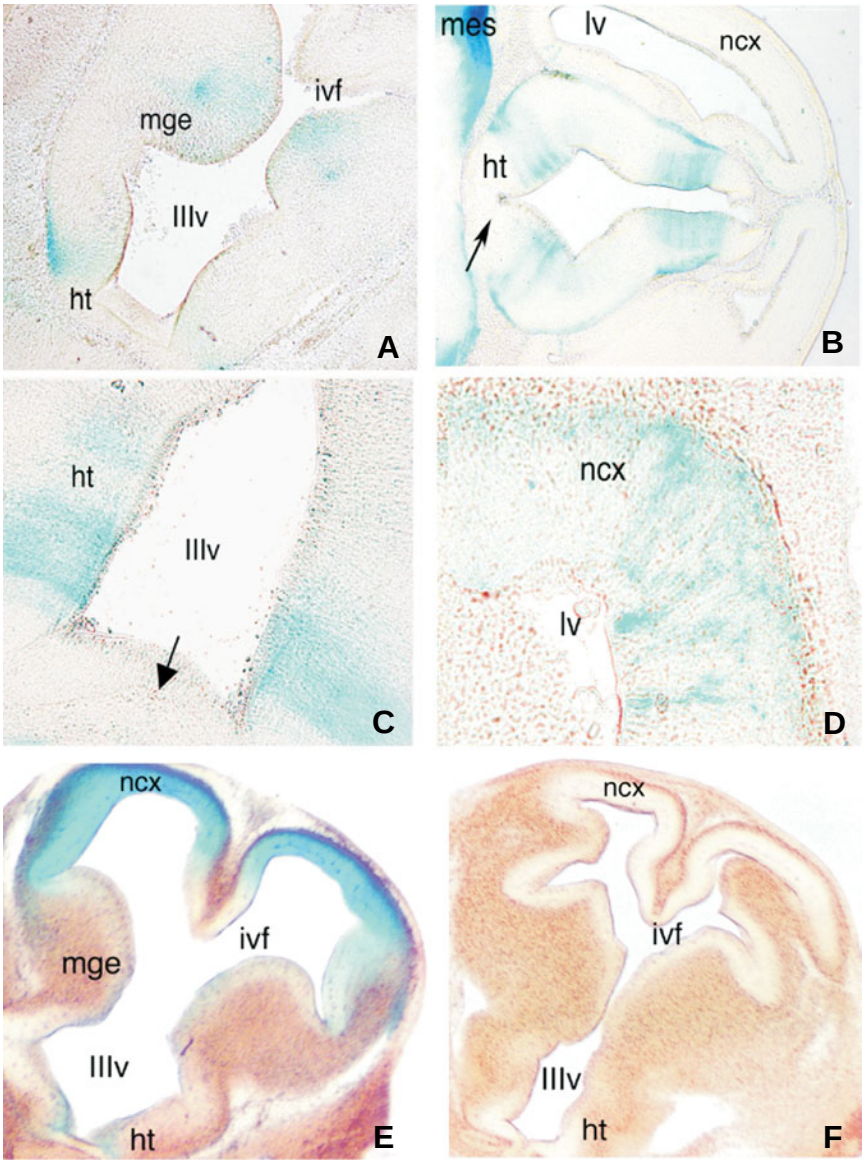

Fig. 5. Transverse sections of E13 transgenic mouse embryos showing β-galactosidase staining in *TTF-1/NKX2.1*-expressing domains in the forebrain. (A) *β-Gal staining is observed in the medial ganglionic eminence and the hypothalamic region of a pNes.OligoC-LacZ-bearing embryo.* **(B)** *A more anterior section of a pNes.OligoC-LacZ embryo (slightly skewed laterally) showing also β-Gal positivity in the thalamus.* **(C)** *Enlargement of the posterior hypothalamic area of the same embryo shown in (B).* **(D)** *Enlargement of the rostralmost presumptive neocortex positive for β-Gal expression. Sections are all from different embryos (apart from B,C).* **(E)** *β-Gal staining of a pNes-LacZ-bearing embryo (= wt CNS-enhancer) showing also a strong staining in the telencephalic vesicles.* **(F)** *Absence of β-Gal staining in the forebrain of a pNes.mut-LacZ (=randomly mutated enhancer) embryo. ivf, interventricular foramen; ht, hypothalamus; lv, lateral ventricle; mge, medial ganglionic eminence; mes, mesencephalon; ncx, presumptive neocortex; t, thalamus; IIIv, third ventricle. Rostral is to the right/top in (A,C,E,F), and right in (B,D).*

recognize a mutated NestBS site in the CNS-specific enhancer if it contains a 5'-CAAG-3' core sequence, but not a random sequence.

