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Comparative Distribution of NURR1 and NUR77 Nuclear Receptors in the Mouse Central Nervous System

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Abstract

NURR1 and NUR77 are members of the nuclear receptor superfamily of transcription factors. Both proteins can interact with common enhancer elements to regulate target gene expression. In order to establish whether both transcription factors are likely to regulate overlapping genes, we have used an *in situ* hybridization approach to relate the constitutive expression pattern of these mRNAs with functionally defined regions of the adult mouse brain. By Western analysis, NURR1 mRNA expressed by brain cells appeared to be translated.

Here we show that both transcripts display a differential but partially overlapping pattern of expression within the central nervous system (CNS). The expression of NURR1 is more restricted than NUR77 and is localized predominantly in sensory neuronal structures associated with the limbic system and in the cerebellum. In contrast, the expression pattern of NUR77 is more widespread. Positively staining cells for NUR77 appear to overlap with NURR1-containing cells in the limbic system and cerebellum, suggesting overlapping roles for these proteins in mediating behavioral and cognitive function as well as equilibrium maintenance. However, the differential expression of NUR77 in motor areas of the cortex and basal ganglia suggest a selective role for this transcription factor in regulation of motor function at the constitutive level. Our data indicates that these nuclear receptors are likely to have both shared and independent gene regulatory roles in neuronal cells.

Index Entries: Orphan receptors; *in situ* hybridization.

Introduction

The nuclear receptor superfamily comprises a group of structurally related transcription factors that program developmental, physiological, and behavioral responses to a variety of chemical signals. The family includes receptors for steroids, certain vitamins, and thyroid hormone (Evans, 1988; Beato, 1989; Tsai and O'Malley, 1994) in addition to a growing number of orphan members whose physiological function and cognate ligand, if any, remain to be established (O'Malley and Conneely, 1992). Nuclear receptors regulate the expression of specific target genes by interacting

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as either monomers (Harding and Lazar, 1993; Wilson et al., 1993a) or dimers (Hard et al., 1990; Naar et al., 1991; Cooney et al., 1992; Kliewer et al., 1992; Perlmann et al., 1993) with specific DNA elements based on extensions or repeated spatial variations of two types of core sequence motifs, AGGTCA and AGAACA (Klein-Hitpass et al., 1986; Martinez et al., 1987; Strahle et al., 1987; Umesono and Evans, 1989). DNA sequence-specific binding by nuclear receptors is specified by a highly conserved DNA binding domain that consists of two zinc finger structural motifs and is the hallmark of this transcription factor family (Evans, 1988; Freedman et al., 1988). Binding of nuclear receptors to DNA and regulation of transcription through specific *cis*-acting sequences may occur in a ligand-dependent or -independent manner (Denner et al., 1990; Aronica and Katzenellenbogen, 1991; Power et al., 1991; Ignar-Trowbridge et al., 1992; Lydon et al., 1992).

We previously reported the isolation of a murine orphan receptor NURR1 (also termed RNR-1 and NOT) that is expressed predominantly in brain tissue (Law et al., 1992; Scearce et al., 1993; Mages et al., 1994). The protein exhibits a close structural relationship to a previously characterized orphan nuclear receptor, NUR77/NGFIβ (Hazel et al., 1988; Milbrandt, 1988). Both NURR1 and NUR77 are part of a subclass of nuclear receptors that function as constitutively active transcription factors and thus do not appear to require binding of a ligand for activity (Davis et al., 1991; Wilson et al., 1991; Scearce et al., 1993). As predicted by the strong amino acid conservation (99%) between the DNA binding domains of NURR1 and NUR77, both proteins bind as monomers to the same *cis*-acting enhancer DNA sequence to regulate target gene expression (Wilson et al., 1993a,b; Scearce et al., 1993; Murphy et al., 1996). These observations suggest that both transcription factors may regulate overlapping target gene networks.

Like NURR1, NUR77 is expressed in mouse brain tissue but spatial expression pattern is less restricted than NURR1 and the transcript is found in a variety of tissues (Law et al., 1992). Unlike most nuclear receptors, NURR1 and NUR77 also are the products of immediate early genes whose expression is induced in response to distinct stimuli both within (Watson and Milbrandt, 1989; Chan et al., 1993) and outside (Scearce et al., 1993; Davis and Lau, 1994; Mages et al., 1994) the central nervous system (CNS), indicating that both constitutive and inducible expression of these transcription factors contribute to their tissue-specific functional roles. In the case of NUR77, induced expression of this transcript has been demonstrated in response to a variety of extracellular signals, including growth factors (Hazel et al., 1988; Milbrandt, 1988; Williams and Lau, 1993), neurotransmitters (Arenander et al., 1989; Watson and Milbrandt, 1989), seizure activity (Watson and Milbrandt, 1989), polypeptide hormones (Wilson et al., 1993b; Davis and Lau, 1994), and T-cell antigens (Liu et al., 1994; Woronicz et al., 1994). In at least two of these cases, the transcription factor has been shown to play an important role in stimulus transcription coupling. For example, the induction of NUR77 by adrenocorticotrophic hormone (ACTH) in adrenal cortical cells can mediate steroidogenesis by regulating the expression of the steroidogenic enzyme steroid-21-hydroxylase (Wilson et al., 1993b). The protein also mediates programmed cell death in self-reactive T-lymphocyte cells in response to apoptotic signals (Liu et al., 1994; Woronicz et al., 1994). Thus, some of the physiological roles of this transcription factor are beginning to unravel.

The selective constitutive expression of NURR1 in brain tissue predicts an important role for this transcription factor in regulation of target genes within the CNS. In support of this hypothesis, specific DNA binding sites for NURR1 have recently been identified in the promoter region of several neuronal potential target genes (Murphy et al., 1995). Therefore, in order to gain further insight into the neural pathways that may be influenced by NURR1 and to establish whether NURR1 and NUR77 may regulate overlapping target genes, we have analyzed their spatial distribution within the CNS by *in situ* hybridization. In the present article we have focused on the comparative constitutive expression of NURR1 and NUR77 mRNAs in mouse brain tissue, where both mRNAs are expressed, to determine whether they are localized in overlapping or independent neural structures.

Here we demonstrate that both transcripts show a differential but overlapping pattern of expression throughout the mammalian brain. NURR1 is expressed in sensory neurons associated with the hypothalamus, olfactory, and limbic systems as well as limbic-associated cortical areas, whereas

NUR77 is more widely expressed in a variety of neural structures, including both motor and sensory pathways

The overlapping expression of NUR77 in sensory pathways that express NURR1 suggests that these transcription factors may have shared roles in mediating limbic-associated behavioral and cognitive functions. However, the selective expression of NUR77 in motor areas suggests that this protein may play an independent role in regulation of motor function.

Materials and Methods

Tissue Preparation

A total of five males and 5 females were used in these studies. The mice were sacrificed by decapitation and the brains were removed from the skull, frozen with Tissue-Tek on dry ice, and stored at –70°C until cryostat sectioning. Serial sections of 10 μm thickness were thaw-mounted onto gelatin-coated microscope slides, dried for 15 min by air, and fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. These slides were then stored at –20°C until use.

Probe Preparation

The NURR1 probe was derived from a 371-bp *Dra*II–*Ava*I fragment between positions 367 and 738 of the NURR1 cDNA. The NUR77 probe was derived from a 287-bp *Mae*III fragment between positions 168 and 455 of the NUR77 cDNA. Both fragments were cloned in the pBluescript KS vector (Stratagene, Los Angeles, CA). The [^{35}S]UTP-labeled antisense strand RNA probes for NURR1 and NUR77 were synthesized using T7 polymerase after linearizing with *Dra*II and *Hind*III, respectively. The [^{35}S]UTP-labeled sense strand RNAs used as controls were synthesized using T3 polymerase after linearizing with *Bam*HI. All probes were hydrolyzed to a length of approx 150 nucleotides before use.

In Situ Hybridization

Before hybridization, sections were warmed to room temperature and allowed to dry. Sections were then permeabilized with proteinase K (20 μg/mL), postfixed in 4% paraformaldehyde, treated with acetic anhydride in 0.1*M* triethanolamine, pH 8.0, and dehydrated through graded ethanol. The hybridization was carried out with a 6×10^6 cpm RNA probe in a volume of 70 μL hybridization buffer containing 0.1*M* DTT, 50% formamide, 10% dextran sulfate, 4X SSC, 1X Denhardt's solution, and 250 μg/mL yeast tRNA at 58°C for 18 h in a humid chamber. Slides were washed in high stringency with 0.1*M* DTT, 2X SSC, and 50% formamide at 60°C, digested with RNase A (20 μg/mL) solution at 37°C for 30 min, and then rinsed to a final stringency of 0.1X SSC at room temperature for 15 min. Sections were dehydrated and air-dried at room temperature. Autoradiographic localization of bound probe was performed by apposition of the sections to X-ray film for 3 d. For higher resolution, the sections were dipped into NTB2 autoradiography emulsion (Eastman Kodak, New Haven, CT). After an appropriate exposure period ranging 6–10 d, slides were developed by using Kodak D-19 developer followed by Kodak fixer, stained with hematoxylin, dehydrated, and coverslipped. The sections were photographed with a Zeiss Axiophot microscope.

Western Blot Analysis

A fusion between six histidines and an amino terminal portion of NURR1 protein was made and expressed in *Escherichia coli*, allowing purification over nickel-chelated agarose beads. Briefly, a 0.7-kb fragment of NURR1 cDNA coding sequence spanning amino acids 9–236 was ligated into the *Kpn*I and *Pst*I sites of PQE-30 (Qiagen, Chatsworth, CA). The 30-kDa fusion protein was expressed from the strong *E. coli* phage T5 promoter in strain M15[pREP4] and purified on nickel-chelated agarose according to the manufacturer's instructions (Ni-NTA; Qiagen). Polyclonal antibodies were raised by injection of the fusion protein into New Zealand white rabbits. All immunization procedures were carried out by Bethyl Laboratories, Inc (Montgomery, TX). Tissue samples were treated by sonication in TESH 0.4*M* NaCl buffer. After two centrifugations (10,000*g* for 10 min and 100,000*g* for 30 min at 4°C) the supernatant proteins were quantified by Bradford protein assay. Samples (25 μg) were separated on a 7.5% acrylamide gel run for 5 h at constant voltage (100 V) at

room temperature and were transferred to Immobilon-P membrane (Millipore, Bedford, MA) for 17 h in 25 m*M* Tris-HCl, 192 m*M* glycine, and 20% methanol; the membrane was blocked for 1 h in 10 m*M* Tris HCl, pH 7.5, 100 m*M* NaCl, containing 2% nonfat dried milk. The immunological reactions were performed with antibody at a 1:4000 dilution for 2 h at room temperature. The antigen–antibody complex was revealed by chemiluminescence (ECL Western blotting kit, Amersham, Arlington Heights, IL).

Results

Comparative Distribution of NURR1 and NUR77 in Adult Mouse Brain

Since our previous analysis demonstrated that both NURR1 and NUR77 are expressed in the adult mouse brain we have compared the spatial expression of both mRNAs within the brain to establish whether these transcription factors are expressed in overlapping or distinct neural structures. In these studies, we analyzed coronal, horizontal, and sagittal brain sections from adult mouse by *in situ* hybridization with antisense and sense NURR1 or NUR77 RNA probes.

Two general observations can be made from these analyses. First, both NURR1 and NUR77 mRNAs show a restricted pattern of expression within the CNS with positively staining cells in every major brain division. Second, NURR1 and NUR77 show a differential pattern of expression throughout the brain, although staining for both transcripts appears to overlap in some neural structures. The comparative distribution of these mRNAs within the major brain divisions is described below and summarized in Table 1.

