CHAPTER 6

Characterization of the NOR-1 gene

- Structure, mapping and transcriptional regulation

of the human NOR-1 gene -

Introduction

Many steroid/thyroid receptors have recently been cloned and analyzed at the cDNA sequence level (Hollenberg, Weinberger *et al.*, 1985; Miesfeld, Rusconi *et al.*, 1986; Walter, Greene *et al.*, 1985; Green, Walter *et al.*, 1986; Greene, Gilna *et al.*, 1986; Krust, Green *et al.*, 1986; Conneely, Sullivan *et al.*, 1986). Specific regions of these molecules share extensive homologies. Functional analysis of some receptors has implicated the conserved regions in DNA-binding and in ligand-binding (Giguère, Hollenberg *et al.*, 1986; Danielsen, Northrop & Ringold, 1986; Kumar, Green *et al.*, 1986; Hollenberg, Giguère *et al.*, 1987; Godowski, Rusconi *et al.*, 1987; Miesfeld, Godowski *et al.*, 1987). A resulting concept is that nuclear receptors are a family of evolutionarily related factors that are important in the trans-activation of specific genes (Green & Chambon, 1986). Since NOR-1 is also classified as a trans-activating factor which binds to the specific *cis*-acting DNA element, it is of interest to explore the evolutional correlation between the NOR-1 gene and steroid/thyroid receptor superfamily genes.

Recently, Maruyama *et al.* (1995) revealed that NOR-1 belongs to the immediate early genes, since its induction is not required *de novo* protein synthesis. Other members of the Nur77/NGFI-B family are also thought to be the early response genes immediately induced by several external stimuli (Hazel, Nathans & Lau, 1988; Milbrandt, 1988; Scearce, Laz *et al.*, 1993). The 5'-flanking region of the Nur77 gene contains one Dyad symmetry element (DSE) which could explain its immediate response to external stimulus (Ryseck, Bravo *et al.*, 1989). Furthermore, sequence analysis of the 5'-flanking region of Nur77 and NGFI-B genes suggested these genes may contain some potential *cis*-acting elements, which may play a role in growth factor stimulation (Ryseck, Bravo *et*

al., 1989; Watson & Milbrandt, 1989).

To better understand the evolution of the Nur77/NGFI-B family in eukaryotes and to facilitate analysis of transcriptional regulation of NOR-1, chromosomal genes must be characterized. Therefore, in this chapter I report the structure of a chromosomal gene for the human orphan nuclear receptor NOR-1. Moreover, in order to study the transcriptional regulation, I cloned and sequenced the 5'-flanking region, and examined its promoter activity by using a series of deletion constructs.

Materials and Methods

Isolation of the NOR-1 genomic clones

The genomic library was generated by cloning fragments from a partial digestion of human DNA with Sau3AI into the λ EMBL3 SP6/T7 vector (Clontech). Recombinant clones of the genomic DNA library were screened using the human NOR-1 cDNA fragment as a probe. The DNA probe was labeled with DIG-dUTP by PCR amplification. Restriction fragment analysis and hybridization with probes from the NOR-1 cDNA were performed using standard protocols.

Construction of luciferase expression vectors

To compare the transcriptional efficiency of potential regulatory regions, gene constructs were made that placed regions of the human NOR-1 gene upstream of the luciferase (Luc) gene in the vector pBS-Luc (WAKO pure chemical industries, pGV-B). Sequential deletion mutants of the 5'-flanking region were prepared by using restriction enzyme sites or PCR amplification. The constructs were named pNOR/-n, where n is the distance in nucleotides from the transcription initiation site. The plasmid containing the Luc gene alone (pBS-Luc) was used as a control. All constructs were confirmed by restriction enzyme analysis and sequence analysis.

