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Nestin Is a Neuroepithelial Target Gene of Thyroid Transcription Factor-1, a Homeoprotein Required for Forebrain Organogenesis*

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Thyroid transcription factor-1 (TTF-1, also known as NKX2.1 and T/EBP), a transcription factor belonging to the NKX-2 family of homeodomain-containing genes, plays an essential role in the organogenesis of the thyroid gland, lung, and ventral forebrain. Nestin is an intermediate filament protein strongly expressed in multipotential neuroepithelial stem cells and rapidly down-regulated during postnatal life. Here we show that stable fibroblastic clones expressing TTF-1 acquire a phenotype reminiscent of neuroepithelial cells in culture and up-regulate the endogenous nestin gene. TTF-1 transactivates in HeLa and NIH3T3 cells a reporter gene driven by a central nervous system-specific enhancer element from the second intron of the rat nestin gene, where it recognizes a DNA-binding site (NestBS) whose sequence resembles a nuclear hormone/cAMP-responsive element very different from canonical TTF-1 binding sites. Nuclear extracts from the head of mouse embryos form a retarded complex with NestBS of the same mobility of the extracts obtained from TTF1-expressing clones, which is either abolished or supershifted in the presence of two different antibodies recognizing the TTF-1 protein. Thus, the neuroepithelial marker nestin is a direct central nervous system-specific target gene of TTF-1, leading to the hypothesis that it might be the effector through which TTF-1 plays its role in the organogenesis of the forebrain.

Thyroid transcription factor-1 (TTF-1),[1] also known as NKX2.1 and T/EBP, is a member of the NKX family of homeodomain-containing genes related to NK *Drosophila* genes (1). TTF-1 plays a fundamental role in the tissue-specific expression of several thyroid-specific (*i.e.* thyroglobulin, thyroperoxidase, thyrotropin receptor, and sodium iodide symporter) and lung-specific (*i.e.* surfactant proteins and the Clara cell secretory protein) genes (2–7). TTF-1 has been reported to be expressed also in the adult rat parathyroid cells and several other adult tissues (including skin, esophagus, retina, anterior pituitary, cerebellum, and hippocampus) (8, 9). Recently, it has been shown that postnatal hypothalamic TTF-1 expression is strongly and transiently up-regulated immediately before puberty in female rats and is associated with the neuroendocrine process of female sexual development (10).

TTF-1 is expressed at the onset of thyroid and lung organogenesis, and in restricted areas of the developing forebrain, namely within the diencephalon (*i.e.* in the hypothalamus and neurohypophysis) and the telencephalon (*i.e.* in the medial ganglionic eminence) (11). The crucial role exerted by TTF-1 in thyroid, lung, and ventral forebrain organogenesis has been directly demonstrated by the analysis of the phenotype of TTF-$1^{-/-}$ mice. In fact, homozygous mutant mice were born dead and lacked completely the thyroid gland, lung parenchyma, and the entire pituitary, and extensive defects were found in the ventral region of the forebrain (12). In addition, it has been reported that, in TTF-1 knockout mice, a ventral-to-dorsal transformation of the pallidum primordium into a striatal-like anlage takes place (13).

TTF-1 is one of the genes involved in the early organization of the vertebrate rostral brain, and it has been suggested that it might play a role in the early patterning of the developing head in longitudinal and transverse domains (14, 15). Whereas TTF-1 in mice is expressed in thyroid and lung up to adulthood, its expression in the ventral forebrain begins at 8.75 dpc, *i.e.* when the peak of neurogenesis takes place in mice, and is considerably down-regulated in the postnatal life (15, 16). However, at very early developmental stages, only few markers are known to be specifically expressed in the central nervous system (CNS), the most known among these being nestin, an intermediate filament type VI protein (17). Nestin is a cytoskeleton component strongly expressed in multipotential neuroepithelial stem cells of the developing CNS, and is rapidly down-regulated during postnatal life, when the expression of glial-fibrillar-acidic protein and neurofilaments occurs in differentiated astrocytes and neurons, respectively. However, during embryogenesis, nestin is also expressed in myogenic precursors (17, 18), developing heart (19), differentiating testis (20), and developing tooth bud (21).

Recently, it has been demonstrated that TTF-1 is required for the specification of nearly all striatal interneurons (22), of roughly 50% of the GABA-ergic interneurons of the neocortex (13) and of hippocampal GABA-ergic interneurons expressing neuropeptide Y and somatostatin (23). However, apart from these data, the function of TTF-1 in the developing forebrain still remains obscure; in particular, it is not known if any CNS-specific gene is regulated by TTF-1 during embryogenesis.

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[1] The abbreviations used are: TTF-1, thyroid transcription factor-1; CNS, central nervous system; dpc, day(s) postcoitum; bp, base pair(s); PCR, polymerase chain reaction; PBS, phosphate-buffered saline; HRE, hormone-responsive element; CRE, cAMP-responsive element; GABA, γ-aminobutyric acid; RT, reverse transcription; EMSA, electrophoretic mobility shift assay; DMEM, Dulbecco's modified Eagle's medium; CMV, cytomegalovirus.

We analyzed NIH3T3 cells stably transfected with an expression vector encoding for TTF-1 to assess whether an up-regulation of the nestin protein occurred. The morphology of the TTF-1-expressing stable clones resembled that one of neuroepithelial cells *in vitro*, and the altered cell shape suggested a possible change in the cytoskeleton components. In addition, we showed that overexpressing TTF-1 activates the endogenous rat nestin gene transcription. Co-transfection and DNA-binding assays with TTF-1 and an already characterized (24) CNS-specific enhancer in the second intron of the rat nestin gene strongly suggest a direct interaction between TTF-1 and a DNA-binding site essential for proper CNS-specific expression whose sequence resembles a nuclear hormone/cAMP element.