Transgenic mouse embryos carrying the 5'-CAAG-3' DNA-recognition sequence of TTF-1/NKX2.1 *in* NestBS *showed β-galactosidase staining within the expression domain of* TTF-1/NKX2.1

In order to test whether the mutation in NestBS containing the *TTF-1/NKX2.1* canonical DNA-binding site present in the pNes.OligoC-Luc construct is recognized *in vivo* by the endogenous *TTF-1/NKX2.1* protein in E13 mouse embryos, transgenic mouse embryos were generated carrying the same three NestBS sites described above (one wild-type and two mutated either randomly or with the 5'-CAAG-3' core sequence). The CNS-specific enhancer of the *nestin* II intron containing the wild-type or either one of the two mutated NestBS sites were inserted upstream of a β-globin minimal promoter driving the expression of a LacZ reporter gene. The three obtained constructs (called pNes-LacZ, pNes.mut-LacZ and pNes.OligoC-LacZ, respectively) were introduced into fertilized FVBxFVB mouse eggs by pronuclear injection. The resulting mouse embryos were harvested and analyzed for lacZ expression by whole-mount β-galactosidase (β-Gal) histochemistry (Fig. 4). As previously reported (Zimmerman *et al.,* 1994; Josephson *et al.,* 1998), E13 transgenic mouse embryos bearing the pNes-LacZ construct showed β-Gal staining along the entire length of the CNS (Fig. 4). Whole-mount β-Gal staining of transgenic embryos bearing the pNes.mut-LacZ or pNes.OligoC-LacZ constructs showed the

TABLE 1

TRANSGENIC EMBRYO ANALYSIS OF THE CNS-SPECIFIC ENHANCER OF THE *NESTIN* GENE CONTAINING A WILD-TYPE OR A MUTATED NESTBS

	Total mouse embryos	Transgenic mouse embryos (% of total)	β-Gal expression[a] (rostral forebrain) TTF1+	ncx/t	β-Gal expression (midbrain/hindbrain)	β-Gal expression (thyroid/lung)	Ectopic whisker expression
pNes.LacZ	12	4 (30%)	4	4	4	0	0
pNes.mut-LacZ	15	5 (33%)	0	0	5	0	0
pNes.OligoC-LacZ	26	7 (27%)	6	5	6	0	5

[a] N. of embryos showing β-galactosidase staining in forebrain regions expressing *TTF-1/NKX2.1* (TTF-1+) or in the presumptive neocortex and thalamus (ncx/t) negative for *TTF-1/NKX2.1*.

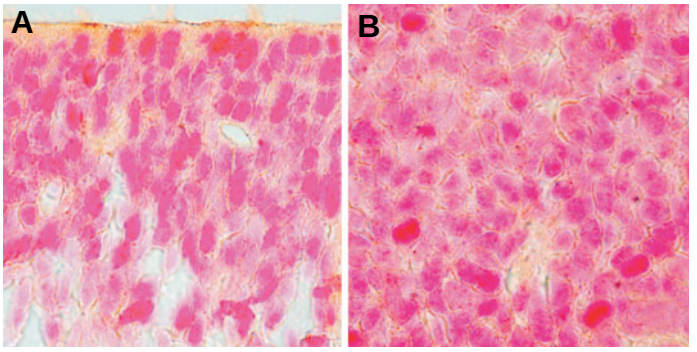

Fig. 6. E13 forebrain embryo bearing the *pNes.OligoC-LacZ* construct double immunostained for *TTF-1/NKX2.1* and *β-galactosidase*. *Positivity for* TTF-1/NKX2.1 *(red, nuclear) and for* β-galactosidase *(brown, cytoplasmic) in cells of the hypothalamus* **(A)** *and medial ganglionic eminence* **(B)**. *Ventricular surface is on the top in (A).*

