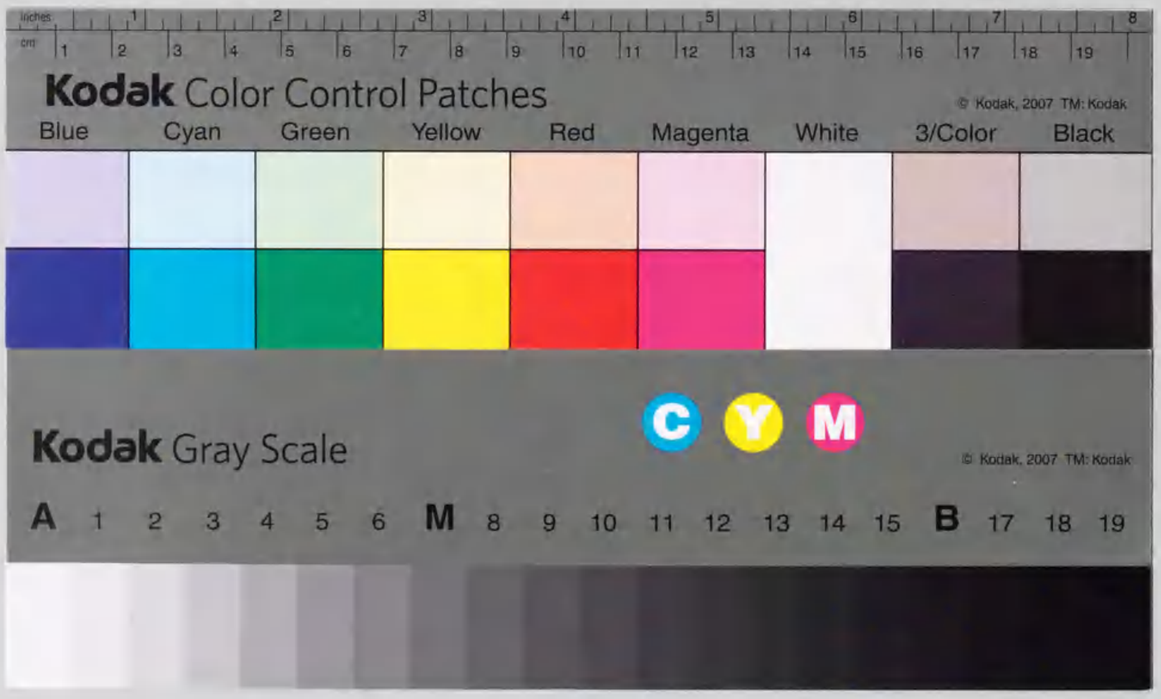
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NOR-1, A NOVEL MEMBER OF THE STEROID/THYROID RECEPTOR SUPERFAMILY

by

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A Dissertation submitted to the Graduate School of Agricultural Sciences,
Nagoya University, in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

1996

ABSTRACT

The present dissertation describes identification and characterization of a novel orphan nuclear receptor belonging to the steroid/thyroid receptor superfamily.

In Chapter 3, by using PCR amplification with primers designed from conserved sequences among the nuclear receptors, a novel member of the steroid/thyroid receptor superfamily gene was identified from primary cultured rat forebrain cells undergoing apoptosis. It was designated as NOR-1 (*n*euron derived *o*rphan *r*eceptor). A cDNA clone of NOR-1 encodes a 68 kDa, 628 amino acid residue-containing protein, with an amino acid sequence highly homologous to the Nur77/NGFI-B family (Nur77/NGFI-B and RNR-1) in its DNA-binding domain and moderately so in its putative ligand-binding domain. Predominant and marginal expression of rat NOR-1 were found in the fetal and adult brain, respectively. It was also revealed with mobility shift assay that the NOR-1 protein binds to the B1a response-element which has been identified as the target sequence of the Nur77/NGFI-B family, suggesting that NOR-1, Nur77/NGFI-B and RNR-1 may transactivate common target gene(s) at different physiological situations.

The experiment of Chapter 4 was designed to clarify the biological role of NOR-1 in primary cultured forebrain cells by selectively inhibiting NOR-1 expression by addition of antisense oligonucleotide to the culture media. Treating cells with the antisense oligomer resulted in the following dramatic morphological changes: (i) cell migration, (ii) extension of processes, and (iii) formation of cell aggregates. These morphological changes were evident after 24 h of antisense-treatment and with better effects obtained at 24 to 72 h. Furthermore, well-developed processes between cellular aggregates were immunopositive for microtubule-associated protein 2 antibody, suggesting that suppression of NOR-1 would enhance neurite outgrowth in forebrain neuronal cells. To determine whether NOR-1 is associated with a process of apoptosis, antisense-treated cells were also examined for some typical phenomena associated with apoptotic cell

death. Treating cultured cells with antisense oligonucleotide alone had no effect on the survival, DNA fragmentation or nuclear morphology, in contrast to its pronounced effect on cell morphology. These findings suggest that NOR-1 is involved in the molecular mechanisms regulating neural differentiation, rather than apoptosis.

In Chapter 5, to further confirm the existence of the NOR-1 gene in vertebrates, a human homologue of NOR-1 was isolated from the human fetal brain. There are two transcripts for human NOR-1, encoding 626 amino acid residues with a calculated molecular mass of 68 kDa. The high homology between rNOR-1/hNOR-1, mNur77 /rNGFI-B/hTR3, and mNURR1/rRNR-1/hNOT indicated that these three types of orphan receptors form a distinct subfamily belonging the steroid/thyroid receptor superfamily. Human NOR-1 mRNA was detected in the adult heart and skeletal muscle as well as in the fetal brain, indicating that its expression is not restricted to events that occur during neural development.

In Chapter 6, chromosomal gene for human NOR-1 and its transcriptional regulation were examined. The hNOR-1 gene is more than 35 kilobases long and interrupted by seven introns. The exon-intron structure of the gene is generally conserved when compared with the steroid/thyroid receptor superfamily and is remarkably similar to that of the Nur77/NGFI-B genes. This suggests that the Nur77 /NGFI-B family has evolved from a common ancestral gene. The 5'-flanking region of NOR-1 is characteristic of a promoter found in housekeeping genes, as indicated by the presence of three copies of Sp1 motifs and the absence of TATA and CCAAT boxes. Deletion analysis of the 5'-flanking region of NOR-1 revealed several positive-acting elements in addition to the core promoter region, as well as highly negative regulatory elements downstream of the NOR-1 transcriptional start site. Fluorescence in situ hybridization (FISH) revealed that the gene is located on chromosome 9q.

In conclusion, the findings in the present thesis demonstrate that (i) a novel orphan nuclear receptor, NOR-1, was cloned in both rat and human, (ii) NOR-1 belongs to the Nur77/NGFI-B family within the steroid/thyroid receptor superfamily, (iii) NOR-1 mRNA is expressed abundantly in the fetal brain, (iv) NOR-1 might be involved in the molecular mechanisms regulating neuronal differentiation, (v) the human NOR-1 gene, which is more than 35 kb long and split into seven introns and eight exons, is located on chromosome 9q, (vi) the 5'-flanking region of NOR-1 is characteristic of housekeeping gene promoters, and (vii) NOR-1 and other members of the Nur77/NGFI-B family might evolved from a common ancestral gene.

CONTENTS

ACKNOWLEDGMENTS

I would like to express my sincere thankfulness to Dr. Kei-ichiro Maeda, who originally introduced me the field of Endocrinology and taught me the foundation of science. I appreciate very much his help during the preparation of this dissertation.

I would like to express my deepest sense of gratitude to the members of TERUMO R&D Center, Institute of Biomedical Science, especially to Drs. Keizaburo Miki, Ken-ichi Urakami and Takashi Kitajima for their valuable advice, suggestions and criticisms and for their kindness throughout this study.

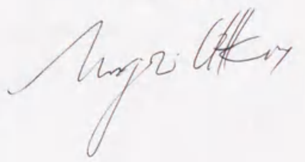
I would like to express my deep thankfulness to Drs. Akira Yokoyama and Hiroko Tsukamura for their valuable advice and suggestions, and for their kindness. Thanks to their excellent education at the Nagoya University, I was able to write this dissertation.

I am grateful to the members of the National Cancer Center Research Institute, especially to Drs. Ken Yamaguchi, Toshihiko Tsukada, Kazuki Sasaki, Shuji Bandoh and Kouji Maruyama for their valuable advice, constructive suggestions and criticisms, and for their kindness throughout this study.

I am heartily thankful to my colleagues, Midori Hijikuro, Mikiko Ito for their skillful technical assistance and valuable suggestions.

Finally, I express my sincere gratitude to my partner Tamami for her warm understanding and support.

The Author



LIST OF ABBREVIATIONS

ATP	adenosine 5'-triphosphate
cDNA	complementary deoxyribonucleic acid
cAMP	cyclic adenosine 3',5'-monophosphate
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
dUTP	2'-deoxyuridine 5'-triphosphate
DTT	dithiothreitol
DNA	deoxyribonucleic acid
FISH	fluorescence in situ hybridization
FBS	fetal bovine serum
HRE	hormone response element
kb	kilobase(s)
MAP2	microtubule-associated protein 2
mRNA	messenger ribonucleic acid
NGF	nerve growth factor
nt	nucleotide(s)
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
TPA	12-*O*-tetradecanoylphorbol-13-acetate
5'-UTR	5'-untranslated region

CHAPTER 1

General introduction

The steroid/thyroid receptor superfamily

Differential control of gene expression has become a central theme in modern molecular biology. The lipophilic hormones, because they are able to diffuse from a source and reach to a target, are candidates to serve as regulators of this process (Evans, 1988). These hormones include the adrenal steroids (cortisol and aldosterone), the sex steroids (progesterone, estrogen, and testosterone) and vitamin D_3. They have been shown to be indispensable for development and physiology in vertebrates (Baxter & Tyrrell, 1981). The adrenal steroids widely influence body homeostasis, controlling glucose and mineral metabolism as well as mediating various kinds of stress responses (Legrand, 1979). At present, their physiological roles are expanding to the control of the immune and nervous systems and influence on the growth and differentiation (Dussault & Ruel, 1987; Oppenheimer, Schwartz *et al.*, 1987). The sex steroids control the development, the embryonic reproductive system, puberty and reproductive functions including behavior in the adult (Clark & Markaverich, 1988; Gurpide & Murphy, 1994; Rories & Spelsberg, 1989). Vitamin D is necessary for normal bone development and plays a critical role in calcium metabolism and bone differentiation (Morrison, Qi *et al.*, 1994; Riggs, Nguyen *et al.*, 1995; Palmieri, Pitcock *et al.*, 1989).

Unlike the water-soluble peptide hormones and growth factors binding to cell surface receptors, the fat-soluble steroid hormones can pass through the lipid bilayer of the cell membrane and interact with intracellular proteins (Evans, 1988). The first clue to clarify how those molecules elicit such a diversity of complex response was provided by the identification of steroid and thyroid hormone receptors (Green & Chambon, 1988). The development of high-affinity synthetic analogs of the ligands overcame many of the difficulties of receptor isolation and has revolutionized both clinical and biochemical studies (Krieger, 1981; Baxter & Tyrrell, 1981). By the early 1980s almost all the

steroid receptors were purified (Yamamoto, 1985; Ringold, 1985). Each receptor undergoes a structural alteration upon hormone binding, which in turn enables DNA binding. Consequently, it was postulated that the binding of hormone to its receptor induces an allosteric change in the receptor that enables the hormone-receptor complex to bind to high affinity sites in chromatin and modulate transcription (Mangelsdorf, Thummel *et al.*, 1995). The subsequent identification of hormonally responsive target genes make a great contribution to the initial characterization of a steroid hormone signaling pathway.

Analysis of the steroid receptors also revealed that DNA and hormone binding properties could be separated by limited proteolysis, leading to the first suggestion of a domain structure (Wrange & Gustaffson, 1978; Carlstedt-Duke, Okret *et al.*, 1982). Furthermore, the cloning and sequencing of cDNAs for several nuclear receptors has indicated that they share a similar structure (Weiringa, Hofer & Weissmann, 1984). Although steroid and thyroid hormones are neither structurally nor biosynthetically related, the existence of a common structure for their receptors supports the proposal that there is a large "superfamily" of genes whose products are ligand-responsive transcription factors (Evans, 1988).

Characteristics conserved among the nuclear receptors

Nuclear receptors share a common modular structure composed of four major domains that have originally been defined by amino acid sequence conservation and function (Giguère, Hollenberg *et al.*, 1986; Krust, Green *et al.*, 1986). The central DNA-binding domain is the most conserved among nuclear receptors and is composed of two zinc finger motifs that serve as interfaces in DNA-protein interaction (Freedman, 1992). The ligand-binding domain, located at the carboxy-terminal end of nuclear

receptors, shows moderate conservation and performs a number of functions that include ligand binding, transcriptional activation and repression, nuclear translocation, and dimerization (Truss & Beato, 1993). In contrast, both the amino-terminal domain and the hinge region separating the DNA- and ligand-binding domains are poorly conserved between the nuclear receptors and their functions remain to be fully delineated. The amino-terminal region of several types of nuclear receptors has been shown to contain a trans-activation domain that may specify target gene activation (Tora, Gronemeyer *et al.*, 1988; Negpal, Saunders *et al.*, 1992).

The DNA-binding domain is included within the highly conserved central core of the protein. The most striking feature is the conservation of Cys residues (Evans, 1988). A comparison of the amino acid sequences between DNA-binding domains of the hormone receptors revealed significant identity and similarity over these evolutionary divergent molecules. The positioning of the residues is similar to a motif originally observed in the 5S gene transcription factor TFIIIA in which multiple Cys- and His-rich repeating units apparently fold into a "fingered" structure coordinated by a zinc ion (Miller, McLachlan & Klug, 1985; Tso, van den Berg & Korn, 1986). This finger of amino acids is proposed to interact with a half turn of DNA. In addition, comparative studies show that the more NH2-terminal "first finger" is more highly conserved among receptors than the more COOH-terminal "second finger" (Evans, 1988). The first finger is relatively more hydrophilic and has few basic amino acids that might be expected to interact with DNA. Although attention has been focused on zinc fingers, the residues between the two fingers and the residues immediately after the second finger are also highly conserved. This raises the possibility that these stretches mediate part of the DNA-binding function.

Nuclear receptors control the activity of primary target genes by binding to short

DNA sequences known as hormone response elements (HREs). These sequences function in a position- and orientation-independent fashion and thus behave like transcriptional enhancers (Chandler, Maler & Yamamoto, 1983; Bonoist & Chambon, 1981). Because of the amino acid conservation within the DNA-binding domains, characteristics of HREs are fundamentally conserved among the superfamily. Therefore, those DNA-binding proteins can be categorized according to the types of HREs they recognize and physical interactions displayed between receptor monomers (Mangelsdorf, Thummel *et al.*, 1995). The first group (Class I) includes steroid hormone receptors such as glucocorticoid and estrogen receptors that bind DNA as homodimers and recognize HREs configured as inverted repeats of the consensus half-sites AGAACA or AGGTCA spaced by 3 bp (Klock, Strähle & Schätz, 1987; Martinez, Givel & Wahli, 1987). The second group (Class II) is composed of thyroid hormone, vitamin D_3 and retinoic acid receptors that bind DNA as heterodimers with the retinoic X receptor (Yu, Delsert *et al.*, 1991; Bugge, Pohl *et al.*, 1992; Kliewer, Umesono *et al.*, 1992; Leid, Kastner *et al.*, 1992; Marks, Hallenbeck *et al.*, 1992; Zhang, Hoffmann *et al.*, 1992b) and recognized HREs configured as direct or everted repeat of the core half-site motif PuGGTCA separated by spacers of defined length (Näär, Boutin *et al.*, 1991; Umesono, Murakami *et al.*, 1991; Tini, Otulakowski *et al.*, 1993). The third group (Class III) comprises receptors such as RXR and COUP-TF that display the ability to bind DNA as homodimers to direct repeat HREs (Mangelsdorf, Umesono *et al.*, 1991; Tran, Zhang *et al.*, 1992). The fourth group (Class IV) includes Nur77, RNR-1 and ROR that bind DNA as monomers to extended half-sites (Watson & Milbrandt, 1989; Nathans, Lau *et al.*, 1988; Scearce, Laz *et al.*, 1993).

