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A Divergent Role of COOH-Terminal Domains in Nurr1 and Nur77 Transactivation

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Orphan nuclear receptors such as Nurr1 and Nur77 have conserved amino acid sequences in the zinc finger DNA binding domains and similar COOH-terminal regions, but have no known ligands. These receptors can bind DNA sequences (response elements) as monomers and can also heterodimerize with the retinoid X receptor to activate transcription. We report here the identification and initial characterization of a novel COOH-terminal truncated isoform of Nurr1, Nurr1a. Internal splicing of Nurr1 generates a frame-shift such that a stop codon is prematurely encoded resulting in a naturally occurring COOH-terminal truncation. Embryonic and postnatal mouse brain showed both Nurr1 and Nurr1a mRNAs expressed during development. To characterize essential COOH-terminal elements that may be deleted from Nurr1a and determine function in putative ligand binding, we created COOH-terminal deletion mutants. Nurr1, Nur77, and 3′-truncated mutants bind in gel mobility shift assays to the monomeric Nur77 response element (B1A-RE). However, in transient transfection assays, a truncation of as little as 15 Nurr1 COOH-terminal amino acids diminished transcriptional activation of B1A-thymidine kinase-chloramphenicol acetyltransferase reporter. This result was not seen for a similar Nur77 deletion mutant, Nur77-586. Unlike full-length Nurr1 and Nur77, transactivation by Nur77-586 was not augmented in response to the presence of retinoid-like receptor and 9-*cis*-retinoic acid. Thus, the interaction of putative ligand binding and transactivation for Nurr1 and Nur77 may function differently.

Nurr1 Nur77 Transcriptional regulation RXR 9-*cis*-RA Nurr1a Alternative splicing

MEMBERS of the steroid/thyroid hormone nuclear receptor superfamily play an important role in complex processes such as differentiation, development, and oncogenesis (29,43,45). Receptors function by linking cellular signals directly to the nucleus where they alter the rate of transcription. Shared structural components have been used to classify these receptors such as the DNA binding domain (DBD), a Cys2-Cys2 zinc finger structure, which is highly conserved among family members and allows for DNA-protein interaction. Many family members have been isolated based on their conserved DBD sequence (17). The COOH-terminal region is conserved to a lesser degree. This region is often referred to as the ligand binding domain, but other functions such as dimerization, nuclear localization, and transactivation/repression are also associated with the COOH-terminal domain. Ligand-bound receptors directly interact with nuclear DNA at specific sites usually found in promoter regions. These specific DNA sequences, known as response elements (RE), are also used to differentiate among family members.

Orphan nuclear receptors are transcription fac-

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tors that share sequence homology with the steroid/thyroid receptor superfamily but have no known ligand (28,33). Although it is thought that ligands might be identified for most of the orphan receptors, some may not require ligand for activation of transcription. Nur77 [also known as NGFI-B (31), N10 (40), and TR3 (7)], an immediate-early response gene, is thought to be an example of a ligand-independent receptor (19). Nur77 mRNA increases in response to a variety of stimulants such as serum growth factors, epidermal growth factor, nerve growth factor, and membrane depolarization factors (2,31,40,52). Post-translational modifications such as phosphorylation of Nur77 have also been shown to affect receptor–RE interaction (10,13,18), although gene-targeting experiments resulting in disruption of the Nur77 gene show no discernible phenotype (9,26).

Another orphan nuclear receptor, Nurr1 [also known as RNR-1 (42), HZF-3 (37), and NOT (27)], may also function as a ligand-independent receptor (24). Nurr1 is highly homologous with Nur77 in the DBD and COOH-terminal regions and can bind specifically to the same response element (34,42). Both Nurr1 and Nur77 have been shown to heterodimerize with RXR utilizing the B1A-RE (16) as well as the retinoic acid response element composed of direct repeats spaced by 5 nucleotides (38). Although Nur77 and Nurr1 are structurally similar (5), differences have been reported in their tissue distribution, responses to various stimuli, and developmental expression of mRNAs. Nur77 is expressed postnatally in a variety of tissues, whereas Nurr1 transcripts are primarily localized in brain tissue throughout fetal development into adulthood (24,51,54,55). Nurr1 has also been found in peripheral blood cells (27), regenerating liver (42), and adrenal gland (11). Like Nur77, Nurr1 responds to membrane depolarization factors, but does not respond to growth factor stimulation (24). Recently reported Nurr1 gene disruption experiments show that Nurr1 produces a mouse phenotype that is dramatically different from Nur77 knockouts (4,53). Mice deficient in Nurr1 die soon after birth and have selectively abolished dopamine biosynthesis in the substantia nigra/ventral tegmental area of the brain.