EXPERIMENTAL PROCEDURES

Generation of TTF1-expressing Stable Fibroblastic Clones—NIH3T3 or COS-7 fibroblasts were cultured in high glucose DMEM supplemented with 10% fetal calf serum (all from Life Technologies, Inc.) in 5% CO_2 in air humidified atmosphere and transfected at 50% confluence by the calcium phosphate precipitation method with 20 μg of the pCMV-TTF1 construct containing the entire TTF-1 cistron (25)/10-cm dish. After 56 h the transfected cells were diluted 1:20 and selected for 15–20 days with G418 at 1 mg/ml (Life Sciences), and then several clonal cell lines were isolated. The pRC-CMV-containing (mock-transfected) cells were selected with G418 only as bulk population. Transfected cells were routinely maintained in DMEM supplemented with 10% fetal calf serum and 0.3 mg/ml G418 at 37 °C and 5% CO_2.

Western Blots and Immunofluorescence Assays—Total protein extracts were obtained from confluent cultured cells by adding, after three washes with PBS, 300 μl of 3× Laemmli buffer to the monolayer in the 10-cm dish. The cell lysates was then harvested by scraping the dish and pouring them into an Eppendorf tube, passed several times through a 1-ml syringe with an 18-gauge needle to eliminate the viscosity resulting from DNA released from the nucleus and boiled 1 min before loading. 6–10 μl of the total protein extracts were then subjected to SDS-polyacrylamide gel electrophoresis followed by Western blotting. The membrane was stained by Ponceau to check whether the amount of the loaded material was comparable in all the lanes. Blots were saturated with 3% BSA, 1% horse serum, 0.1% Tween in PBS; incubated for 1 h with a purified anti-TTF1HD rabbit polyclonal antibody (1:10,000) or a monoclonal antibody against rat nestin from PharMingen (1:1,000); and then washed several times with 0.1% Tween in PBS, 1% BSA. We used the goat anti-rabbit biotinylated antibody from Roche Molecular Biochemicals (1:5,000) followed by an incubation with peroxidase-coupled streptavidin (1:500) or the goat anti-mouse antibody from the ECL kit used for the detection (Pierce) of TTF-1 and nestin, respectively.

In the immunofluorescence assays, the cells were fixed with 4% paraformaldehyde in PBS for 10 min on ice followed, only for TTF-1 detection, by 5 min of permeabilization with 0.5% Triton-X in PBS. The cultured cells were then blocked with 2% BSA in PBS and incubated for 1 h with same primary antibodies used for the Western blottings diluted at 1:1,500 (α-TTF-1) or 1:500 (α-nestin), extensively washed with 1% FCS in PBS and then incubated with goat anti-rabbit tetramethylrhodamine B isothiocyanate or goat anti-mouse fluorescein isothiocyanate purified antibodies from Zymed Laboratories Inc. (1:200) for TTF-1 and nestin, respectively.

Northern Blot and RT-PCR Experiments—Total mRNA was obtained from cultured cells by washing the cells in culture three times with PBS and then using the Trizol method (Life Technologies, Inc.). 15 μg of total mRNA from TTF1-cl.5, cl.9 were loaded on 1% denaturing agarose gel and blotted on Hybond-N+ nylon membrane (Amersham Pharmacia Biotech). The RNAs were probed with a ^{32}-P radiolabeled 650-bp-long DNA fragment from the nestin cDNA obtained by PCR amplification, using the M13 universal primers, of the IMAGE expressed sequence tag clone (614992) from mouse embryos at 13.5 dpc, which showed a 87% identity with the published rat nestin cDNA sequence. The amplified band was then cloned and sequenced. Mock-transfected 3T3 cells and brain tissue of mouse embryos at 14 dpc were used as negative and positive controls, respectively. Northern blot hybridizations were carried out by standard techniques.

RT reactions were performed using 20 ng/μl amounts of the same RNAs of the Northern blot experiments, Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.), 5 pmol/μl random examers, 0.2 mM dNTPs at 42 °C for 1 h. PCR reaction was then performed with half of the RT reaction using the following oligonucleotides deduced from the published sequences: 5′-AGTTCGAGGGACAGGCTCTC-3′ and 5′-GGAGAGGGCAGACTGGTGTG-3′ for the m-musashi-1 mRNA, and 5′-ATGGTAGATGCTTTCTGC-3′ and 5′-CTATGCCTTTTCATAACAGCG-3′ for the B-FABP mRNA.

Cell Culture and Co-transfection Assays—NIH3T3 wild type and transfectant cells were cultured in high glucose DMEM supplemented with 10% fetal calf serum (all from Life Technologies, Inc.) in 5% CO_2 in air humidified at 37 °C. The reporter construct pNes-Luc was obtained by PCR-amplifying a DNA fragment from rat genomic DNA using two oligonucleotides flanking the CNS-specific enhancer from the rat nestin II intron (5′-GGTCCAGGGAGCTCTGAGGAATGTAACCAC-3′ and 5′-TATCAGACTCCTCAGATCTGTCTCCGCCTC-3′), which contained a *Sac*I and *Bgl*II site to facilitate the cloning procedure. The resulting 276-bp-long DNA fragment was then cloned into the *Sac*I and *Bgl*II sites of the polylinker of the pGL2-Enhancer vector, which contains a SV40 enhancer downstream of the luciferase reporter gene (Promega). The cDNA expression vector (pCMV-TTF1) was the same used for the generation of the stable clones. The co-transfection assay was performed as described previously (26). 4 μg of pNES-Luc or pC5-E1b (25), a kind gift of Dr. Di Lauro, and 1 μg of pCMV-TTF1 and/or pRC-LUC constructs DNA per dish were transfected. 1 μg of pCMV-βGal was used to normalize the transfection. All transfections were carried out in triplicate batches in at least three separate experiments.