A novel category within the steroid/thyroid receptor superfamily

- orphan nuclear receptors -

By the year of 1990, a total of 15 members of the superfamily have been identified as receptors for all the known fat-soluble hormones. Today, there are more than 30 different subfamilies of nuclear receptors, spanning a large diversity of animals from worm to human (Mangelsdorf & Evans, 1995). While the availability of purified hormones and antibodies enabled the discovery of the first receptors, low stringency hybridization screening and genetic and molecular cloning techniques have allowed the identification of numerous members of the family for which there are no apparent ligands. These were termed "orphan receptors", and found in various species such as nematodes (Larsen, Yeh *et al.*, 1994), arthropods (Lavorgna, Ueda *et al.*, 1991; Palli, Hiruka & Riddiford, 1992), and vertebrates (Chang, Kokontis *et al.*, 1989b; Lazar, Hodin *et al.*, 1989). Both genetic and biochemical analyses have demonstrated that these proteins could regulate specific gene networks and important developmental precesses (Hiromi, Mlodzik *et al.*, 1993; Liu, Smith *et al.*, 1994; Woronicz, Calnan *et al.*, 1994). These transcriptional regulators have been shown to bind as apparent monomers (Giguère, Yang *et al.*, 1994; Wilson, Fahrner & Milbrandt, 1993; Wilson, Paulsen *et al.*, 1992c), homodimers (Sladek, Zhong *et al.*, 1990; Cooney, Tsai *et al.*, 1992), and heterodimers (Baes, Gulik *et al.*, 1994; Keller, Dreyer *et al.*, 1993; Kliewer, Umesono *et al.*, 1992) to diverse hormone response elements composed of a single core half-site PuGGTCA preceded by an AT-rich sequence or direct/inverted repeats of the core half-site separated by spacers of varying length (Giguère, Yang *et al.*, 1994; Harding & Lazar, 1993; Wilson, Fahrner *et al.*, 1991; Mangelsdorf, Umesono *et al.*, 1991). The recognition sites of orphan receptors often overlap with DNA targets for ligand-activated nuclear receptors. Consequently, orphan receptors such as COUP-TF and ARP-1 have

been shown to modulate the activity of ligand-activated nuclear receptors by competition for cognate binding sites (Cooney, Tsai *et al.*, 1992; Tran, Zhang *et al.*, 1992).

Functions of orphan nuclear receptors have been elusive and subject to speculation, but another activation mechanisms and functions have been proposed in recently identified orphan receptors. Retinoid X receptors (RXR) and peroxisome proliferator-activated receptors (PPARs) could be activated by the retinoid metabolite *9-cis*-retinoic acid and peroxisome proliferators, respectively (Kiewer, Umesono *et al.*, 1992c; Dreyer, Keller *et al.*, 1992). Other orphan receptors may have no ligands but could be activated by signal transduction pathways, as has been proposed for TR2 orphan receptor (Chang, Kokontis *et al.*, 1989a). Some are constitutive transactivators, such as Nur77/NGFI-B (Chang, Kokontis *et al.*, 1989b; Hazel, Nathans & Lau, 1988), or repressors, such as COUP-TF and ARP-1 (Cooney, Tsai *et al.*, 1992; Kliewer, Umesono *et al.*, 1992a; Mietus-Snyder, Sladek *et al.*, 1992). Phosphorylation is one potential candidate for mediating receptor function by such pathways. Another mechanism by which receptors can modulate gene expression is by cross-coupling to augment or inhibit signaling pathways mediated by other classes of transcription factors. An intriguing property of some orphan receptors is their ability to facilitate or modify ligand-mediated signaling by interaction with other members of the steroid hormone receptor superfamily at the protein or DNA level. RXRs are essential for signal transduction mediating the action of retinoids, thyroid hormone, vitamin D_3, and peroxisome proliferators through acting as heterodimerization partners for the respective receptors (Zhang, Hoffmann *et al.*, 1992). COUP-TF also has the above-mentioned potential to modify ligand-mediated signaling (Kiewer, Umesono *et al.*, 1992). However, the main route of its interference is through direct binding to response elements of receptors for retinoids, thyroid hormone, vitamin D_3, and peroxisome proliferators (Cooney, Tsai *et al.*, 1992; Miyata, Zhang *et al.*,

1993).

Several orphan receptors have been detected in the brain, where the most complex cell-cell interactions forms a neuronal network. The orphan receptors Nur77/NGFI-B and RNR-1 were shown to act as immediate early gene products in neural tissues (Chang, Kokontis *et al.*, 1989b; Scearce, Laz *et al.*, 1993; Law, Conneely *et al.*, 1992), and COUP-TF was shown to play a role in the regulation of the oxytocin gene in the hypothalamoneurohypophyseal system (Adan, Cox *et al.*, 1992; Adan, Cox *et al.*, 1993). These properties also indicate that orphan receptors play important roles in signal transduction to control complex cell-to-cell interactions.

Possible physiological functions of the orphan nuclear receptors

A number of orphan nuclear receptors, including FTZ-F1, ERRs, LXR, xONR, MB67s, TR2s, TLL, GCNF, Rev-Erbs, RORs, COUP-TFs, PPARs, HNF4, and Nur77/NGFI-Bs have been identified over this decade (Mangelsdorf, Thummel *et al.*, 1995), and extensively studied in transfected cultured cells. Since *in vitro* approaches could be employed to clarify a part of physiological mechanism, *in vivo* models are always crucial for assigning functions to orphan receptors. Targeted disruption of a gene (knockout) via homologous recombination has recently been used to generate mice lacking various receptors. Expression of dominant negative receptors or antisense mRNA has also been employed. Together with some nuclear receptor mutations associated with pathological condition, knockouts have led us to a significant advance in our knowledge of the physiological functions of several orphan nuclear receptors. As described below, these transcriptional regulators have been shown to regulate much more important biological processes than was expected before, including development, apoptosis, and oncogenesis, and participate in several intracellular signaling pathways.

The FTZ-F1 protein binds to promoter regions of several steroidogenic enzymes (Sadovsky, Crawford *et al.*, 1995) and of the anti-Mülerian hormone (Shen, Moore *et al.*, 1994). FTZ-F1 null mutants lack gonads and adrenals and die during the first days of life, probably due to the absence of adrenal function since they can be rescued by corticosteroids administration (Luo, Ikeda & Parker, 1994; Luo, Ikeda *et al.*, 1995; Sadovsky, Crawford *et al.*, 1995; Shinoda, Lei *et al.*, 1995). These mice also lack the ventromedial hypothalamic nucleus, which is the center for feeding and sexual behavior as well as a region controlling autonomic functions. In addition, luteinizing hormone, follicle-stimulating hormone, and the gonadotropin-releasing hormone are not detected in the pituitary of the mutant (Ingraham, Lala *et al.*, 1994). Thus, FTZ-F1 appears to be critical for the development or regular functions of the hypothalamic-pituitary-gonadal axis.

In transfected cells, peroxisome proliferator-activated receptors α, β and γ (PPAR α, PPAR β, and PPAR γ) are activated by a variety of fatty acids and several synthetic compounds that induce peroxisome proliferation in the liver and hepatocarcinogenesis (Green & Wahli, 1994). As heterodimers with retinoid X receptors (RXRs), PPARs have been broadly implicated in the control of lipid metabolism, since putative PPAR response elements have been characterized in the promoter regions of several genes encoding enzymes involved in fatty acid catabolism (β- and ω-oxidation).

Hepatocyte nuclear factor 4 (HNF4) binds to response elements present in several liver-specific genes (Chen, Manova *et al.*, 1994). HNF4 mutants die at approximately 8.5 days postcoitum and exhibit extensive cell death in the ectoderm at 6.5 days postcoitum (Chen, Manova *et al.*, 1994; Bachvarova & Darnell, 1994). Gastrulation and mesoderm formation appear delayed by 24 h and are greatly impaired. The underlying

mechanisms are unknown, but the primary defect is likely to reside in the visceral endoderm, which selectively express HNF4 at early stages.

Nerve growth factor-induced receptor NGFI-B is an immediate-early protein whose expression is induced by a variety of stimuli. NGFI-B may be involved in the control of the hypothalamic-pituitary-adrenocortical axis, as NGFI-B transcripts are strongly induced by stress in the adrenal cortex and in the paraventricular hypothalamic nucleus (Crawford, Sadovsky *et al.*, 1995). Together with FTZ-F1, NGFI-B has also been implicated in the control of steroidogenic enzyme expression in adrenal gland (Crawford, Sadovsky *et al.*, 1995). NGFI-B might also play a role in the control of activation-induced apoptosis of thymocytes and T-cell hybridomas, since NGFI-B is rapidly and strongly induced after activation of immature thymocytes and since expression of a "dominant negative" NGFI-B or NGFI-B antisense transcripts prevents activation-induced apoptosis of T-cells (Liu, Smith *et al.*, 1994; Woronicz, Calnan *et al.*, 1994). It, therefore, is surprising that NGFI-B null mutants display no detectable defect, with respect to both T-cell apoptosis and function of the hypothalamic-pituitary-adrenocortical axis (Lee, Pineau *et al.*, 1995; Crawford, Sadovsky *et al.*, 1995). The discrepancy between the normal phenotype of the NGFI-B null mice and the possible functions of NGFI-B most probably reflects functional compensation by closely related receptors (Crawford, Sadovsky *et al.*, 1995).

The studies summarized here strongly suggest that the orphan nuclear receptors encode a vast repertoire of transcriptional regulators that play critical roles during development and homeostasis. While apparently all the receptors for each steroid hormones have been cloned, novel orphan receptors should remain to be identified and they are expected to be the largest portion of the nuclear receptor family. The further discovery of orphan receptors leads to the implied existence of new classes of

physiologic regulators having a widespread impact on embryology as well as physiology.

Objectives

This dissertation describes an identification and characterization of a novel nuclear receptor which is expressed in the fetal brain during development of the nervous system.

In the first approach in Chapter 3, to isolate a novel nuclear receptor, I amplified mRNA sequences in apoptotic rat forebrain cells that could encode two zinc finger DNA-binding motif by using RT-PCR with primers designed from conserved sequences among the superfamily. In the experiment, a novel member of the superfamily was identified, and designated as NOR-1. Chapter 4 was designed to clarify the physiological role of NOR-1 in the forebrain at the cellular level. I examined the influence of antisense oligonucleotide to NOR-1 in primary cultured cells dissociated from the rat fetal forebrains. In Chapter 5, I also identified a human homologue of NOR-1 from the fetal brain, and revealed its characteristics. In Chapter 6, the structure of a chromosomal gene for human NOR-1 and its transcriptional regulation were analyzed.

CHAPTER 2

General procedures

Primary culture of neuronal cells

Forebrain cultures were prepared from rat embryos (embryonic day 17) taken from Wistar strain maternal rats under ether anesthesia. The forebrains were chopped into small pieces and disaggregated with 0.25% (w/v) trypsin for 30 min at 37 °C. Cells were suspended in DF medium [Dulbecco's modified Eagle's medium / Ham F-12 containing 10% fetal bovine serum (Gibco BRL, MD USA), 2 mM glutamate, 1 mM pyruvic acid, 20 mM HEPES, 30 nM selenite, penicillin 100 U/ml and streptomycin 100μg/ml] and centrifuged at 1,000 rpm for 5 min. The pellet was resuspended in DF medium containing DNaseI 0.1 mg/ml, and further dissociated with a Pasteur pipette. Cells were washed twice with DF medium and plated onto poly-L-lysine-coated plates (Becton Dickinson Labware, CA USA) at 5×10^5 cells/cm^2.

Detection of DNA fragmentation

Nuclear damages induced in apoptotic cells have shown that cell DNA is cleaved into multimers of about 180-200 bp, which may be visualized as a distinct ladder of bands following agarose gel electrophoresis (Wyllie, 1980). For DNA extraction, cultured forebrain cells were washed and pelleted by centrifugation at $200 \times$g for 10 min. Cell suspensions were transferred to ice-cold lysis buffer containing 5 mM Tris, 20 mM EDTA, 0.5 % Triton X-100, pH 8.0, and samples were allowed to lyse for 15 min on ice before centrifugation for 20 min at $27,000 \times$g to separate intact chromatin (pellet) from DNA fragments (supernatant). Supernatants were extracted with phenol/chloroform and then precipitated with ethanol. Electrophoresis was performed for 45 min at 100 V on agarose gels.

RNA preparation

Total RNA from cultured cells or tissues was prepared using the acid guanidinium-phenol-chloroform method (Chomczynski & Sacchi, 1987). RNAs were resuspended with Diethyl pyrocarbonate (Sigma Chemical Co., MO USA) -treated water, and quantified according to A_{260}. For cDNA library construction, poly(A)$^+$ RNA was separated form total RNA using Oligotex-dT30 (Takara Shuzo Co., Shiga, Japan).

λ ZAP cDNA library construction

Five microgram of poly(A)$^+$ RNA was used for the construction of λ ZAP cDNA library. First strand cDNA was synthesized with reverse-transcriptase (Superscript II, 100 U, Gibco BRL) at 37 ˚C for 1 h in reaction buffer containing dATP, dCTP, dGTP, dTTP, linker primer (5'-GAGAGAGAGAGAGAGAGAGAACTAGTCTCGAGTTTTT TTTTTTTTTTTTT-3'), and RNase block ribonuclease inhibitor (40 U; Toyobo Co., Tokyo, Japan). Second strand was synthesized with DNA polymerase I (100 U; Toyobo Co.) and RNase H (6 U; Toyobo Co.) at 16 ˚C for 2.5 h in solution containing dATP, dCTP, dGTP and dTTP. After second strand synthesis, EcoRI adopters (5'-AATTCGG CACGAG-3' and 3'-GCCGTGCTC-5') were ligated to ends of the cDNA products. It was then followed by kinasing the EcoRI ends, XhoI digestion, size fractionation with the Sephacryl S-400 spin column, and ligating cDNA into the uni-ZAP XR vector arms (Stratagene, CA USA). The concatamers were packaged with Gigapack II packaging extract (Toyobo Co.).

DIG-labeled DNA probes

For the screening of libraries, cDNA fragments were labeled with Digoxigenin-11-dUTP (DIG-dUTP; Boehringer Mannheim GmbH, Germany) by PCR amplification.

PCR labeling mixture was consisted with dATP, dCTP, dGTP (200μM each), 150μM dTTP, 50μM DIG-dUTP, 10pmole PCR-primers, 10mM Tris HCl, 50mM KCl, 1.5mM $MgCl_2$, 0.01% gelatin, 10ng template DNA fragment, and 5U Taq DNA polymerase (Takara Shuzo Co.). It was then subjected to the PCR amplification (30 cycles) by temperature cycling instrument (Perkin Elmer, GeanAmp PCR System 2400, CA USA). PCR cycles are consisted of denaturing at 94 °C for 1 min, annealing at 50 °C for 2 min and extension at 72 °C for 3 min.

Library screening

Recombinant λ phage libraries (λ ZAP XR, λ gt11, and λ EMBL SP6/T7) were screened with DIG-labeled cDNA probes. Libraries were plated on 90×130 mm LB plates to 50,000 pfu/plate with 600μl of host cells (XL1-Blue, Y1090r- and K802 for λ ZAP XR, λ gt11, and λ EMBL SP6/T7, respectively) at an OD_{600} of 0.5/plate and 7 ml of LB top agar/plate. These plates were incubated at 37 °C for 8 - 24 h, and then plaques were transferred onto a nitrocellulose membrane for 2 min. The membrane was denatured with 1.5 M NaCl and 0.5M NaOH for 2 min, neutralized in a 1.5 M NaCl and 0.5 M Tris-HCl (pH 8.0) for 2 × 2 min, and rinsed in 2×SSC buffer [20×SSC; 175.3g NaCl, 88.2g sodium citrate (pH7.0)/litter]. Phage DNA was crosslinked to the membrane using the UV crosslinker (Fluo-Link, Cosmo-Bio Co., Tokyo, Japan).

The membrane was incubated with prehybridization solution [0.1% (w/v) N-lauroylsarcosine Na-salt (Sigma Chemical Co.), 0.02% (w/v) SDS, 1% (w/v) blocking reagent (Boehringer Mannheim)] at 65 °C for 4 h, and then hybridized at 65 °C for 16 h in hybridization solution [prehybridization solution + 10ng/ml DIG-labeled probes]. It was washed 2×5 min in 2×SSC-0.1%SDS and 2×15 min in 0.1×SSC-0.1%SDS at

65 °C. Hybridized DIG-probes were detected by an enzyme-linked color reaction using alkaline phosphatase conjugated anti-DIG antibody (Boehringer Mannheim) and alkaline phosphatase substrates [4-Nitro blue tetrazolium chloride and 5-Bromo-4-chloro-3-indolyl-phosphate (Boehringer Mannheim)].

DNA sequencing

Isolated λ ZAP phage clone was automatically transferred to a plasmid pBlueScript SK(-) by using *in vivo* excision (Stratagene). DNA fragments isolated from λ gt11 were subcloned into the EcoRI site of pBlueScript SK(-) or pUC119. Genomic DNA fragments isolated from λ EMBL3 SP6/T7 and containing the partial NOR-1 gene were subcloned into the site of pUC119 or pBlueScript SK(-). Various restriction fragments derived from those plasmids were then cloned into pBlueScript SK(-) and sequenced by the dideoxy chain termination procedure (Sanger, Nicklen & Coulson, 1977). For the determination of more than 1 kb sequences, a series of deletion constructs was made by kilo sequence deletion kit (Takara Shuzo Co.). Primers for DNA sequencing were synthesized by using DNA synthesizer (392 DNA/RNA Synthesizer, Applied Biosystems, CA USA).