Cell culture and DNA transfection

Mouse fibroblast L929 cells were grown in Dulbecco's modified Eagle's medium supplemented with 2 % fetal bovine serum. For transient expression assays, cells were plated at 2×10^5 cells/6-well plate and cultured for 24 h before transfection by the DEAE-Dextran method (Gonzalez & Joly, 1995) with 0.25 pmol (1 - 2.3 μg) of the appropriate

plasmids purified by banding in CsCl. Cells were harvested 48 h after the addition of DNA. Finally, cells were washed and scraped in 0.2 ml lysis buffer. Cellular debris was removed by centrifugation for 1 min at 4 °C, and the supernatant were analyzed by luciferase assay. For normalization of transfection efficiencies in L929 cells, a β-galactosidase expression plasmid (pSRα-lacZ) was introduced in cotransfections. Luciferase and β-galactosidase assays were performed as previously described (Foster, Jahroudi *et al.*, 1988; Brasier, Tate & Habener, 1989). All transfections were repeated at least four times.

Chromosomal localization

Purified DNA from a genomic clone designated as #EN3 was labeled with biotin dUTP by nick translation. The labeled probe was combined with sheared human DNA and hybridized to normal metaphase chromosomes derived from phytohemagglutinin-stimulated peripheral blood lymphocytes in a solution containing 50 % formamide, 10 % dextran sulfate and 2×SSC. Specific hybridization signals were detected by incubating the hybridized slides in fluorescein-labeled avidin followed by counterstaining with propidium iodide.

Results

Isolation and characterization of the human NOR-1 gene

I screened a human genomic library using DIG-labeled NOR-1 cDNA to obtain NOR-1 genomic clones. Several genomic clones containing the amino acid coding regions of the hNOR-1 gene were isolated and subcloned into plasmid vectors for higher resolution restriction mapping and hybridization studies. Appropriate subfragments were inserted into plasmid vectors and sequenced. Calculations based on seven overlapping clones (17 kb average) yielded a estimated size of >35 kb for the hNOR-1 gene.

A comparison of the genomic and cDNA sequences revealed that the NOR-1 gene is composed of eight exons of 555, 174, 953, 130, 173, 200, 179 and >1438 bp. Schematic representations of the gene are shown in Fig. 6.1. The relevant gene sequence at each exon-intron boundary is shown in Fig. 6.2. The intron/exon boundaries have the canonical splice consensus sequences (Breathnach & Chambon, 1981) and no discrepancies were identified between the sequence of the exons of the gene and the cDNA sequence.

Exons 1 and 2 of the hNOR-1 gene contained most of the 5' untranslated region, and the protein-coding region was located within exons 3 through 8. Exon 3 contains the translation initiation codon, together with the nonconserved N-terminal domain of the molecule. The residues comprising the DNA-binding domain of the steroid/thyroid receptors are encoded in exons 3 and 4. Like other genes of this family, each zinc finger was partitioned into separate exons. Exon 3 contained the amino-terminal domain and the first zinc finger and exon 4 was devoted entirely to the second zinc finger. The position of the boundary between exons 3 and 4, is similar to that in the Nur77/NGFI-B genes (Rysek, Bravo *et al.*, 1989; Watson & Milbrandt, 1989), but different from that in other

members of this superfamily: the human estrogen receptor (Ponglikitmongkol, Green & Chambon, 1988), mineralocorticoid receptor (Arriza, Weinberg *et al.*, 1987), retinoic acid receptors (Brand, Petkovich *et al.*, 1988; Giguère, Ong *et al.*, 1987; Petkovich, Brand *et al.*, 1987), and thyroid hormone receptors (Lazar, Hodin *et al.*, 1989; Weinberger, Thompson *et al.*, 1986; Zahraoui & Cuny, 1987). A putative ligand-binding region in the carboxyl terminus was encoded in exons 6, 7 and 8. Exon 8 also included the termination codon and the 3' untranslated region. This structural arrangement was in general, similar to that reported for several other nuclear receptor genes and remarkably similar to those of the Nur77/NGFI-B family. Alignment of the hNOR-1 and Nur77 amino acid sequence indicated that the location of introns has been conserved during evolution (Fig. 6.3).