Nuclear Extracts and Gel Retardation Experiments—Crude nuclear extracts from NIH3T3 cells and from the head of mouse embryos at 14 dpc were prepared as described previously (26). ^{32}P-Labeled 40-mer double-stranded oligonucleotide NestBS (5′-CTCCCAGAGGATGAGGTCATCGGCCTTGGCCTTGGGTGGG-3′) was used as probe in gel retardation experiment as described previously (27) with 3–5 μl of nuclear extracts. The bacterial extracts containing the TTF-1 homeodomain were already described (1, 28). A 50 molar excess of the double-stranded unlabeled NestBS, ΔCAAG (5′-CTCCCAGAGGATGAGGTCATCGGCCCGGGCCCCGGGGTGGG-3′), HBE1mut (Ref. 26, as unspecific sequence) oligonucleotides was used in competition experiments. Two antibodies, one recognizing the TTF-1 homeodomain and the other recognizing the N terminus portion of the TTF-1 protein were used (1 μl of undiluted antibody/20-μl DNA binding reaction) in the gel retardation experiments.

RESULTS

TTF-1 Overexpression Changes the in Vitro Morphology of Transfected Cell Lines and Up-regulates the Expression of the Endogenous Nestin Gene—Full-length cDNA coding for rat TTF-1 was cloned into the human CMV promoter-based mammalian expression vector Rc-CMV to generate the pCMV-TTF1 construct bearing a neomycin resistance gene (25). NIH3T3 cells were stably transfected with pCMV-TTF1, and 26 clones were isolated. Several clones (60%) exhibited a strikingly changed morphology; they became very thin and elongated, showing two filopodia outgrowing from the opposite sites of the cell. Moreover, TTF-1-transfected cells displayed a peculiar distribution within the plate, in large bundles, parallel to each other (Fig. 1*A*). At lower cell density the cells showed a "net-like" distribution. To test the specificity of this effect, the pCMV-TTF1 construct was stably transfected also into another cell line (COS-7) of simian origin; roughly the same percentage (56%) as 3T3 transfected clones showed an altered morphology *in vitro* (data not shown), ruling out any species-specific effect. We analyzed 14 randomly chosen clones of 26 for the presence of both TTF-1 and nestin proteins. Total protein extracts were prepared from confluent cells, and Western blots experiments were performed using a rabbit polyclonal antibody raised against the TTF-1 homeodomain and a mouse monoclonal antibody against nestin (Fig. 1*B*). All the clones showing the presence of a TTF-1-specific band of approximately 42 kDa, similar to the molecular mass described for TTF-1 in thyroid cell lines (29), were also positive for a nestin-specific 200-kDa band. A second band was also visible, as previously seen for TTF-1 in thyroid (29, 30) and lung (31), which can be caused by different phosphorylation states (29). Only selected clones are shown in Fig. 1*B*. As positive controls for nestin expression, we used total protein extracts from a neuroepithelial cell line (MNS-70) and from embryonic brain of 14.5 dpc mouse em-