^{32}P-labeled DNA probe

For Northern blot analysis, isolated cDNA fragments were labeled through the incorporation of [α-^{32}P]dCTP (Amersham, Buckinghamshire, England) using a random primer DNA-labeling kit (Takara Shuzo Co.). Heat-denatured cDNA fragments (25 ng) were labeled by klenow fragment (2 U) in reaction buffer containing dGTP, dATP, dTTP (0.2 mM each), 1.85 MBq [α-^{32}P]dCTP (111 TBq/mmol, 3000 Ci/mmol), and 8 bp random prime mix. The probes were purified by ethanol precipitation.

Northern blots

Twenty micrograms of heat-denatured total RNA or 2 μg of polyA selected RNA/lane were separated by electrophoresis in a 0.9% agarose, 1.6% formaldehyde, MOPS denaturing gel and transferred to a nylon-membrane (Hybond-N, Amersham). Hybridization buffer consisted of 6×SSC, 5×Denhalt's [50×Denhalt's; 5g Ficoll (Type 400, Pharmacia Biotech, Uppsala, Sweden), 5g polyvinylpyrrolidone (Sigma Chemical Co.), 5g bovine serum albumin (Fraction V, Sigma Chemical Co.)/500ml], 0.5% SDS, heat-denatured herring sperm DNA 100μg/ml (Boehringer Mannheim), for ^{32}P-labeled cDNA probes. Blots were hybridized at 65 °C for 16 h and washed 2×5 min in 2×SSC-0.1%SDS and 2×15 min in 0.2×SSC-0.1%SDS at 65 °C, and then hybridized probes were detected by autoradiography.

Data analysis

The computer program DNASIS-DBREF 50 (Hitachi Software Engineering Co., Tokyo, Japan) was used to the following subjects; open reading frame search, restriction enzyme site search, prediction of molecular weight, and amino acid translation from DNA sequence. Homology search to the databases EMBL, GenBank, SWISS-PROT, and Protein Identification Resource (PIR), was performed with the algolism BLAST or FASTA.

CHAPTER 3

Isolation of a novel nuclear receptor gene

- Molecular cloning of a novel steroid/thyroid receptor superfamily gene from cultured rat neuronal cells -

Introduction

Orphan nuclear receptors in the superfamily of steroid/thyroid hormone receptors share a common domain architecture and a high degree of identity in the DNA-binding domain (Chang & Kokontis, 1989b; O'Malley, 1990). Their functions remain unclear, but recently several potential functions have been explored.

During development in the thymus, immature thymocytes which express self-reactive T-cell antigen receptors are eliminated from the developing T-cell repertoire (Nossal, 1994). This process of clonal deletion, or negative selection, is thought to be mediated by apoptotic cell death (Allen, 1994). To date, several genes which are required for activation-induced apoptosis in T-cell hybridomas have been identified. These are c-myc (Shi, Glynn, *et al.*, 1992), the Fas/Fas ligand receptor pair (Brunner, Mogil *et al.*, 1995; Dhein, Walczac *et al.*, 1995; Ju, Panka *et al.*, 1995), and Nur77 (Liu, Smith *et al.*, 1994; Woronicz, Calnan *et al.*, 1994). Nur77 is an immediate-early gene product encoding an orphan nuclear receptor (Hazel, Nathans & Lau, 1988; Ryseck, Bravo *et al.*, 1989; Milbrandt, 1988). Expression of dominant negative or antisense Nur77 mutations can inhibit apoptosis (Liu, Smith *et al.*, 1994; Woronicz, Calnan *et al.*, 1994), suggesting that Nur77 is necessary for induced apoptosis in T-cell hybridomas.

Apoptosis is also an important biochemical pathway for cell death that occur during the developing nervous system. For example, 40-50 % of lumber spinal cord motor neurons die during normal chick development (Hamburger, 1975) and 80 % of all programmed cell deaths that occur during nematode larvae development affect neurons (Horvitz, Ellis & Sternberg, 1982). Taken together these observations, there is a possibility that Nur77 and/or other members of the nuclear receptors might be activated in apoptotic neuronal cells. Therefore, to characterize the nuclear receptors expressed in

brain neuronal cells undergoing apoptosis, I examined mRNA sequences in apoptotic rat forebrain neuronal cells that could encode nuclear receptors, using RT-PCR.

Materials and Methods

RNA preparation

Total RNA from the above cultures was prepared 20 h after culture initiation, using the acid guanidinium-phenol-chloroform method (Chomczynski & Sacchi, 1987).

Reverse transcriptase-PCR and cDNA cloning

Ten micrograms of total RNA derived from rat brain neuronal cells in culture was reverse-transcribed using an oligo(dT) primer and reverse transcriptase (Superscript II, Gibco BRL). Sequences related to DNA-binding domains of nuclear receptors were amplified by PCR. The single strand cDNA products were denatured and subjected to PCR amplification (40 cycles) with two primers (ARGGCTGCAAGGSYTTCTT as 5' primer and CNYCYTTCAYCATKCCCAC as 3' primer), which were designed from conserved sequences among the thyroid/steroid receptor superfamily. Each PCR cycle consisted of denaturation at 95 °C for 1 min, annealing at 60 °C for 2 min, and extension at 72 °C for 3 min. The PCR products were ligated into plasmid PCRII (Invitrogen, CA USA) and sequenced. The cDNA fragment encoding a sequence similar to those of nuclear receptors was used to screen a rat brain cultured neuronal cell cDNA library in λ ZAP XR (Stratagene) and to isolate the cDNA encoding this novel nuclear receptor.

Mobility shift assay

NOR-1 cDNA was transcribed and translated with rabbit reticulocyte lysate (Amersham) *in vitro*. The mobility shift assay was performed with 4 μl of *in vitro* translation products and double-stranded B1a oligonucleotide (0.1ng, 5'-GAGTTTTAAA AGGTCATGCTCAATTT-3') labeled with [γ-^{32}P]ATP (Amersham) to a specific

activity of 10^5 cpm/ng. The binding reactions were performed in 10 mM HEPES (pH 7.9), 1 mM DTT, 5% glycerol, denatured herring sperm DNA (5ng/ml) and cold double-strand oligonucleotides. These reactions were incubated for 20 min at room temperature. They were loaded on to 4% acrylamide gels in 0.25 ×TBE buffer and run at 150 V for 1.5 h. The gels were dried and autoradiographed.

Results

Isolation of a novel nuclear receptor from apoptotic neuronal cells

Programmed cell death in the nervous system has been studied in primary cultures of neurotrophic factor-deprived neurons (Dipasquale, Marini & Youle, 1991). Cultured neuronal cells which receive insufficient extracellular signals, such as from nerve growth factor (NGF), immediately proceed to apoptosis (Edwards & Tolkovsky, 1994). To ascertain whether cultured rat forebrain neuronal cells undergo to apoptosis, DNA fragmentation was assayed by gel electrophoresis of DNA. Neuronal cells were found to contain fragmented DNA in multiples of approximately 180 base pairs, giving rise to the characteristic DNA " ladder " pattern of apoptosis (Fig. 3.1). DNA ladders were detected in cells by 20 h after plating, and reached a plateau of maximum level by 72 h.

Thirty fragments were isolated when cDNA derived from neuronal cells cultured for 20 h was used as template in a PCR amplification with a pair of degenerated oligonucleotides corresponding to the DNA-binding domain of the thyroid/steroid receptor superfamily. Sequence analysis of them indicated that one fragment out of thirty had a novel sequence with similarity to the nuclear receptors. The PCR product encoding a sequence similar to the nuclear receptors was used to select a nearly full-length cDNA clone from a library prepared from poly(A)$^+$ mRNA isolated from rat brain neuronal cells in culture. Screening 2×10^5 clones with the PCR fragment as a probe revealed six positive clones, one of which was chosen for sequence analysis. This cDNA was designated as NOR-1 (*n*euron-derived *o*rphan *r*eceptor).

The longest nucleotide sequence of NOR-1 contains 4,400 nucleotides, which includes a major open reading frame initiating at position 700 that is 1,884 nucleotides in length, and encodes a protein of molecular weight 68 kDa (Fig. 3.2). The initiation site

of the translation was assigned to the methionine codon at nucleotide 700 because the first ATG triplet (position 347) had a poor flanking sequence for translation initiation according to the consensus sequence derived by Kozak (Kozak, 1986), and was stopped at the in-frame terminator TAA at nucleated 491. Consequently the second ATG, which does have features of the consensus sequence for initiation, would be used.

The NOR-1 reading frame is followed by a 3' untranslated region of 1,814 nucleotides. The 3' noncoding region contained two repeats of polyadenylation signals (positions 3,126 and 4,115), neither of which, however, was located upstream of the poly(A) motif. This region also contains five repeats of the sequence ATTTA, a motif that may facilitate selective mRNA degradation or destabilization by removal of the poly(A) tail (Shaw & Kamen, 1986). These observations would indicate that the NOR-1 cDNA, as isolated, lacked a poly(A) tail, and thus the 4,400 nucleated sequence lacked information as to the 3' end.

Homology analysis

A computer analysis revealed that NOR-1 was closely related to the thyroid/steroid receptor superfamily (Evans, 1988), with the greatest similarity to the nuclear receptors r-RNR-1 and m-Nur77 (and its rat homologue r-NGFI-B) (Hazel, Nathans & Lau, 1988; Milbrandt, 1988; Scearce, Laz *et al.*, 1993). The NOR-1 amino acid sequence shows 40% - 50% identity in its DNA-binding domain when compared to the thyroid/steroid receptor superfamily (Fig. 3.3). Most notable in this region is the presence of eight strictly conserved cysteine residues thought to form two zinc fingers. The carboxyl-terminal of NOR-1 is 15% - 5% homologous to the putative ligand binding domains in the steroid/thyroid receptor superfamily. The homology between NOR-1 and known nuclear receptors indicates that NOR-1 is a member of this superfamily. The alignment

between Nur77, RNR-1 and NOR-1 is shown in Fig. 3.4. In the DNA-binding domain, 91% and 97% of the amino acid residues of NOR-1 are identical with those of Nur77 and RNR-1, respectively. In the P and A Box regions, which are important in DNA sequence recognition (Wilson, Paulsen *et al.*, 1992), NOR-1 shows 100% homology to the other Nur77 family members. In the D Box region, which is essential for the orientation of dimeric receptors (Wilson, Paulsen *et al.*, 1992), NOR-1 was consistent with four-fifths of Nur77, and all of RNR-1.

Characterization of NOR-1

In vitro translated NOR-1 protein was used in a mobility shift assay to test the binding of the B1a response-element, an oligonucleotide that has been identified as an r-NGFI-B/m-Nur77 and r-RNR-1 response element (Scearce, Laz *et al.*, 1993; Wilson, Fahrner *et al.*, 1991). The binding of NOR-1 to the [^{32}P]B1a site was specific, as shown by competition with cold probe (Fig. 3.5). Binding of [^{32}P]B1a to NOR-1 is significantly reduced at a 20-fold excess and completely eliminated at a 100-fold excess of unlabeled oligonucleotide.

Northern analysis revealed that NOR-1 mRNA was expressed at high levels in cultured apoptotic neuronal cells and fetal brain, but at a low level in adult brain (Fig. 3.6). A survey of expression in normal adult tissues indicated that brain is the only other tissue that shows significant NOR-1 expression, although there is marginal expression in thymus, kidney and spleen.

Discussion

I have identified NOR-1, a novel member of the thyroid/steroid receptor superfamily, which is expressed in apoptotic neuronal cells and fetal brain. The NOR-1 gene is similar to those of the Nur77/NGFI-B family, which is induced during the G0/G1 transition or apoptosis. The Nur77/NGFI-B family and NOR-1 are particularly similar in their DNA-binding domains, and moderately so in their putative ligand-binding domains. It is also notable that the amino terminus of NOR-1, the putative transactivation-domain, shows only 25% and 36% homology to the corresponding regions in Nur77 and RNR-1, respectively. Because of the sequence divergence in this region, it is likely that these three proteins will exhibit important differences in whatever function is determined by it. The carboxyl-terminal ligand-binding domain is moderately conserved, the three proteins sharing common structural features.

The high degree of homology in the DNA-binding domains between NOR-1, Nur77, and RNR-1 allowed us to predict that NOR-1 would bind to the B1a response-element identified by genetic selection in yeast (Wilson, Fahrner *et al.*, 1991). Using mobility shift assay, I demonstrated that the NOR-1 protein binds specifically to the B1a response-element. This implies that the function of the three proteins is as transcriptional regulators which bind a similar promoter sequence of target genes. In addition, structural and functional similarities between NOR-1 and the Nur77/NGFI-B family indicate that NOR-1 also belongs to a family of Nur77/NGFI-B.

From the analysis of mRNA structure, polyadenylation is not observed on NOR-1 transcripts found in cultured neuronal cells. Recent work indicates that mitogenic stimulation of cells induces Nur77 mRNA also lacking a detectable poly(A) tail (Liu, Smith *et al.*, 1994). In contrast, the expression of an intact full-length Nur77 gene

product is correlated with the commitment of a cell to undergo apoptosis (Liu, Smith *et al.*, 1994). It remains possible that full-length NOR-1 mRNA could exist in different situations as well.

NOR-1 mRNA was detected in apoptotic neuronal cells and fetal brain, with marginal expression in other adult tissues. A rat homologue of Nur77, NGFI-B, is present in lung, brain, and superior cervical ganglia, and is highly expressed in adrenal tissue (Milbrandt, 1988). The RNR-1 gene is expressed at a low level in lung, spleen, and stomach and at a high level in brain (Scearce, Laz *et al.*, 1993). Although NOR-1 and the Nur77 family share common features, tissue-specific expression pattern of the respective genes suggests that they may have differential functions.