To determine the specific roles of Nurr1 and Nur77 during cellular signaling and transcriptional activation, additional analysis and characterization of the orphan nuclear receptors is necessary. The role of carboxyl-terminal activation function domain (AF2), which contains two sets of heptad leucine repeats, has yet to be fully understood. We report the identification of a carboxyl-truncated isoform of Nurr1, Nurr1a, which is expressed during embryonic and postnatal development in mouse brain tissue. Although 143 amino acids are deleted from the COOH-terminus of Nurr1a, both Nurr1- and Nurr1a-translated mRNAs are able to interact with the same B1A-RE, albeit to different degrees. Isolation of this naturally truncated Nurr1 isoform suggests multiple mechanisms of transcriptional control brought about by variations of the orphan COOH-terminal regions. Additional functional data using NIH3T3 cells and COOH-terminal truncated mutants indicates the last 15 amino acids (aa) of the Nurr1 COOH region are required for full transactivation in association with the B1A-RE. In contrast, a 15-aa truncation of Nur77 retained wild-type-like activation through the B1A-RE and showed no augmented activation in the presence of RXR and 9-*cis*-retinoic acid (RA), unlike results observed with full-length Nur77 receptor. Thus, multiple mechanisms of transcriptional control may be brought about by changes in the orphan COOH-terminal regions.

MATERIALS AND METHODS

Materials

All DNA restriction enzymes were obtained from New England Biolabs, Inc.; [γ-^{32}P]ATP (5000 Ci/mmol) and [^{14}C]chloramphenicol (60 Ci/mmol) were purchased from Amersham Corp. and DuPont NEN, respectively.

Cloning and Sequencing of Nurr1, Nurr1a, Nur77, and Deletion Mutants

A mouse brain cDNA library purchased from Stratagene (La Jolla, CA) was screened for Nurr1 isoforms by PCR as previously described (22). PCR amplification with restriction enzyme tagged primers containing 5′ *Hin*dIII site or 3′ *Hpa*I sites were used for subcloning coding regions of Nurr1, Nurr1a, and Nur77 cDNAs into an RSV expression vector (ATCC, Rockville, MD). A Kozak sequence was also included upstream of the first encoded methionine of Nurr1, Nurr1a, Nur77, and deletion mutants to enhance transcription. Subsequent clones were derived from a *Hin*dIII/*Hpa*I restriction enzyme digest encoding full-length cDNAs. Nested oligonucleotide primer sets were used to sequence both strands of Nurr1 and Nurr1a cDNA clones. Nur77 cDNAs were kindly provided by Dr. Lau.

Nurr1 deletion clones were constructed from pSP72 subclones using naturally occurring restriction enzyme sites and either 5′ *Hin*dIII or 3′ *Hpa*I vector sites. In-frame stop codons were inserted by subcloning double-stranded oligonucleotides of approximately 20 nucleotides with overhanging restriction enzyme sites. The most COOH-terminal deletion mutant, Nurr1-583, was constructed as Nur77-586. Both were cut with restriction enzymes *Kpn*I/*Hpa*I and annealed to identical oligonucleotide sequence containing an in-frame stop codon. All clones were sequenced as described above to confirm truncation. Nurr1 R342G was isolated from the original pool of full-length Nurr1 PCR clones and found to have a substitution of an adenine for a guanine at nucleotide 1361 (reference accession sequence S53744) resulting in an amino acid change of an arginine to a glycine at amino acid 342.

RNA Isolation, Reverse Transcription, and PCR

Fetal, neonatal, and young adult mice were sacrificed and used for tissue collection. Brain tissue was immediately frozen in liquid nitrogen and stored at −80°C until analyzed. Total RNA was isolated from frozen tissue samples by homogenization and extraction with Stat-60 (Friendswood, TX) according to the manufacture's suggestions. Hybridization of 150 ng of oligonucleotide B (5′-accccattattgaaagtcac-3′; nucleotides 1951-1971) to 6 μg of total RNA was performed by heating at 95°C for 5 min followed by fast cooling on ice. Partial Nurr1 cDNAs were synthesized according to the Super Script preamplification system (Gibco/BRL, Gaithersburg, MD) at 42°C for 1 h.

Oligonucleotide primers used to amplify cDNAs were complementary to Nurr1 coding regions located upstream and downstream of the deleted region in Nurr1a. Primer A (150 ng, 5′-cagagaagatccctggctttg-3′; nucleotides 1598-1618) was paired with primer B (150 ng) for PCR amplification with 1 unit of *Taq* DNA polymerase (Promega, Madison, WI). The reaction was brought to a final volume of 100 μl with 1 × PCR buffer and topped with a thin layer of mineral oil. Each reaction was subjected to 40 PCR cycles as previously described (35) using a Perkin Elmer thermal cycler 480. Mineral oil was subsequently removed by extraction with chloroform and amplified DNA fragments were separated on 2% agarose gel and sized using a 100-bp DNA ladder (Gibco/BRL).

To verify the identity of the PCR products, DNA fragments were transferred by Southern blotting to Magna nylon membranes (MSI, Westborough, MA). Blots were hybridized with a ^{32}P-end-labeled oligonucleotide C (5′-tctctctgtgacca tagccag-3′; nucleotides 1861-1881) specific to Nurr1, which is nested between primers A and B, washed according to the protocol recommended by MSI, and exposed to Kodak XAR-5 film with intensifying screens at −80°C. PCR products were cloned into pCRscript according to Stratagene and sequenced as described above.