presence of a signal in the entire neural tube in roughly 30% of the total embryos analyzed, with the notable exclusion of the telencephalic portions (Fig. 4 and Table 1). However, in 6 out of 7 (~86%) of the pNes.OligoC-LacZ-bearing embryos a faint staining was also detected in the rostralmost telencephalic regions. In addition, in 5 out of 7 (71%) of the pNes.OligoC-LacZ transgenic embryos an ectopic expression in whisker pad primordia was also present (Fig. 4 and Table 1). To better identify the CNS regions displaying the β-Gal staining, 125 μM thick transverse sections were obtained from E13 forebrains of transgenic mice bearing the three constructs. Forebrains of transgenic mice bearing the pNes.OligoC-LacZ construct showed an intense staining in the medial ganglionic eminence and hypothalamus (Fig. 5 A,B), not observed in pNes.mut-LacZ-bearing embryos (Fig. 5F). However, a strong expression in the thalamus was also observed, not present in the pNes.mut-LacZ-bearing mice or in the domains of expression of the wild type *TTF1/NKX2.1* protein (Fig. 5B). Moreover, a faint but clear β-Gal expression was present in the rostralmost regions of the cortical plate, as already suggested by whole-mount β-Gal staining (Fig. 4D). For comparison, a similar transverse section from a transgenic mouse bearing a pNes-Luc construct (containing a wt CNS-specific enhancer) showed β-Gal expression in the telencephalic vesicles, medial ganglionic eminence and hypothalamus (Fig. 5E). More posterior CNS regions, i.e. mesencephalon (Fig. 5B) and prospective spinal cord (data not shown), showed the same β-Gal expression pattern with all the three constructs. Remarkably, all the regions strongly expressing *TTF-1/NKX2.1* within the CNS during mouse development are positive for lacZ expression (Price *et al.,* 1992; Sussel *et al.,* 1999), with the sole exception of the more ventral regions of the hypothalamus, in which no β-Gal expression was observed (Fig. 5 B,C, arrows).

Finally, in order to demonstrate that *TTF-1/NKX2.1* and β-Gal actually co-exists in the same cell/s of transgenic embryos, we performed a double immunostaining on forebrain sections of E13 pNes.OligoC-LacZ-expressing embryos: at high magnification a clear co-localization was shown with two antibodies recognizing the β-Gal and the TTF-1/NKX2.1 proteins (in the cytoplasm and nucleus, respectively) in the hypothalamus (Fig. 6A) and in the medial ganglionic eminence (Fig. 6B).

The transactivating effect of* TTF-1/NKX2.1 *on the CNS-specific enhancer is unaffected by Retinoic Acid Receptor-α

Several recombinant nuclear hormone receptors known to control important events during development (i.e., TRs, RXR, RAR-α, and COUP-TF) are able to bind *in vitro* to HRE/CRE-like (=NestBS) sequences (Lothian *et al.,* 1999). Moreover, it has been shown that retinoic acid stimulation of the human surfactant protein B promoter is dependent on both Retinoic Acid Receptor-α (RAR-α) and *TTF-1/NKX2.1* binding elements strictly juxtaposed among them, and that *TTF-1/NKX2.1* and RAR-α directly interact in two hybrid system assay (Naltner *et al.,* 2000). To test whether *TTF-1/NKX2.1* and RAR-α also interact in the context of the CNS-specific enhancer of the *nestin* gene, we co-transfected in 3T3 cells the pNes-Luc reporter construct together with the *TTF-1/NKX2.1*-encoding pCMV-TTF1 vector, and/or pSG-RAR-α, encoding for RAR-α (Fig. 7). The analysis of the luciferase activity showed that pCMV-TTF1 alone induced a ~3-fold activation over the basal activity of pNes-Luc alone (as previously reported), whereas pSG-RAR-α caused a negligible increase. However, when the two nuclear factors were transfected together, they failed to show any synergistic or even additional effect on the extent of transcription of the reporter gene, suggesting that the transactivating activity of *TTF-1/NKX2.1* on the CNS-specific enhancer of the *nestin* gene was unaffected by RAR-α (Fig. 7).