The 5'-flanking region of the NOR-1 gene

To identify the DNA elements that direct human NOR-1 gene transcription, I sequenced the 5' flanking region of NOR-1. Examination of the human NOR-1 sequence 5' to the major transcription initiation site (designated +1) revealed several notable features. As shown in Fig. 6.4, neither TATA (Groudine, Peretz & Weintraub, 1981) nor CCAAT (Maniatis, Goodbourn & Fischer, 1987) promoter elements were found within the region containing the proximal major transcriptional initiation sites. The sequence GGGCGG is found at three positions -312, -904 and -1298 and corresponds to the binding site for the transcription factor Sp1 (Dynan & Tjian, 1983), commonly seen in the promoters of viral and cellular housekeeping genes. In this region, there are two copies of the consensus sequence of the transcription factor AP1 (Fisch, Prywes & Roeder, 1989), and three copies of cAMP response element (CRE) (Montminy, Sevarino *et al.*, 1986). Moreover, it contained a 5'-GTTCAACACGTGTGTG-3' motif which

closely resembles the consensus MyoD response element which is present in the muscle specific gene promoters (Buskin & Hauschka, 1989; Ephrussi, Church *et al.*, 1985; Lenardo, Pierce & Baltimore, 1987).

Promoter activity of the human NOR-1 gene

In order to study the transcriptional activity of this putative promoter region, mouse L929 cells were used for transient transfections of reporter gene constructs. This cell line has been shown to have the basal level of expression of NOR-1 and induction of its mRNA by treatment of TPA or forskolin (Bandoh, Tsukada *et al.*, 1995). Progressively shorter fragments of the 5'-flanking region fused with the coding region of the luciferase gene in the plasmid pBS-Luc were transfected into L929 cells. The transfection assay data shown here were means of at least four independent experiments and normalized by using a cotransfected β-galactosidase expression vector (pSR α-lacZ). As shown on Fig. 6.5, the transfection of the pNOR/-8.0k construct resulted in more than 100-fold increase in Luc activity relative to the control pBS-Luc construct. Further, it showed about 50 % activity relative to pGV-C (WAKO pure chemical industries), driven by the simian virus 40 early promoter (data not shown). Deletions in the 5' to 3' direction resulted in a stepwise decrease in Luc gene expression. Deletion to nucleotide -4.0k resulted in about 30% decrease in expression. An additional deletion to nucleotide -301 caused a decrease to 50% of the pNOR/-8.0k. Extending deletion to nucleotide -94 caused a decrease to 75 % of the pNOR/-8.0k, and a further deletion of 52 bp (to position -42) resulted in a reduction in Luc activity to about 5 %. Further deletion of the next 42 nucleotides (to position +1) did not alter Luc activity. The most dramatic change in activity was seen with deletion of the region -94 to -42 containing the putative first positive regulatory element. Examination of this region revealed at least three copies of

the cAMP response element (positions -53, -64 and -79 nt). Finally, the shortest construct pNOR/+1 demonstrated a significant level of activity compared with the control pBS-Luc construct, indicating the presence of a functional positive regulatory element.

Negative regulatory elements in the NOR-1 gene

In order to determine other important elements responsible for transcriptional regulation, I also examined the downstream region of the NOR-1 transcriptional initiation site. Insertion of either intron 1 (3.2 kb) or 2 (2.3 kb) to the upstream of the NOR-1 5'-flanking region (pNOR/-1703) demonstrated a approximately 80 or 50 % decrease in Luc activity, respectively (Fig. 6.6). These findings indicate that the downstream from the transcriptional initiation site includes at least two negative-acting elements.

The gene for NOR-1 is located on chromosome 9q

To determine the chromosomal localization of the hNOR-1 gene, I performed fluorescence in situ hybridization (FISH) using human metaphase spreads. The human genomic fragment #EN3 containing exons 2, 3, 4, and 5 of the hNOR-1 gene was used as a probe. FISH of the genomic DNA with the #EN3 probe resulted in the specific labelling of the long arm of a group C chromosome. I then co-hybridized a genomic clone from the locus of p16, a specific inhibitor of CDK4 and CDK6 previously assigned to 9p21, with #EN3. This procedure specifically labeled 9p21 as well as the mid portion of 9q (Fig. 6.7). Measurements of ten specifically hybridized chromosomes 9 indicated that #EN3 is located at about 40 % of the distance from the heterochromatic-euchromatic boundary on 9q to the telomere of chromosome arm 9q, an area that corresponds to band 9q22. A total of 80 metaphase cells was analyzed with 52 exhibiting specific labeling. These observations strongly suggest that the NOR-1 gene is located on chromosome 9q.