Telencephalon

The expression of NURR1 and NUR77 mRNAs overlaps most extensively in the telencephalon (Fig. 1). NURR1 expression in this brain region was more restricted than NUR77 and was localized primarily in the olfactory areas, the limbic system, and limbic-associated cortical structures. The expression of NUR77 appeared to overlap with NURR1 in the olfactory (Fig. 1B,C) and limbic areas but unlike NURR1, NUR77 staining was widespread throughout all regions of the cortex and also was observed in the basal ganglia (Fig. 1E,F).

Within the olfactory areas, positively staining cells for both transcripts were observed in the olfactory bulb and piriform cortex, although staining for NUR77 was more intense than NURR1 in these areas. However, unlike NURR1, NUR77 was also observed in the olfactory tubercle and accumbens nucleus (Fig. 1).

Both transcripts were expressed at high levels in the major components of the limbic system and limbic-associated cortical structures. In these areas, positively staining cells were localized in the hippocampus and hippocampal-associated structures, including the presubiculum, subiculum, perirhinal and entorhinal cortex, and amygdaloid areas. Within the hippocampus, intense staining for both transcripts was detected in the CA fields of Ammon's horn whereas weak staining for both mRNAs was observed in the dentate gyrus (Fig. 2).

In addition, positively staining cells for both transcripts were found in the frontal, temporal, and parietal cortices (Figs. 1 and 2). Low-level staining for both mRNAs was found in the septohypothalamic nucleus. However, only NUR77 was detected in the lateral septal nucleus. Overlapping expression of NURR1 and NUR77 also was observed in the Bed nucleus of the stria terminalis and in the claustrum (Fig. 1E,F), suggesting a role for these proteins in mediating autonomic and visual responses.

The most striking differences in expression pattern within the telencephalon were observed in the cortex and basal ganglia (Fig. 1E,F). Intense staining for NUR77 was observed throughout the frontal lobe as well as in the caudate putamen, ventral pallidum, and globus pallidus, suggesting a selective role for this factor in mediating motor responses. In contrast, NURR1 was localized to the supralimbic layer of the parietal and temporal lobes and was concentrated in the deepest layer (VI) of the cortex (Figs. 1B,E, 2B,E).

Diencephalon

NURR1 and NUR77 showed mostly a differential pattern of staining in the diencephalon. Positively staining cells that express NURR1 were observed in all major diencephalic structures,

Table 1
Distribution of NURR1 mRNA in the Mouse Central Nervous System

Region	NURR1	NUR77
Telencephalon		
Neocortex		
Cingulate cortex (Cg)	++[a]	++
Frontal cortex (Fr)	+	+++
Parietal cortex (Par)	++	++
Temporal cortex (Te)	+++	++
Inner layer of the cortex (IL)	++	+
Entorhinal cortex (Ent)	++	+
Dorsal endopiriform nucleus (DEn)	++	++
Perirhinal cortex (PRh)	++	++
Piriform cortex (Pir)	+	+++
Subiculum (S)	+++	++
Hippocampus		
Field CA1	+++	+++
Field CA2	++	++
Field CA3	++	++
Dentate gyrus (DG)	+	+
Septohypothalamic n. (SHy)	+	+
Bed nu of the stria terminalis (BSTM)	+	+
Amygdala		
Amigdalohip area (AHi)	++	++
Posteromed cortical amyg nu (PMCo)	++	++
Amigdopiriform transition (Apir)	++	++
Olfactory bulb (OB)	++	+++
Olfactory tubercle (Tu)	–	++
Claustrum (Cl)	+++	++
Caudate putamen (CPu)	–	+++
Globus pallidus (GP)	–	++
Ventral pallidum (VP)	–	++
Accumbens nucleus (Acb)	–	++
Lateral septal nu (LS)	–	++
Diencephalon		
Thalamus		
Anteromedial thalamic nu (AM)	++	–
Parafasicular nucleus (PF)	++	–
Anterodorsal thalamic nu (AD)	++	++
Paraventricular thal nu, anterior (PVA)	++	–
Precommissural nu (PrC)	+	–
Medial habenular nu (MHb)	+++	–
Lateral habenular nu (LHb)	+++	–
Medial geniculate body (MG)	–	+
Hypothalamus		
Periventricular zone		
Paraventricular hypothalamic nu (Pa)	++	–
Periventricular hypothalamic nu (Pe)	++	–
Medial zone		
Medial preoptic area (MPA)	+	++
Medial preoptic nucleus (MPO)	–	++

(continued)

Table 1 *(continued)*

Region	NURR1	NUR77
Anterior hypoth area (AH)	+	-
Posterior hypoth area (PH)	+++	+
Premammillary body (PMV)	+++	+
Lateral zone		
Lateral preoptic areas (LPO)	+	+
Lateral hypoth area (LH)	++	–
Pituitary gland		
Anterior pituitary (APit)	++	+++
Posterior pituitary (PPit)	++	+
Mesencephalon		
Pontine nuclei (Pn)	++	+
Tegmentum		
Ventral tegmental area (VTA)	+++	+
Dorsal nucleus raphe (DR)	++	–
Caudal linear nu raphe (CLi)	++	–
Dorsal tegmental decussation (dtgx)	++	+
Retrorubral field (RRF)	++	+
Pedunculopontine tegmental nu (PPTg)	+++	+
Interstitial nu of the mlf (IMLF)	++	–
Rostral interstitial nu mlf (RI)	++	++
Interpeduncular nucleus (IL)	–	+
Edinger-Westphal nucleus (EW)	++	–
Central gray (CG)	+	+
Rhombencephalon		
Pons		
Medial vestibular nu (MVe)	+	–
Intermediate reticular nu (IRt)	++	–
Raphe nucleus (R)	++	–
Pontine reticular nu, oral (PnO)	+	–
Cerebellum (Cb), granular layer	+++	+++
Medulla oblongata	+	+
Spinal cord (SP)		
Lamina X of the gray matter	+	–
Dorsal horn	–	+

[a]Semiquantitative estimates of the signals are indicated: +, weak; ++, moderate; +++, strong.

including the thalamus, habenula, and hypothalamus (Fig 2B,E). Intense staining for NURR1 was observed in the medial and lateral habenular region whereas lower levels of staining were observed in several thalamic nuclei, including the anteromedial, parafasicular, anterodorsal, paraventricular, and precommissural nuclei. Within the main hypothalamic zones, moderate to high levels of NURR1 staining were observed in at least nine discrete nuclei (Table 1). The most intense hybridization signal was observed in premammillary nuclei, which are associated with emotive responses and in the posterior hypothalamic area (Fig. 1E). A role for NURR1 in mediating neuroendocrine and autonomic responses is supported by its expression in the paraventricular, periventricular (Fig. 3), anterior, posterior, and lateral hypothalamic areas (Fig. 2B,E). The medial and lateral preoptic areas also showed a faint and diffuse NURR1 expression (Fig. 1E).

In contrast to NURR1, staining for NUR77 was weak in the diencephalon with positive cells

observed in fewer thalamic and hypothalamic nuclei. NUR77 staining was absent from the habenula and was detected at low levels in the anterodorsal (Fig. 2C) and medial geniculate nucleus of the thalamus. Within the hypothalamus, moderate to weak staining was observed in the medial preoptic nucleus, posterior hypothalamic area, premammillary body, and lateral preoptic areas (Figs. 1F, 2F).

The pituitary gland showed differential staining of NURR1 and NUR77 between the posterior and anterior regions (Fig. 4). Widespread expression of NURR1 was observed in both the anterior and posterior regions, whereas positively staining cells for NUR77 were localized predominantly in the anterior region, with few NURR1-positive cells in the posterior lobe.

Mesencephalon

The expression of NURR1 and NUR77 in the midbrain is shown in Fig. 5. NURR1 was expressed at high levels in the cerebral peduncles where positively staining cells were observed in several nuclei with fiber pathway connections to the limbic system, ventral striatum, and hypothalamus. These included the ventral tegmental area, raphe nuclei, pedunculopontine tegmental nucleus, interstitial nucleus of the medial longitudinal fasciculus, retrorubral field, and central gray (Fig. 5B,E).

Several nuclei with major projections to the cerebellum (pontine, reticulotegmental, and lateral reticular) also expressed high levels of NURR1 mRNA. The Edinger-Westphal nucleus showed moderate NURR1 expression, suggesting a pos-

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Fig. 1. *(Figs. 1–6 on pp. 58–59)* *In situ* hybridization analysis of NURR1 and NUR77 mRNA expression in the telencephalon of the mouse adult brain. Bright field (**A**) and dark field photomicrographs of adjacent sagittal sections hybridized to NURR1 (**B**) and NUR77 (**C**) antisense ^{35}S probes through the olfactory bulb. Bright field (**D**) and dark field photomicrographs of adjacent coronal sections hybridized to NURR1 (**E**) and NUR77 (**F**) antisense ^{35}S probes at rostral level. No hybridization was detected using the sense probes. Note that NUR77 distribution is widespread in this rostral region of the brain. OB, olfactory bulb; Cl, claustrum; Fr, frontal cortex; Pir, piriform cortex; Ahi, amigdalohip area; Cpu, caudate putamen; GP, globus pallidus; Acb, Accumbens nu; VP, ventral pallidus; Tu, olfactory tubercle, Cg, cingulum; Shy, septohypothalamic nu; BSTM, bed nucleus of the stria terminalis; MPO, medial preoptic nucleus; Den, dorsal endopiriform nucleus; IL, inner layer of the cortex.

Fig. 2. Distribution of *NURR1* and *NUR77* gene expression through diencephalon. *Bright field* (**A**) *and dark* field photomicrographs of adjacent horizontal sections hybridized to NURR1 (**B**) and NUR77 (**C**) antisense probes through the habenula of the thalamus. Bright field (**D**) and dark field photomicrographs of adjacent coronal sections hybridized to NURR1 (**E**) and NUR77 (**F**) antisense probes through posterior part of thalamus and hypothalamus regions. Hb, habenula; Prc, precommissural nucleus; AD, anterior dorsal thalamus; PH, posterior hypothalamic nu; PMV, premammillary body; Ahi, amigdalohip area; PMCo, posteromed cortical amyg nu; Apir, amigdopiriform transition; Cg, cingulum cortex; Par, parietal cortex; Te, temporal cortex; Prh, perirhinal cortex; S, subiculum; CA-3, hippocampus fields; DG, dentate gyrus.

Fig. 3. Mouse coronal section showing the distribution of NURR1 and mRNA in the hypothalamus. (**A**) Bright field; (**B**) dark field. Pa, paraventricular hypothalamic nucleus; Pe, periventricular hypothalamic nucleus.

Fig. 4. Pituitary gland showing the expression pattern of NURR1 and NUR77. Top panels show the bright (**A**) and dark field (**B**) of NURR1 mRNA. Bottom panels show the bright (**C**) and dark (**D**) field of NUR77 mRNA. APit, anterior pituitary; PPit, posterior pituitary.

Fig. 5. Distribution of NURR1 and NUR77 gene expression through midbrain. Bright field (**A**) and dark field photomicrographs of adjacent horizontal sections hybridized to NURR1 (**B**) and NUR77 (**C**) antisense probes through the raphe nucleus of the brain. Bright field (**D**) and dark field photomicrographs of adjacent coronal sections hybridized to NURR1 (**E**) and *NUR77* (**F**) antisense probes through the ventral tegmental area of the midbrain. PVA, Paraventricular thal nu, anterior; PF, parafasicular nu; RI, rostral interstitial nu mlf; DR, dorsal nu raphe; Pptg, pedunculopontine tegmental nu; VP, ventral pallidus; GP, globus pallidus; Cpu, caudate putamen; LS, lateral septal nu; S, subiculum; Ent, entorhinal cortex; Te, temporal cortex.