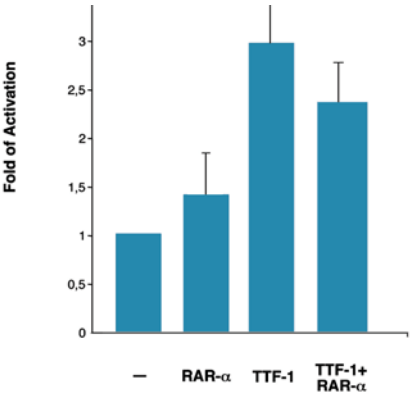

Fig. 7. TTF-1 and RAR-α do not interact within the CNS-specific nestin enhancer. *Co-transfection of expression vectors encoding for* TTF-1/NKX2.1 *and/or RAR-α together with pNes.Luc in 3T3 cells does not reveal any synergistic or additional effect. Luciferase activity is expressed as fold of induction on the basal transcriptional activity of the* pNes.Luc *construct.*

Discussion

We have previously shown that *TTF-1/NKX2.1* is able to activate the transcription of the CNS-specific enhancer in the second intron of the *nestin* gene in 3T3 cells (Lonigro *et al.,* 2001). Here we provide evidence that the same transactivation is indeed achieved *in vivo* in the developing mouse embryos. Immunohistochemistry and immunofluorescence assays shows that *TTF-1/NKX2.1* and *nestin* are co-expressed in the same cell population: in fact, in E13 wild type mouse embryos and in cells dissociated from brains of E14 mouse embryos, most of the *TTF-1/NKX2.1*-expressing cells are also positive for *nestin* (Fig.1). EMSA assays

performed with NestBS oligonucleotide and nuclear extracts prepared from E14 mouse brains already showed that *TTF-1/NKX2.1* does not bind to NestBS if mutagenized into a random sequence (Lonigro *et al.,* 2001). These data are here confirmed and extended by co-transfection assays in 3T3 cells demonstrating that the *TTF-1/NKX2.1*-mediated transactivation of the *nestin* gene is abolished by a randomly mutagenized NestBS (pNes.mut-Luc), while it is still present if NestBS is replaced by *TTF-1/NKX2.1* canonical DNA-recognition site (5'-GTCAAGTG-3'), even if the wild-type NestBS displays a completely different DNA sequence (5'-TGAGGTCA-3'). Thereof, we generated transgenic mouse embryos carrying a construct bearing the canonical DNA-binding site of *TTF-1/NKX2.1* (or a random sequence) in the NestBS site within the CNS-enhancer of the *nestin* gene. Transgenic mice analysis showed that the endogenous *TTF-1/NKX2.1* protein is still able to recognize its DNA-recognition site in the mouse embryo and, consequently, to restore a β-galactosidase expression in *TTF-1/NKX2.1*-expressing regions, which instead remains abolished in transgenic embryos bearing a random mutation of the same site. On the contrary, β-Gal staining in telencephalic districts where *TTF-1/NKX2.1* is not expressed (i.e., most of the telencephalic vesicles) is absent in pNes.OligoC-LacZ-bearing embryos as in pNes.mut-LacZ-bearing embryos, suggesting that the activity of NestBS cannot be fully replaced by the DNA-binding site consensus for *TTF-1/NKX2.1*, and that other NestBS-binding factor/s are needed for *nestin* expression in these telencephalic regions. In addition, as expected, the reporter gene expression driven by the two mutagenized constructs is indistinguishable in more posterior regions of the neural tube (i.e., mesencephalon, rhombencephalon and spinal cord), suggesting that NestBS does not influence *nestin* expression in these districts and that other factors binding to other DNA-binding sites within the CNS-enhancer (see also Josephson *et al.,* 1998) are necessary for proper *nestin* expression along the more posterior neural tube. Similarly, more ventral hypothalamic regions are devoid of β-Gal expression, suggesting that other transcription factors are needed, either alone or in conjunction with *TTF-1/NKX2.1*, to achieve a proper *nestin* expression in other developing diencephalic regions.