Amplification of Genomic Nurr1 DNA

Partial cloning of the Nurr1 gene was carried out by PCR amplification. Putative exon/intron junctions were predicted by sequence alignment between the Nur77 gene (accession number X16995) and Nurr1 cDNA. Oligonucleotide primers were designed to flank alternatively spliced region in Nurr1 (primer 1 5′-tctatggagatcatcagaggg tgggcagagaagatc-3′ and 3′ primer 2 5′-tttgtagct cttccactctcttgggttccttgagcc-3′). Amplification was carried out as described by Barnes (1). In brief, 100 ng of mouse genomic DNA was PCR amplified with a mixture 15:1 of Klentaq1 (Ab Peptides, St. Louis) and Pfu (Stratagene) polymerase for 24 cycles of 30 s at 99°C, 30 s at 67°C, 15 min at 68°C. PCR fragments were cloned into pCRscript vectors and sequenced.

In Vitro Transcription, Translation, and Mobility Shift Assay

In vitro transcription and translation of Nurr1, Nurr1a, Nur77, and deletion mutants were performed according to Promega TnT Coupled Reticulocyte Lysate System manufacturer's recommendation. DNA clones were transcribed with SP6 or T7 RNA polymerase from pSP72 or pBluescript recombinant plasmid and used to program rabbit reticulocyte lysate either in the presence or absence of [^{35}S]methionine. Radiolabeled proteins were separated on 10% sodium dodecyl sulfate, Tris-glycine gels (NOVEX, San Diego, CA) and sized to confirm protein truncations and similar quantity.

Synthetic oligonucleotides complementary to the B1A response element (5′-gagttttaaaaggtcat gctcaatttggat-3′), used as probes for mobility gel-shift assays, were end-labeled with [γ-^{32}P]ATP, annealed, and gel purified. Mobility shift assays were performed with up to 4 μl of programmed rabbit reticulocyte lysate in 20 mM HEPES (pH 7.9), 1 mM dithiothreitol, 10% (v/v) glycerol, 80 mM KCl, 1 mM EDTA, 0.5 mM $MgCl_2$, and 1 μg of poly(dI-dC) (Midland) in a total volume of 20

μl. Reactions were incubated 10 min on ice, after which radiolabeled, double-stranded B1A-RE oligonucleotides were added (5–10 × 10^3 cmp). The reaction continued for 20 min on ice. Samples were loaded onto 6% Protogel (National Diagnostics, Atlanta, GA) and run with 0.5% × TBE buffer for 2 h at 130 V. Gels were dried and exposed to Kodak XAR-5 film with intensifying screens at room temperature.

Cell Culture and Transfection Analysis

NIH3T3 cells, a mouse fibroblast cell line, were cultured and transfected as previously described (12,14). Briefly, 10 μg expression vector plasmids containing RSV driving expression of full-length Nurr1 and Nur77 mutants were cotransfected by electroporation with 1 μg pCMV-β-Gal and 20 μg of reporter gene containing one copy of the B1A-RE, thymidine kinase promoter, and chloramphenicol acetyltransferase (B1A-TK-CAT). Cells were cultured in four 60-mm dishes for 72 h with medium containing either 10% resin-stripped or full serum, harvested, lysed, and assayed for β-Gal and CAT activity (32,44). Background counts from cells transfected with pUC19 alone were subtracted from CAT activity, which was then normalized to β-Gal activity (12). In addition, cells also electroporated with 10 μg pRSVRXRβ were grown in the presence or absence of 1 × 10^{-6} M 9-*cis*-RA in 10% resin-stripped (41) fetal bovine serum. Data shown were normalized to the appropriate control assays of B1A-TK-CAT reporter alone, plus RXR, or plus RXR and 9-*cis*-RA.

RESULTS

Isolation and Characterization of the Nurr1a cDNA

During PCR amplification with primers specific to the 5′ and 3′ ends of Nurr1-untranslated regions, Nurr1a was coamplified from a mouse cDNA library. Subsequent cloning and sequencing of the PCR fragments revealed clones either identical in sequence to the previously described mouse Nurr1 cDNA (24) or containing an internal deletion of 121 nucleotides (Nurr1a).

Nurr1a coding sequence (455 aa) is identical to Nurr1 (598 aa) for the first 454 aa including the N-terminal and DNA binding domain before the sequence diverges. The addition of a Nurr1a isoleucine at aa 455 is encoded before the stop codon (Fig. 1A). Although sequence alignment by the best-fit program (GCG, Madison, WI) indicates an internal deletion of 121 nucleotides from Nurr1a, the nucleotide sequence following the deleted region aligns with the COOH-terminal sequence of Nurr1. This internal deletion causes a frameshift resulting in a stop codon following aa 455 of Nurr1a. Thus, the Nurr1a COOH-terminal contains only the first four leucine zipper residues and has lost the last 143 aa, including the second group of four leucine zipper residues.