Discussion

To further investigate the relationship of hNOR-1 to other members of the steroid/thyroid receptor superfamily, I determined the organization of the human NOR-1 gene. The chromosomal human NOR-1 gene is also similar to members of the steroid /thyroid receptor superfamily in terms of exon-intron structure. The putative trans-activating domain of the hNOR-1 protein is completely encoded by a single exon and the DNA-binding domain is composed of two zinc fingers, each of which is encoded by separate exons. Furthermore, the putative ligand-binding domain of hNOR-1 is encoded in multiple exons like several of the nuclear ligand receptor molecules. These share a similar structure, confirming that the genes are a family of evolutionarily related factors that are important in the trans-activation of specific genes.

Unlike other steroid hormone receptor genes, exons 1 and 2 of hNOR-1 contain only 5' untranslated sequences. Such exons often contain elements involved in the regulation of expression. Thus, it is possible that these untranslated exons confer additional post-transcriptional regulation of NOR-1 expression. Another notable finding in the structure of the gene is the position of intron 3, which separates the first zinc finger from the second zinc finger, with the Phe-Phe-Lys residues being in one exon and the Arg residue in the next. The position is identical to that of the Nur77 and NGFI-B genes (Ryseck, Bravo *et al.*, 1989; Watson & Milbrandt, 1989), but unlike that of the other members of the steroid/thyroid receptor superfamily (Ponglikitmongkol, Green & Chambon, 1988; Arriza, Weinberg *et al.*, 1987; Brand, Petkovich *et al.*, 1988; Giguère, Ong *et al.*, 1987; Petkovich, Brand *et al.*, 1987; Lazar, Hodin *et al.*, 1989; Weinberger, Thompson *et al.*, 1986; Zahraoui & Cuny, 1987). One position is typical for all the members of the steroid hormone receptor family, the other for the thyroid/retinoic

receptor family. The ligand-binding domains of several steroid hormone receptors are divided into four exons separated by three large introns. In hNOR-1 and Nur77, however, the same regions are divided into three exons. Comparisons of those genes revealed that the exon-intron structure is highly conserved between the hNOR-1 and the Nur77/NGFI-B family, suggesting that the members of the Nur77/NGFI-B family evolved from a common ancestral gene.

Sequence analysis of the NOR-1 5'-flanking region reveals multiple regulatory sites homologous to known transcription factor consensus sequences. The upstream of the transcriptional initiation site contains three copies of both CRE motifs and GC boxes, and lacks TATA and CCAAT boxes. This promoter region exhibits features typical of housekeeping genes (Dynan, 1986); absence of TATA or CCAAT promoter elements and a high G+C content. It should be noted that the promoter region of the human TR3 also lacks an apparent TATA or CCAAT box (Uemura, Mizokami & Chang, 1995).

To determine the promoter activity, the NOR-1 5'-flanking region and Luc chimeras were constructed and transfected into the L929 cells. In the experiment, its 5'-flanking region show significant promoter activity compared to the control construct, indicating that the NOR-1 gene was actuary transcribed from the gene. Furthermore, to clarify the essential promoter region, I also examined transcriptional activity of its promoter region by using a series of deletion constructs. Sequential deletion analyses using the NOR-1 and Luc chimeras indicated that a minimal region exhibiting promoter activity is located between nt -94 and -42, where there are three copies of cAMP response elements. Based on Luc assay, a Luc reporter plasmid, pNOR/-1703, which contains three copies of GC boxes, exhibits very high Luc activity. Further, sequential deletion of this region showed a step-wise decrease in Luc activity, suggesting that its flanking region nt -1703 to -94 are responsible for at least 50 % of basal activity from the -1703

bp promoter region. This result also indicate that the upstream region of the transcriptional initiation site may play a role as positive-acting element in human NOR-1 gene transcription.