On the other hand, we found in pNes.OligoC-LacZ-bearing embryos a β-Gal signal in two nestin-expressing regions of active cellular proliferation where no *TTF-1/NKX2.1* expression has ever been described: the thalamus and - even if at a minor extent - in a subset of the most rostral part of the telencephalic vesicles (Fig. 5D), where lacZ expression resembles radial glial cells, previously shown to express *nestin* during development (Dahlstrand *et al.,* 1995). These findings might be explained by the presence in these two forebrain regions of factor/s (possibly belonging to the NK2 gene family) still able to recognize the CNS-specific enhancer when it bears the *TTF-1/NKX2.1* recognition site, but not the randomly mutated NestBS. However, we cannot rule out a more simple ectopic effect: in fact, lacZ expression also in the whisker pad primordium is strongly associated with the expression in the basal forebrain, suggesting that it is probably due to an ectopic recognition of the exogenous DNA-sequence by factor/s present in this region.

Josephson and co-authors (1998) have shown that the mutation in the HRE (=NestBS) site had detectable effects only from 12.5 d.p.c. onwards, but the TTF-1/NKX2.1 protein is present in the developing forebrain already from 9 d.p.c. on (Lazzaro *et al.,* 1991; Price *et al.,* 1992). A similar discrepancy is also observed in thyroid, where *TTF-1/NKX2.1* elicits tissue-specific expression of the thyroglobulin and thyroperoxidase genes only from 14 d.p.c. onwards, even if the protein is already present from 9 d.p.c. (Lazzaro *et al.,* 1991). For the above mentioned thyroid-specific promoters, it has already been shown that down-regulation of a thyroid-specific repressor (TTF-2) between E13 and E15 is the major event leading to a complete transcriptional activation mediated by TTF-1 in conjunction with Pax8, another thyroid-specific nuclear factor (Zannini *et al.,* 1997). It might be conceivable that, also in the case of the CNS-enhancer, either a relief from a concurrent negative regulation or a positive interaction of other factor/s with *TTF-1/NKX2.1* is necessary to achieve a complete transcriptional activity of the *nestin* gene.

Finally, the extent of the transactivation with *TTF-1/NKX2.1* alone is, as already reported, not very high, suggesting that other factors present in the proper cellular environment are required for a full expression of the *nestin* gene. Therefore, since RAR-α is able to bind *in vitro* to NestBS (Lothian *et al.,* 1999) and also to establish a protein-protein interaction with TTF-1 in the context of another tissue-specific promoter (Naltner *et al.,* 2000), we co-transfected in 3T3 cells *TTF-1/NKX2.1* and *RAR-α* in order to find a synergistic or additional effect of the two proteins on the CNS-specific enhancer of the *nestin* gene, but we did not observe it. We are going to try other nuclear hormone receptors (in particular, 9-*cis*-retinoic receptor RXR, thyroid hormone receptor and chicken ovalbumin upstream promoter transcription factor COUP-TF) already shown to bind to the CNS-specific enhancer in order to see a cooperation or even a protein-protein interaction with *TTF-1/NKX2.1*.

In conclusion, we have demonstrated that in transgenic mouse embryos endogenous *TTF-1/NKX2.1* is co-expressed with *nestin*, it is able to recognize its canonical DNA-binding site in the context of the CNS-specific enhancer of the *nestin* gene and to drive β-galactosidase reporter gene expression in *TTF-1/NKX2.1*-expressing regions, suggesting that *TTF-1/NKX2.1* -in conjunction with other transcription factors binding to distinct sites within the same enhancer- regulates the transcription of the *nestin* gene in the developing embryo in these regions. These data suggest that nestin might be at least one of the effectors through which *TTF-1/NKX2.1* plays its pivotal role in the organogenesis of the forebrain.

Materials and Methods

Construction of reporter plasmids

To obtain pNes-LacZ construct we amplified with GL2.1 (5'-GTATCTTATGGTACTGTAACT-3') and LUC-XN3' (5'-ATCCTCTAGAGGATAGAATGGCGCCGGG-3') oligonucleotides the pNes.Luc construct already described (Lonigro *et al.,* 2001) to obtain a 415 bp-long DNA fragment (containing also ~60 bp from the luciferase gene coding sequence in its 3'-portion). This 415-bp-long fragment was cut with SmaI and XbaI and the resulting 380-bp long fragment cloned in NotI (filled-in) and SpeI sites of the pGS-100 LacZ-containing reporter plasmid, a modified version of the pBGZ40 vector (Yee and Rigby, 1993), kindly provided by S. Brunelli. The NestBS site was mutagenized by polymerase chain reaction (PCR) with the above mentioned GL2.1 and LUC-XN3' oligonucleotides and the mismatched primers 5'-AGAGGAGTAGGCCTTCGGCCTTGGCCTTGG-3' to obtain the pNes.mut-LacZ construct, and with the mismatched primers 5'-CCAGAGGAGTCAAGTGTCGGCCTTGGCCTT-3' to obtain the pNes.OligoC-LacZ construct. The amplified fragments were cloned in the pGS-100 vector as described for pNes.LacZ.