Alternative Splicing Generates an Additional Nurr1 Isoform, Nurr1a

Sequence comparison of both Nurr1 and Nurr1a cDNAs with the genomic sequence of Nurr1 (5) and Nur77 (46) suggests Nurr1a may be a result of alternative splicing. In order to further characterize the structure of Nurr1a, we partially sequenced the Nurr1 gene to determine the location of donor and acceptor splice sites for both Nurr1 and Nurr1a transcripts. Oligonucleotides used in PCR amplification of the Nurr1 gene were designed specifically to flank the exon region expected to contain an alternative splice site, which deleted 121 nucleotides from the Nurr1a transcript. Predicted oligonucleotide primers were based on known gene structure of Nur77 and its sequence alignment with Nurr1 and Nurr1a cDNAs. PCR amplification, cloning, and sequencing of a fragment of approximately 800 nucleotides revealed that alternative splice sites were used to generate Nurr1a messages (Fig. 1B). Exon regions were separated by two short introns of 323 and 112 nucleotides for Nurr1 and 444 and 112 nucleotides for Nurr1a. Inspection of the splice sites shows that Nurr1a transcripts are generated by splicing to a 3′ acceptor site, which splits the coding sequence within exon 7 at amino acid 494 of the Nurr1 gene (Fig. 1C). Both donor and acceptor sites have conserved essential ends of the intron and conform to the GT-AG rule, which is characteristic for eukaryotic genes. The sequence surrounding the splice site in exon 7 is 5′-CAG GTC-3′, where the underlined AG nucleotides represent intronic acceptor splice sites. This is identical in human, mouse, and rat Nurr1, human and *Xenopus* Nur77, and rat NOR-1, but mouse and rat Nur77 homologues have distinctive consensus sequences at this corresponding region (5′-CCGATC-3′) that would not favor alternative splicing. PCR amplification of two other conserved splice sites found in the Nurr1 gene indicate structural homology between the Nurr1 and Nur77 genes (46).

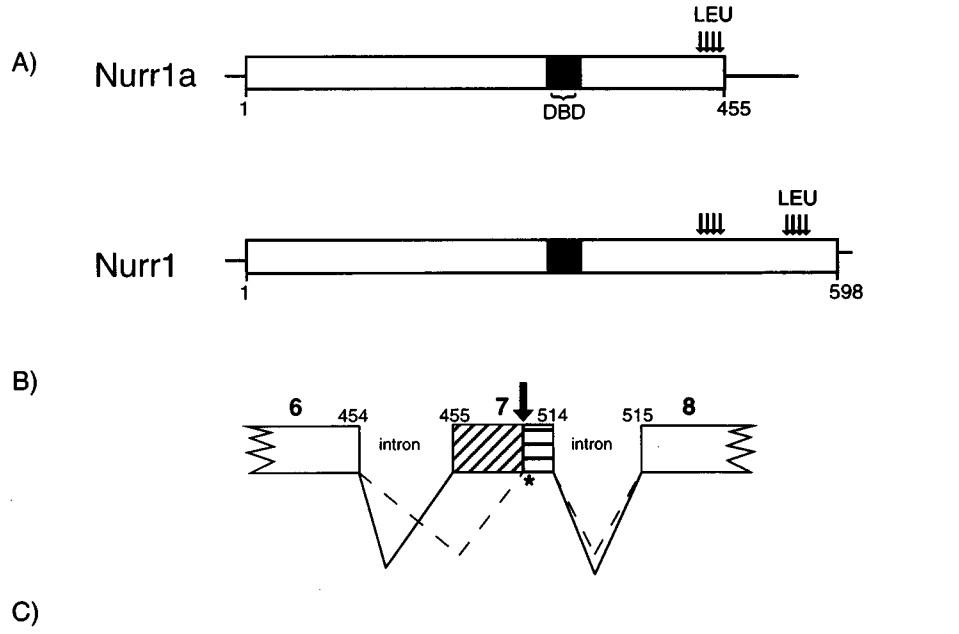

FIG. 1. Comparison of Nurr1 and Nurr1a cDNAs and a partial genomic map. (A) Schematic representation of Nurr1a and Nurr1 coding regions. The DBD and putative leucine zipper residues are represented by the shaded box and groups of four vertical arrows, respectively. (B) A diagram of alternative splicing of the Nurr1 gene. Alternative splicing of Nurr1 or Nurr1a (depicted by the vertical arrow) RNA is indicated by either solid or dashed lines, respectively. Patterned boxes represent exon 7 associated with Nurr1 transcript (diagonal slash and horizontal striped) and Nurr1a (horizontal striped). The small numbers above the exons correspond to Nurr1 amino acid sequence and the asterisk below exon 7 denotes the location of the Nurr1a stop codon. (C) Partial genomic sequence of the Nurr1 gene. Exon nucleotides are capitalized and shown in bold. Two vertical lines mark alternative splice sites for Nurr1 and Nurr1a in exon 7. The stop codon of Nurr1a is indicated by boxed asterisks and the GT-AG intron donor and acceptor sites are underlined. Numbers on the left indicate derived Nurr1 amino acid sequence.