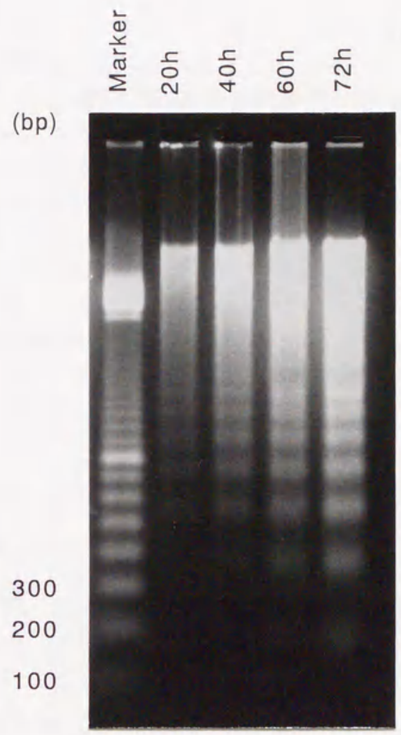


Fig. 3.1. Agarose gel electrophoresis of DNA fragmentation in cultured forebrain cells. Cells were cultured for 20h , 40h , 60h , and 72h (lane 2 - 5). A 100-bp DNA ladder is shown for comparison (lane 1). The gels shown are representative data obtained form the three independent experiments.

```
   1                                                          CCGAGTCTCCTGCCTCCCGCCCCCCACCC
  30 CTCCAGCGCCTGCTCCTCCTCCGCTCCCCATACACAGACACGCTCACACCCGCTCCTTCACTTGCAC
  97 ACACAGACACACGCGCGCTCACACGCTCCGCACACACACTCCACTCTCTCCCGCGCGCTCACACCCC
 164 TCTCTCTCGGCGCCCTCGCCGGTGTCGCGCCGCGCCGCGCCGCAGCCGGACGCCCCTCCAGGGCTCA
 231 CTTTGCAACGCTGACAGAGCGGGCAGTGGCCGTGGAGGTGGGAAACGTGGCGACATCCTAGCCCCTG
 298 GTCGCAGCCGGAGACTGGACGCTGCGGAACCTCTCGGCGGCGCTCTCCCATGAGTTGGGATCGCAGC
 365 ATCCCCAGCCAGCCGCTGCTCACCGCCTCTGGGAGCCGCTGGGTTTGTGCACCGCAGCCCTTCCGGG
 432 ACAGCAGCTGTGACTCTCCCCCAATCCAGATTTCGGGGTCGCTCTCTAGAAACTCGCTCTAAAGACG
 499 GAACCTCCACAGAACCCAAAGCCCACTGCGGGAGAGCGCAGCCCGACAAGCCCGGGCGCTGAGCCTG
 566 GACCCTCAACAGAGCGGGCCAGCACAGCGGCGGCGGCTGCTTCGCCTATCCCGACGTCCCCGCCTCC
 633 TACACTCTCAGCCTCCGCTGGAGAGACCCCCAGCCCCACCATTCAGCGCGCAAGATACCCTCCAGAT
 700 ATG CCC TGC GTG CAA GCC CAA TAT AGC CCT TCG CCT CCG GGG TCC ACT TAT
       M   P   C   V   Q   A   Q   Y   S   P   S   P   P   G   S   T   Y  17
 751 GCC ACG CAG ACT TAT GGC TCG GAA TAC ACC ACA GAA ATC ATG AAC CCC GAC
       A   T   Q   T   Y   G   S   E   Y   T   T   E   I   M   N   P   D  34
 802 TAT GCC AAG CTG ACC ATG GAC CTC GGT AGC ACG GGG ATC ATG GCC ACG GCC
       Y   A   K   L   T   M   D   L   G   S   T   G   I   M   A   T   A  51
 853 ACG ACG TCC CTG CCC AGC TTC AGT ACC TTC ATG GAG GGC TAC CCC AGC AGC
       T   T   S   L   P   S   F   S   T   F   M   E   G   Y   P   S   S  68
 904 TGC GAA CTC AAG CCC TCC TGC CTG TAC CAA ATG CCG CCT TCT GGG CCT CGG
       C   E   L   K   P   S   C   L   Y   Q   M   P   P   S   G   P   R  85
 955 CCT TTG ATC AAG ATG GAA GAG GGT CGC GAG CAT GGC TAC CAC CAC CAC CAC
       P   L   I   K   M   E   E   G   R   E   H   G   Y   H   H   H   H 102
1006 CAC CAT CAC CAT CAT CAT CAC CAC CAC CAC CAC CAG CAG CAG CAG CCG TCC ATT
       H   H   H   H   H   H   H   H   H   H   Q   Q   Q   Q   P   S   I 119
1057 CCT CCT CCC TCT GGC CCC GAG GAC GAG GTA CTG CCC AGC ACC TCC ATG TAC
       P   P   P   S   G   P   E   D   E   V   L   P   S   T   S   M   Y 136
1108 TTC AAG CAG TCT CCG CCG TCT ACG CCG ACC ACT CCA GGC TTC CCC CCG CAG
       F   K   Q   S   P   P   S   T   P   T   T   P   G   F   P   P   Q 153
1159 GCG GGG GCG CTG TGG GAC GAC GAG CTG CCC TCT GCG CCT GGC TGC ATC GCT
       A   G   A   L   W   D   D   E   L   P   S   A   P   G   C   I   A 170
1210 CCG GGA CCG CTG CTG GAC CCG CAG ATG AAG GCA GTG CCC CCA ATG GCC GCT
       P   G   P   L   L   D   P   Q   M   K   A   V   P   P   M   A   A 187
1261 GCT GCG CGC TTC CCG ATC TTC TTC AAG CCC TCA CCG CCA CAC CCT CCC GCG
       A   A   R   F   P   I   F   F   K   P   S   P   P   H   P   P   A 204
1312 CCC AGC CCA GCC GGC GGC CAC CAC CTG GGC TAT GAC CCC ACG GCC GCA GCT
       P   S   P   A   G   G   H   H   L   G   Y   D   P   T   A   A   A 221
1363 GCG CTC AGT CTA CCC CTG GGA GCC GCG GCC GCC GCG GGC AGC CAA GCT GCT
       A   L   S   L   P   L   G   A   A   A   A   A   G   S   Q   A   A 238
1414 GCG CTC GAG GGC CAT CCG TAC GGG CTC CCG CTG GCC AAG AGG ACG GCC ACG
       A   L   E   G   H   P   Y   G   L   P   L   A   K   R   T   A   T 255
1465 TTG ACC TTC CCT CCG CTG GGC CTC ACA GCG TCC CCT ACC GCG TCC AGC CTG
       L   T   F   P   P   L   G   L   T   A   S   P   T   A   S   S   L 272
1516 CTG GGA GAG AGC CCC AGC CTA CCA TCG CCA CCC AAT AGG AGC TCA TCA TCC
       L   G   E   S   P   S   L   P   S   P   P   N   R   S   S   S   S 289
1567 GGC GAG GGC ACG TGT GCT GTG TGC GGG GAC AAT GCT GCC TGC CAG CAC TAC
       G   E   G   T   C   A   V   C   G   D   N   A   A   C   Q   H   Y 306
1618 GGA GTC CGC ACC TGC GAG GGC TGC AAG GGC TTC TTC AAG AGA ACG GTG CAG
       G   V   R   T   C   E   G   C   K   G   F   F   K   R   T   V   Q 323
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1669  AAA AAC GCA AAA TAT GTT TGC TTG GCA AAT AAA AAC TGC CCG GTA GAC AAG
        K   N   A   K   Y   V   C   L   A   N   K   N   C   P   V   D   K   340
1720  AGA CGT CGA AAT CGA TGT CAG TAC TGC AGG TTT CAG AAG TGT CTC AGT GTC
        R   R   R   N   R   C   Q   Y   C   R   F   Q   K   C   L   S   V   357
1771  GGG ATG GTG AAG GAA GTT GTG CGT ACA GAT AGT CTG AAA GGG AGG AGA GGT
        G   M   V   K   E   V   V   R   T   D   S   L   K   G   R   R   G   374
1822  CGT CTG CCT TCC AAA CCA AAG AGC CCA CTA CAA CAG GAG CCC TCG CAG CCC
        R   L   P   S   K   P   K   S   P   L   Q   Q   E   P   S   Q   P   391
1873  TCC CCA CCA TCT CCT CCG ATC TGT ATG ATG AAC GCC CTT GTC CGA GCT TTA
        S   P   P   S   P   P   I   C   M   M   N   A   L   V   R   A   L   408
1924  ACA GAC GCA ACG CCC AGA GAC CTT GAT TAC TCC AGA TAC TGT CCC ACC GAC
        T   D   A   T   P   R   D   L   D   Y   S   R   Y   C   P   T   D   425
1975  CAG GCC ACT GCG GGC ACA GAC GCT GAG CAC GTG CAG CAG TTC TAC AAC CTT
        Q   A   T   A   G   T   D   A   E   H   V   Q   Q   F   Y   N   L   442
2026  CTG ACG GCC TCC ATC GAC GTG TCC AGA AGC TGG GCA GAA AAG ATC CCC GGA
        L   T   A   S   I   D   V   S   R   S   W   A   E   K   I   P   G   459
2077  TTC ACT GAT CTC CCC AAA GAA GAT CAG ACG TTA CTT ATA GAA TCA GCC TTT
        F   T   D   L   P   K   E   D   Q   T   L   L   I   E   S   A   F   476
2128  TTG GAG CTG TTC GTT CTT AGA CTT TCT ATC AGG TCA AAC ACT GCT GAA GAT
        L   E   L   F   V   L   R   L   S   I   R   S   N   T   A   E   D   493
2179  AAG TTT GTG TTC TGC AAT GGA CTT GTC CTG CAC CGA CTT CAG TGC CTT CGC
        K   F   V   F   C   N   G   L   V   L   H   R   L   Q   C   L   R   510
2230  GGA TTT GGG GAG TGG CTC GAC TCC ATT AAA GAC TTT TCT TTA AAT TTG CAG
        G   F   G   E   W   L   D   S   I   K   D   F   S   L   N   L   Q   527
2281  AGC CTG AAC CTT GAT ATC CAA GCC TTA GCC TGC CTG TCA GCA CTG AGT ATG
        S   L   N   L   D   I   Q   A   L   A   C   L   S   A   L   S   M   544
2332  ATC ACA GAG CGA CAT GGG TTA AAA GAA CCA AAG AGA GTG GAG GAG CTA TGC
        I   T   E   R   H   G   L   K   E   P   K   R   V   E   E   L   C   561
2383  AAC AAG ATC ACA AGC AGC TTA AAG GAC CAC CAG AGG AAG GGA CAG GCT CTG
        N   K   I   T   S   S   L   K   D   H   Q   R   K   G   Q   A   L   578
2434  GAG CCC TCA GAG CCC AAG GTC CTT CGC GCA CTG GTG GAA CTG AGG AAG ATC
        E   P   S   E   P   K   V   L   R   A   L   V   E   L   R   K   I   595
2485  TGC ACC CAG GGC CTC CAG CGT ATC TTC TAC CTG AAG CTG GAG GAC TTG GTG
        C   T   Q   G   L   Q   R   I   F   Y   L   K   L   E   D   L   V   612
2536  TCC CCA CCT TCT GTC ATC GAC AAG CTC TTC CTT GAT ACC CTG CCT TTC TGA
        S   P   P   S   V   I   D   K   L   F   L   D   T   L   P   F   *   628
2587  GCAGGGGAAGCCTGAGCAGAGAGCTACTTGCTCTGCTGGCACTGGTCATTAAGTGAGCAAAAGGATG
2654  GGTTTGAACACCTGCCCCTCTATCCTTCCTCCAGGGGAAAAAGCAGCTCCCATAGAAAGCAAAGACT
2721  TTTTTTTTTCCTGGCACCTTTCCTTACAACCTAAAGCCAGAAACCTTGCAGAGTATTGTGTTGGGGT
2788  TGTGTTTTATATTTAGGCTTTGGTGGGTGGGCTGGGAGGGGGTAAAATAGTTCATGAGGCTTTTCTA
2855  AGAAATTGCTGACGAAGCACTTTTGGATGATGCTATCCCAGCAGTGGGGTGGGGAGAAAGGATAATA
2922  TAACTGTTTTAAAAACTCTTTCCGGGGGAATATGACTATGGTTGCTTTGTATTTAAAAATAAGAACA
2989  GCCAAGGGCTGTTTTACCAGGGTAGGGCTGTGTCTTAAGACTGATCCCTTTAGTATGTACTTCCCGG
3056  ATCGAGGCACATAAGTGGTGCAAATGAGGCGGGGAAATTCTTCATTTCTTCATTTCTTTCTTCTTCT
3123  TAAAATAAAATGGCAAAAAAAAAAAAGATGGAAGATTATCTACAAATCAGACTTAGCAAAATGATAAT
3190  GGCTATTCGCTTCCACATACAAGTGCAATTTTTTAGAGTGCTGTCTTACTAAGTCTTGTTTGTGAAC
3257  TCTCCCTCATTTTATATGAAAATAAGAAGGAGGCAGTCATGTTATCAAACGGCGTGCTCATTTTCCT
3324  AGCTCACCCTTGGTCCACCTGCCCTGTAGAACCCTTCGGAGGTATGGCCCTTCTAAGACTTTCAGGC
3391  CACTCTTGATGGAATTCGACACCCCTCCCCTCAACCCATGACTATCCAGATGTCCTGAATGGGGATC
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3458 AGGTTATAAAATGGATTGCATATGACTGTGTTCGCTGTGTGTTTGTCAACCTGGACAGAGTTCTCTA
3525 AACCTTCTTTAGTTGTAGCAAGTTCCTGATTCCTCCATTCAGAAGCCCAAGGAGCATTGGGTGACTC
3592 GATCAAGGGTTAACCCTAGGAGAACATGCAAATAAGTAGGAACTGGGTCAGACAGGGTAAGCACCAG
3659 AGATGATAAGGATTTATATATAAATATATATAAAATTAATTTTTGTTATTGGTTATAGACAATTTTG
3726 GAAAGCAAGAGAATCATCTCTTTTTTTTTTTTAAAGAGGAAAAGATAGTATTGATGTATTAGCAAAG
3793 ATTAGTGGGGTACGGTTCAACATTCCGTGTTTGTGCCCCCTTTTCTATGTTTCTACTGTTGATGGCA
3860 TATTATTATGAAATGATTCGTTGCATAGTGTCCTTATTTGTATGAACATTTGTATGCACGTTCTATT
3927 GTAATCGCTTTGCCTGTATTTATTGCAAGACCACCAGCTCCTGGAGGCTGAGTTACAGAATAATCAA
3994 ATGGGGTGTTCGTGGTGACTTGGATACACCGGTTAGAAATTAAATAAGCATATATATATATATAAAA
4061 ACATAGCAGGTTACATATATATTTATAATGTGTCTTTTTATTAACCATTTGTACAATAAATGTCACT
4128 TCCCACGCAGTTATTTTATCCTTTGTTTGCAGTGACCTTTAAGGCAGCACTGTTTAGCACTTTGATA
4195 TGAAATTTTTTGCTTATTTTTTTGCTAAATTCAAATAACGTTTGAAGATTTTTAGGTCTAAAAGTCT
4262 TTATATTATATACACTGTATCAAGTCAAGATACCTTTGGCCGTTTTGCTAAGACTCAAACTTTGAAT
4329 GTCAAACCAATGTCACGGTAGCTTCTGTTAGCTTTTAATCATTTTTGCTTTAGTCTTTTTTTTTAAA
4396 AAAAA

Fig. 3.2. Nucleotide sequence and predicted amino acid sequence of NOR-1 cDNA. Numbers at left refer to the first nucleotides on the lines, and numbers at right refer to the last amino acids on the lines. The ATTTA sequence motifs are shadowed.

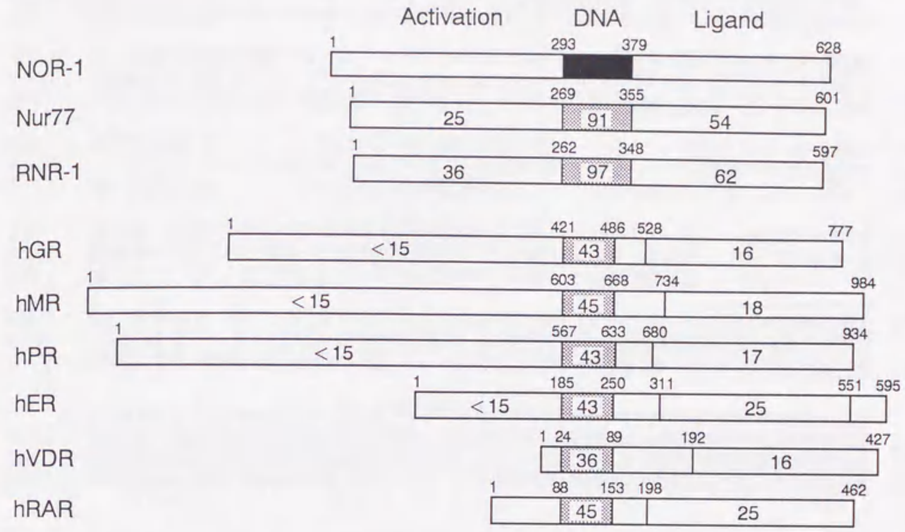

Fig. 3.3. Schematic amino acid comparison of NOR-1 and the thyroid/steroid receptor superfamily. The percentage of amino acid identity is indicated for each region in relation to the corresponding region of NOR-1. The amino acid position of each domain boundary is shown. Designations are as follows: Activation, the putative transactivation domain; DNA, the DNA-binding domain; Ligand, the putative ligand-binding domain; hGR, human glucocorticoid receptor; hMR, human mineralocorticoid receptor; hPR, human progesterone receptor; hER, human estrogen receptor; hVDR, human vitamin D receptor; hRAR, human retinoic acid receptor.

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NOR-1   MPCVQAQYSPSP-PGSTYATQTYG--S--EYTTEIMNPDYAKLTMDLGST-GIMATATTSLPSFSTFMEGYP   66
Nur77   MPCIQAQYGTPA-TSPG-PRDHLT--G--D-PLAL-E--FGKPTMDLASPETA-PAAPATLPSFSTFMDGYT   61
RNR-1   MPCVQAQYGSSPQGASP-ASQSYSYHSSGEYSSDFLTPEFVKFSMDLTNT-EI-TATTS-LPSFSTFMDNYS   68

NOR-1   SSCELKPSCLYQMPPSGPRPLIKMEEGREHGYHHHHHHHHHHHHHHHQQQQPS-IPPPSGPEDEVLP-STSMY  136
Nur77   GEFD--TF-LYQLPGTT-------Q-PC--S-------------------SA-CSSASSTS----S-SSS--   95
RNR-1   TGYDVKPPCLYQMPLSGQQSSIKVEDI-QM--H-------------NYQQHSHLPPQS--EE-MMPHSGSVY  132

NOR-1   FKQSPPSTPTTP---------G-FPPQ-AGALWDDELPSAPG------C-----IAPGPLL-D--P-QMKAV  182
Nur77   ---SATSPASASFKFEDFQVYGCYPGTLSGPL-DETLSSSGSEYYGSPC-----SAPSPSTPNFQPSQLSPW  156
RNR-1   YKPSSPPTPSTP---------G-FQVQ-HSPMWDD--PGSLH------NFHQNYVATTHMI-E----QRKT-  168

NOR-1   -PPMA--AAAR-F-PIF-FKPSP-PHPPAPSPAGGHHLGYDPTAAAALSLPLG-AA----AAAGS-QAAALE  241
Nur77   DGSFGHFSPSQTYEGLWAW-TEQLPKASSGPPPPPTFFSFSPPTGPS-P-SLAQSSLKLFPPPATHQLG--E  225
RNR-1   -P-VS-----R-L-SLFSFKQSA-P-GTPVSSCQMR---FDGP----LHVPMN-PE----PAGSH-HV--VD  215

                                                                  DNA Binding domain
NOR-1   GHPYGLPLAKR-TATLTFPPLGLTASPT--ASSLLGESPSLPSP-PNRSSSSG--EGTCAVCGDNAACQHYG  307
Nur77   GESYSMP-A----A---FPGLA-PTSPNRDTSGIL-DAP-VTSTKS-RSGASGGSEGRCAVCGDNASCQHYG  283
RNR-1   GQTFAVPNPIRKPASMGFPGLQIGHASQ-----LL-DTQ-VPPS-PSRGSPSN--EGLCAVCGDNAACQHYG  276

                                                                  DNA Binding domain
NOR-1   VRTCEGCKGFFKRTVQKNAKYVCLANKNCPVDKRRRNRCQYCRFQKCLSVGMVKEVVRTDSLKGRRGRLPSK  379
Nur77   VRTCEGCKGFFKRTVQKSAKYICLANKDCPVDKRRRNRCQFCRFQKCLAVGMVKEVVRTDSLKGRRGRLPSK  355
RNR-1   VRTCEGCKGFFKRTVQKNAKYVCLANKNCPVDKRRRNRCQYCRFQKCLAVGMVKEVVRTDSLKGRRGRLPSK  348
            P Box                 D Box                                       A Box

NOR-1   PKSPLQQEPSQPSP---PSPPICMMNALVRALTDATPRDLDYSRYCPTDQATAG-TDAEHVQQFYNLLTASI  447
Nur77   PK----Q-PPDASP---TNLLTSLIRAHLDS--GPSTAKLDYSKFQELVLPRFGKEDAGDVQQFYDLLSGSL  417
RNR-1   PKS-----PQDPSPPSPPGSDQCPRQTHVDS--NPAMTSLDYSRFQANPDYQMSGDDTQHIQQFYDLLTGSM  413

NOR-1   DVSRSWAEKIPGFTDL-PKEDQTLLIESAFLELFVLRLSIRSNTAEDKFVFCNGLVLHRLQCLRGFGEWLDS  518
Nur77   DVIRKWAEKIPGFIELCPGD-QDLLLESAFLELFILRLAYRSKPGEGKLIFCSGLVLHQLQCARGFGDWIDN  488
RNR-1   EIIRGWAEKIPGFADL-PKASQDLLFESAFLELFVLRLAYRSNPVEGKLIFCNGVVLHRLQCVRGFGEWIDS  484

NOR-1   IKDFSLNLQSLNLDIQALACLSALSMITERHGLKEPKRVEELCNKITSSLKDH--QRKGQALEPS-EPKVLR  587
Nur77   ILAFSRSLHSLGVDVPAFACLSALVLITDRHGLQDPRRVEELQNRIASCLKEHMATVAGDPQPASCLSRLLG  560
RNR-1   IVEFSSNLQNMNIDISAFSCIAALAMVTERHGLKEPKRVEELQNKIVNCLKDHVTFNNGGLNRPNYLSKLLG  556

NOR-1   ALVELRKICTQGLQRIFYLKLEDLVSPPSVIDKLFLDTLPF                                  628
Nur77   KLPELRTLCTQGLQRIFCLKLEDLVPPPPIVDKIFMDTLSF                                  601
RNR-1   KLPELRTLCTQGLQRIFYLKLEDLVPPPAIIDKLFLDTLPF                                  597
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Fig. 3.4. The amino acid translation of NOR-1 is compared with that of m-Nur77 and rRNR-1. Numbers on the right refer to the last amino acid on each line. The amino acids that are homologous in two or more proteins are shadowed. P,D, and A boxes and the DNA-binding domain are indicated.

Fig. 3.5. NOR-1 binds specifically to the B1a response-element. NOR-1 mRNA was translated in vitro with reticulocyte lysate and bound to radiolabeled B1a (Nur77 DNA binding element) and run on a native acrylamide gel. Lane 1, reticulocyte lysate programmed with H_2O ; lane 2, reticulocyte lysate programmed with NOR-1 mRNA; lanes 3 and 4, reticulocyte lysate as in lane 2 with 20× and 100×unlabeled B1a oligonucleotide, respectively.

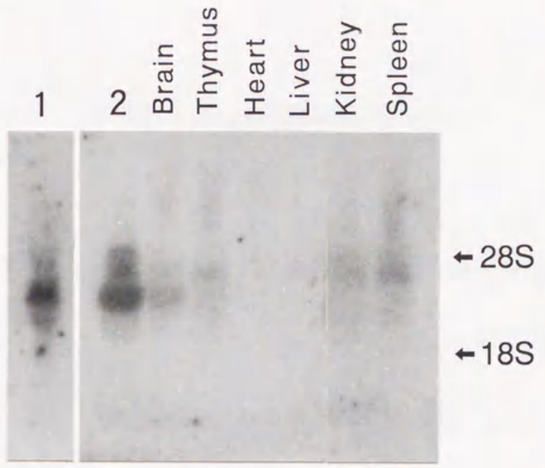


Fig. 3.6. Tissue distribution of NOR-1 mRNA. Northern blots containing 20 μg of total RNA/lane were immobilized on nylon-membrane and hybridized with a [^{32}P] labeled NOR-1 cDNA probe. The locations of the 28S and 18S RNAs are indicated. Lane 1; fetal forebrain (E17), lane 2; forebrain neuronal cells undergoing apoptosis in primary culture. RNAs of the other tissues were prepared from adult rats.

CHAPTER 4

Physiological role of NOR-1 in the fetal brain

- Effects of antisense oligonucleotide to NOR-1

in primary cultured forebrain cells -

Introduction

In the previous chapter, I identified a novel member of the orphan nuclear receptors referred to as NOR-1. Intracellular nuclear receptors such as estrogen and progesterone receptors, through binding to their specific ligands, act to coordinate complex events involved in development, differentiation, and physiological response to diverse stimuli (Evans, 1988). The hormone-receptor complex may function as a key constituent in determining commitment to specific cell lineages, as well as provoking differentiation in already determined cells (Mangelsdorf, Thummel *et al.*, 1995). Therefore, orphan nuclear receptors within the steroid/thyroid receptor superfamily may also coordinate the components of physiological responses by activating/repressing the expression of gene networks.

Analysis of NOR-1 mRNA expression in normal tissues revealed that it is expressed at a high level in fetal forebrain at embryonic day 17 (E17), the developmental stage of the nervous system, but at a low level in the adult forebrain. While a few members of the nuclear receptor show tissue specific expression (Shen, Moore *et al.*, 1994), NOR-1 is expressed typically in the fetal brain in which neural networks have been constructed through developmental processes. These findings suggest a possibility that NOR-1 may be involved in the functions of neural development processes, including differentiation, proliferation and embryogenesis. Its mRNA is also expressed in cultured forebrain neuronal cells that show the characteristic DNA ladder pattern of apoptosis. During nervous system development many neurons die by apoptosis, which is used for both coordination of neuronal population size and removal of incorrectly connected neurons (Hamburger, 1975). In addition, Liu *et al.* (1994) revealed that Nur77, whose amino acid sequence is highly homologous to NOR-1, might act in the control of

apoptosis of thymocytes and T-cell hybridomas. Therefore, it remains possible that NOR-1 might be associated with apoptosis of neuronal cells.

The present chapter was designed to clarify the physiological role of NOR-1 in forebrains at the cellular level. I examined the influence of antisense oligonucleotide to NOR-1 in primary cultured cells dissociated from the rat fetal forebrains.

Materials and Methods

Oligonucleotides

Phosphorothioate antisense oligonucleotides were designed to hybridize to initiation site on the rat NOR-1 mRNA. They are 21-mers (5'-CATATCTGGAGGGTATCTTGC-3', antisense[A]; and 5'-AGGGCATATCTGGAGGGTATC-3', antisense[B]) that are complementary to nucleotides 682-702 and 686-706 of NOR-1 mRNA (nucleotide 700 begins with the AUG codon). The sense oligonucleotide (5'- GATACCCTCCAGATAT GCCCT-3') used as control had a sequence corresponding to that of the mRNA (position 686-706). In addition, random oligonucleotide (5'-GACATGAGGACTATAGCTACC-3') was synthesized having the same sequence as the antisense[A] oligomer except for the presence of ten mismatches. They were synthesized on an Applied Biosystems DNA synthesizer and then purified by HPLC (Simazu Seisakujyo, Kyoto, Japan) with a C_{18} reverse-phase column (Tosoh Co., Tokyo, Japan). Oligonucleotide (1 μM) was added to the culture media at 20 h after the culture initiation.

RNA preparation and reverse transcriptase-PCR

Total RNAs from the forebrain cultures in the presence or absence of oligonucleotide were prepared 40 h after the treatment. Zero point two microgram of total RNA was reverse-transcribed using an oligo (dT) primer and reverse transcriptase (Superscript II, Gibco BRL). The single strand cDNA products were denatured and subjected to PCR amplification (40 cycles) with two primers (5'-CTCTGCGCCTGGCT GCATCG-3' as 5' primer and 5'-TGTCTACCGGGCAGTTTTTA-3' as 3' primer) for NOR-1 cDNA amplification. For glyceraldehyde 3-phosphate dehydrogenase (G3PDH) cDNA amplification, as control, the cDNA products were subjected to PCR amplification

(35 cycles) with two primers (5'-ACCACCATGGAGAAGGCT -3' as 5' primer and 5'-GGATCCTTACTCCTT-GGAGGC -3' as 3' primer). Each PCR cycle consisted of denaturing at 94 °C for 1 min, annealing at 60 °C for 2 min and extension at 72 °C for 3 min. The PCR products were electrophoresed on agarose gels.

Immunocytochemistry of MAP 2

Forebrain cells were plated on poly-L-lysine coated chamber slides (Becton Dickinson Labware) and treated with the oligonucleotide. After 72 h of the treatment, the cells were fixed with 4 % paraformaldehyde in 0.1 M phosphate buffer, and transferred to ice-cold 90 % methanol + 5 % acetic acid for 10 min. The cultures were incubated with MAP2 monoclonal antibody (Amersham) for 15-18 h at 4 °C in phosphate buffer containing 2 % (w/v) bovine serum albumin. The bound mouse immunoglobulin was detected using biotinylated horse anti-mouse immunoglobulin (Vector Labs., CA USA), and then visualized by streptoavidin conjugated with fluorescein isothiocyanate (FITC, Gibco BRL). The cells were observed with a fluorescence microscope.

Measurement of cell viability

The alamarBlue assay (Alamar Biosciences, CA USA) was used to measure quantitatively the viability of cultured forebrain cells. The alamarBlue consists of an oxidation-reduction indicator that yields calorimetric change in response to metabolic activity of cultured cells (Ahmed, Gogal & Walsh, 1994). Cells received alamarBlue at a volume of 50 μl/well (24-well tissue culture plates) in culture media, and further incubated for 3 h at 37 °C. Absorbances of culture medium were measured at a wavelength of 570 nm, and subtracted background absorbance at 600 nm.

Electron microscopic observation

After treatment with the oligonucleotide for 72 h, the cells were briefly washed with phosphate-buffered saline and fixed with 2.5 % glutaraldehyde in 0.1 M phosphate buffer at 4 °C for 10 min. The samples were post-fixed with 1 % osmium, dehydrated through a graded ethanol series, and then embedded in plastic resin (Quetol 812, Nisshin EM Co., Tokyo, Japan). All the steps were carried out in the culture dishes. Ultrathin sections were cut vertical to the bottom of the plates, stained with uranyl acetate and lead citrate, and observed under an electron microscope (JEOL 1200 EX, Nihon densi Co., Tokyo, Japan).

Results

Effect of the antisense oligonucleotides on NOR-1 expression

Treatment of rat forebrain cells with the antisense oligomer (1 μM) led to a reduction in the amount of NOR-1 mRNA as assayed by RT-PCR amplification (Fig. 4.1). The NOR-1 levels were decreased after 40 h of antisense-treatment (ANTI[A] and [B]), compared to the level in untreated cells (control). Cultured cells treated with the antisense[B] oligomer exhibited no detectable level of NOR-1, whereas antisense[A]-treated cells possessed a small number of NOR-1 PCR products. After a 40 h incubation with the sense or random oligomer, the NOR-1 levels did not show any apparent changes compared to the untreated cells. PCR amplifications with primers designed from G3PDH cDNA sequence, as control, were not changed in any group. For this reason, the antisense[B] oligomer was used in all of the other experiments.

Effect of antisense-treatment on the morphological changes

Treatment of forebrain cells with the antisense oligonucleotide led to dramatic morphological changes (Fig. 4.2). Primary embryonic day 17 rat forebrain cultures were used to determine the effects of the antisense oligomer to NOR-1. Cells in complete medium containing 10 % FBS sent out short neurites in untreated, random-, or sense-treated groups after 24 h (Fig. 4.2 A, D, G). Treatment with the antisense oligomer to NOR-1 (1 μM; antisense[B]) induced cell migration, and increased a number of cells with processes, compared to the morphology in the other groups (Fig. 4.2 J).

After 72 h in culture, antisense-treated cells acquired well-developed processes, and cellular clustering appeared. Neurite bundles were frequently observed between the aggregates. Furthermore, antisense-treatment markedly increased the length of processes

(Fig. 4.2 B, E, H and K), which were about 50 - 200 μm in control groups and 200 - 500 μm in the antisense-treated group. In untreated, random- and sense-treated groups, cells were distributed in a nearly homogenous fashion with very few or no aggregates. Neurites were relatively short, with frequently branched processes, and neurite bundles were rarely seen (Fig. 4.2 B, E, H).