To obtain the pNes.mut-Luc and pNes.OligoC-Luc construct, the pNes.mut-LacZ and pNes.OligoC-LacZ constructs were cut with SacI and BglII endonucleases and the resulting 271 bp-fragment was cloned in the SacI and BglII sites of the pGL2-Enhancer vector (Promega).

Immunofluorescence assays and flow cytometric analysis

For immunofluorescence assays, whole brains from E14 embryos were mechanically dissociated, plated, cultured overnight and fixed as previously described (Guazzi *et al.,* 2003). Cells were then incubated for 10 min at room temperature with 0.1% Triton-X in 0.1 M phosphate buffer, pH 7.4 (PBS), blocked in 2% bovine serum albumin in PBS incubated for 1 hour with a purified anti-TTF1 rabbit polyclonal antibody, a kind gift from R. Di Lauro's lab (1:1000) and with a mouse monoclonal antibody raised against rat *nestin* (1:500; Pharmingen, USA). After extensive washing in PBS+0.1 mg/ml bovine serum albumin, donkey TRITC-coupled anti-rabbit and FITC-coupled anti-mouse immunoglobulins secondary antibodies from Jackson Laboratories (1:200) were used for visualization. For flow cytometric analysis, cells were dissociated as described above, aliquots of 10^6 cells were directly immunostained (without any plating procedure) as described above, and fluorescence signals were collected using a FACScalibur (Becton Dickinson. USA).

Cell culture and co-transfection Assays

Neuro-2A cells were grown in DMEM+10%FCS+ 2mM L-Glutamine in humidified atmosphere, 5% CO2 at 37°C and NIH 3T3 cells were cultured as previously described (Lonigro *et al.,* 2001). 5 μg of the reporter constructs pNes.Luc, pNes.mut-Luc and pNes.OligoC-Luc (described above) were co-transfected with 0.25 μg of pCMV-TTF1, a mammalian expression vector encoding for *TTF1/NKX2.1* (De Felice *et al.,* 1995), or with the empty expression vector in 6 cm culture dishes by standard Ca^+/phosphate method. Data were normalized by co-transfection with an expression vector for β galactosidase (pPGK-β gal) and standard β-gal assay. The pSG-RAR-α vector, encoding for *RAR-α,* was a gift from P. Chambon. All transfections were carried out in duplicate batches in at least three separate experiments.

Generation of transgenic mice, whole-mount β-gal procedure and immunohistochemistry

Transgenic mouse embryos were generated by pronuclear microinjection of fertilized eggs from FVBxFVB crosses and implantation in CD1 foster mothers. All the embryos were harvested, washed in PBS and fixed in cold 2% paraformaldehyde, 0.1% glutaraldehyde in PBS for 15 min. Embryos were washed three times in cold PBS for 15 min and stained with 1 mg/ml X-Gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6{\cdot}H_2O$, 2 mM $MgCl_2$, 0.02% NP-40, 0.01% Na-deoxicholate in PBS at 37°C for 2-6 hours. Embryos were then washed extensively with PBS at room temperature and post-fixed with 4% paraformaldehyde in PBS for storage at 4°C. Embryos positive for β-Gal staining were then washed three times in PBS for 10 min at room temperature, included in 2% agarose in PBS and sectioned with a vibratome in 125 μM thick transverse sections. Double immunohistochemistry experiments were performed with the same polyclonal antibody used for the IF assay (anti-TTF-1) and either a mouse monoclonal antibody against beta-galactosidase (Zymed, San Francisco, USA) or the mouse monoclonal anti-nestin (Pharmingen), using the "PicTure Double Immunohistochemistry Kit" from Invitrogen, USA following the manufacturer's instructions.

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