Fig. 6. Localization of NURR1 mRNA in a coronal section of the mouse hindbrain bright field (**A**) and dark field (**B**) photomicrographs. Cb, cerebellum; R, raphe nucleus; MVe, medial vestibular nucleus; IRt, intermediate reticular nucleus.

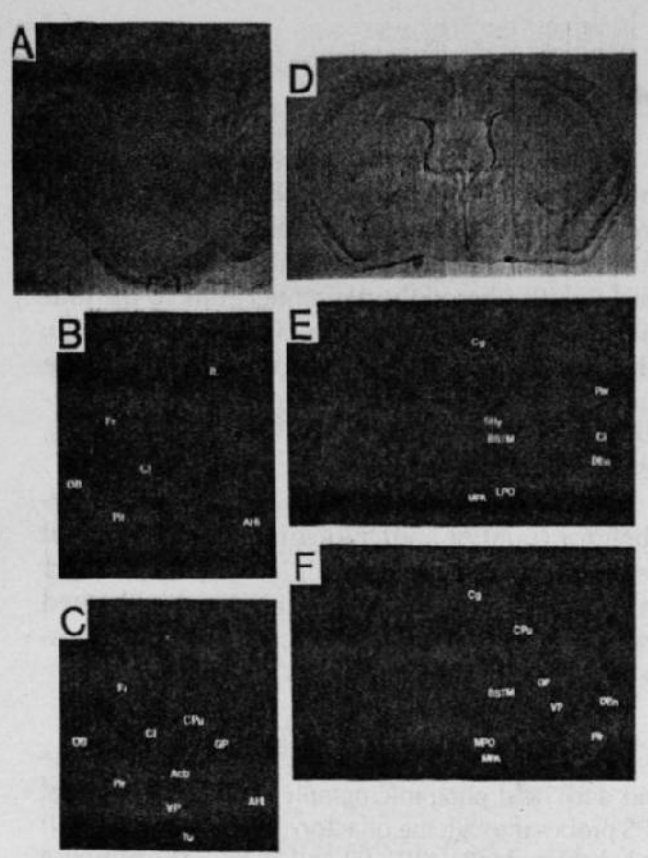

Fig. 1.

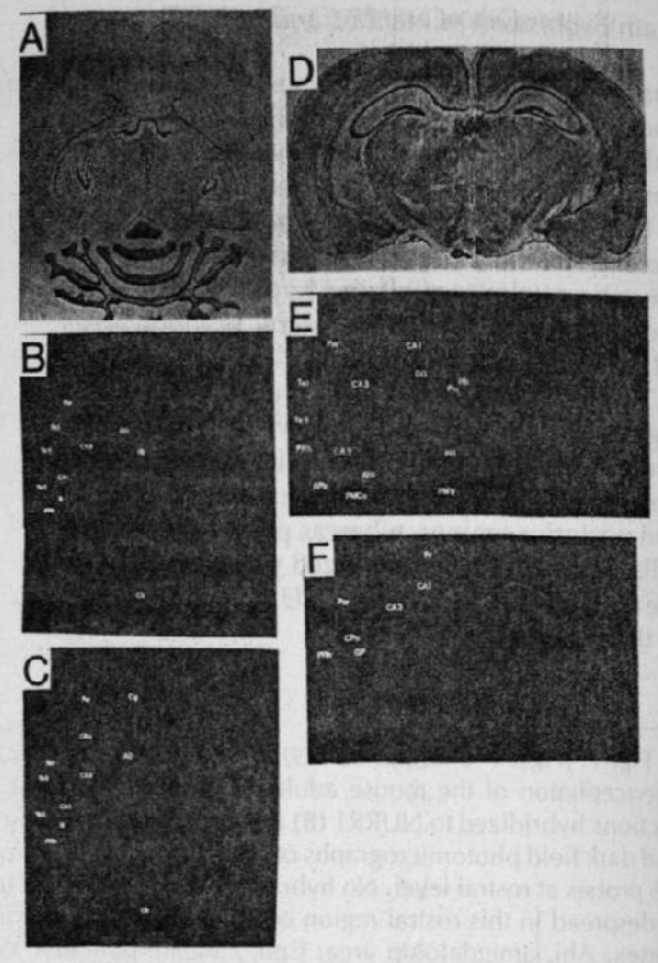

Fig. 2.

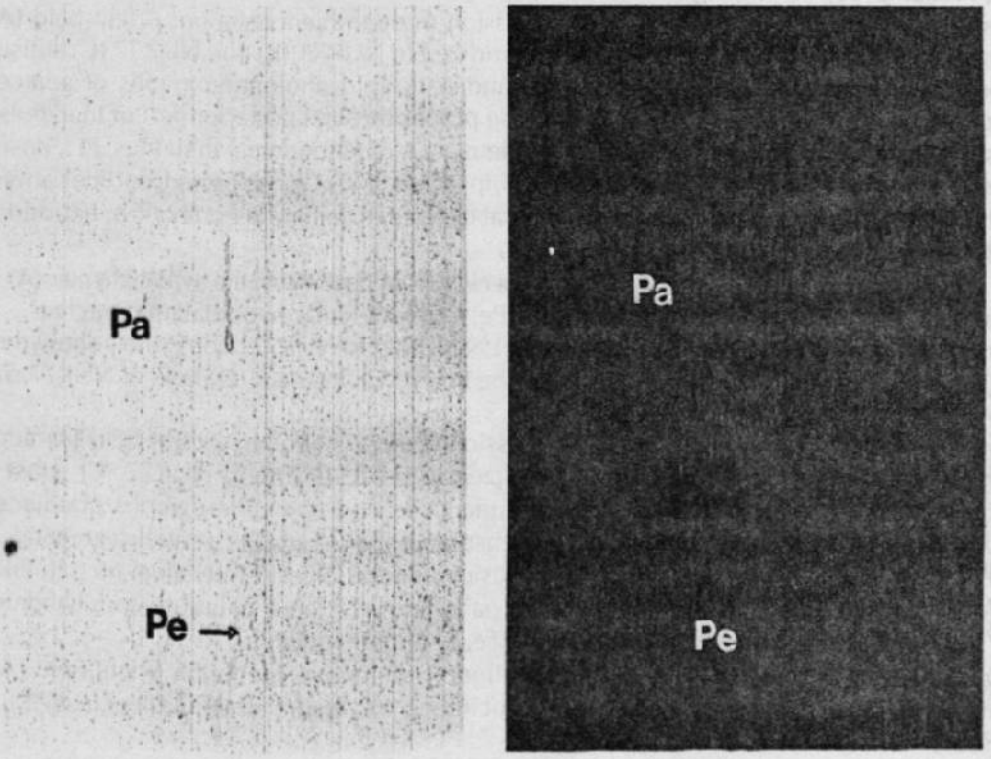

Fig. 3.

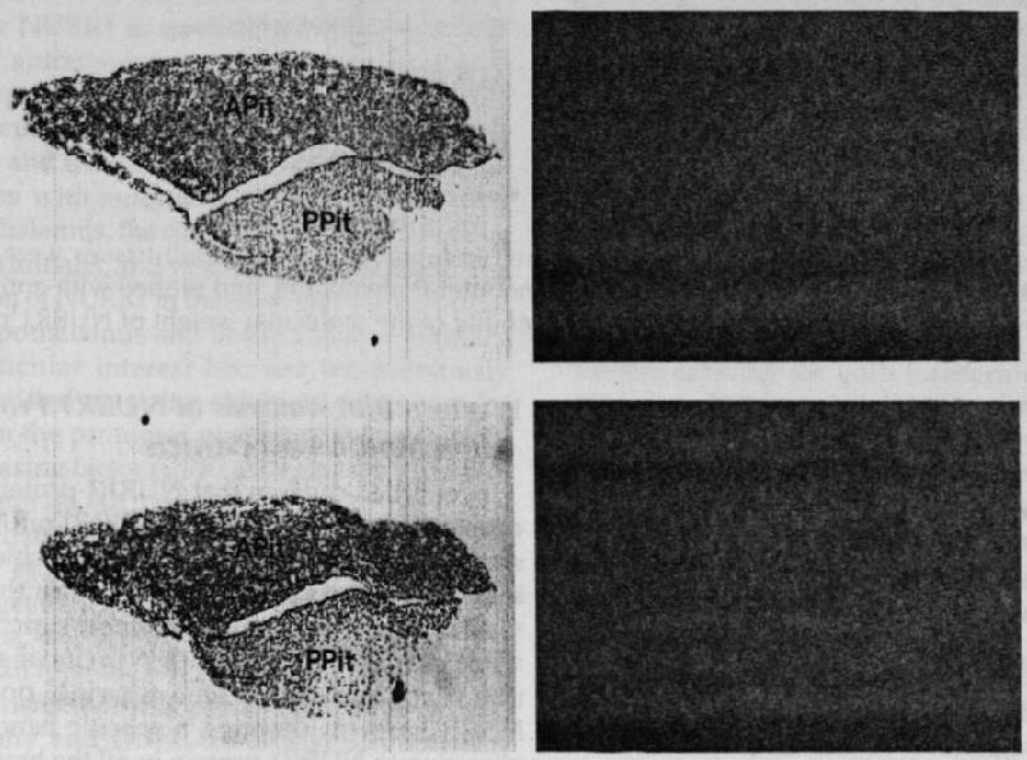

Fig. 4.

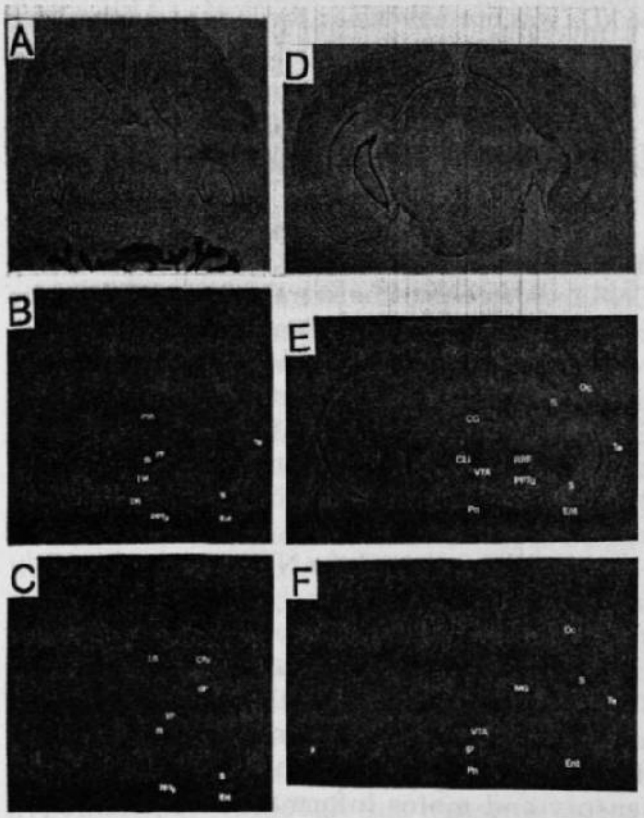

Fig. 5.