Expression of Nurr1 and Nurr1a in Developing Mouse Brain

Northern blot analysis showed that only a single-size Nurr1 transcript has been identified [(24); data not shown], although different sizes have been reported for different species: 3.5 kb in mouse (24), 3.5 (42) and 2 kb (37) in rat, and 4.2 kb in humans (27). A deletion of 121 nt, as found in the Nurr1a transcript, would be difficult to detected by Northern blotting of a large transcript.

In order to determine whether Nurr1a tran-

scripts occur naturally, we analyzed Nurr1a mRNA expression in brain tissue during development. Total RNA from mouse brain tissue was reverse transcribed with a Nurr1-specific oligonucleotide primer B (Fig. 2A) located downstream from the point of cDNA sequence divergence. Partial Nurr1 cDNAs were PCR amplified with oligonucleotides B and A, common to both Nurr1 and Nurr1a cDNAs. Both predicted Nurr1 (384 nt) and Nurr1a (264 nt) PCR products were detected in embryonic day 18, postnatal days 1, 3, 5, 8, and 14 (Fig. 2B), as well as 4-week (data not shown) brain tissue samples and were confirmed by probing a blot with an oligonucleotide primer nested between primers A and B. Although the RT-PCR assay is not strictly quantitative, in repeated amplifications, 1-day-old brain samples consistently contained the greatest number of Nurr1 and Nurr1a transcripts with Nurr1 messages outnumbering Nurr1a messages. Similar early postnatal expression of Nurr1 has also been shown by Northern blot analysis by Law et al. (24) and has been repeated in our laboratory (data not shown).

Interactions of Nurr1a, Nurr1, Nur77, and COOH-Terminal Deletion Mutants With the B1A Response Element and Transactivation Properties

Because Nurr1a is a naturally occurring form of Nurr1 truncated in the COOH-terminus, it was of interest to determine DNA binding and transcriptional properties of COOH deletion mutants of Nurr1 and for comparison purposes Nur77. Although the DBDs of Nurr1 and Nur77 are nearly identical in amino acid sequence (approximately 92%), changes in the length of the COOH-terminal are known to affect transactivation activity of other steroid/thyroid receptor family members including Nur77 (3,6,39,47). To determine if COOH-terminal deletions of Nurr1 and Nur77 alter interactions with the response element B1A known to bind and function in Nurr1 and Nur77 transactivation (42,50), gel mobility shift and transient transfection assays were performed. In gel mobility shift assays, both Nurr1 and Nur77 bound to B1A-RE efficiently, contrary to the clones containing deletions of the last 15 aa (Nurr1–583 and Nur77–586) and Nurr1a, which gave a weaker binding (Fig. 3A). Several additional Nurr1 COOH-terminal deletion constructs (data not shown) all had similar results to the Nurr1 mutant encoding a terminal deletion of as little as 15 aa (Nurr1–583).

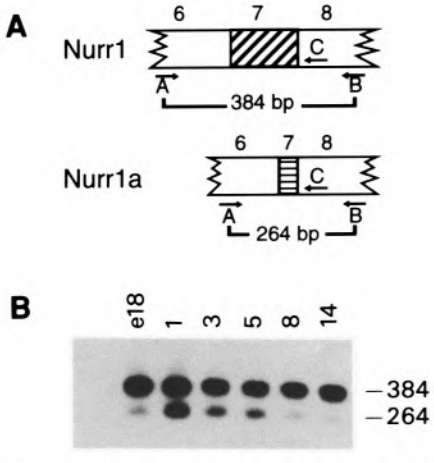

FIG. 2. Expression analysis of Nurr1 and Nurr1a transcripts by RT-PCR. (A) Total RNA from embryonic day 18, (e18), and postnatal days 1, 3, 5, 8, and 14 mouse brain tissue subject to RT-PCR with primers A and B as depicted by the schematic representation. Nurr1 and Nurr1a transcripts were identified by amplification of 384- or 264-nucleotide bands, respectively. Nurr1 and Nurr1a exons, represented by boxed regions, are numbered corresponding to homologous Nur77 exons. (B) Autoradiograph of a Southern blot probed with an exon 8-specific oligonucleotide. Fragment sizes were estimated from a 100-bp ladder on an ethidium bromide-stained agarose gel.

In addition, a Nurr1 clone containing a base substitution resulting in mutation of an arginine for a glycine at amino acid 342 in the A-box (Nurr1 R342G) was tested for binding to the B1A-RE. This A-box region has been previously shown to be essential for Nur77–DNA interaction and function (50). The single point mutation of the arginine in the wild-type Nurr1 completely eliminated detectable binding (Fig. 3A, lane 2). Thus, the A-box of Nurr1 also plays a pivotal role in DNA binding. Estimated size and quantity of Nurr1, Nurr1 R342G, Nurr1–583, Nurr1a, Nur77, and Nur77–586 in vitro transcription and translation proteins are shown in Fig. 3B.