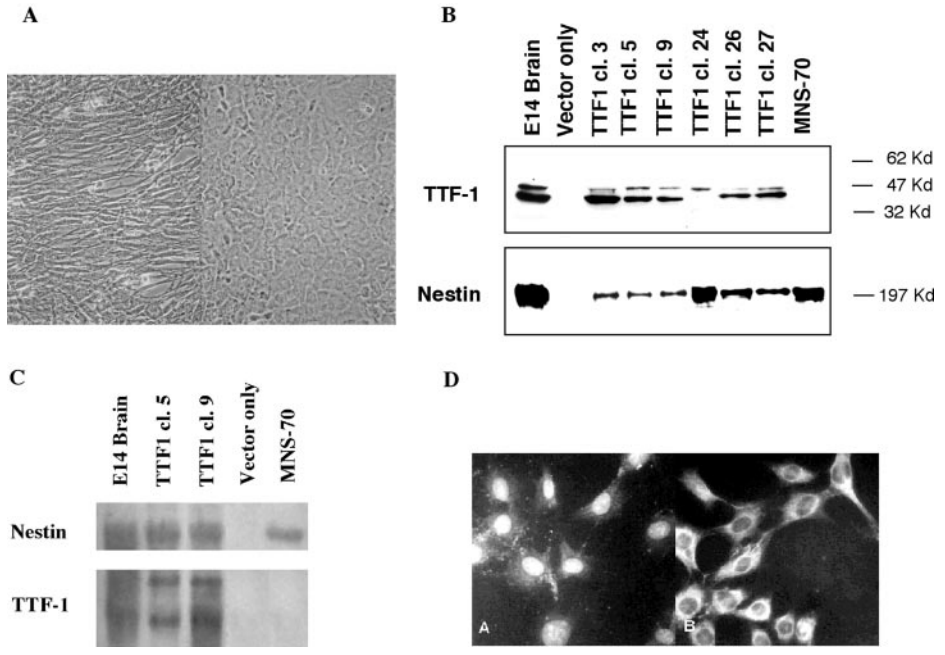

FIG. 1. **TTF-1 ectopic expression up-regulates the transcription of the endogenous nestin gene.** *A*, NIH 3T3 cells (3T3-TTF1 cl.5) stably transfected with the pCMV-TTF1 construct encoding for the full-length cDNA of TTF-1 (*left*), but not cells stably transfected with the empty vector (*right*), show an altered cell morphology. *B*, total protein extracts from stably transfected NIH3T3 cells were assayed in Western blot for TTF-1 and nestin. Molecular size markers are on the *right*. The experiments were performed on separate blots. Total protein extracts from brains of mouse embryos at 14 dpc (*E14 brain*) and from the neuroepithelial cell line MNS-70 were used as positive controls. Vector only, protein extract from cells transfected with empty vector used as negative control. *C*, Northern blots experiments were performed with 15 μg of total mRNA from two TTF-1-expressing clones (cl.5 and cl.9). Total mRNA from brain of mouse embryos at 14 dpc (*E14 Brain*) or from MNS-70 cells were used as positive control and RNA extracted from NIH3T3 cells transfected with the empty vector as negative. *D*, immunofluorescence assay on 3T3-TTF1 cl.5 transfectant cells with an anti-TTF1 homeodomain purified antiserum and a tetramethylrhodamine B isothiocyanate-coupled secondary antibody (*panel A*) and the same clone labeled with an anti-nestin monoclonal antibody and a fluorescein isothiocyanate-coupled secondary antibody (*panel B*).

bryos. Nestin expression was absent in the mock-transfected cells. A Western blot performed with 3T3-TTF1 cl.24 total protein extracts obtained from cells in culture for different length of time (from 1 day to 2 months) showed that the amount of nestin protein among these extracts did not change (data not shown).

To confirm these data, Northern blot experiments were also performed with total mRNA from two TTF1-expressing clones; a nestin-specific band was present, whereas it was absent in the mock-transfected 3T3 cells (Fig. 1*C*). The presence of different TTF-1-specific mRNAs has already been shown in all the TTF-1-expressing tissues (1), but was unexpected in a cell line stably transfected with a CMV promoter-driven expression vector encoding for TTF-1. However, a previously described (32) autoregulatory mechanism in the transcription of the TTF-1 endogenous gene might explain the presence of a second mRNA in the transfected cells.

We also stained 3T3-TTF1 cl.5, one of the clones found positive by Western and Northern blots, with the antibody against the TTF-1 homeodomain (Fig. 1*D*, *panel A*) and against nestin (Fig. 1*D*, *panel B*); as expected, a clear nuclear localization was found for the TTF-1 protein, whereas nestin was exclusively found in the cytoplasmic compartment.

Neuronal and Other Neuroepithelial Markers Are Not Up-regulated in NIH 3T3 Cells—Very few molecular markers are known to be present in the developing murine CNS at 8.75 dpc, when neurogenesis takes place. Apart from nestin, two molecules are known to show a similar pattern of expression: B-FABP and musashi-1. B-FABP is expressed during the peak of neurogenesis in the ventricular zone, but not in the floorplate and dorsal midline, and is shut down as the cells migrate away (33). m-musashi-1 is a RNA-binding protein, which is preferentially expressed in mitotically active neural precursor cells within the CNS (34). In addition, nestin, B-FABP, and m-musashi-1 are all expressed also in adult neural stem cells (35, 36). We then decided to examine the expression of these genes in 3T3 stable TTF1 transfectants by RT-PCR, but none of them was positive, whereas mRNA from 14.5 dpc murine brains showed a specific DNA amplification using the same assay conditions (data not shown). Moreover, immunofluorescence experiments showed that markers of more differentiated neuronal phenotype (MAP-2, neurofilament proteins, and GFAP) were not expressed in transfectant clones (data not shown).

TTF-1 Transactivates a CNS-specific Enhancer in the Rat II Intron of the Nestin Gene in NIH 3T3 and HeLa Cells—It has been reported that a 257-bp-long enhancer within the II intron of the rat nestin gene is essential in transgenic mice for proper CNS-specific expression, and the relevant elements for its activity were identified; one was bound by POU proteins, whereas the other showed similarity in its DNA sequence with hormone-responsive (HRE) or cAMP-responsive (CRE) elements, although the actual DNA binding activity for this site was not clearly identified (24). We PCR-amplified the 257-bp-long DNA fragment from rat genomic DNA and cloned it in front of the luciferase reporter gene in the pGL2-enhancer expression vector, containing also an SV40 enhancer downstream of the luciferase gene, and the resulting construct was named pNes-Luc. To assess whether the 257-bp-long sequence was able to mediate the up-regulation by TTF-1, we co-transfected both in NIH-3T3 and HeLa cells the pNes-Luc construct together with the same pCMV-TTF1 expression vector used for the stable transfection assay. The pNES-Luc reporter construct showed a maximum of 5- and 3.6-fold increase in transcriptional activity when co-transfected with pCMV-TTF1 construct in 3T3 and HeLa cells, respectively, in a TTF1-dosage dependent manner (Fig. 2). The reporter construct alone pGL2-enhancer failed to