In a number of nuclear receptor genes such as progesterone (Huckaby, Conneely *et al.*, 1987) and estrogen receptors (Ponglikitmongkol, Green & Chambon, 1988), the 5' UTR region and transactivating domain are encoded in a single exon. However, those regions of NOR-1 are composed of three exons divided by two introns (introns 1 and 2). An explanation for the existence of the introns localizing in the 5'-UTR might be that the downstream region contains the important elements responsible for transcriptional regulation. To address this possibility, the transcriptional activity of the pNOR construct containing a single copy of intron 1 or 2 was assayed. pNOR/+int.1 (containing intron 1) and pNOR/+int.2 (containing intron 2) demonstrated a approximately 80 and 50 % decrease in Luc activity, indicating that the presence of strong negative regulatory elements within those regions.

Another potentially important sequence within human NOR-1 promoter region is the imperfect MyoD recognition sequence including the E-box (Buskin, & Hauschka, 1989; Ephrussi, Church *et al.*, 1985; Lenardo, Pierce & Baltimore, 1987). MyoD, which is a skeletal muscle-specific protein that is able to induce myogenesis in a wide variety of cell types (Tapscott, Davis *et al.*, 1988), recognizes a element containing an inner core sequence, CANNTG, known as the E-box. In the previous chapter, I showed by Northern blots that hNOR-1 mRNA is abundantly expressed in skeletal muscles. This result might be due to the motif in the 5'-flanking region (position -793), but further analysis is required to determine if this site is functional.