Transient transfection assays were used to investigate the role of the last 15 aa of Nurr1 and Nur77. Transcriptional activation of B1A-TK-CAT reporter was observed when Nurr1, Nur77, and the mutants were individually transfected in NIH3T3 cells (Fig. 4). As expected, the Nurr1 R342G lost transactivation activity. Thus, these transfection data coupled with the gel mobility shift results indicate the importance of the intact Nurr1 A-box for wild-type-like activity.

The induction mediated by Nurr1a was similar to the COOH-terminal deletion mutant Nurr1–583. Both Nurr1a and Nurr1–583 activation was reduced about 50% compared to full-length Nurr1. These results further underscore the importance of the most COOH-terminal amino acids in Nurr1 transactivation. On the other hand, deleting the last 15 aa from the COOH-terminal of

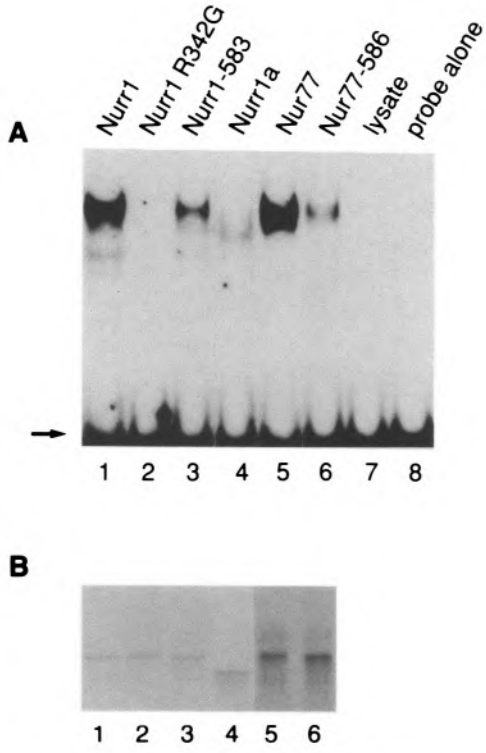

FIG. 3. Interaction of Nurr1, Nurr1a, Nur77, and COOH-terminal mutant constructs with the B1A response element analyzed by gel mobility shift assay. (A) A γ-^{32}P-labeled double-stranded oligonucleotide containing the B1A response element sequence was incubated with Nurr1 or Nur77 programmed rabbit reticulocyte lysates and the resulting complexes were resolved on a 6% native acrylamide gel. Lysate programmed with Nurr1 (lane 1); Nurr1 R342G (lane 2); Nurr1-583 (lane 3); Nurr1a (lane 4); Nur77 (lane 5); Nur77-586 (lane 6); lysate and probe alone (lanes 7 and 8, respectively). The arrow indicates the location of free probe. (B) [^{35}S]Methionine programmed reticulocyte lysates of Nurr1, Nurr1a, Nur77, and deletion mutants were size separate on Tris-glycine gels to determine similar quantity.

Nur77 (Nur77-586 mutant) had no effect on the activity. This outcome is similar to results reported by Paulsen et al. (36) using a 7-aa Nur77 COOH-terminal deletion.

*Transactivation of Nurr1, Nurr1a, Nur77, and COOH-Terminal Deletion Mutants in the Presence of RXR and 9-*cis*-RA*

Nurr1, Nurr1a, Nur77, and the 3′ truncation mutants (Nurr1-583 and Nur77-586) were tested for transactivation activity in the presence of RXR with or without 9-*cis*-RA. Both Nurr1 and Nur77 activated transcription through the B1A-RE either in the presence or absence of coexpressed RXR to the same degree (about threefold) (Fig. 5). The reporter alone containing a single copy of the B1A-RE with RXR and 9-*cis*-RA was not activated above background. Unlike results reported by Forman et al. (16) using CV1 cells, we observed no suppression of Nurr1 or Nur77 activity in NIH3T3 cells when cotransfected with RXR alone. However, in the presence of 9-*cis*-RA both Nurr1-RXR and Nur77-RXR complexes became more responsive, reaching about sevenfold activation.

The transactivation of B1A-RE mediated by Nurr1-583 (Fig. 5) and Nurr1a (data not shown) in the presence of RXR and 9-*cis*-RA was comparable to Nurr1-583 alone. Although a slight increase in activation was observed, these values were minor compare to full-length Nurr1 response. In contrast, the Nur77-586 mutant retained Nur77-like activity when coexpressed with RXR but had little response to the addition of 9-*cis*-RA. Repeated attempts were made to show interactions of RXR with Nurr1 and Nur77 using gel mobility shift assays and the B1A-RE. Nonetheless, only monomeric Nurr1 and Nur77 receptors bound to the B1A-RE were detected (data not shown).

DISCUSSION

Several lines of evidence indicate that Nurr1 and Nur77 might regulate overlapping sets of genes by activating transcription through the same response element. B1A, the response element derived from an in vivo selection system in yeast (48), has been shown to function in transactivation of both Nurr1 (42) and Nur77 (48). Understanding this complex process provides a potential for determining specific regulation of target gene expression.