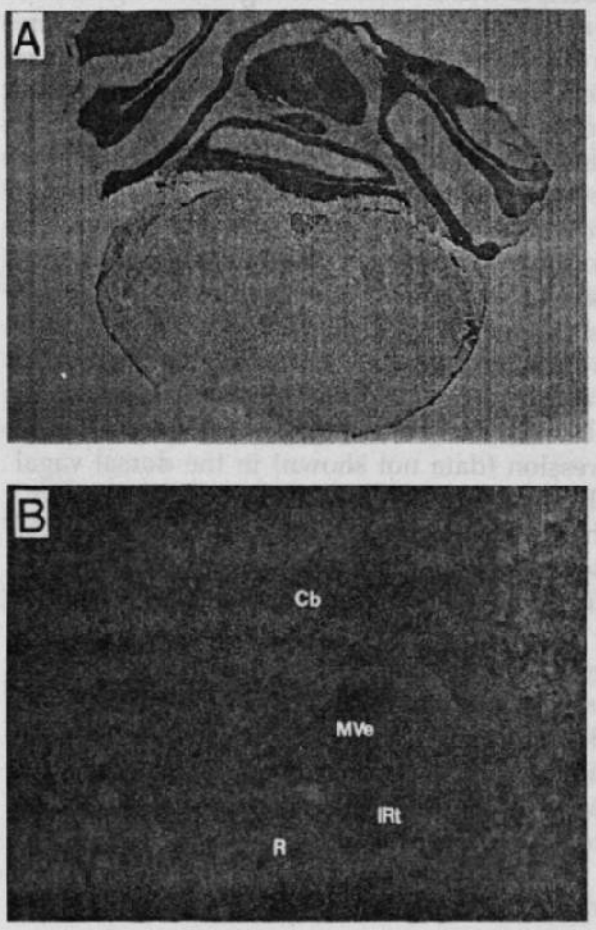

Fig. 6.

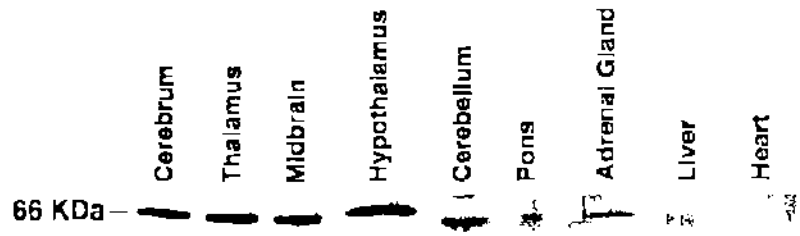

Fig. 7. Expression of NURR1 protein. Extracts prepared from various mouse adult tissues were electrophoresed on SDS-polyacrylamide gel, transferred to immobilon-P membrane, and probed with anti-NURR1 (immune) serum. The number at the left indicates the mobility of the molecular weight of NURR1 protein.

sible role for NURR1 in control of eye muscle activity. In contrast, we observed only faint expression for NUR77 within these tegmental regions and this transcript was not expressed in the raphe nucleus (Fig. 5C). Low levels of NUR77 mRNA also were observed in the medial geniculate and interpeduncular nucleus (Fig. 5F).

Rhombencephalon and Spinal Cord

The highest levels of expression of NURR1 and NUR77 in this region were observed in the cerebellum (Fig. 2B,C), where intense staining for both transcripts was detected throughout the granular layers. The most striking difference in the expression pattern of NURR1 and NUR77 in this brain region was observed in the pons and medulla, which lacked detectable expression of NUR77. The pons showed moderate NURR1 staining (Fig. 6) in several nuclei, including the medial vestibular and intermediate reticular nucleus, which is involved in four general types of function: motor control, sensory control, visceral control, and control of consciousness. Consistent with its midbrain expression pattern, positively staining cells for NURR1 were observed in the pontine nuclei and raphe nuclei. The medulla showed moderate NURR1 expression (data not shown) in the dorsal vagal nucleus involved in sensory, motor, and parasympathetic functions. The remaining portions of these nuclei were unlabeled.

Finally, very low intensity staining for NURR1 and NUR77 was observed in the spinal cord, where the transcripts showed a differential non overlapping expression pattern. Expression of NUR77 was localized in the dorsal horn whereas NURR1 staining was confined to the lamina X region of the gray matter surrounding the central canal (data not shown).

Western Blot Analysis of NURR1 Protein in Mouse Tissue Extracts

In order to confirm that NURR1 protein is also detected in areas that express NURR1 mRNA transcripts, we carried out Western immunoblot analysis of tissue extracts from cerebrum, thalamus, midbrain, hypothalamus, cerebellum, pons, adrenal gland, liver, and heart. A polyclonal antibody that recognizes the amino terminal portion of NURR1 protein detected a specific band, corresponding to NURR1 protein in all the brain tissue extracts and in the adrenal gland. This band of 66 kDa was not detected in the liver and heart tissue extracts (Fig. 7).

Discussion

The present analysis of the comparative spatial distribution of NURR1 and NUR77 within the CNS indicates that these transcripts may have both shared and independent functions in neuronal cells. Both mRNAs show a different but partially overlapping pattern of expression within the CNS. The function of NURR1 may be more specific since its expression is more restricted than NUR77 and appears to be mainly localized to sensory neural structures.

Positively staining cells for NURR1 were observed in all major brain divisions. However, its expression within these areas was found predominantly in structures associated with the limbic system. The association of NURR1 with the hypothalamus and limbic system predicts a role for this transcription factor in the integration and processing of sensory and motor information required for expression of complex motivational and emo-

tional behaviors as well as learning and memory. A role for NURR1 in mediating both neuroendocrine and autonomic components of these behaviors is supported by the localization of NURR1 within neuroendocrine structures of the hypothalamus and the anterior pituitary gland, and its association with autonomic structures, including the hypothalamus, the cirgulate gyrus, Bed nucleus of stria terminalis, and ventral tegmental area. The localization of NURR1 in the paraventricular nucleus of the hypothalamus and in the anterior pituitary is of particular interest because we previously have identified *cis*-acting enhanced sequences for NURR1 in the promoter regions of the corticotrophin-releasing factor (CRF) gene and the pro-opiomelanoxortin (POMC) gene, which are expressed in the hypothalamic and pituitary areas, respectively (Murphy et al., 1995). Furthermore, using synthetic target genes containing the CRF and POMC promoters, we recently have demonstrated that NURR1 can regulate the expression of these genes when transfected into pituitary cells in culture (E. Murphy and O. M. Conneely, unpublished results). These data, together with the previous demonstration by others that NUR77 upregulation by ACTH can mediate regulation of expression of the steroidogenic enzyme steroid-21-hydroxylase (Wilson et al., 1993b), provide the first indication that the NURR1/NUR77 subfamily of nuclear receptors may play an important neuroendocrine role in the coordinate regulation of activity of the hypothalamic–pituitary–adrenal axis.

The expression of NUR77 overlaps extensively with NURR1 in the limbic system but also shows significant differences in its expression pattern in all major brain divisions. Most striking is the widespread expression of NUR77 throughout all regions of the cerebral cortex and its selective expression in telencephalic structures within motor areas. The expression of NUR77 in the basal ganglia, nucleus accumbens, and olfactory tubercle suggests that this transcription factor may participate in the regulation of motor activity.

Significant differences in expression patterns of the two transcripts also are observed in the diencephalon (Fig. 2). Most notable is the lack of appearance of positively staining cells for NUR77 in the habenula and its low expression in the hypothalamus. Although this transcript is not constitutively expressed in the endocrine hypothalamus, its expression in the anterior pituitary together with the previously reported stress-inducible expression of NUR77 in the paraventricular hypothalamic nucleus (Chan et al., 1993) support a role for this protein in mediating neuroendocrine responses.

Unlike NURR1, which shows significant expression in mid- and hind-brain structures, particularly those with fiber connections to the limbic system, the expression of NUR77 in these areas is extremely low to undetectable. However, the intense staining for both transcripts throughout the cerebellum suggests that they may have overlapping roles in mediating maintenance of equilibrium and coordination of muscle action.

Our analysis of the expression patterns of NURR1 and NUR77 in this study identifies functionally defined brain regions in which constitutive expression of these transcription factors is likely to contribute to maintenance of a base-line or constitutive level of neuronal activity. The ability of these transcripts to respond in an inducible manner to a variety of stimuli provides an additional level of contribution of these transcription factors to neuronal activity under specific stimulatory conditions that are not detected in this study.

We did not see a discrepancy between expression of the NURR1 mRNA and its protein in the main structures of the brain. The overlapping expression of NURR1 and NUR77 in the limbic system together with their ability to interact with similar enhancer elements (Scearce et al., 1993) clearly predicts that both proteins may interact with similar gene networks in behavioral and cognitive pathways. Whether this overlapping interaction leads to a redundancy of function of both proteins or a differential regulation of gene transcription remains to be established.

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Cloning and structural organization of the gene encoding the murine nuclear receptor transcription factor, NURR1

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Abstract

NURR1 is an immediate early gene product and a member of the nuclear receptor superfamily of transcription factors. Using the NURR1 cDNA as a probe, we isolated the genomic DNA encoding NURR1 from a mouse 129SvEv genomic library. The NURR1 gene is approximately 6.2 kb long and is organized into 7 exons separated by 6 introns. Structural analysis of the NURR1 reveals that this gene shares a similar structure with that of the nuclear receptor NUR77/NGFI-B. As in NUR77, the promoter region of NURR1 lacks an identifiable TATA box, but is GC-rich. The proximal promoter region also contains an ATF/CREB consensus binding site that may participate in cAMP-mediated induction of this immediate early gene product. Isolation and structural characterization of the NURR1 gene provides information for further developmental and transcriptional regulation studies of this gene.

Keywords: Promoter; Consensus sequence; NUR77

1. Introduction

We previously reported the isolation of a murine orphan receptor NURR1 (also termed RNR-1 and NOT) that is expressed predominantly in the main regions of the central nervous system (Law et al., 1992; Scearce et al., 1993; Mages et al., 1994; Saucedo-Cardenas and Conneely, 1996). NURR1 is a member of the nuclear receptor superfamily. This family comprises a group of structurally related transcription factors that program developmental, physiological and behavioral responses to a variety of chemical signals. The family includes receptors for steroids, certain vitamins and thyroid hormone (Evans, 1988; Beato, 1989; Tsai and O'Malley, 1994) in addition to a growing number of orphan members whose physiological function and cognate ligand, if any, remain to be established (O'Malley and Conneely, 1992).

NURR1 cDNA encodes a 66-kilodalton protein and exhibits close structural relationship to previously characterized orphan nuclear receptors, NGFI-B/NUR77/N10/NAK-1 (Milbrandt, 1988; Hazel et al., 1988; Ryseck et al., 1989; Nakai et al., 1990) and NOR-1/MINOR/TEC (Ohkura et al., 1994; Hedvat and Irving, 1995; Labelle et al., 1995). These receptors are part of a subclass of nuclear receptors that are immediate early gene products and function as constitutively active transcription factors that do not appear to require binding of a ligand for activity (Chalepakis et al., 1988; Davis et al., 1991; Wilson et al., 1991). In addition, these three transcription factors are able to bind to the same cis-acting enhancer DNA sequence to regulate target gene expression (Chalepakis et al., 1988; Ohkura et al., 1994; Hedvat and Irving, 1995; Wilson et al., 1993a,b; Murphy et al., 1995). These observations suggest that these transcription factors may regulate overlapping gene networks.

To understand the regulation of the NURR1 gene and its relationship to other members of the nuclear receptor superfamily, we have determined its structural organization. The exon intron arrangement is similar to that of the NGFI-B/NUR77 nuclear receptor supporting the conclusion that this subclass of gene products have a close evolutionary relationship within the nuclear receptor superfamily. Analysis of the proximal promoter region of NURR1 reveals at least one potential cis-acting sequence that may participate in signal induced transcription of this immediate early gene.