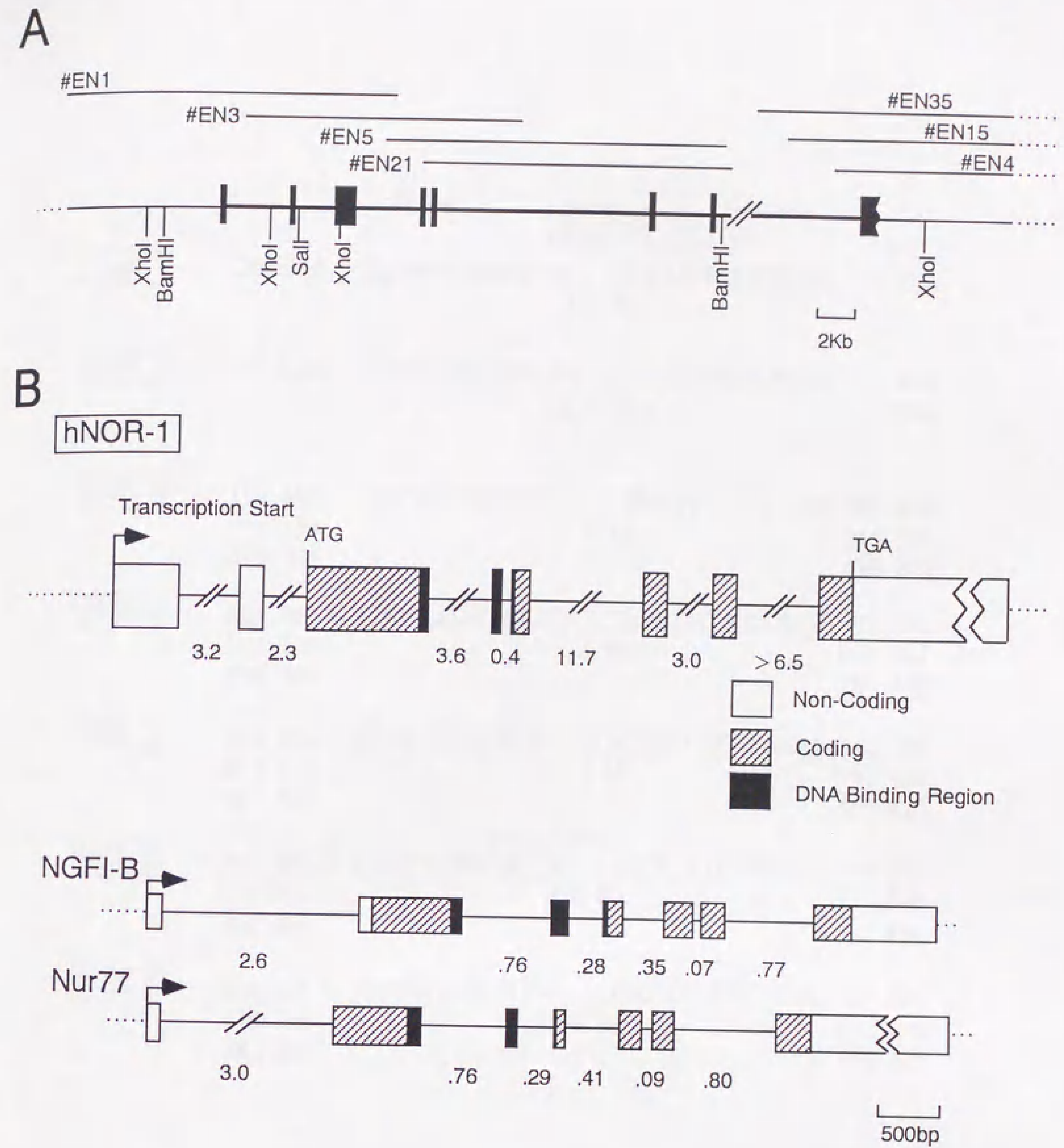

Fig. 6.1. Genomic structure of the hNOR-1 gene. (A) A restriction map of 35 kb of the hNOR-1 gene encompassed by the λ genomic clones (#EN1 - 35) is presented. The introns are indicated by thick lines and the exons by boxes. (B) Comparison of the hNOR-1 gene structure with those of Nur77 and NGFI-B. Numbers indicate the intron length in kilobases.