By 7 days, without any medium change, cells in untreated, random- or sense-treated groups were dissolved or damaged, and processes were not observed. A number of bright and round cells appeared in cultures, at which point they had stopped developing. In contrast, cells treated with the antisense oligomer appeared as phasebright neuronlike cells, exhibiting morphology characteristic of developing neurons (Fig. 4.2 L). They formed large aggregates, which were interconnected by well-developed processes. Increasing numbers of neurite bundles were also present in those cultures.

Consistent with studies on the ability of oligonucleotides in inhibiting NOR-1 expression, antisense[B]-treatment was more effective in extension of processes compared with antisense [A]-treatment. Treatment with a 5-fold lower concentration of the antisense oligonucleotide (0.2 μM; [A] or [B]) had no detectable effect on forebrain cells, and treatment with a 5-fold higher concentration of the sense, random, antisense[A] or antisense[B] oligonucleotide (5 μM) showed a significant neurotoxic response as exemplified by swelling of cell bodies and neurite fragmentation, without any apparent efforts.

Identification of neurites in antisense-treated cells

To determine whether the processes extended in response to the antisense oligomer include neurites, immunocytochemical analysis was performed with a monoclonal antibody to the rat MAP2. MAP2 has proven to be a useful neuritic marker, because its

cellular location was restricted to the processes of neurons (Bloom, Schoenteld & Vallee, 1984). Fig. 4.3 shows the results of MAP2 immunocytochemical staining after 72 h of the treatment. In antisense[B]-treated cells, almost all the processes connecting between aggregates were stained for MAP2, indicating that the processes were filled with neurites growing from forebrain neuronal cells. In addition, the aggregates induced by antisense-treatment were filled with immunoreactive MAP2, which were densely packed, and had a fibrillary structure. In sense-treated cells, MAP2 was positive for a large proportion (40-60 %) of cells. These neurons, most of which were round, measured 20 - 30 μm in diameter and had two to four processes stained up to a length of 200 μm. Cells in untreated or random-treated groups showed staining pattern similar to that of the sense-treated cells (not illustrated).

Effect of antisense-treatment on apoptotic cell death

To investigate whether a physiological role of NOR-1 is associated with a process of apoptosis, antisense-treated cells were examined for some typical phenomena in apoptotic cell death. DNA fragmentation that occurs in apoptotic cells was observed among untreated, random-, sense-, antisense[A]- or antisense[B]-treated groups after 72 h of the treatment (Fig. 4.4). In all of the groups, no apparent differences were detected. Viability of forebrain cells treated with the oligonucleotide was determined by alamarBlue assay. After a 72 h incubation with the oligonucleotide, the alamarBlue values (mean ± S.E.M., n=4) did not significantly differ between untreated control (0.12 ± 0.0097), 1 μM sense-treated control (0.11 ± 0.0064) and 1 μM antisense[B]-treated experimental (0.11 ± 0.0061) groups. Since nuclear morphological changes have been known to be associated with apoptosis, I investigated the nuclear morphology on cultured cells treated with the oligonucleotide by electron microscopy. No apparent differences were observed

between sense- and antisense[B]-treated groups (Fig. 4.5). In both groups, condensed chromatin was often found in the neuronal cells. The features of cells in the untreated or random oligomer-treated groups were essentially similar to those treated with the sense oligonucleotide, as well as the antisense oligonucleotide (not illustrated).

Discussion

The present chapter has demonstrated that neurite extension as well as cell migration of cultured rat forebrain cells was dramatically induced by treatment of the antisense oligonucleotide to NOR-1.

The specific effect of the antisense oligonucleotide on the expression of NOR-1 was demonstrated by RT-PCR amplification with primers designed from NOR-1 cDNA sequence. Treatment of cells with the phosphorothioate antisense oligonucleotide promoted a reduction in NOR-1 mRNA, suggesting that this reduction was due, in part, to RNase H-mediated cleavage of target mRNA (Chiang, Chan *et al.*, 1991). The partial-overlapping oligonucleotides, designated antisense[A] and antisense[B] oligonucleotides, had apparent effects on NOR-1 expression, whereas antisense[A]-treatment was less effective in inhibiting NOR-1 expression compared with antisense[B]-treatment. This observation is consistent with the morphological changes induced by those oligonucleotides, which showed that antisense[A]-treatment was less effective in extension of processes compared with antisense[B]-treatment. The difference in their inhibition of NOR-1 expression may be due to the secondary structure of the target RNA sites. Cells treated with a high dosage of the oligonucleotides (5 μM) showed a neurotoxic response (*e. g.*, swelling of cell bodies and neurite fragmentation) in all of sense-, random-, antisense[A]-, or antisense[B]-treated groups, probably because of the neurotoxicity of the phosphorothioate oligonucleotides.

Under the present experimental conditions, significant enhancement of cell migration, neurite extension, and formation of cellular aggregates was induced with only 24 h of antisense-treatment, with better effects at from 24 to 72 h. This suggests that the effects of antisense-treatment have a positive influence on forebrain cells, and can be

induced by brief exposure without any supplement of neurotrophic agents. The increase in length of the processes and numbers of the neurite bundles with longer exposure to the antisense oligonucleotide indicate that the NOR-1 antisense oligomer exerts a major effect on the formation of neuronal processes. In contrast, effects on survival rates were not evoked by exposure to the antisense oligonucleotide, suggesting that its effect might be specific to the cellular morphology. Results from MAP2 immunofluorescence study show that both the aggregates and processes, induced by antisense-treatment, were filled with neurites growing from neuronal cells. It seems, therefore, that antisense-treatment caused forebrain cells to undergo a morphological change that could be considered to be a form of differentiation. During nervous system development, neuronal cells have been reported to be migrating, extending many neuronal fibers, and showing the formation of axonal pathways and synaptic connections (Purves & Lichtman, 1985). The present observations are consistent with the concept that NOR-1 is important in the functions of neural development processes.

To investigate whether NOR-1 is also associated with a process of apoptosis, antisense-treated cells were examined for some typical phenomena in apoptotic cell death. However, no differences were observed with regard to apoptotic DNA ladders, survival rates, and nuclear morphological changes between antisense-, sense-treated and control groups. These results indicate that the antisense oligonucleotide to NOR-1 has essentially no effect on apoptosis or survival in forebrain cells, in contrast to its pronounced effect on cell morphology. Although there are many similarities between NOR-1 and the Nur77/NGFI-B family, the physiological role of NOR-1 might differ from those of the other Nur77/NGFI-B family members.

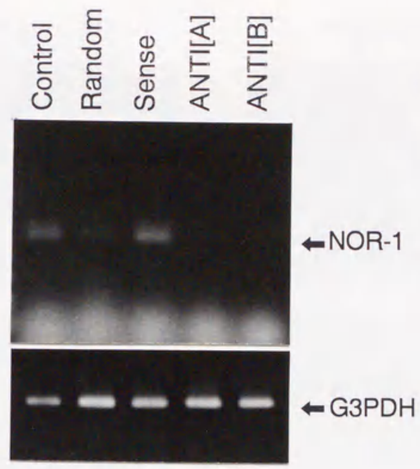
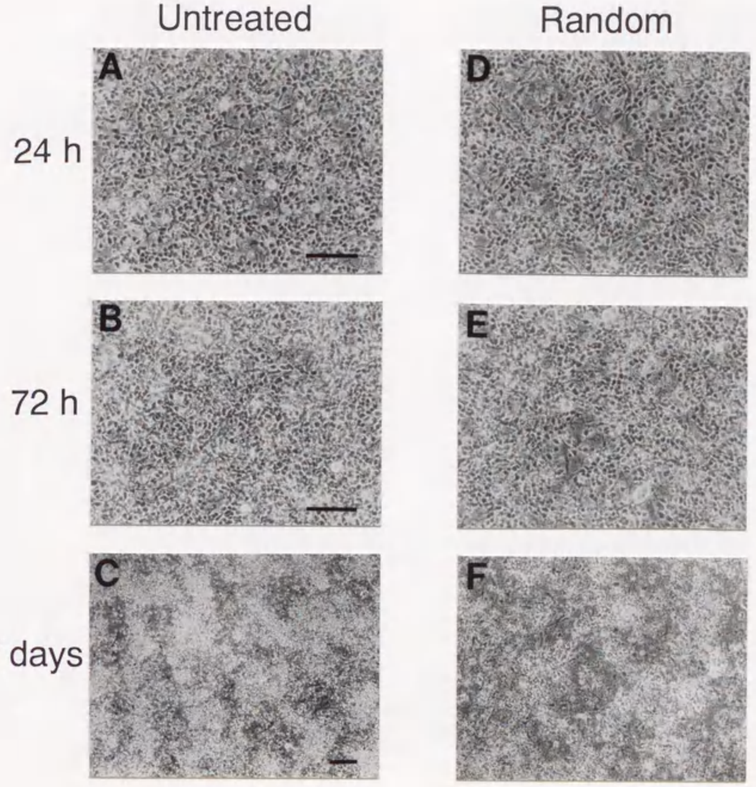


Fig. 4.1. NOR-1 levels decline by treating cells with the antisense oligonucleotide. NOR-1 as well as G3PDH cDNA were amplified by RT-PCR from primary cultured forebrain cells treated with 1 μM oligonucleotide. The PCR products were electrophoresed on agarose gels. Cultures were untreated (lane 1, control), treated with the random (lane 2), sense (lane 3), antisense[A] (lane 4, ANTI[A]), or antisense[B] (lane 5, ANTI[B]) oligonucleotide for 40 h.


Untreated
Random
24 h
72 h
7 days
A
B
C
D
E
F

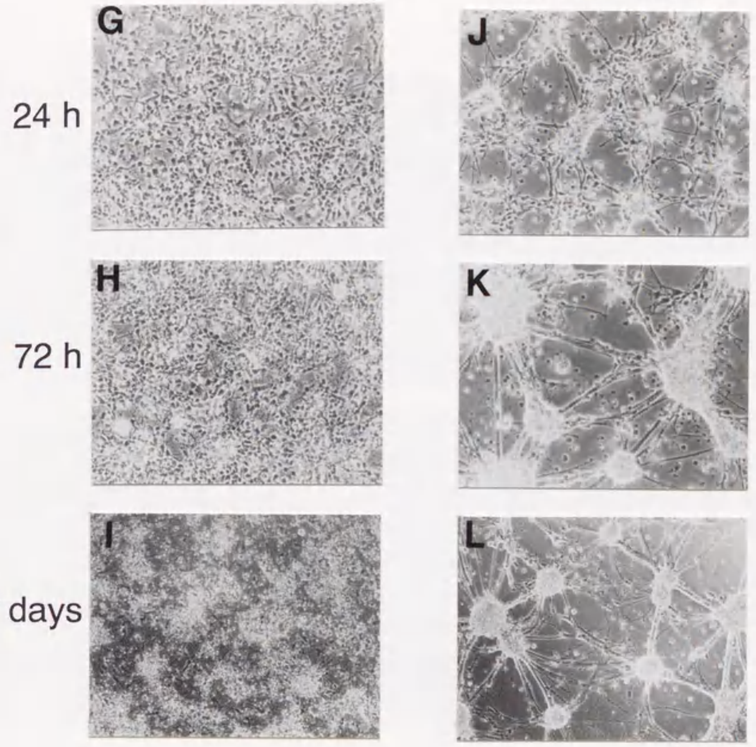


Fig. 4.2. Morphological changes in primary cultured rat forebrain cells after antisense treatment. E17 rat forebrain cells were grown in complete medium in the presence or absence of phosphorothioate oligonucleotide. Cultures were untreated (A-C), treated with the 1 μM random (D-F), sense (G-I) or antisense[B] (J-L) oligonucleotide for 24 h (upper panels), 72 h (middle panels), or 7 days (low panels). The experiment was repeated three times with similar results. They were photographed by a phase-contrast microscope. Bars=200 μm.

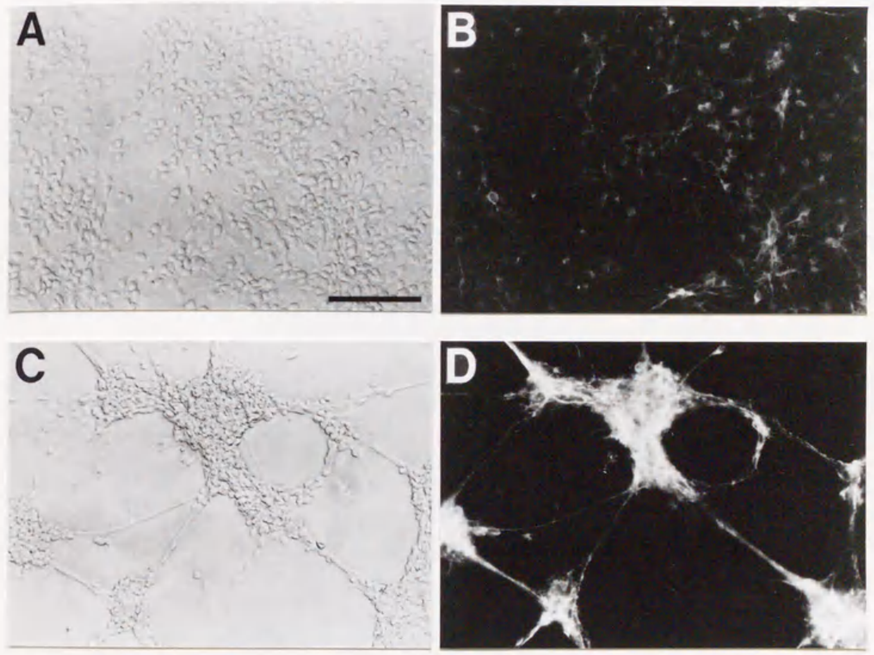


Fig. 4.3. MAP2 immunoreactivity in antisense-treated cells. Primary cultured rat forebrain cells were treated with 1 μM sense (A and B), or antisense (C and D) oligonucleotide for 72 h. Bright field (A and C). Immunofluorescence for MAP2 in the same fields (B and D). Bar=200 μm.

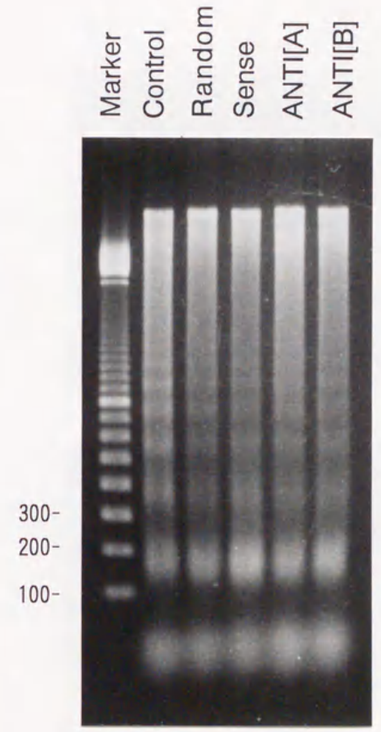


Fig. 4.4. Agarose gel electrophoresis of DNA fragmentation in cultured forebrain cells. Cultures were untreated (lane 2, control) , treated with 1 μM random (lane 3), sense (lane 4), antisense[A] (lane 5, ANTI[A]), or antisense[B] (lane 6, ANTI[B]) oligonucleotide for 72 h. A 100-bp DNA ladder is shown for comparison (lane 1). The gels shown are representative data obtained from the three independent experiments.

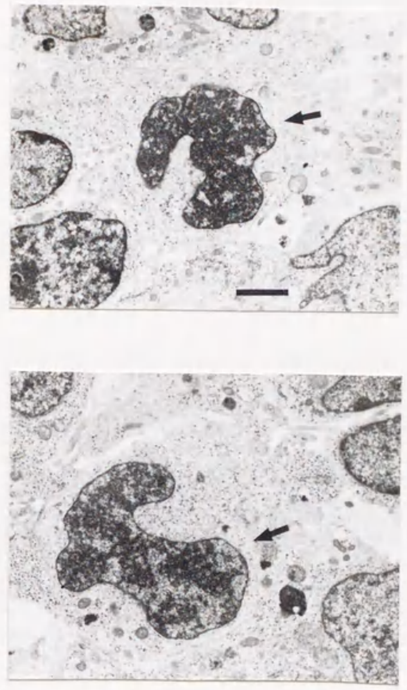


Fig. 4.5. Electron micrographs of primary cultured forebrain cells. The cells were treated with the sense (upper panel) or antisense (low panel) oligonucleotide for 72 h. Arrows indicate the condensed chromatin in the neuronal cells. Bar=2 μm.

CHAPTER 5

Identification of a human homologue of NOR-1

Introduction

The cDNA that encode rat NOR-1 nuclear receptor has been cloned by a PCR-based strategy, and physiological studies, in chapter 4, demonstrated that NOR-1 might be associated with neural development processes.

NOR-1 shares amino acid sequence similarity with the members of the Nur77 /NGFI-B family, composed of Nur77/NGFI-B and RNR-1. Nur77 is first identified as a set of genes that are expressed during the G_0/G_1 transition in mouse fibroblasts, with no known function (Hazel, Nathans & Lau, 1988). It is also expressed in the pheochromocytoma cell line PC12 after KCl-mediated membrane depolarization or NGF treatment (Bartel, Sheng *et al.*, 1989). These agents result in divergent biological effects: the G_0/G_1 transition is mitogenic for fibroblasts; in PC12 cells NGF causes neuronal differentiation. RNR-1 is one of the immediate early genes induced during liver regeneration, but little is known of its specific role in physiological processes (Scearce, Laz *et al.*, 1993). Nur77/NGFI-B and RNR-1 have been identified in rat [NGFI-B (Milbrandt, 1988) and RNR-1 (Scearce, Laz *et al.*, 1993)] , mouse [Nur77 (Hazel, Nathans & Lau, 1988) and NURR1(Law, Conneely *et al.*, 1992)] and human [TR3 (Chang, Kokontis *et al.*, 1989b) and NOT (Mages, Rilke *et al.*, 1994)]. Their functional and structural similarity indicates that elements of transcriptional regulation are likely to be conserved among rat, mouse and human, and therefore will probably be conserved in all vertebrates. Thus, to further characterize the NOR-1 gene, it is important to determine whether NOR-1 is also existed in other vertebrates, especially in human.

In this chapter I describe the isolation and characterization of a human homologue to rat NOR-1 from the fetal brain. I present its nucleotide, deduced amino acid sequence, tissue distribution and Southern blot analysis, and I conclude that its homologue is

defined as a novel nuclear receptor to be called human NOR-1.

Materials and Methods

Cell culture and RNA isolation

The human neuroblastoma cell line, NB-OK-1, was maintained in RPMI 1640 medium supplemented with 10 % fetal calf serum (Mitsubishi Kasei, Tokyo, Japan). Total RNA fractions were isolated using acid guanidinium thiocyanate-phenol-chloroform method.

Reverse Transcriptase-PCR and cDNA cloning

One microgram of total RNA derived from human neuroblastoma cells was reverse-transcribed using an oligo(dT) primer and reverse transcriptase (Superscript II, Gibco BRL). Sequences related to rat NOR-1 were amplified by PCR. The single strand cDNA products were denatured and amplified by PCR using two primers (CGAGCTTTAACAGACGCAAC as 5' primer and TCCTCCAGCTTCAGGTAGAA as 3' primer) designed from the sequence of rat NOR-1. The PCR products were ligated into plasmid PCRII (Invitrogen) and sequenced. The cDNA fragment encoding a sequence similar to that of rat NOR-1 was labeled with DIG-dUTP (Boehringer Mannheim) by PCR. It was then used to screen a human fetal brain λ gt11 cDNA library (age; 19 - 23 weeks, Clontech) and to isolate the cDNA encoding the sequence similar to that of rat NOR-1.

Northern blotting

The tissue specificity of isolated cDNA fragments was determined by Northern blotting using commercial filters (Clontech). One blot contained about 2 μg of poly(A)$^+$ mRNA per lane from four human fetal tissues (brain, lung, liver and kidney), and

another blot contained 2 μg poly(A)$^+$ mRNA from adult tissues (heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas).

Southern blotting

Genomic DNA derived from human placental tissue (Clontech) was digested with various restriction enzymes, separated on 0.6 % agarose gels and transferred to a nylon membrane (Amersham) according to standard protocols. "A' and "B" probes contain the human NOR-1 cDNA at 1113 - 1649 and 2507 - 3802 nt, respectively.

Amplification of the 5'-end of NOR-1 cDNA

I determined the transcriptional start site of the cDNA by means of 5'-Ampli-FINDER RACE (Clontech). One microgram of Poly(A)$^+$ RNA derived from human skeletal muscle (Clontech) was reverse-transcribed with the antisense NOR-1 gene-specific primer (P1; GGTGTATTCCGAGCTGTATGTCTGCGC). The cDNA was purified and concentrated by ethanol precipitation. A specific single-stranded anchor oligonucleotide (CACGAATTCACTATCGATTCTGGAACCTTCAGAGG) was ligated to the 3'-end of the cDNA. Following anchor ligation, a portion of the cDNA was then used as a template for PCR amplification, using a primer complementary to the anchor (CTGGTTCGGCCCACCTCTGAAGGTTCCAGAATCGATAG) and a nested gene-specific primer (P2; AACCTGGAGGGGAAGGGCTATATTGGG or P3; GTGAGCGCGTCTGTGTATGGGGAGC). The PCR products were ligated into plasmid PCRII and sequenced.

Results

Isolation of the human NOR-1 cDNA

One fragment was isolated when cDNA derived from human neuroblastoma cells was used as a template in a PCR amplification with a pair of degenerate oligonucleotides corresponding to the rat NOR-1 sequence. The sequence was similar to that of rat NOR-1. The PCR product was then used to select a nearly full-length cDNA clone from a human fetal brain cDNA library. Screening with the PCR fragment as a probe revealed several positive clones. Restriction enzyme mapping and cDNA sequencing showed that the isolated clones had overlapping sequences which formed a 3.8 kb nuclear receptor gene. This cDNA was designated as human NOR-1 (hNOR-1; *n*euron derived *o*rphan *r*eceptor).

The isolated cDNA consisted of 3762 nucleotides (Fig. 5.1; positions 41 - 3802 nt), and it contained a major open reading frame of 1881 nt starting with an initiation ATG codon at 732 nt. The 5' untranslated region of the NOR-1 cDNA contained two ATG triplets, but they had a poor flanking sequence for translation initiation according to the consensus sequence derived by Kozak (Kozak, 1986) and it was stopped at the in-frame terminator. Consequently the third ATG (position 732 nt), which has features of the consensus sequence for initiation, would be used. The open reading frame of the NOR-1 cDNA encodes a protein of 626 amino acids, with a predicted molecular mass of 68 kDa. The 3'-untranslated region of the cDNA contained three copies of the sequence ATTTA (positions 2989, 3671 and 3765 nt), which have been identified in many short-lived mRNAs and may accelerate poly(A) degradation (Shaw & Kamen, 1986). Moreover, none of the cDNA contained a poly(A) tail, indicating a possibility that the hNOR-1 mRNA does not have a detectable poly(A) tail.

A comparison of the hNOR-1 amino acid sequence with known protein sequences revealed that hNOR-1 is the human homologue of the rat NOR-1 gene and it has

significant similarity in sequence and structure to the steroid/thyroid hormone receptors. Human and rat NOR-1 orphan receptors have identical amino acid sequences in the DNA binding domain. The overall homology between the two receptors was 93%.

The most striking feature of the NOR-1 orphan receptor was the high homology with the Nur77/NGFI-B family (Fig. 5.2). In the DNA-binding domain, 93 and 98% of the amino acid residues of hNOR-1 were identical with those of human TR3 (Chang, Kokontis *et al.*, 1989b) [also called Nur77 (Hazel, Nathans & Lau, 1988), NGFI-B (Milbrandt, 1988), TIS1 (Herschman, 1991), N10 (Ryseck, Bravo *et al.*, 1989), NAK1 (Nakai, Kartha *et al.*, 1990) and ST-59 (Bondy, 1991)] and NOT (Mages, Rilke *et al.*, 1994) [also called NURR1 (Law, Conneely *et al.*, 1992) and RNR-1 (Scearce, Laz *et al.*, 1993)], respectively. Overall, hNOR-1 showed 43 and 57 % homology to the TR3 and NOT, respectively. The value is higher than that for any other member of the steroid /thyroid receptor superfamily, indicating that hNOR-1, TR3, and NOT form a distinct subclass. The carboxyl-terminal region of the hNOR-1 protein, which corresponded to the ligand binding domain of several steroid/thyroid receptors, was again highly homologous to TR3 and NOT. This region of the protein also contains a leucine-zipper, a motif known to mediate protein-protein interactions (Landschulz, Johnson & McKnight, 1988). This structural feature, which is unusual for steroid receptors, is also present in the Nur77/NGFI-B family.

Determination of the transcriptional start site

The transcriptional start site of the hNOR-1 gene was determined by 5'-Ampli-FINDER RACE. Reverse-transcription was performed with an oligonucleotide primer (P1) located from nt 786 to 812. PCR amplification proceeded using an anchor primer and a nested gene-specific primer located from nt 747 to 774. Sequence analysis of these PCR products revealed that the major transcription initiation site is 40 nt upstream of the 5' end of the hNOR-1 cDNA obtained from a λ gt11 library (Fig. 5.1; position 1 - 40 nt,

and Fig. 5.3). Similar results were obtained with a primer (P3) located at 97 - 121 nt.

Tissue distribution of NOR-1

I determined the tissue distribution of hNOR-1 by Northern blotting RNAs prepared from a variety of human tissues. The results are shown in Fig. 5.4. In fetal tissues, NOR-1 expression was apparently confined to the brain and lung with a small amount being localized in the kidney and liver. In adult tissues, NOR-1 mRNA was detected in the heart, abundantly in skeletal muscle, and marginally in the brain and kidney. Two bands of 4.0 and 5.5 kb were detected in RNAs from the fetal lung and adult heart and skeletal muscle, whereas one band of 5.5 kb was found in the fetal and adult brains.

The human NOR-1 gene is encoded by a single-copy gene

A gene very closely related to rNOR-1, called rat NOR-2, has been identified from a rat brain cDNA library (Petropoulos, Part *et al.*, 1995). Rat NOR-2 has identical amino acid sequences to rNOR-1 in the trans-activating and DNA-Binding domains, but not in the 5' and 3' untranslated regions. I searched for other genes closely related to NOR-1 by Southern blotting human genomic DNA using ^{32}P-labeled cDNA probes generated from the trans-activating domain (A probe) or the 3' untranslated region (B probe). The results are shown in Fig. 5.5. Human genomic DNA digested with several different restriction enzymes was transferred to nylon membranes and hybridized with labeled "A" or "B" cDNA probes. A single band was detected by both probes in all lanes. These results indicate that the human NOR-1 gene is represented as a single copy in the haploid genome.

Discussion

In this chapter I described a human orphan nuclear receptor gene designated as hNOR-1. A comparison with the sequences of known proteins showed that hNOR-1 is the human homologue of the orphan receptor, ratNOR-1. In fact, hNOR-1 is 93 % homologous to rNOR-1, and differs from the rNOR-1 protein by 44 amino acids. This very high evolutionary conservation of the sequence suggests that all the domains of the molecule have a critical functional role.

The nuclear receptors directly modulate transcription by interaction with their target genes. A comparison of the domain structure of hNOR-1 with that of the Nur77/NGFI-B family indicated that hNOR-1 shares high similarity in the DNA binding domain. Moreover, the rat NOR-1 protein bound to the B1a response-element (5'-AAAAGGTCA-3') that is a target sequence of the Nur77/NGFI-B family (Chapter 3; Wilson, Fahrner *et al.*, 1991). High affinity binding to the B1a response element requires, in addition to the DNA-binding domain, the participation of the A-box region on the C-terminal flanking side (Wilson, Paulsen *et al.*, 1992). The identical homology between rat and human NOR-1 in the DNA-binding domains together with the A box regions indicates that hNOR-1 will also bind with high affinity to the target sequence of the Nur77/NGFI-B family. The hNOR-1 amino acid sequence also shared similarity in the carboxyl-terminal region, which represents the putative dimerizing domain or ligand-binding domain.

The tissue distribution of hNOR-1 showed predominant and marginal expression in the fetal and adult brain, respectively. This expression profile was similar to that of rat NOR-1, indicating a role for hNOR-1 in neural development processes. However, it was also found in adult heart and skeletal muscle, suggesting that hNOR-1 expression is not restricted to the events that occur during neural development. In chapter 3, I described that rat NOR-1 was constitutively expressed only in brain tissue. The discrepancy between these results is probably due to the difference of species and/or the existence of

the poly(A) tail.

Northern blots of hNOR-1 revealed two bands of 4.0 and 5.5 kb in RNAs from the adult heart and skeletal muscle at various levels, while one band of 5.5 kb was detected in the fetal and adult brains. Since I did not find evidence supporting the presence of additional genomic sequences closely related to the hNOR-1 gene, they are probably generated by alternative splicing, specific cleavage of the mRNA, or the alternative use of two different poly(A) additional signals.