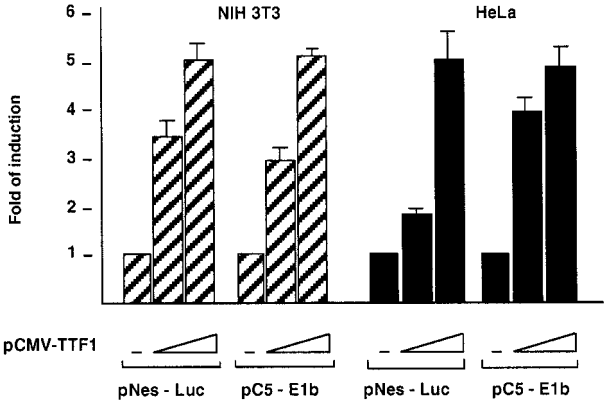

FIG. 2. **Transactivation of a CNS-specific enhancer from the II intron of the rat nestin gene (pNes-Luc) by TTF-1 in HeLa and NIH3T3 cells.** A reporter construct containing five TTF-1-binding sites from the thyroglobulin promoter cloned upstream of the E1b TATA box (pC5-E1b) was used as positive control of the transactivating activity of the TTF-1 protein. 4 μg of the reporter construct (pNes-Luc or pC5-E1b) were co-transfected in NIH 3T3 (*hatched bars*) or HeLa (*black bars*) cells together with increasing amounts (0.5 and 1 μg) of expression vector encoding for TTF-1. The transactivating activity is expressed as -fold of induction, and the basal transcriptional activity is arbitrarily considered equal to 1.

show any increase in transcriptional activity in co-transfection assays (data not shown). The extent of transactivating effect on the pNes-Luc reporter was similar to that obtained with the pC5-E1b construct containing five TTF-1-binding sites from the thyroglobulin promoter cloned upstream of the E1b TATA box, which was already been shown to be efficiently transcribed upon co-transfection of a TTF-1 expression vector alone (25), suggesting that the transactivating activity observed was dependent only upon the presence of the TTF-1 protein.

TTF-1 Is the Nuclear Factor from 14 dpc Mouse Embryos Binding to the CNS-specific Enhancer of the Nestin Gene—Nuclear extracts prepared from 3T3-TTF1 cl.5 were able to bind in EMSA to a 40-mer double-stranded oligonucleotide (NestBS, Fig. 3*A*) containing one of the footprinted regions on the nestin 257-bp-long enhancer and encompassing the HRE/CRE-like site (Fig. 3*B*, *arrow* on the *left*). The binding activity was specifically competed by a 500× molar excess of an unlabeled oligonucleotide containing the same site (Fig. 3B, +*spec.*), but not of an unrelated one (Fig. 3B, +*unspec.*). An EMSA assay was performed as well with nuclear extracts obtained from the head of 14 dpc mouse embryos; a retarded complex with the same mobility of the complex formed by nuclear extracts from the TTF-1 transfected clone was detected and specifically competed (Fig. 3*B*, *lanes 4–6*), suggesting that the factor binding *in vivo* to this site in the rat nestin gene might indeed be TTF-1. A much fainter retarded complex was formed also by 3T3 mock-transfected cells, but the complex showed a faster mobility in the gel (Fig. 3*B*, *smaller arrow*).

To assess whether the nuclear factor from mouse embryos binding to the NestBS oligonucleotide in EMSA assay was TTF-1, we performed the assay in the presence of a purified rabbit polyclonal antibody that recognizes an epitope within the homeodomain of TTF-1. The antibody specifically interfered with the TTF1/NestBS complex formation (Fig. 3*C*, *lanes 1–3*). The observed interference did not depend on whether the antibody was added before or after the DNA-protein complex formation in the binding assay, suggesting that binding of TTF-1 to the DNA or to the antibody was mutually exclusive. In addition, a gel retardation assay was also performed at the presence of a different polyclonal antibody recognizing the N terminus portion of the TTF-1 protein; although the TTF1/NestBS retarded complex was almost completely abolished, a supershift of the same retarded complex was observed (Fig. 3*C*, *lanes 4–6*), showing that TTF-1 was indeed at least one of the murine embryonal nuclear factors binding to the NestBS site.

TTF-1 Binds to the Nestin CNS-specific Enhancer with a Novel DNA-binding Specificity—To better define the DNA-binding specificity of TTF-1 on the nestin II intron, we performed a competition experiment in an EMSA assay with nuclear extracts from the head of 14 dpc mouse embryos and end-labeled NestBS; the retarded complex TTF1/NestBS was competed out by a 500× molar excess of unlabeled NestBS oligonucleotide (Fig. 4*A*, *lane 2*), but not by the same oligonucleotide in which the 5′-TGAGGTCA-3′ site was mutagenized in 5′-GTAGGCCT-3′ (Fig. 4*A*, *lane 3*). Moreover, partially purified bacterial extracts containing the TTF-1 homeodomain (*TTF-1 HD b.ex.*) completely failed to bind to the NestBSmut oligonucleotide (data not shown). The same mutagenized DNA sequence (5′-GTAGGCCT-3′) present in the NestBSmut oligonucleotide has already been shown by transgenic mice analysis to abolish the nestin CNS-specific expression within the telencephalon and dorsal mesencephalon from 12.5 dpc of mouse development until at least 14.5 dpc (24). TTF-1 HD-containing bacterial extracts were able to specifically bind to the NestBS sequence, and the TTF1 HD/NestBS complex formation was completely inhibited by 50-molar excess of unlabeled NestBS oligonucleotide but not by an equal amount of an unrelated unlabeled oligonucleotide (Fig. 4*B*, *lane 3 versus lane 5*).