We report here functional characterization of various carboxyl-terminal Nurr1 and Nur77 mutants in conjunction with the identification and initial characterization of a novel COOH-terminal truncated isoform of Nurr1, Nurr1a. Nurr1a is generated as a result of alternative splicing of the Nurr1 gene and is found expressed during development in the brain. Nurr1a also binds to the B1A-RE, although to a lesser degree than full-length Nurr1, but has little transactivation activity. Experiments were designed such that COOH-terminal and A-box mutations of both Nurr1 and Nur77 help define putative functional role of Nurr1a in vivo and clarify transactivational difference between Nurr1 and Nur77.

DNA-protein interactions between the DBD of the receptors and B1A-RE are dependent on a functional A-box region located 36 aa downstream from the DBD. The A-box was first identi-

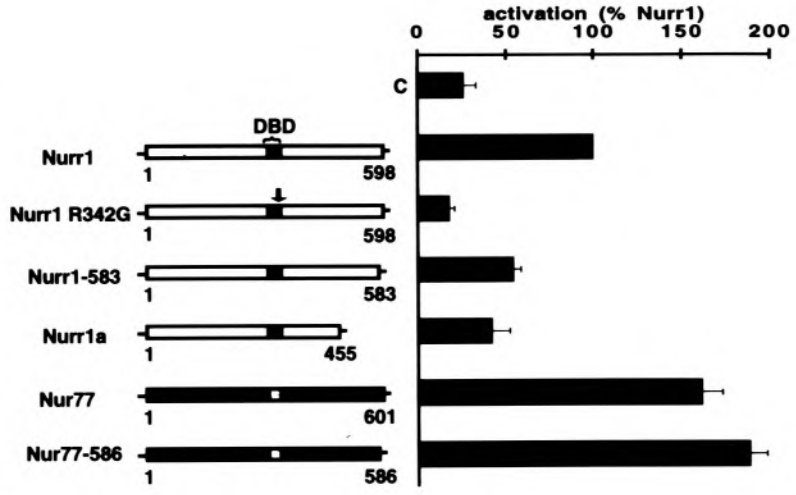

FIG. 4. Transcriptional activity of Nurr1, Nurr1a, Nur77, and the COOH-terminal deletion mutants. Coding regions of Nurr1, Nur77, and deletion constructs are shown schematically on the left where the sequence length is indicated below each bar. The vertical arrow over Nurr1 R342G DBD indicates the location of an A-box mutation were a glycine (G) was substituted for an arginine (R) at amino acid residue 342. NIH3T3 cells were cotransfected with either a Nurr1 or Nur77 expression construct, a β-Gal control plasmid, and a TK-CAT-reporter construct that carried a single copy of the B1A response element. Control (C) indicates transfection of B1A-TK-CAT alone. Relative transactivation activity is expressed as a percentage of full-length Nurr1 with activity expressed as mean ± SD and is depicted by the histogram on the right.

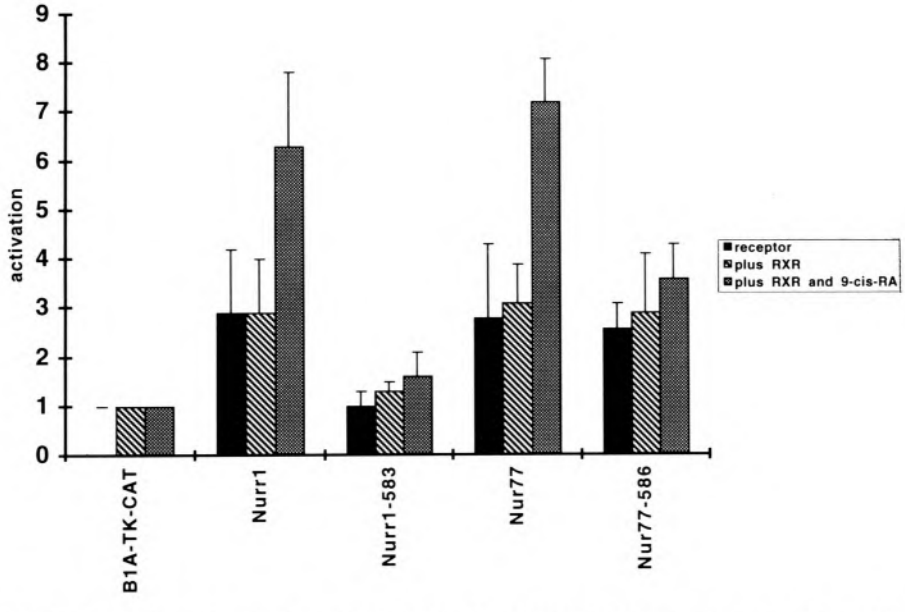

FIG. 5. Transcriptional activation of Nurr1, Nur77, and the COOH-terminal deletion mutants cotransfected with RXR and 9-*cis*-RA. NIH3T3 cells were cotransfected with Nurr1, Nurr1–583, Nur77, or Nur77–586 expression constructs, a β-Gal control plasmid, and a B1A-TK-CAT-reporter construct that carried a single copy of the B1A response element. Open bars: reporter alone; black bars: receptors alone; hatched bars: in the presence of RXR expression vector; stippled bars: receptors cotransfected with RXR and treated with 1×10^{-6} M 9-*cis*-RA. Relative transactivation activity is expressed as fold activation relative to cell transfected with reporter alone. Transfection data are the averages of at least two independent transfection assays performed in duplicate and expressed as mean ± SD.