```
1       ATAAATGACGTGCCGAGAGAGCGAGCGAACGCGCAGCCGGGAGAGCGGAGTCTCCTGCCTC
62  CCGCCCCCCACCCCTCCAGCTCCTGCTCCTCCTCCGCTCCCCATACACAGACGCGCTCACACCCGCT
129 CCCTCACTCGAACACACAGACACAAGCGCGCACACAGGCTCCGCACACACACACTTCGCTCTCCCGC
196 GCGCTCACACCCCTCTTGCCCTGAGCCCTTGCCGGTGCAGCGCGGCGCCGCAGCTGGACGCCCCTCC
263 CGGGCTCACTTTGCAACGCTGACGGTGCCGGCAGTGGCCGTGGAGGTGGGAACAGCGGCGGCATCCT
330 CCCCCCTGGTCACAGCCCAAGCCAGGACGCCCGCGGAACCTCTCGGCTGTGCTCTCCCATGAGTCGG
397 GATCGCAGCATCCCCCACCAGCCGCTCACCGCCTCCGGGAGCCGCTGGGCTTGTACACCGCAGCCCT
464 TCCGGGACAGCAGCTGTGACTCCCCCCCAGTGCAGATTTCGGGACAGCTCTCTAGAAACTCGCTCTA
531 AAGACGGAACCGCCACAGCACTCAAAGCCCACTGCGGAAGAGGGCAGCCCGGCAAGCCCGGGCCCTG
598 AGCCTGGACCCTTAGCGGTGCCGGGCAGCACTGCCGGCGCTTCGCCTCGCCGGACGTCCGCTCCTCC
665 TACACTCTCAGCCTCCGCTGGAGAGACCCCCAGCCCCACCATTCAGCGCGCAAGATACCCTCCAGAT