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Abbreviations: bp, base pair(s); kb, kilobase(s) or 1000 bp; nt, nucleotide(s).

2. Experimental and discussion

2.1. Isolation of the NURR1 gene

Two mouse 129/SvEv genomic libraries prepared in λDash II (Stratagene) and P1 (Genome Systems Inc) were used to isolate the NURR1 gene. Screening of the λDash II library was performed (Grunstein and Hogness, 1975) using as probe a *Dra*II-*Pst*I (680-bp) fragment from the N-terminal region of NURR1 cDNA (Law et al., 1992). The probe was radiolabeled using [^{32}P] dCTP to a specific activity of 2×10^9 cpm/μg. Using this approach, one positive clone was identified from the λDASH II genomic library. Two fragments of this clone, 5.5-kb *Bam*HI (p20) and 6.5-kb *Eco*RI (p10), corresponding to the 5′ portion of the NURR1 gene, were subcloned into pSP72 (Promega). The P1 library was initially screened by PCR using oligonucleotides specific for the same N-terminal region of the NURR1 gene. Two NURR1 positive clones were identified. One of these clones, 7.8-kb *Bgl*II (p30), containing the 3′ end of the NURR1 gene was subcloned into *Bam*HI site of pUC19 (New England Biolabs, Inc.). The three overlapping genomic fragments (p10, p20 and p30) were characterized by Southern analysis (Fig. 1). Together, these fragments spanned the entire NURR1 gene and were used to establish the intron/exon organization by PCR amplification and sequence analysis.

2.2. Analysis of exons, introns and exon/intron boundaries

To identify and sequence the exon/intron boundaries, amplification of individual regions of the NURR1 gene was performed. This was done by using 10 ng of NURR1 genomic DNA (p20 and p30) as template and a variety of 20–27-mer oligonucleotide primers. These primers were designed from the NURR1 cDNA and by comparison to sequences flanking the published NUR77/NGFI-B intron/exon boundaries (Watson and Milbrandt, 1989). The PCR products were analyzed by agarose gel electrophoresis and sequenced directly (Sanger et al., 1977; Casanova et al., 1990).

The structural organization of the NURR1 gene is shown in Fig. 1. Sequences of the exon/intron junctions, as well as the size of each exon and corresponding encoded amino acids, are shown in Table 1. A comparison of the genomic and cDNA sequences revealed that the NURR1 gene is composed of seven exons. Exon sizes ranged from 130 to 866 bp, the largest being the second exon which contains the first ATG codon. The introns vary in size from 350 bp to 1 kb. All of the exon-intron boundaries satisfy the GT-AG intron donor-acceptor splice rule (Mount, 1982). Based on this data, the physical map of the NURR1 gene spans approximately 6.2 kb.

Comparison of the NURR1 gene structure with its closely related family member NGFI-B (Watson and Milbrandt, 1989) reveals that the exon/intron boundaries are identical. The two genes have seven exons which are similar in length and in the distribution of the protein-coding sequence. Both contain the amino terminal transactivation domain in exon 2. The DNA binding domain, common to all members of the nuclear receptor superfamily, is encoded by exons 2 and 3. Exon 4 encodes a nonconserved domain thought to function as a hinge region transactivation domain between the DNA- and the putative ligand-binding domains (Evans, 1988). The exons 5, 6, and 7 encode the dimerization and putative ligand binding domains. In addition, like NGFI-B, the NURR1 gene contains in exon 7, the termination codon and the 3′ untranslated region, which contains multiple AUUUA motifs that may play a role in the regulation of NURR1 mRNA stability (Milbrandt, 1988). While the gene structure shares a similar general organization with other members of the nuclear receptor superfamily (Hazel et al., 1988), the NURR1 and NUR77/NGFI-B genes contain an addi-

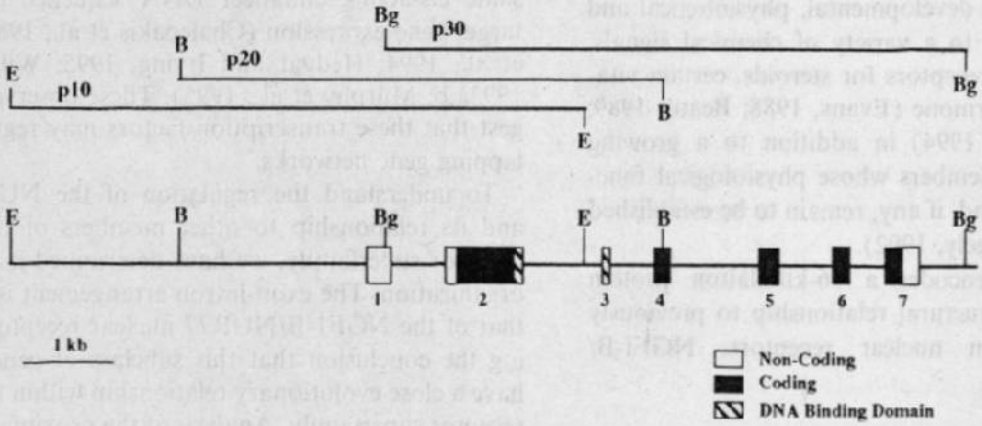

Fig. 1. Structural organization of the NURR1 gene and its relationship to the three overlapping genomic clones identified from hybridization screening. The boxes in the map denote exons with the exon number shown below each box and the line connecting each box denotes introns. The coding region and DNA binding domain of NURR1 are indicated. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI.

Table 1
Location of introns and exon-intron boundaries of NURR1 gene

EXON	(SIZE)	EXON 5'	(•)	INTRON(SIZE)	EXON 3'
1	334bp	GAA G	334	GTCAGT..INTRON 1(~600bp)..TTCCAG	CC ATG*
					Met
2	866bp	TTT AAG	1201	GTGAGC..INTRON 2(~900bp)..CTACAG	CGC ACG
		Phe Lys			Arg Thr
3	130bp	GAA G	1330	GTAGGT..INTRON 3(~500bp)..TTACAG	TG GTT
		Glu V			al Val
4	164bp	TCC AGG	1494	GTAAGA..INTRON 4 (~1 kb)..TTCCAG	TTC CAG
		Ser Arg			Phe Gln
5	203bp	TAC AG	1697	GTAATG..INTRON 5(~600bp)..TTGCAG	G TCC
		Tyr Ar			g Ser
6	176bp	ACA G	1876	GTCAGT..INTRON 6(~350bp)..CTGCAG	AG AGA
		Thr G			lu Arg
7	>347bp				

Exon-intron boundaries and the size of each exon and intron are shown. The ATG* codon is located in exon 2; (●) indicates the nucleotide number of the NURR1 cDNA at which the intron is located

tional intron in the amino terminal portion of the gene 5′ to the DNA binding domain.

2.3. Determination of the transcription initiation site and 5′ sequence analysis

Total RNA was prepared from adult 129/C57 mouse brain (Kingston, 1989). An 18-mer antisense oligonucleotide primer was synthesized corresponding to nucleotides 97–114 of exon 1 of NURR1. The oligonucleotide was labeled with T4 polynucleotide Kinase and (γ^{32}P) to a specific activity of 10^8 cpm/μg). The extension reactions were performed as described (Wong et al., 1989). As is typical of genes with TATA-less promoters, some heterogeneity was observed in the transcription start site. However, five potential initiation sites were detected (Fig. 2), one of which was clearly identified as the major transcriptional start site located 322 bp upstream of the translation initiation ATG in the NURR1 cDNA. We sequenced ~500 bp of the 5′ flanking sequence of NURR1 gene (Fig. 3). Like NUR77/NGFI-B, the NURR1 gene lacks identifiable TATA and CAAT boxes and contains several GC boxes. Computer analysis of this 5′ flanking sequence revealed that there is an ATF/CREB consensus cis-acting binding site located at −165 nucleotide (Fig. 3). The identification of this consensus CREB site in the proximal promoter region of the NURR1 gene is of particular interest in light of the known induction of NURR1 expression in the adrenal glands by ACTH (Wilson et al., 1993b) and our recent observation that NURR1 mRNA is induced in primary pituitary cells by corticotrophin releasing factor (CRF) (Murphy E. and Conneely O.M., in preparation). Both CRF and ACTH play an important role in neuroendocrine regulation of the hypothalamic/pituitary/adrenal axis and the signal/

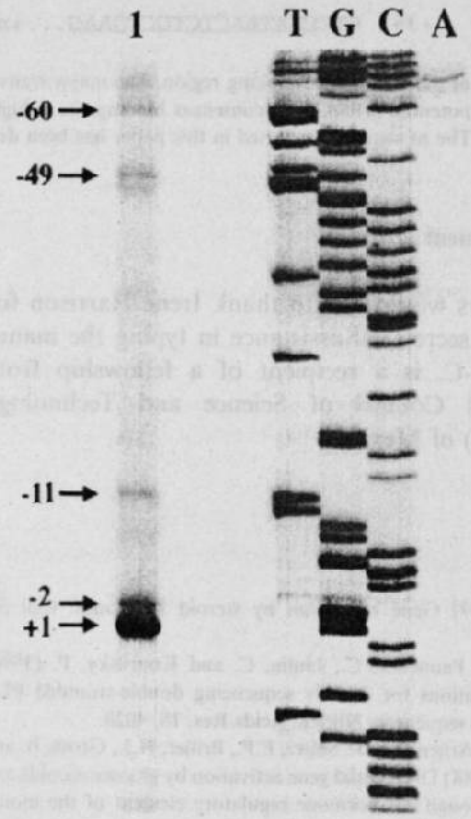

Fig. 2. Identification of the transcriptional start site of NURR1. Primer extension analysis of the NURR1 gene is shown in lane 1. The arrows show the five potential transcription initiation sites.

transcription coupling by these effectors is mediated by activation of a cAMP dependent pathway.

Cloning and sequence characterization of the NURR1 gene provides an important tool to facilitate elucidation of the mechanisms that underlie the restricted temporal and spatial expression of this gene as well as its differential regulation by extracellular signals.

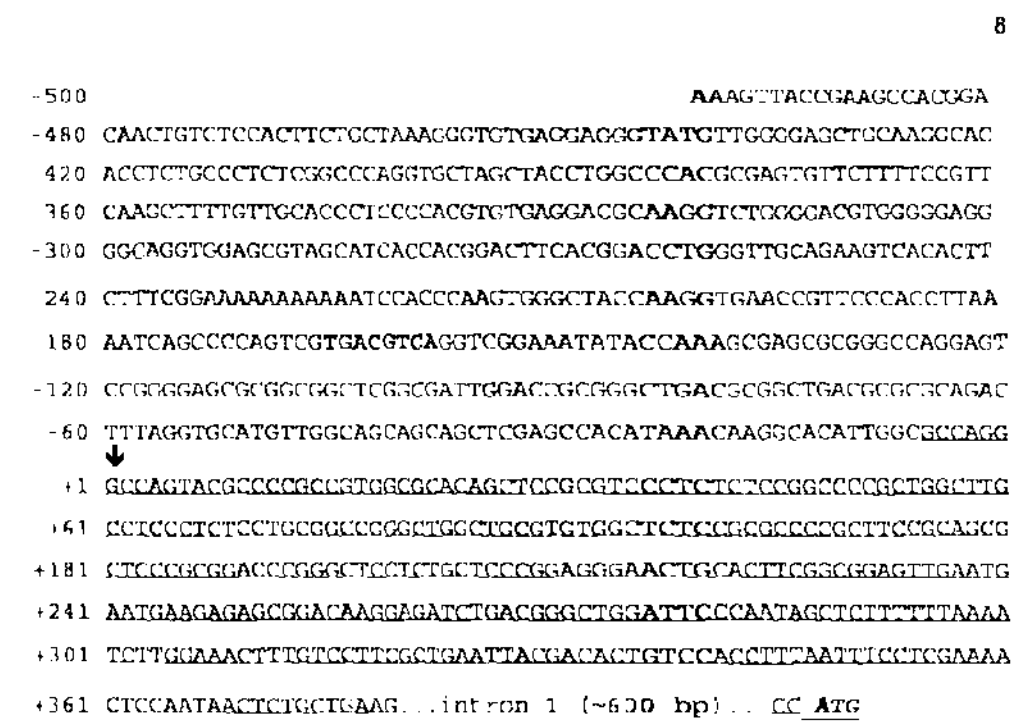

Fig. 3. Sequence of the NURR1 5′ flanking region. The major transcription initiation site is indicated by an arrow. The NURR1 cDNA sequence is underlined. A potential ATF/CREB consensus binding site is highlighted in bold letters. The ATG translation initiation codon is at the 3′ end of the sequence. The nt sequence reported in this paper has been deposited in GenBank/NCBI under accession No. L67738.