fied in Nur77 and has been shown to be essential for Nur77 transactivation (50). We show that the A-box of Nurr1, like that of Nur77, is also essential for DNA binding and transactivation. An amino acid substitution of a glycine for an arginine in the second position of the A-box eliminated detectable DNA binding and diminished transactivation activity of Nurr1, suggesting that similar conformational structures may be formed during Nurr1-DNA and Nur77-DNA transactivation. With the completion of extensive X-ray crystal structures and nuclear magnetic resonance imaging, a third helix has been identified in the region located proximate to the zinc finger DBD domain of RXR homodimers (25). It has been hypothesized that this extended surface of interaction between DNA and protein may help stabilize monomeric receptor binding much like that predicted for orphan receptors such as Nurr1, Nur77, and ROR (28,30,49). Thus, a mutation in the A-box region may destabilize the necessary conformational structure essential for transcriptional activation.

Although the mechanism of transactivation through DNA binding seems to be similar for Nurr1 and Nur77, the role of the COOH-terminal region in activation suggests differences. Our results show the COOH-terminus of Nurr1 affects transcriptional activity when assayed by transient transfection. A truncation of as little as 15 aa from the COOH-terminus of full-length Nurr1 reduced receptor transactivation. Nurr1 and Nur77 are identical in 10 of the 15 residues and deletion of the last 15 aa did not remove any of the four heptad leucine repeats, which have been shown by others to greatly control transcriptional activity of some receptors of the superfamily (15,21,23,47). Interestingly, a corresponding deletion in Nur77 had no effect on the activity of the receptor, indicating a significantly different requirement between the transactivational activity of Nurr1 and Nur77. Unlike some other characterized receptors such as those for estrogen (23) and glucocorticoid (3) as well as Nur77 (10,36), additional deletions in Nurr1 (as found in Nurr1a) never restored transactivation activity. Thus, an important domain responsible for transactivation through the B1A-RE by Nurr1 is located within the last 15 aa of the COOH-terminal.

We have shown this same region also has important properties for the transactivation by RXR-Nur77 complex in the presence of 9-*cis*-RA, in contrast to Nur77 alone. Both Nurr1 and Nur77 become stronger activators in the presence of RXR and 9-*cis*-RA in NIH3T3 and CV1 cells (16). However, deleting the last 15 aa from Nur77 abolished RXR- and 9-*cis*-RA-dependent transactivation enhancement as observed for full-length Nur77. These findings indicate that in NIH3T3 cells the pathway of 9-*cis*-RA signaling functions through the extreme COOH-terminal domain of Nur77, whereas the same region is not required for a full activation of Nur77 in the absence of RXR and 9-*cis*-RA. Although a similar pathway of RXR and 9-*cis*-RA signaling may occur during Nurr1 activation, it is difficult to assess the function with the truncated Nurr1a and Nurr1-583 receptors because the level of activation was well below Nurr1 basal activity. In contrast, the transactivation activity of both Nurr1 and Nur77 in the presence of RXR alone was not repressed in NIH3T3 cells, as reported by Forman et al. (16) using CV1 cells. This discrepancy may result from the presence or absence of cellular-specific coenhancers or repressors (6,8,20). Because we were unable to show, and others have not shown, the direct interaction of RXR with Nurr1 and Nur77 by in vitro gel mobility shift assays with the B1A-RE, the interaction of the receptors could be weak or involvement of an essential coactivator may be necessary for protein-protein interactions. Our results suggest as little as 15 aa of the COOH-terminal of these orphan receptors may be fundamental for this type of interaction.

Despite the overlapping spatial and temporal expression of Nurr1 and Nur77 in some brain regions, Nur77 is expressed almost exclusively in adults whereas Nurr1 is expressed during embryonic development. Mice disrupted for Nurr1 (4,53) and Nur77 (9,26) genes result in two phenotypically unique animals: Nurr1 knockout mice die shortly after birth, whereas mice with a Nur77 targeted disruption have no known distinguishing phenotype and appear normal. It has been proposed that functional redundancy of Nurr1 and Nur77 working through the same RE may compensate for the normalcy of the Nur77 knockout mice. Our study helps to clarify putative differences in activation for Nurr1, Nurr1a, and Nur77 during target gene expression. The multiple mechanisms of transcriptional control as described may be regulated by not only structural variation of Nurr1 and Nur77 COOH-terminal, evidenced by naturally occurring Nurr1a mRNAs, but also cell-specific differences of transcriptional regulatory factors.

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