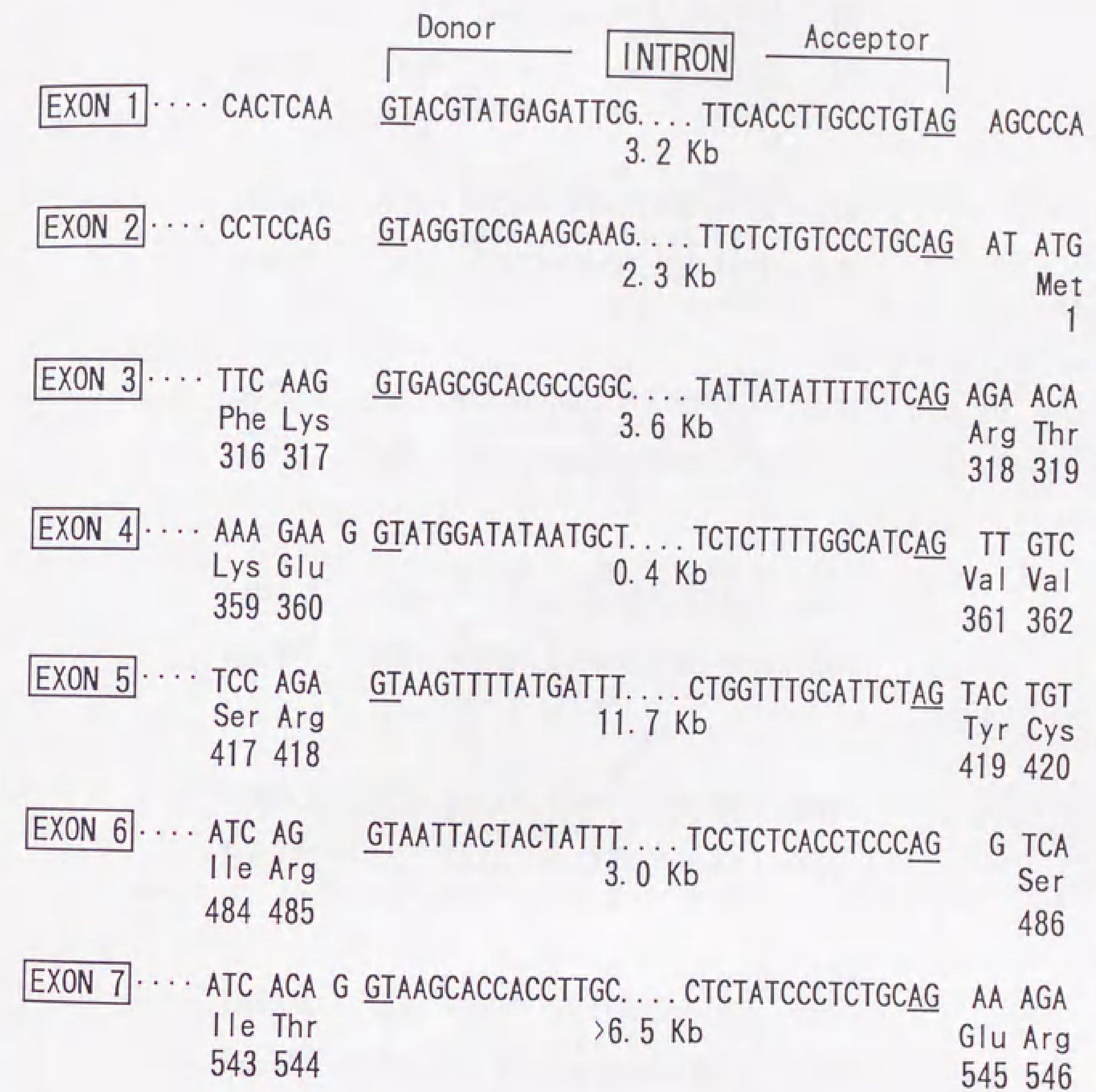

Fig. 6.2. Intron/exon boundaries of the hNOR-1 gene. The sequence of each of the intron/exon boundaries is shown and the position of the intron with the amino acid sequence is indicated. The highly conserved GT and AG sequences at the donor and acceptor sites respectively, are underlined.

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                     exon2      ▼       exon3
hNOR-1       1-   .........MPCVQAQYSP   -10
                           *** ****
Nur77        1-   .........MPCIQAQYGT   -10
                  exon1     ▲       exon2

                  exon3     ▼       exon4
hNOR-1     308-   TCEGCKGFFKRTVQKNAKYV  -327
                  *************** ***
Nur77      286-   TCEGCKGFFKRTVQKSAKYI  -315
                  exon2     ▲       exon3

                  exon4     ▼       exon5
hNOR-1     351-   KCLSVGMVKEVVRTDSLKGR  -370
                  *** ****************
Nur77      329-   KCLAVGMVKEVVRTDSLKGR  -348
                  exon3     ▲       exon4

                  exon5     ▼       exon6
hNOR-1     409-   P--RDLDYSRYCPTDQAAAG  -428
                  *    ****
Nur77      380-   PSTAKLDYSKFQELVLPRFG  -398
                  exon4     ▲       exon5

                  exon6     ▼       exon7
hNOR-1     475-   LELFVLRLSIRSNTAEDKFV  -494
                  **** ***  **    * *
Nur77      447-   LELFILRLAYRSKPGEGKLI  -466
                  exon5     ▲       exon6

                  exon7     ▼       exon8
hNOR-1     535-   ACLSALSMITERHGLKEPKR  -554
                  ******  ** ****   * *
Nur77      507-   ACLSALVLITDRHGLQDPRR  -526
                  exon6     ▲       exon7
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Fig. 6.3. Comparison of intron/exon boundaries of hNOR-1 and Nur77 genes. The amino acid sequences (single letter code) of hNOR-1 and Nur77 are aligned with respect to the position of the intron (arrowheads). Optimal alignment was obtained by introducing gaps (-) in the sequence. Asterisks indicate the sequence identity between hNOR-1 and NGFI-B.