732 ATG CCC TGC GTC CAA GCC CAA TAT AGC CCT TCC CCT CCA GGT TCC AGT TAT
      M   P   C   V   Q   A   Q   Y   S   P   S   P   P   G   S   S   Y   17
783 GCG GCG CAG ACA TAC AGC TCG GAA TAC ACC ACG GAG ATC ATG AAC CCC GAC
      A   A   Q   T   Y   S   S   E   Y   T   T   E   I   M   N   P   D   34
834 TAC ACC AAG CTG ACC ATG GAC CTT GGC AGC ACT GAG ATC ACG GCT ACA GCC
      Y   T   K   L   T   M   D   L   G   S   T   E   I   T   A   T   A   51
885 ACC ACG TCC CTG CCC AGC ATC AGT ACC TTC GTG GAG GGC TAC TCG AGC AAC
      T   T   S   L   P   S   I   S   T   F   V   E   G   Y   S   S   N   68
936 TAC GAA CTC AAG CCT TCC TGC GTG TAC CAA ATG CAG CGG CCC TTG ATC AAA
      Y   E   L   K   P   S   C   V   Y   Q   M   Q   R   P   L   I   K   85
987 GTG GAG GAG GGG CGG GCG CCC AGC TAC CAT CAC CAT CAC CAC CAC CAC CAC
      V   E   E   G   R   A   P   S   Y   H   H   H   H   H   H   H   H   102
1038 CAC CAC CAC CAC CAT CAC CAG CAG CAG CAT CAG CAG CCA TCC ATT CCT CCA
      H   H   H   H   H   H   Q   Q   Q   H   Q   Q   P   S   I   P   P   119
1089 GCC TCC AGC CCG GAG GAC GAG GTG CTG CCC AGC ACC TCC ATG TAC TTC AAG
      A   S   S   P   E   D   E   V   L   P   S   T   S   M   Y   F   K   136
1140 CAG TCC CCA CCG TCC ACC CCC ACC ACG CCG GCC TTC CCC CCG CAG GCG GGG
      Q   S   P   P   S   T   P   T   T   P   A   F   P   P   Q   A   G   153
1191 GCG TTA TGG GAC GAG GCA CTG CCC TCG GCG CCC GGC TGC ATC GCA CCC GGC
      A   L   W   D   E   A   L   P   S   A   P   G   C   I   A   P   G   170
1242 CCG CTG CTG GAC CCG CCG ATG AAG GCG GTC CCC ACG GTG GCC GGC GCG CGC
      P   L   L   D   P   P   M   K   A   V   P   T   V   A   G   A   R   187
1293 TTC CCG CTC TTC CAC TTC AAG CCC TCG CCG CCG CAT CCC CCC GCG CCC AGC
      F   P   L   F   H   F   K   P   S   P   P   H   P   P   A   P   S   204
1344 CCG GCC GGC GGC CAC CAC CTC GGC TAC GAC CCG ACG GCC GCT GCC GCG CTC
      P   A   G   G   H   H   L   G   Y   D   P   T   A   A   A   A   L   221
1395 AGC CTG CCG CTG GGA GCC GCA GCC GCC GCG GGC AGC CAG GCC GCC GCG CTT
      S   L   P   L   G   A   A   A   A   A   G   S   Q   A   A   A   L   238
1446 GAG GGC CAC CCG TAC GGG CTG CCG CTG GCC AAG AGG GCG GCC CCG CTG GCC
      E   G   H   P   Y   G   L   P   L   A   K   R   A   A   P   L   A   255
1497 TTC CCG CCT CTC GGC CTC ACG CCC TCC CCT ACC GCG TCC AGC CTG CTG GGC
      F   P   P   L   G   L   T   P   S   P   T   A   S   S   L   L   G   272
1548 GAG AGT CCC AGC CTG CCG TCG CCG CCC AGC AGG AGC TCG TCG TCT GGC GAG
      E   S   P   S   L   P   S   P   P   S   R   S   S   S   S   G   E   289
1599 GGC ACG TGT GCC GTG TGC GGG GAC AAC GCC GCC TGC CAG CAC TAC GGC GTG
      G   T   C   A   V   C   G   D   N   A   A   C   Q   H   Y   G   V   306
1650 CGA ACC TGC GAG GGC TGC AAG GGC TTT TTC AAG AGA ACA GTG CAG AAA AAT
      R   T   C   E   G   C   K   G   F   F   K   R   T   V   Q   K   N   323
1701 GCA AAA TAT GTT TGC CTG GCA AAT AAA AAC TGC CCA GTA GAC AAG AGA CGT
      A   K   Y   V   C   L   A   N   K   N   C   P   V   D   K   R   R   340
1752 CGA AAC CGA TGT CAG TAC TGT CGA TTT CAG AAG TGT CTC AGT GTT GGA ATG
      R   N   R   C   Q   Y   C   R   F   Q   K   C   L   S   V   G   M   357
1803 GTA AAA GAA GTT GTC CGT ACA GAT AGT CTG AAA GGG AGG AGA GGT CGT CTG
      V   K   E   V   V   R   T   D   S   L   K   G   R   R   G   R   L   374
1854 CCT TCC AAA CCA AAG AGC CCA TTA CAA CAG GAA CCT TCT CAG CCC TCT CCA
      P   S   K   P   K   S   P   L   Q   Q   E   P   S   Q   P   S   P   391
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1905  CCT TCT CCT CCA ATC TGC ATG ATG AAT GCT CTT GTC CGA GCT TTA ACA GAC
        P   S   P   P   I   C   M   M   N   A   L   V   R   A   L   T   D   408
1956  TCA ACA CCC AGA GAT CTT GAT TAT TCC AGA TAC TGT CCC ACT GAC CAG GCT
        S   T   P   R   D   L   D   Y   S   R   Y   C   P   T   D   Q   A   425
2007  GCT GCA GGC ACA GAT GCT GAG CAT GTG CAA CAA TTC TAC AAC CTC CTG ACA
        A   A   G   T   D   A   E   H   V   Q   Q   F   Y   N   L   L   T   442
2058  GCC TCC ATT GAT GTA TCC AGA AGC TGG GCA GAA AGG ATT CCG GGA TTT ACT
        A   S   I   D   V   S   R   S   W   A   E   R   I   P   G   F   T   459
2109  GAT CTC CCC AAA GAA GAT CAG ACA TTA CTT ATT GAA TCA GCC TTT TTG GAG
        D   L   P   K   E   D   Q   T   L   L   I   E   S   A   F   L   E   476
2160  CTG TTT GTC CTC AGA CTT TCC ATC AGG TCA AAC ACT GCT GAA GAT AAG TTT
        L   F   V   L   R   L   S   I   R   S   N   T   A   E   D   K   F   493
2211  GTG TTC TGC AAT GGA CTT GTC CTG CAT CGA CTT CAG TGC CTT CGT GGA TTT
        V   F   C   N   G   L   V   L   H   R   L   Q   C   L   R   G   F   510
2262  GGG GAG TGG CTC GAC TCT ATT AAA GAC TTT TCC TTA AAT TTG CAG AGC CTG
        G   E   W   L   D   S   I   K   D   F   S   L   N   L   Q   S   L   527
2313  AAC CTT GAT ATC CAA GCC TTA GCC TGC CTG TCA GCA CTG AGC ATG ATC ACA
        N   L   D   I   Q   A   L   A   C   L   S   A   L   S   M   I   T   544
2364  GAA AGA CAT GGG TTA AAA GAA CCA AAG AGA GTC GAA GAG CTA TGC AAC AAG
        E   R   H   G   L   K   E   P   K   R   V   E   E   L   C   N   K   561
2415  ATC ACA AGC AGT TTA AAA GAC CAC CAG AGT AAG GGA CAG GCT CTG GAA CCC
        I   T   S   S   L   K   D   H   Q   S   K   G   Q   A   L   E   P   578
2466  AAC GAG TCC AAG GTC CTG GTT GCC CTG GTA GAA CTG AGG AAG ATC TGC ACC
        N   E   S   K   V   L   V   A   L   V   E   L   R   K   I   C   T   595
2517  CTG GGC CTC CAG CGC ATC TTC TAC CTG AAG CTG GAA GAC TTG GTG TCT CCA
        L   G   L   Q   R   I   F   Y   L   K   L   E   D   L   V   S   P   612
2568  CCT TCC ATC ATT GAC AAG CTC TTC CTG GAC ACC CTA CCT TTC TAA TCAGGAG
        P   S   I   I   D   K   L   F   L   D   T   L   P   F   *               626
2620  CAGTGGAGCAGTGAGCTGCCTCCTCTCCTAGCACCCTGCTTCTACGCAGCAAAGGGATAGGTTTGGA
2687  AACCTATCATTTCCTGTCCTTCCTTAAGAGGAAAAGCAGCTCCTGTAGAAAGCAAAGACTTTCTTTT
2754  TTTTCTGGCTCTTTTCCTTACAACCTAAAGCCAGAAAACTTGCAGAGTATTGTGTTGGGGTTGTGTT
2821  TTATATTTAGGCATTGGGGGATGGGGTGGGAGGGGGTTATAGTTCATGAGGGTTTTCTAAGAAATTG
2888  CTAACAAAGCACTTTTGGACAATGCTATCCCAGCAGGAAAAAAAAGGATAATATAACTGTTTTAAAA
2955  CTCTTTCTGGGGAATCCAATTATAGTTGCTTTGTATTTAAAAACAAGAACAGCCAAGGGTTGTTCGC
3022  CAGGGTAGGATGTGTCTTAAAGATTGGTCCCTTGAAAATATGCTTCCTGTATCAAAGGTACGTATGT
3089  GGTGCAAACAAGGCAGAAACTTCCTTTTAATTTCCTTCTTCCTTTATTTTAACAAATGGTGAAAGAT
3156  GGAGGATTACCTACAAATCAGACATGGCAAAACAATAATGGCTGTTTGCTTCCATAAACAAGTGCAA
3223  TTTTTTAAAGTGCTGTCTTACTAAGTCTTGTTTATTAACTCTCCTTTATTCTATATGGAAATAAAAA
3290  GGAGGCAGTCATGTTAGCAAATGACACGTTAATATCCCTAGCAGAGGCTGTGTTCACCTTCCCTGTC
3357  GATCCCTTCTGAGGTATGGCCCATCCAAGACTTTTAGGCCATTCTTGATGGAACCAGATCCCTGCCC
3424  TGACTGTCCAGCTATCCTGAAAGTGGATCAGATTATAAACTGGATTACATGTAACTGTTTTGGTTGT
3491  GTTCTATCAACCCCACCAGAGTTCCCTAAACTTGCTTCAGTTATAGTAACTGACTGGTATATTCATT
3558  CAGAAGCGCCATAAGTCAGTTGAGTATTTGATCCCTAGATAAGAACATGCAAATCAGCAGGAACTGG
3625  TCATACAGGGTAAGCACCAGGGACAATAAGGATTTTTATAGATATAATTTAATTTTTGGTAATTGGG
3697  TTAAGGAGACCAATTTTGGAGAGCAAGCAAATCTTCTTTTTAAAAAATAGTATGAATGTGAATACTA
3759  GAAAAGATTTAAGAAATAGTATGAGTGTGAGTACTAGGAAGGAT
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Fig. 5.1. Nucleotide sequence and predicted amino acid sequence of the human NOR-1 cDNA. Numbers at left refer to the first nucleotides on the lines, and numbers at right refer to the last amino acids on the lines. The ATTTA sequence motifs are shadowed.