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Neurobiology

Nurr1 is essential for the induction of the dopaminergic phenotype and the survival of ventral mesencephalic late dopaminergic precursor neurons

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ABSTRACT **Nurr1 is a member of the nuclear receptor superfamily of transcription factors that is expressed predominantly in the central nervous system, including developing and mature dopaminergic neurons. Recent studies have demonstrated that Nurr1 is essential for the induction of phenotypic markers of ventral mid-brain dopaminergic neurons whose generation is specified by the floor plate-derived morphogenic signal sonic hedgehog (SHH), but the precise role of Nurr1 in this differentiative pathway has not been established. To provide further insights into the role of Nurr1 in the final differentiation pathway, we have examined the fate of dopamine cell precursors in Nurr1 null mutant mice. Here we demonstrate that Nurr1 functions at the later stages of dopamine cell development to drive differentiation of ventral mesencephalic late dopaminergic precursor neurons. In the absence of Nurr1, neuroepithelial cells that give rise to dopaminergic neurons adopt a normal ventral localization and neuronal phenotype characterized by expression of the homeodomain transcription factor and mesencephalic marker, Ptx-3, at embryonic day 11.5. However, these late precursors fail to induce a dopaminergic phenotype, indicating that Nurr1 is essential for specifying commitment of mesencephalic precursors to the full dopaminergic phenotype. Further, as development progresses, these mid-brain dopamine precursor cells degenerate in the absence of Nurr1, resulting in loss of Ptx-3 expression and a concomitant increase in apoptosis of ventral midbrain neurons in newborn null mutant mice. Taken together, these data indicate that Nurr1 is essential for both survival and final differentiation of ventral mesencephalic late dopaminergic precursor neurons into a complete dopaminergic phenotype.**

The catecholamine neurotransmitter dopamine plays a central role in the control of voluntary movement, cognition, and emotive behaviors (1). The majority of neurons that produce dopamine originate in the ventral midbrain in the substantia nigra (A9) and the ventral tegmental area (A10). Neurons arising from the substantia nigra project to the striatum to regulate motor control and their degeneration is associated with Parkinson's disease (1–3). Neurons from the ventral tegmental area give rise to a distinct system that projects to the limbic system and cortex, and regulates emotional and reward behavior and motivation (4). Disturbances in this system are implicated in schizophrenia and addictive behavioral disorders (5–7).

While the physiological relevance and clinical significance of dopaminergic neurons are well recognized, the mechanisms underlying their development are poorly understood and are the subject of intense investigation. The development of mid-brain dopaminergic neurons is initiated at embryonic day 10 in the mouse in the ventrolateral neural tube adjacent to the floor plate and is regulated by the floor plate-derived morphogenic signal, sonic hedgehog (SHH) (8–11). SHH initially induces a general ventral cell fate characterized by the induction of ventral markers, including the SHH receptor, ptc, the winged helix, and zinc finger transcription factors, HNF3β and Gli-1 (11–14). Subsequently, these ventralized cells further differentiate to adopt different specific cell fates along the anterior–posterior axis. Indeed, ectopic expression of SHH in the dorsal neural tube of transgenic mice is sufficient to drive induction of dopaminergic and serotonergic neurons of the mid- and hindbrain, respectively (11). At the level of the midbrain, this differentiation leads ultimately to the expression of dopamine synthetic enzymes including tyrosine hydroxylase (TH) and L-aromatic amino acid decarboxylase (AADC). The expression of these enzymes in the ventral midbrain at embryonic day 11.5 in the mouse is characteristic of the emergence of a dopaminergic phenotype (15).

Nurr1, a member of the nuclear receptor superfamily of transcription factors (16–18), is expressed predominantly in the central nervous system in limbic areas and the ventral midbrain, including dopamine neurons (19–21). The onset of Nurr1 expression in the ventral midbrain occurs at embryonic day 10.5 before the appearance of the dopaminergic marker enzyme, TH, at embryonic day 11.5. Expression of Nurr1 continues in mature dopaminergic neurons during adulthood, suggesting that the protein may also be required for normal function of mature dopaminergic neurons. Using Nurr1 null mutant mice, Zetterstrom *et al.* (19) recently demonstrated that ablation of Nurr1 leads to agenesis of midbrain dopaminergic neurons as evidenced by an absence of the dopaminergic cell markers, TH, the retinoic acid converting enzyme, ADH2 and the receptor tyrosine kinase, c-ret, and a loss of striatal dopamine neurotransmitter. However, the precise role of nurr1 in this developmental cascade has not been established.

The objective of this study was to examine the role of Nurr1 in mediating the final differentiation of ventral dopaminergic neurons. Here we demonstrate that in the absence of Nurr1, neuroepithelial cells undergo normal ventralization, differentiate into neurons, and adopt a specific mesencephalic phenotype that is identified by the expression of the homeodomain protein, Ptx-3 (22). However, these dopamine precursor cells are arrested in a developmental state described by a lack of dopamine phenotypic markers. Further, these dopaminergic precursors do not survive and die as development progresses to the neonatal stage. Together, these data indicate that Nurr1

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Abbreviations. SHH, sonic hedgehog; TH, tyrosine hydroxylase; AADC, L-aromatic amino acid decarboxylase; Chat, choline acetyltransferase; ES cell, *embryonic stem cell*.

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regulates dopaminergic cell development by promoting both survival of ventral mesencephalic neurons and their differentiation into the final dopaminergic phenotype.

MATERIALS AND METHODS

Gene Targeting. The Nurr1 genomic DNA fragment (~7.6 kb) that comprised the gene targeting vector was isolated from mouse 129Sv λ Dash II genomic library (Stratagene) using as a probe a fragment from the *N*-terminal region of Nurr1 cDNA (16, 23). This mNurr1 genomic fragment contained exons 2–8 of the Nurr1 gene. Exon 3 encodes the nonconserved N-terminal domain of the receptor that contains the initiating methionine residue, ATG. The *neo*^{r} gene PGKNEObpA (24) was inserted into a unique *Nco*I restriction site in exon 3 that was located downstream from the initiator codon ATG but upstream of the DNA-binding domain in the Nurr1 gene (16). The insertion of the *neo*^{r} gene into exon 3 divides the 7.6-kb Nurr1 genomic fragment into 5′ and 3′ arms of Nurr1 homology that are 1.9 kb and 5.7 kb in size, respectively. The herpes simplex virus thymidine kinase (HSV-TK) gene (25) was attached 5′ to exon 3 and inserted with a transcriptional orientation opposite to both the *neo* and Nurr1 genes. The cloning plasmid used in this vector construction was PSP72 (Promega). The targeting vector was linearized by the restriction enzyme *Not*I located in a synthetic linker at the 3′ end of the long arm of homology.

Introduction of Targeting Vector into Mouse Embryonic Stem (ES) Cells. The general procedures for culturing and manipulating ES cells before and after the electroporation step were followed as described (26). Briefly, 10^7 ES cells were electroporated with 25 μg of linearized targeting vector in 0.9 ml of PBS at 230 V and 500 μF with a Bio-Rad Gene Pulser. Electroporations were performed routinely using the actively growing ES cell line AB-1 (27). ES cells were cultured in the presence of G418 (350 μg/ml) and 1-(2-deoxy-2-fluoro-β-D-arabinofurnaosyl) 5-iodouracil (FIAU) drug selection. Drug-resistant ES cell colonies were picked and expanded in 96-well SNL76/7 feeder plates (master plates). A duplicate gelatinized 96 well (no feeder layer) of each master plate was also prepared to identify targeted events by Southern blot analysis. The master plates containing the ES cell clones for blastocyst microinjection were frozen at −70°C until identification of those ES cells scoring positive for the targeted event.

Generation of Chimeric Mice and Germ-Line Transmission of the Nurr1 Mutation. Four targeted ES cell clones were tested for germ-line transmission of the Nurr1 mutation. ES cells (13–15) were microinjected into the blastocoel of 3.5-day-old blastocyst stage embryos derived from C57BL/6 females. Embryos were transferred unilaterally into the uterine horn (six to seven embryos per horn) of pseudopregnant F_1 (CBA × C57BL/6) foster mothers. Approximately 10 days after birth, the sex of the offspring was determined and the extent of agouti coat color was evaluated. Male chimeras with 60% to 100% agouti coat color were backcrossed to C57BL/6 females, and germ-line transmission was determined by the presence of agouti offspring.

Screening of ES Cells and Mouse Tail DNA for Targeted Events. To identify the Nurr1 mutation in ES cells, Southern blot analysis was performed on genomic DNA isolated from ES cells colonies. DNA samples were digested with *Bam*HI overnight, resolved by electrophoresis, and transferred onto nylon membranes for hybridization with a radiolabeled 0.9-kb *Hin*dIII–*Eco*RV genomic DNA fragment located outside but immediately 5′ to the disrupted nurr1 genomic fragment (see Fig. 1*A*). Hybridization and washing conditions were as described (28). To detect germ-line transmission of the null mutant allele, PCR analysis of tail DNA from agouti offspring from chimeric mice was carried out. Three oligonucleotides were used in a single PCR for genotyping. They consisted of a 5′ primer (GGCACTCCTGTGTCTAGCTGCC) located on the 5′-end of the *neo*^{r} gene in exon 3 and two 3′ primers, one (CTGCCTTGGGAAAAGCGCCTCC) located in the *neo*^{r} gene to generate a 200-bp PCR product representing the mutated allele and the other (CAGCCCTCACAAGTGCGAACAC) located in a 3′ portion of exon 3 that was deleted in the targeting vector to allow selective detection of the wild-type allele as a 300-bp product.

Determination of TH Activity, Catecholamine, and Related Compounds. TH was measured using coupled nonenzymatic decarboxylation of L dopa (29). Briefly, mouse striatal tissues after dissection were homogenized in 50 mM of cold Tris·HCl buffer containing 1 mM EDTA and 0.2% Tween 20, pH 7.2 (1:20 vol/vol) with a Teflon homogenizer. Twenty-five microliter aliquots of homogenate were incubated in a microwell culture plate with reaction solution containing [^{14}C]tyrosine [NEN, specific activity 48.6 mCi/mmol (1 Ci = 37 GBq)] and cofactors at 37°C for 20 min. The L-[^{14}C]dopa formed was decarboxylated by adding 33 mM potassium ferricyanide and heating the mixture at 55°C for 30 min. The [^{14}C]CO_2 released was absorbed on filter paper impregnated with benzethonium hydroxide and quantified by counting the radioactivity on the paper covering each well.