On the opposite DNA strand, within the footprinted sequence immediately flanking the NestBS site, there is a double-repeat of the 5′-CAAG-3′ sequence, which is the canonical core motif of the TTF-1 DNA binding activity (Fig. 3*A*, *bold*). However, an excess of an oligonucleotide containing the two mutagenized 5′-CAAG-3′ sites (ΔCAAG) efficiently competed the binding of the TTF1 HD to the NestBS site (Fig. 4*B*, *lane 4*). In addition, the labeled ΔCAAG oligonucleotide was able to bind the TTF-1 homeodomain at a similar extent than the wild type NestBS oligonucleotide (Fig. 4*B*, *lanes 6* and *7*). Taken together these data suggest that the TTF1/NestBS interaction occurs, at least *in vitro*, exclusively within the 5′-TGAGGTCA-3′ (HRE/CRE-like) site and that the two 5′-CAAG-3′ sites present within the NestBS sequence are not recognized by TTF-1.

DISCUSSION

Little is known about the function of TTF-1 in the forebrain during embryogenesis. It is conceivable that TTF-1 might act through the transcriptional activation of target genes, but none of these genes has been identified yet. TTF-1 expression in the ventral forebrain begins at the peak of neurogenesis and is considerably down-regulated during adulthood, rising the possibility that also putative brain-specific TTF-1 target genes have a similar developmentally regulated expression. Here, we provide evidence that nestin (17), an intermediate filament type VI protein found in multipotent neuroepithelial stem cells of the developing CNS that is rapidly down-regulated during postnatal life, is, to our knowledge, the first embryonal target gene of TTF-1 in the forebrain to be identified.

TTF-1-expressing NIH3T3 clones show a dramatically altered morphology, and concomitantly a strong up-regulation of the endogenous nestin gene, as documented by Western/Northern blots and immunofluorescence assays. Intermediate filament proteins represent, together with microtubules and microfilaments, the major structure in the cytoskeleton. Therefore, the dramatic change in cell shape of the TTF-1-transfected cells may be a direct consequence of the overexpression of nestin.

Goldman and co-workers (37) reported that a clearly detect-

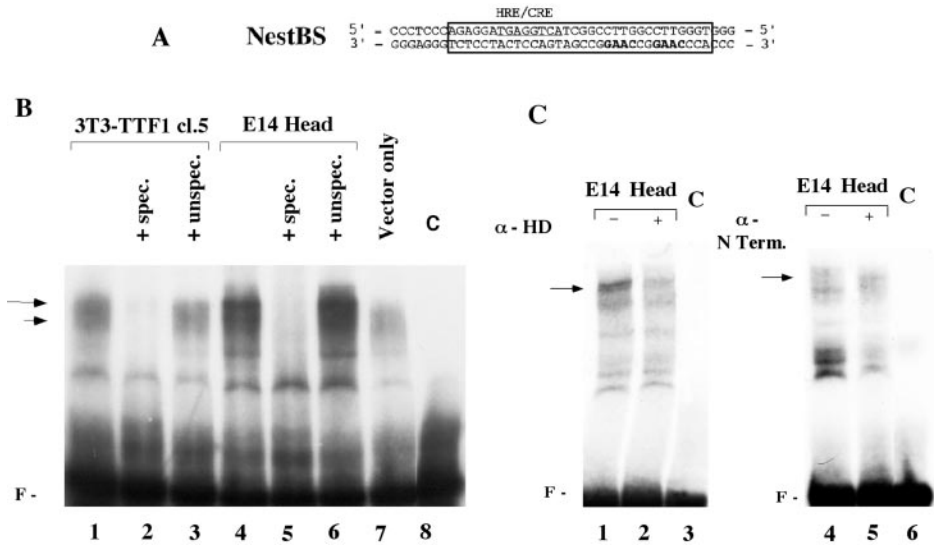

FIG. 3. **TTF-1 binds to the NestBS site within the CNS-specific enhancer of the nestin rat II intron.** *A*, DNA sequence of the NestBS oligonucleotide used in the DNA-binding assays as derived from the sequence of the CNS-specific enhancer of the rat nestin gene. The sequence identified in Ref. 24 as essential for CNS-specific expression is *boxed*, and the HRE/CRE-like site is *underlined*. Two 5′-CAAG-3′ TTF-1 core motif sequences are also shown in *bold*. *B*, nuclear extracts from TTF1-transfected 3T3 cells (3T3-TTF1 cl.5, *lanes 1–3*), from head of 14 dpc mouse embryos (*E14 Head*, *lanes 4–6*) and from mock-transfected cells (*Vector only*, *lane 7*) were used in EMSA with ^{32}P-labeled NestBS. A 500-fold molar excess of unlabeled NestBS (*lanes 2* and *5*) or of an unrelated (*lanes 3* and *6*) double-stranded oligonucleotide were used as competitors. The specifically retarded complexes are shown by an *arrow* on the *left*. The *small arrow* indicates the faster retarded complex formed by mock-transfected cells. *C*, the nuclear factor present in the murine embryonal extracts binding to NestBS is TTF-1. Gel retardation assays of labeled NestBS double-stranded oligonucleotide bound by nuclear extracts from the head of 14 dpc mouse embryos (*E14 Head*) in the absence (−) or presence (+) of two antibodies recognizing either the homeodomain (*α-HD*, *lanes 1–3*) or the N terminus portion (*α-N Term.*, *lanes 4–6*) of the TTF-1 protein. The *arrows* on the *left* indicate the TTF1/NestBS retarded complex, which is abolished at the presence of the homeodomain antibody or the supershifted complex obtained in the presence of the N terminus portion antibody, respectively. *C*, no protein added to the assay. *F-*, unbound probe.

able nestin-specific band was present on Western blots in NIH3T3 and other fibroblastic cell lines; this discrepancy very likely results from the fact that in their assays these authors performed Western blots experiments with protein extracts highly enriched in cytoskeletal components, whereas in our assays we used total unfractionated cellular extracts, although a clonal variation among different batches of NIH3T3 cells cannot be excluded.