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hNOR-1  MPCVQAQYSPSPPGSSYAAQTYS----SEYTTEIMNPDYTKLTMDLGSTEITATATTSLPSISTFVEGYS   66
rNOR-1  MPCVQAQYSPSPPGSTYATQTYG----SEYTTEIMNPDYAKLTMDLGSTGIMATATTSLPSFSTFMEGYP   66
TR3     MPCIQAQYGTPAPSPG---------PRDHLASDPLTPEFIKPTMDLASPEITATATTSLPSISTFVEGYS   61
NOT     MPCVQAQYGSSPQGASPASQSYSYHSSGEYSSDFLTPEFVKFSMDLTNTEI--TATTSLPSFSTFMDNYS   68

hNOR-1  SNYELKPSCVYQMQ----RPLIKVEEGRAPSYHHHHHHHHHHHHHHHQQQHQQPSIPPASSPEDEVLPSTS  132
rNOR-1  SSCELKPSCLYQMPPSGPRPLIKMEEGREHGYHHHHHHHHHHHHHHHQQ--QQPSIPPPSGPEDEVLPSTS  134
TR3     GEFD---TFLYQLPGTVQPCSSASSSASSTSS-------------------SSATSPASASFKFEDFQV  108
NOT     TGYDVKPPCLYQMPLSGQQSSIKVEDIQMHNY----------QQH--SHLPPQS---EEMMPHSG  118

hNOR-1  M-YFKQ-SPPSTPTTPAFPPQAGALWDE--ALPS-APGCIAPGPLLDPPMKAVPTVAGA-RFPLFHFKPS  196
rNOR-1  M-YFKQ-SPPSTPTTPGFPPQAGALWDD--ELPS-APGCIAPGPLLDPQMKAVPPMAAAARFPIF-FKPS  198
TR3     YGCYPGPLSGPVDEALSSSGSDYYGSPCSAPSPSTPSFQPPQLSPWDGSFGHFSPSQTYEG--LRAWTEQ  176
NOT     SVYYKPSSPP-TPTTPGFQVQHSPMWDDPGSLHNFHQNYVATTHMIEQRKTPVS------RLSLFSFKQS  181

hNOR-1  PPHPPAPSPAGGHHLGYDPTAAAALSLPLGAAAAAGSQAAALEGHPYGLPLA-KRAAPLAFPPLGLTPSP  265
rNOR-1  PPHPPAPSPAGGHHLGYDPTAAAALSLPLGAAAAAGSQAAALEGHPYGLPLA-KRTATLTFPPLGLTASP  267
TR3     LPKASGPPQPPAFFSFSPPTGPSPSLAQSPLKLFPSQATHQLGEGESYSMPTA-------FPGLAPTSPH  239
NOT     PP----GTPVSSCQMRFD----GPLHVPMNPEPAGSHHV--VDGQTFAVPNPIRKPASMGFPGLQIGH--  239

                                        DNA Binding Domain
hNOR-1  -TASSLLGESPSLPSPPSRSSSSGEGTCAVCGDNAACQHYGVRTCEGCKGFFKRTVQKNAKYVCLANKNC  334
rNOR-1  -TASSLLGESPSLPSPPNRSSSSGEGTCAVCGDNAACQHYGVRTCEGCKGFFKRTVQKNAKYVCLANKNC  336
TR3     LEGSGILDTPVTSTKARSGAPGPSEGRCAVCGDNASCQHYGVRTCEGCKGFFKRTVQKNAKYICLANKDC  309
NOT     --ASQLLDT--QVPSPPSRGSPSNEGLCAVCGDNAACQHYGVRTCEGCKGFFKRTVQKNAKYVCLANKNC  305
                                        P Box                   D Box
                                                                A Box
hNOR-1  PVDKRRRNRCQYCRFQKCLSVGMVKEVVRTDSLKGRRGRLPSKPKSPLQQEPSQPSPPSPPICMMNALVR  404
rNOR-1  PVDKRRRNRCQYCRFQKCLSVGMVKEVVRTDSLKGRRGRLPSKPKSPLQQEPSQPSPPSPPICMMNALVR  406
TR3     PVDKRRRNRCQFCRFQKCLAVGMVKEVVRTDSLKGRRGRLPSKPKQP---------PDASPANLLTSLVL  370
NOT     PVDKRRRNRCQYCRFQKCLAVGMVKEVVRTDSLKGRRGRLPSKPKSP-----QEPSPPSPPVSLISALVR  370

                                                                    ★       ★
hNOR-1  ALTDSTP--RDLDYSRYCPTDQ-AAAGTDAEHVQQFYNLLTASIDVSRSWAERIPGFTDLPKEDQTLLIE  471
rNOR-1  ALTDATP--RDLDYSRYCPTDQ-ATAGTDAEHVQQFYNLLTASIDVSRSWAEKIPGFTDLPKEDQTLLIE  473
TR3     AHLDSGPSTAKLDYSKFQELVLPHFGKEDAGDVQQFYDLLSGSLEVIRKWAEKIPGFAELSPADQDLLLE  440
NOT     AHVDSNPAMTSLDYSRFQANPDYQMSGDDTQHIQQFYDLLTGSMEIIRGWAEKIPGFADLPKADQDLLFE  440

          ★       ★
hNOR-1  SAFLELFVLRLSIRSNTAEDKFVFCNGLVLHRLQCLRGFGEWLDSIKDFSLNLQSLNLDIQALACLSALS  541
rNOR-1  SAFLELFVLRLSIRSNTAEDKFVFCNGLVLHRLQCLRGFGEWLDSIKDFSLNLQSLNLDIQALACLSALS  543
TR3     SAFLELFILRLAYRSKPGEGKLIFCSGLVLHRLQCARGFGDWIDSILAFSRSLHSLLVDVPAFACLSALV  510
NOT     SAFLELFVLRLAYRSNPVEGKLIFCNGVVLHRLQCVRGFGEWIDSIVEFSSNLQNMNIDISAFSCIAALA  510

hNOR-1  MITERHGLKEPKRVEELCNKITSSLKDHQS-KGQALEPNE--SKVLVALVELRKICTLGLQRIFYLKLED  608
rNOR-1  MITERHGLKEPKRVEELCNKITSSLKDHQR-KGQALEPSE--PKVLRALVELRKICTQGLQRIFYLKLED  611
TR3     LITDRHGLQEPRRVEELQNRIASCLKEHVAAVAGEPQPASCLSRLLGKLPELRTLCTQGLQRIFYLKLED  580
NOT     MVTERHGLKEPKRVEELQNKIVNCLKDHVTFNNGGLNRPNYLSKLLGKLPELRTLCTQGLQRIFYLKLED  580

hNOR-1  LVSPPSIIDKLFLDTLPF                                                    626
rNOR-1  LVSPPSVIDKLFLDTLPF                                                    628
TR3     LVPPPPIIDKIFMDTLPF                                                    598
NOT     LVPPPAIIDKLFLDTLPF                                                    598
```

Fig. 5.2. The amino acid translation of hNOR-1 compared with that of rNOR-1, TR3 and NOT. Numbers on the right refer to the last amino acid on each line. The amino acids that are homologous to two or more proteins are shadowed. P, D, and A boxes and the DNA-binding domain are indicated. Stars indicate leucine residues in the putative leucine-zipper conserved among the Nur77/NGFI-B family.

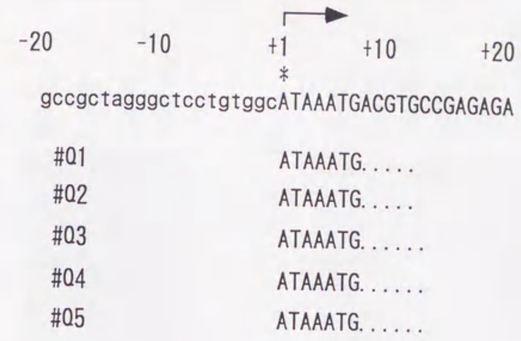

Fig. 5.3. Analysis of the transcriptional start site of hNOR-1. We performed 5'-AmpliFINDER RACE on poly(A)+ mRNA isolated from human skeletal muscle. The relevant sequence around the transcriptional start site is shown on the top. The 5'-end sequences (#Q1 - 3) were obtained by PCR amplification with an anchor primer and the P2 primer, and the sequences (#Q4 - 5) were obtained using the P3 primer. Asterisk represents the initiation site determined according to those of the PCR products. Each PCR amplification was performed independently.

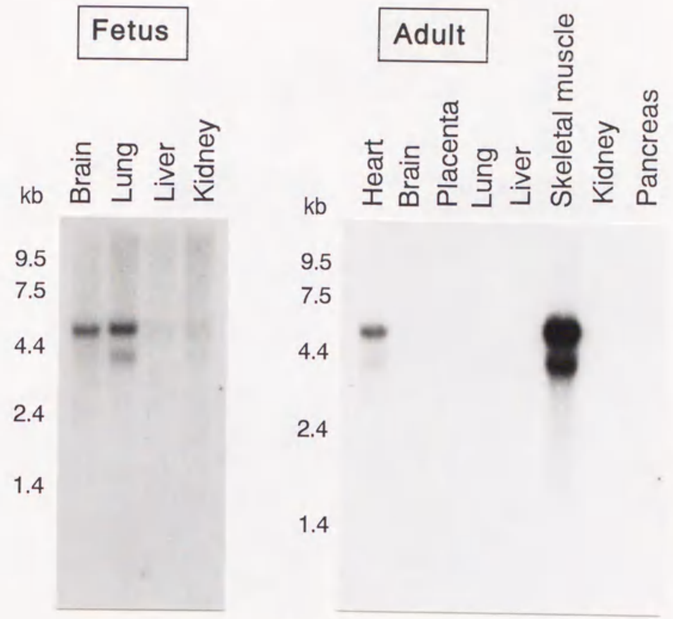

Fig. 5.4. Northern blots of hNOR-1 mRNAs from various human tissues. Samples containing 2 μg of poly(A)$^+$ mRNA/lane were immobilized on nylon-membranes and hybridized with a [^{32}P]-labeled hNOR-1 cDNA probe. The locations of size markers are indicated.

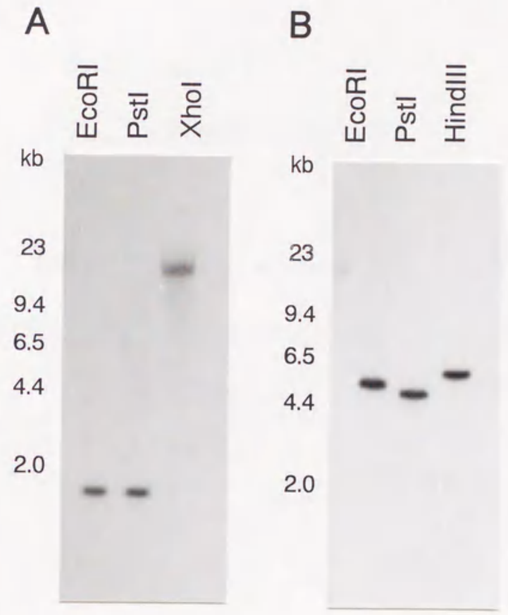

Fig. 5.5. Southern blots of human genomic DNA digested with various enzymes. (A) Hybridization with a [^{32}P]-labeled hNOR-1 cDNA probe "A" generated from the trans activating domain, and (B) shows hybridization with probe "B" generated from the 3' untranslated region.