Catecholamines and related compounds in homogenized tissue were extracted with 10% perchloric acid (1:10 vol/vol), clarified by centrifugation, and chromatographed by HPLC on a BAS P/N reversed-phase cartridge column (Phase-II ODS 3 μm × 100 × 3.7 mm). The acid extract was applied isocratically and detected electrochemically according to published procedures (30).

Measurement of Choline Acetyltransferase (Chat) Activity. Chat activity was assayed in the caudate nucleus, hippocampus, and frontal cortex, using a slight modification of Fonnum's method (31). After brain tissues were homogenized in 10 mM (1:50 vol/vol) of cold PBS containing 1 mM of EDTA (pH 7.4), and centrifuged at 10,000 × *g* for 10 min, 25 μl of supernatant was incubated with 75 μl of assay medium (150 mM NaCl/0.5 mM EDTA/0.15 mM eserine/0.2 mM [^{3}H]acetyl-CoA/5 mM choline chloride/50 mM PBS) in 96-well plate microwells. After 45 min incubation at 37°C, reactions were terminated by transferring 80 μl of incubation mixtures into vials containing Fonnum's scintillation solution, and were counted in the Rockbeta LKB scintillation counter.

Immunohistochemistry and *in Situ* Hybridization. For immunohistochemical studies, brains of wild-type and mutant newborns were fixed in 4% paraformaldehyde for 12–24 hours at 4°C and soaked in 30% sucrose overnight, frozen, and cut on a cryostat to 20 μm. Frozen sections were stained according to standard avidin–biotin immunohistochemical procedures (Vector Laboratories). Primary antisera included polyclonal rabbit antiserum against TH and AADC diluted 1:600 (Eugene Tech International, Inc.) and mouse monoclonal antiserum 3A10 diluted 1:200 (Developmental Studies Hybridoma Bank). *In situ* hybridization with TH, Ptx-3, HNF3β, and Nurr1 riboprobes were performed as described previously (20, 22).

Apoptosis Detection. Embryos were obtained by dissection of pregnant mice at specific stages of pregnancy [noon of the day on which the copulatory plug was detected was designated day 0.5 of gestation (E0.5)]. The embryos and newborn brains were fixed in 4% paraformaldehyde, dehydrated by washing in graded alcohol solutions, embedded in paraffin, and sectioned at 5 μm. To identify apoptotic cells, we used the trevigen apoptotic cell system *in situ* kit (TACS) from Trevigen (Gaithersburg, MD) according to manufacturer's recommendations.

RESULTS

Targeted Disruption of the *Nurr1* Gene in Mice. The *Nurr1* gene was disrupted in mouse embryonic stem cells using the targeting vector shown in Fig. 1. The vector contained a 7.6-kb

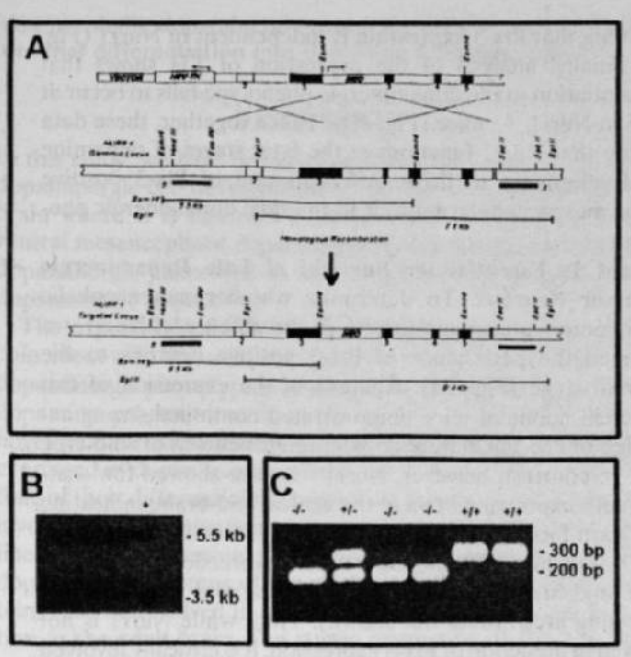

FIG. 1. Targeted inactivation of Nurr1 gene in mouse ES cells and generation of mutant mice. (*A*) Schematic diagram of the strategy used to target Nurr1 gene. (*Top*) The 13.7-kb targeting vector used for electroporation. Numbered boxes represent exons. The PGK-neobpA and the HSV-thymidine kinase cassettes are indicated by open boxes. The arrows indicate the direction of transcription. (*Middle*) Genomic structure of the Nurr1 gene. (*Bottom*) Representation of the structure of the inactivated Nurr1 gene. (*B*) Southern blot analysis of the DNA from G418-resistant ES cells. The DNA was digested with *Bam*HI and hybridized to a 900-bp genomic probe located upstream of the 5′ homologous arm. This probe hybridized to a 5.5-kb and a 3.5-kb fragment from wild-type and mutant alleles, respectively. (*C*) PCR analysis of mice derived from heterozygous Nurr1 (+/−) crosses. Two PCR products are shown at 300 bp and 200 bp correspond to the wild-type and mutant alleles, respectively.

fragment of the mouse *nurr1* gene interrupted within exon 3 by the introduction of a neomycin resistance cassette (neo^r) downstream of the initiator ATG and upstream of the DNA-binding domain of *nurr1*. Targeted integration of this vector into the *nurr1* genomic locus was detected by Southern blot analysis of *Bam*HI-digested ES cell DNA using a ^{32}P-labeled genomic probe (900 bp) located 5′ to the *nurr1* sequences used in the targeting vector (Fig. 1*A*). Using this strategy, the wild-type *nurr1* gene is represented by a 5.5-kb radioactive band containing exons 2–4, whereas the mutated *nurr1* allele is represented by a shorter hybridizing band at 3.5-kb due to the presence of an additional *Bam*HI site within the neo^r gene (Fig. 1*B*). Targeted ES cell clones were microinjected into blastocysts to generate chimeric mice and germ-line transmission of the mutant allele was detected by PCR analysis of tail DNA from agouti offspring of chimeras derived from one ES cell clone. In these analyses (see *Materials and Methods*), the wild-type *nurr1* gene is represented by a 300-bp PCR product while the mutated allele is represented by a 200-bp PCR product (Fig. 1*C*). Genotype analysis indicated that homozygote Nurr1 null mutant ($Nurr1^{-/-}$) mice were born at the expected frequency but consistent with previous findings these mice died within 12 hours after birth (19).

Ventral Midbrain Dopaminergic Neurons Specifically Require Nurr1 for Final Differentiation. To examine the role of nurr1 in development of the dopaminergic system, we analyzed the expression of two dopaminergic cell markers, TH and AADC, in the substantia nigra and ventral tegmental area of wild-type and $nurr1^{-/-}$ neonatal mice by immunohistochemistry (Fig. 2 *A–D*). Consistent with previous findings, ablation of nurr1 resulted in a complete absence of both markers, confirming that nurr1 is essential for expression of a mesencephalic dopaminergic cell phenotype. Analysis of striatal neurotransmission confirmed that this defect is associated with a significant decrease in TH activity at the striatal axon terminals (Fig. 2*F*), a complete loss of dopamine from the striatum of $Nurr1^{-/-}$ mice and a significant decrease in dopamine levels in heterozygote mice (Fig. 2*E*). Furthermore, analysis of the levels of catecholamines, serotonin, and cholinergic activity demonstrated that the defect in striatal neurotransmission was specific to dopamine because levels of norepinephrine, serotonin, and Chat were uneffected (Fig. 2 *E* and *G*). Finally, to determine whether the defect was specific to the mesencephalic dopamine neurons, we examined TH expression and dopamine levels in neural crest-derived cells of the adrenal medulla that also express Nurr1 (Fig. 3*A*). Comparison of TH immunoreactivity (Fig. 3 *C* and *D*) and dopamine transmitter levels (Fig. 3*B*) in wild-type and $Nurr1^{-/-}$ mice revealed no abnormalities associated with Nurr1 ablation, indicating that Nurr1 is not required for development of these cells. Similarly, no changes in TH expression were observed in dopaminergic neurons of the periglomerular region of the olfactory bulb in nurr1 null mutant mice (Fig. 3 *E* and *F*) even though these neurons normally express nurr1 (21).

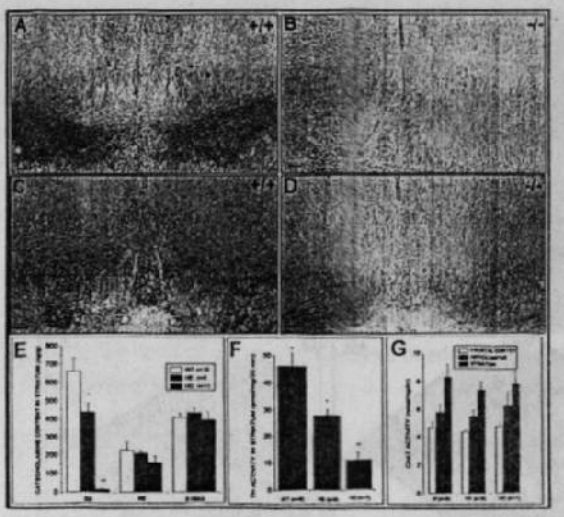

FIG. 2. Analysis of monoamines and Chat levels in newborn mice. Coronal sections (*A* and *C*) show the TH and AADC immunostaining, respectively, in the substantia nigra (A9) and ventral tegmental (A10) area of wild-type brain. (*B* and *D*) Loss of both markers at the same level in coronal sections of $nurr1^{-/-}$ mice. (*E*) Monoamine levels were measured in the striatum of newborn wild-type, heterozygous, and homozygous mice by HPLC. A complete loss of dopamine levels in the homozygous mice and a significant decrease in the heterozygous mice was detected. The TH activity in the striatum (*F*) was significantly decreased in the homozygous and heterozygous mice, while choline acetyltransferase activity (*G*), norepinephrine, and serotonin (*E*) were uneffected. (Bars = 100 μm.)