Homozygous TTF-1 $^{-/-}$ mice die at birth and have severe thyroid, lung, and pituitary defects (12). Extensive defects were found in the ventral region of the forebrain; the third ventricle no longer reached the floor of the diencephalon, the infundibular and mammillary recesses were not developed, and, at the level of the tuberal hypothalamus, the ventromedial and dorsomedial nuclei were either reduced in size and fused in the midline. Finally, the premammillary and the arcuate nuclei were not developed, whereas in the posterior hypothalamus the mammillary body and the supramammillary nucleus were not found. Remarkably, in forebrain regions, which in control mice lack completely or show a weak TTF-1 expression, the extent of the defects observed was greatly absent or reduced, respectively. Our data demonstrating a direct regulatory interaction between the CNS-specific enhancer of the nestin gene and TTF-1 suggest the intriguing hypothesis that a reduced, if not absent, amount of nestin protein within the districts where TTF-1 is expressed might be the cause of the defects found in the forebrain of TTF-1$^{-/-}$ mice. In other words, there is the possibility that nestin is the effector through which TTF-1 plays its fundamental role in the organogenesis of the forebrain in mouse embryos. Other experiments are needed to clarify this issue.

However, it has already been reported that, in TTF-1 knockout mice, a ventral-to-dorsal transformation of the pallidum primordium into a striatal-like anlage takes place (13). Therefore, the nestin expression in TTF-1$^{-/-}$ mice cannot be easily recognized as absent or reduced, because other nuclear factor/s present in the re-specified more dorsal CNS regions might substitute TTF-1 in the regulation of the nestin CNS-specific enhancer. If this is the case, the only detectable sign in TTF-1$^{-/-}$ mice would be the reduced overall size of the brain, as it is actually observed (13).

The up-regulation of the endogenous nestin gene by TTF-1 is mediated through DNA binding to a 257-bp-long enhancer within the rat second intron, which has already been shown to be essential for proper CNS expression. This enhancer contains one site similar to a HRE or CRE, although the actual DNA binding activity at that site was not clearly identified (24). Moreover, it has also been reported that several recombinant nuclear hormone receptors (*i.e.* thyroid hormone receptors, retinoid X receptor, retinoic acid receptor, and chicken ovalbumin upstream promoter-transcription factor) were able to bind *in vitro* to the HRE/CRE-like sequence (41). Nuclear extracts prepared from a TTF-1-expressing 3T3 clone were able to specifically bind in EMSA to the footprinted region on the nestin 257-bp-long enhancer encompassing the HRE/CRE-like sequences. Nuclear extracts from head of 14 dpc mouse embryos formed a retarded complex of identical mobility with the same region. Moreover, two different polyclonal anti-TTF1 antibodies confirmed that TTF-1 is indeed the murine embryonal nuclear factor binding to the nestin CNS-specific enhancer.

The NestBS site contains a core 5′-TGAGGTCA-3′ sequence, very similar to or overlapping a HRE or CRE, whereas the two copies of the 5′-CAAG-3′ site present within the NestBS site are not recognized by TTF-1. Therefore, the sequence recognized by TTF-1 in the CNS-specific enhancer is different from the canonical 5′-CAAG-3′ TTF-1 binding site. TTF-1 has already been shown to bind to a 38-bp-long silencer from the major histocompatibility complex class I containing a putative cAMP-responsive element and not a 5′-CAAG-3′ site (38). Moreover, we have already shown by sequential selection and