Nurr1 Is Essential for Terminal Differentiation of Late Mesencephalic Precursor Neurons into a Full Dopaminergic Phenotype. To examine the stage in mesencephalic dopaminergic cell development at which Nurr1 functions, we examined the phenotype of cells in the ventral midbrain of wild-type and Nurr1 null mutant embryos at embryonic day 11.5 using specific markers of the dopaminergic developmental cascade. First, we determined whether the cells have responded to the SSH inductive signal by adopting a general neuronal phenotype. Comparison of the expression of the ventral marker, HNF3β, and the general neuronal marker, 3A10 (32), in wild-type and $Nurr1^{-/-}$ mice showed similar expression of both markers in wild-type and $Nurr1^{-/-}$ mice demonstrating that ventralization and general neuronal induction of neuroepithelial cells in the ventrolateral neural plate are uneffected by Nurr1 ablation (Fig. 4 *A* and *B*). Next, we examined the

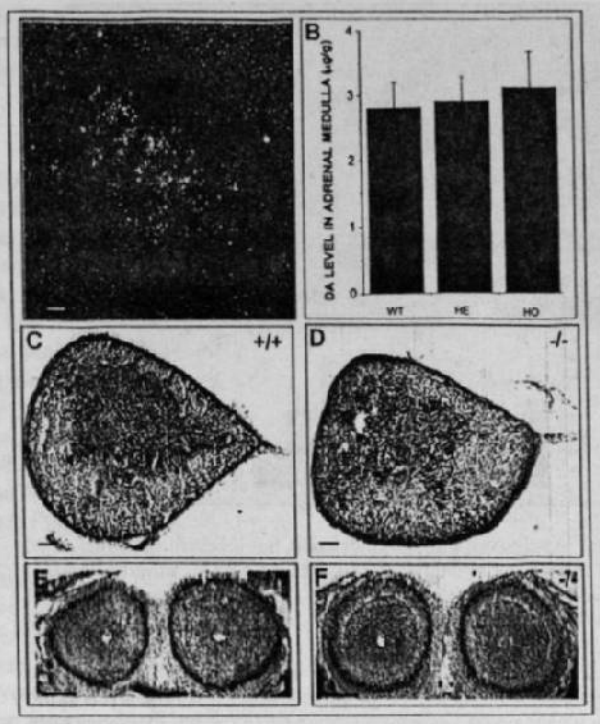

FIG. 3. TH expression and dopamine levels in neural crest-derived cells of the newborn adrenal medulla. (*A*) *In situ* hybridization analysis of Nurr1 mRNA expression in the adrenal gland of a wild-type mouse. Nurr1 mRNA was detected in the medulla of the adrenal gland. (*B*) Dopamine levels in adrenal medulla of wild-type, heterozygous, and homozygous mice. No significant differences in dopamine levels were detected. TH immunoreactivity of wild-type (*C*) and mutant adrenal gland from newborn mice (*D*). (*E* and *F*) No detectable differences in TH immunoreactivity in the periglomerular dopaminergic cells of the olfactory bulb in wild-type and nurr1 null mutant mice, respectively. (Bar = 50 μm.)

expression of the recently identified homeodomain protein, Ptx-3, as a marker for mesencephalic dopaminergic progenitor cells whose onset of expression at embryonic day 11 is subsequent to that of Nurr1 (22). Surprisingly, both wild-type and Nurr1$^{-/-}$ mice showed similar expression patterns of Ptx-3 that appeared to be in all cells of the ventral midbrain, indicating that Ptx-3 expression is independent of Nurr1 (Fig. 4*C*). Finally, analysis of the expression of TH shows that differentiation to the dopaminergic phenotype fails to occur at E11.5 in Nurr1$^{-/-}$ mice (Fig. 4*D*). Taken together, these data indicate that Nurr1 functions at the later stages of dopamine cell development to drive differentiation of Ptx-3 positive ventral mesencephalic neurons to the final dopaminergic phenotype.

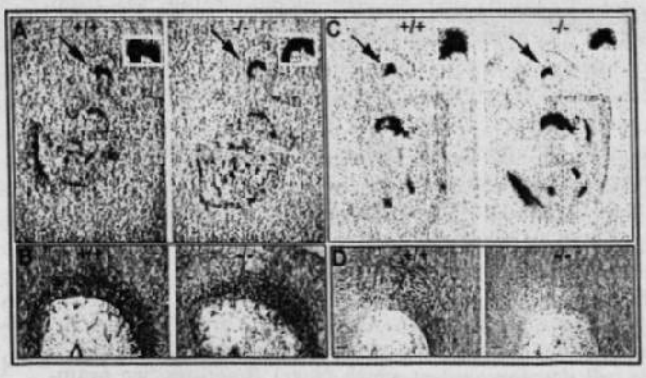

FIG. 4. Analysis of the phenotype of cells in the ventral midbrain of wild-type and Nurr1 mutant embryos. (*A*) Autoradiographic localization of hybridization to the ventral marker, HNF3β. The arrow shows the strong staining in the ventral part of the midbrain of both 12.5 days wild-type (*Left*) and mutant (*Right*) mice embryo. The square on the top shows a higher magnification of this expression. (*B*) Immunostaining for the general neuronal marker, 3A10, in 11.5 day wild-type and mutant mouse embryos. (*C*) *In situ* hybridization analysis of Ptx-3 mRNA expression in 11.5 day mouse embryo. The arrow indicates the positive staining in the ventral midbrain. Wild-type and mutant mice showed similar staining. (*D*) Immunohistochemical localization of TH expression in the ventral midbrain of 12.5 day wild-type embryo and lack of expression in the nurr1$^{-/-}$ midbrain. (Bars = 20 μm.)

Nurr1 Is Essential for Survival of Late Dopaminergic Precursor Neurons. To determine whether mesencephalic dopaminergic precursors survive in the absence of Nurr1, we examined the persistence of Ptx-3 positive neurons to the neonatal stage (Fig. 5*A*). Analysis of the expression of this marker in neonatal mice demonstrated continued strong expression of Ptx-3 in mature dopaminergic neurons of wild-type mice. In contrast, however, Nurr1$^{-/-}$ mice showed few scattered cells expressing Ptx-3 in the ventral mid-brain, indicating significant loss of Ptx-3 positive cells in the neonate (Fig. 5 *C* and *D*). Furthermore, the loss of Ptx-3 expression was specific to the mid-brain region and was not observed in other Ptx-3 expressing areas (data not shown). Thus, while Nurr1 is not involved in induction of Ptx-3 expression, it is critically involved in maintenance of Ptx-3 expressing cells. To determine whether the loss in PTX-3 expression was associated with a loss of ventral midbrain cells, we compared the levels of apoptosis in wild-type and Nurr1$^{-/-}$ mutant neonates using a TUNEL assay. The results of these analyses demonstrated that the loss in Ptx-3-expressing cells was associated with an increase in the number of apoptotic and dying cells that was specific to the ventral midbrain of the Nurr1 null mutant mice (Fig. 5 *E* and *F*). Quantitation of apoptotic cells in the substantia nigra and VTA regions in three independent wild-type and Nurr1 null mutant neonates confirmed an increase in apoptotic cells from 0.5% in wild-type mice to 7% in Nurr1 null mutant mice. Finally, the increase in cell death was consistent with an obvious decrease in the number of cells observed in the neonatal ventral midbrain (see Fig. 5 *C* and *E* versus *D* and *F*). These data indicate that Nurr1 is required for survival of

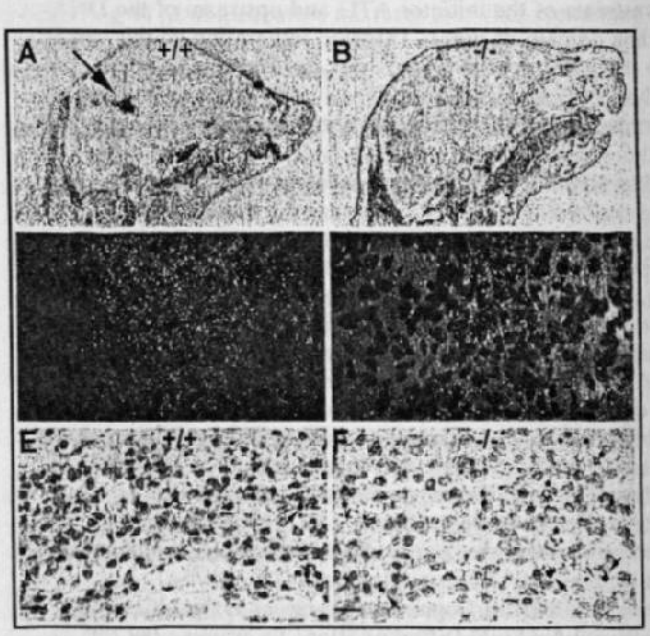

FIG. 5. Loss of Ptx-3 expression and increased cell death specifically in the ventral midbrain region of newborn Nurr1$^{-/-}$ mice. (*A* and *C*) Localization of Ptx-3 expression in the ventral midbrain (arrow) of wild-type neonate. (*B* and *D*) Ptx-3 staining is almost depleted in the Nurr1$^{-/-}$ midbrain. TUNEL staining in the ventral midbrain region of wild-type (*E*) and Nurr1$^{-/-}$ mice (*F*). Notice the increase in TUNEL positive nuclei (arrowheads) and dying cells (asterisk) in the Nurr1$^{-/-}$ mice. (Bars = 20 μm.)

mesencephalic dopaminergic precursor cells as well as their terminal *differentiation into dopamine producing cells.*

DISCUSSION

In this study, we have demonstrated that the role of Nurr1 in dopaminergic cell development is specific to the ventral midbrain where it is essential to induce final differentiation of ventral mesencephalic dopaminergic precursor neurons into a dopaminergic *phenotype as well as for survival of these late* dopaminergic precursors.

The expression of Ptx-3 in developing dopaminergic cells coincident with the onset of expression of a fully differentiated dopaminergic phenotype provides an excellent marker for late dopamine progenitor cells in the mid-brain (22). Ptx-3 is a novel bicoid-related homeobox gene product that is strongly expressed in dopaminergic cells of the ventral midbrain at the time of their differentiation, suggesting that the protein may be involved in determination of the mesencephalic dopaminergic lineage (22). Expression of Ptx-3 and nurr1 persists in midbrain *dopaminergic neurons of the adult, indicating that* both proteins may be involved in maintenance of dopamine cell function in the adult brain. The strong expression of Ptx 3 in the ventral mid-brain of Nurr1 $^{-/-}$ embryos indicates that induction of expression of this protein and the development of late dopamine precursor neurons are independent of Nurr1. In contrast, Nurr1 is essential for induction of a dopaminergic phenotype in Ptx-3 positive precursor neurons. In this regard, it is possible that Ptx-3 and Nurr1, although regulated independently, may function in a cooperative manner to regulate factors required for terminal differentiation of dopaminergic neurons. *Consistent with this hypothesis*, cooperative interactions between a nuclear orphan receptor (FTZ-F1) and a homeodomain protein (FTZ) have been demonstrated recently (33, 34). In the case of Nurr1, such cooperative interactions may explain the specificity of the neuronal defects in midbrain dopaminergic neurons because Ptx-3 is not expressed in other Nurr1-containing neurons, including the limbic system and dopaminergic neurons of the olfactory bulb and hypothalamus (20, 21).

The observation that Nurr1 is essential for maintenance of Ptx 3 positive progenitors raises speculation as to the role of *Nurr1 in dopaminergic cell survival.* Increased cell death in Nurr1 $^{-/-}$ neonates may simply be due an inability of undifferentiated dopamine progenitors to make synaptic contacts with their targets. Previous studies using mutant mice in which TH was ablated in dopaminergic neurons have indicated that dopamine-mediated signaling does not appear to be required for functional synaptogenesis of neuronal projections from the substantia nigra to the striatum (35). However, it cannot be ruled out that loss of all markers of the dopaminergic phenotype as is observed in the Nurr1 null mutant mice has an impact on the establishment of such connections. A second possibility *is that Nurr1 may be required for the* expression of factors that promote survival as well as differentiation of dopamine progenitors. Dopaminergic cell survival is known to be regulated by the neurotrophins, brain-derived *neurotrophic factor* (BDNF) and neurotrophin 4/5 (36–38) and by members of the TGFβ family of trophic factors, most notably glial-derived neurotrophic factor (GDNF) (39, 40). The trophic effects of these factors on dopamine cell survival are apparently redundant because ablation of individual members of these families or their receptors does not result in dopamine cell loss (41–43). However, the role of these factors in promoting survival and differentiation of *late dopamine precursor* neurons is unknown. A role for nurr1 in regulation of survival of both dopamine precursors and differentiated dopaminergic cells at least in part through regulation of these factors and/or their receptors is supported by the observation that ablation of Nurr1 results in loss of expression of the tyrosine kinase signal transducing receptor for GDNF, c-ret (19) and by the identi*fication of binding sites for* Nurr1 in the promoter region of the BDNF gene (44). Thus, it is possible that Nurr1 may be a transcriptional regulator of several of these genes. Further studies to examine the role of Nurr1 in the regulation of neurotrophic factors and their receptors should provide im*portant* insights into the role of Nurr1 in dopaminergic cell commitment and survival.

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