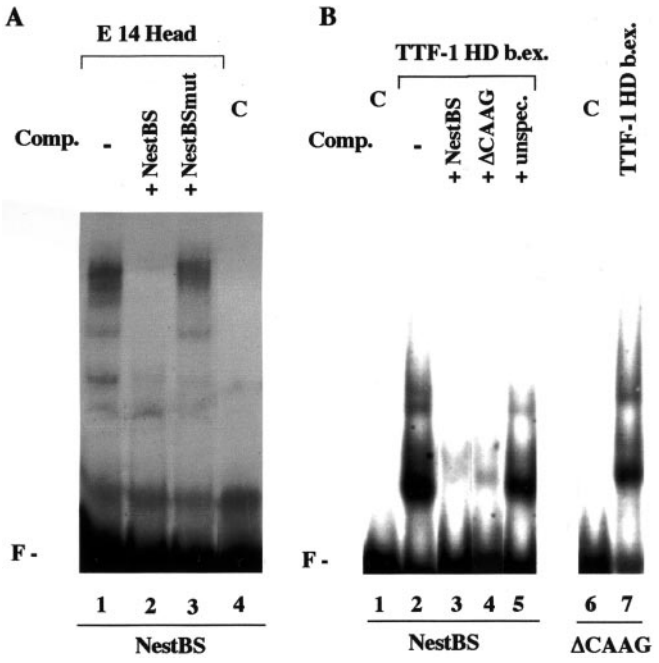

FIG. 4. **TTF-1 binds to the 5′-TGAGGTCA-3′ site within the NestBS sequence with a novel DNA-binding specificity.** *A*, nuclear extracts from head of 14 dpc mouse embryos bind the NestBS (*lane 1*) and the TTF-1/NestBS retarded complex is specifically competed by an excess of unlabeled NestBS oligonucleotide (*lane 2*) but not by the NestBSmut oligonucleotide in which the 5′-TGAGGTCA-3′ HRE/CRE-like motif was mutagenized in 5′-GTAGGCCT-3′ (*lane 3*). *B*, gel retardation assay of the oligonucleotides NestBS (*lanes 1–5*) or ΔCAAG, identical to NestBS apart from the presence of two mutated 5′-CAAG-3′ sites (*lanes 6* and *7*, see also Fig. 3) bound by partially purified bacterial extracts containing the TTF-1 homeodomain (*TTF-1 HD b.ex.*). The TTF1HD/NestBS retarded complex formation is specifically competed by an excess of specific NestBS oligonucleotide but not by an unspecific oligonucleotide (*lane 3 versus lane 5*). The ΔCAAG unlabeled oligonucleotide is able to compete the retarded TTF-1HD/NestBS complex formation (*lane 4*). Moreover, the ΔCAAG oligonucleotide is able to bind the TTF-1 HD (*lanes 6* and *7*), showing that the two 5′-CAAG-3′ sites are not recognized by TTF-1. *C*, no protein added to the assay. *F-*, unbound probe.

amplification of TTF-1 HD binding sites that several of the selected sequences did not contain the 5′-CAAG-3′ core motif and still were efficiently bound (39). Finally, lung- and thyroid-specific promoters have already been reported to contain a CRE-like element close to TTF-1 binding sites; among them, the human surfactant protein B (huSP-B) and the rat T1α promoters show a striking sequence homology to the NestBS site (Fig. 5) (3, 31). Interestingly enough, it has recently been reported that retinoic acid stimulation of the human surfactant protein B promoter is dependent on strictly juxtaposed retinoic acid receptor and TTF-1 binding elements (40).

Mutation of the 5′-TGAGGTCA-3′ site in the 257-bp-long enhancer indicates that this element is critical for a portion of the nestin CNS-specific expression pattern *in vivo* (the telencephalon and dorsal mesencephalon) from 12.5 dpc of mouse development until at least 14.5 dpc (24). In contrast, mutagenesis of the octamer/POU site abolishes reporter expression in most of the neuroepithelium, indicating that all the additional transcriptional elements within the second intron (including the 5′-TGAGGTCA-3′ site) are dependent upon the activity of this POU site. These data suggest that the interaction of TTF-1 on the nestin regulatory region is not essential for proper CNS-specific nestin expression, but might be relevant for the nestin expression within the telencephalon from 12.5 dpc onward, whereas other factors might play a similar role in other compartments (*i.e.* dorsal mesencephalon).

```
Rat T1α      -135   CTGGCCTGAGGTCATCTTGTGATGCTGGCCAA     -104
                       :  ::::::::::       ::
Rat Nestin  -1463   AGAGGATGAGGTCATCGGCCTTGGCCTTGGGT    -1494
                    : :   :::::::: :     :       :
Mo  SP-B     -86    AAACACTGAGGTCAGCCTGTTCCCATGGAGTC     -55
```

FIG. 5. **Alignment of the regulatory region of the nestin gene with the rat T1a (rat T1α) and the mouse surfactant protein-B (mo SP-B) lung-specific promoters.** *Colon* (:), nucleotide identities. The bases at which methylation interferes with protein binding from Ref. 24 are *underlined*.

The extent of the transactivation of the 257-bp-long enhancer is not very high, even though significant, because the effect is similar to what is observed with a TTF1-dependent artificial promoter used as positive control. In fact, it has already been reported that the interplay of TTF-1 with other nuclear factor/s present in the proper cellular environment is necessary to achieve a complete transcriptional activation of thyroid-specific promoters (25). Therefore, it appears likely that other nuclear factors are also necessary to achieve a complete transactivation of the nestin CNS-specific enhancer together with the TTF-1 protein.

The finding that TTF-1 is involved in the regulation of the nestin gene is supported by the observation that TTF-1 is expressed through all the layers of the developing neuroepithelium (16) in which also nestin is expressed, including the layers lining the lumen of the ventricle where active cell proliferation takes place. Moreover, TTF-1 expression in the adult includes at least four sites where the presence of a self-renewing stem cell population, *i.e.* skin, hippocampus, cerebellum, and retina, is known (8); it would be interesting to check whether, in these structures, a co-localization within the same cells of TTF-1 and nestin proteins occurs.

Finally, an fascinating hypothesis is that TTF-1 might reprogram NIH3T3 cells toward a neuroepithelial state, as suggested by the up-regulation of the nestin protein in the transfected clones. In other words, TTF-1 stably transfected 3T3 clones might mimic a multipotential neural stem cell population "frozen" at a very early developmental stage. However, the absence of other neuroepithelial markers like B-FABP and m-musashi-1 in the transfected cells renders unlikely a more global change in the genetic program of the TTF-1-transfected clones. Other experiments are needed to better elucidate this issue.

In conclusion, we have shown that overexpression of the TTF-1 protein is able to change the morphology of transfected fibroblastic cell lines and up-regulates the endogenous nestin gene. TTF-1 is able to transactivate a CNS-specific enhancer of the nestin gene through direct DNA binding to a HRE/CRE-like site bearing the 5′-TGAGGTCA-3′ sequence both *in vivo* and *in vitro*.

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Nestin Is a Neuroepithelial Target Gene of Thyroid Transcription Factor-1, a Homeoprotein Required for Forebrain Organogenesis

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