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-1703                                                      TGG CGCCGGCCGG GGTCCCGGCC
-1680  AACCGCCGAA TTTAGTAACA TCGCCTGCGT CAATCACGCG CCTCGGTGCG TCAGGCCGCG
-1620  CGGCTCCAGG TCCTGCTCCC CCCCTTCAAG CCTTTGAATG GATACAATGT AGCAGCGCCC
-1560  TCCTTCCTTC CGAGGCTGGA TTGGAACCGC CGCAGTGCAG AGACTCGGTT GCTCTCGGCT
-1500  GGGTCAACTT TCGGGGCATT CTCCCACGAT CCTCTCCGCA CCACCGTGTC TGAATTGGAA
-1440  GTGGAGGCGA AGAAAGATAT ACATGCCATA TTTACCTATA TGTAGTTTGT TTTCAAGTTT
-1380  CTGGTCCTAG CTCGAACCTT CTTCGATTCT GAAATGTGTG CTGTCTACAA AGGAATCTTG
-1320  TATCTCCCCT CGGCGCAGCC CCCCGCCCCG CCACACACAC ACAAATTGGG ACAGGTCAAA
                               Sp1
-1260  CATATAAAAC GGTATTTGTG ATTCAAGCGG ACCACATGGG GACCACTCTA TCTGCATTGT
                             AP1
-1200  TTCACTCAAA TATTTTCTCC TGTCCAAAAA TTCATTTCTG AAAGAGACTG CGTTCACTCA
-1140  GCAGCAACCT TTGGGACTAG GGGTCTTTAA CTCTGATAAA TTTTGTTTTC ATCAAGAAAT
-1080  TTACACTTAA ATTTATCATT TCCAGGAAGA AATTGCTCTC CTTCATACAG TCACCCAGGC
-1020  TTTCGGCACA CCATTTCATG ACAAATGTGT CCGAGGAGAC CAAAGCAAAT CCCCTAGCGA
 -960  GGGACTGACT AATAAGTCCT GTTGATTGAT TTCGAAATGT TTAATTTGGG AGATGTGGGC
          AP1                                                   Sp1
 -900  GGAGGGCATC TACAACCATC AAAAAGTGAA AGTGCTAGTT GAGAGTTCCA TTTCTGACCC
 -840  GGTGCCGGGG AGGAGGAATG ATTTGCAATA GTCAGACCCG CTCAGCTGTT CAACACGTGT
                                                          MyoD-like
 -780  GTGTTTGTTT TACACACAGA GTAGTTTCTG CTGCAGCCGC GTGTGCATGA TGGATGTGCA
 -720  CTTCGCTGGG TTATAACGTG TCCAGTTAAG AAACCCACGC CGTACGTGTA AAGAAATCAA
 -660  ACCTTATCCC CGGAACCATC TGCATCCCTG TGTGAACACG CACCCAGTAA ATGATGCGGG
 -600  GAGGGGGGAT TAGCCTGGGC GCAGAGGACC GGAGCAACGT AAACAGCTTT AGAACCTATG
 -540  CAAGAGGAAA GTGCAGCTGC ACCTCAGGGC GTCTTCGGGC TGGTGCCAGA CGCCTTCTGC
 -480  ACCGGCTGCC AGGTCACTGG AGCTGGTCAG AAGCTGGCTG GCGGAGCTTC CCTTTCGGAA
 -420  GAGCTGTCCT CTCCCTTACC CCCCTCGCCC TGGCTCCGTG CCTCGGGGCA GCCTCGGAGG
 -360  CGCGCCAGCA GCACTCCTCC AACTCTACTC CACCCGAGCC TGACAGCTGG GCGGTCCCGC
                                                     Sp1
 -300  CTGACCCGTG GGCAGGCCGC TGCACCCTCC CGCAGACGCA CGCCCTGGCG AGCGGTTCCG
 -240  CTGCAAAAAG AGAAGCCCCC AGGCCGGGGC CGGCCGTGCG GCGGAGTTTC CATTGTGCGG
 -180  CCGTGCGACT GGCCGAGGAA CGCGCGCGCG CGCGCACACG AACACACACA CCCTCCCTCG
 -120  CACACGCGGA ACCGGCTGGG CCAGGGGAGG GAGGAGGAGG GTGACGTAGC GTCCCATGGC
                                                 CRE             CRE
  -60  GTCACATTGA CGTCTCGCAT TCCAGGCACT CTATGGAGAG GCCGCTAGGG CTCCTGTGGC
       ──────►      CRE
   +1  ATAAATGACG TGCCGAGAGA GCGAGCGAAC GCGCAGCCGG GAGAGCGGAG TCTCCTGCCT
  +61  CCCGCCCCCC ACCCCTCCAG CTCCTGCTCC TCCTCCGCTC CCCATACACA GACGCGCTCA
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Fig. 6.4. Sequence of the NOR-1 5'-flanking region from -1703 to +120. Nucleotides are numbered relative to the transcriptional start site of the NOR-1. All sequences with potential cis-acting elements are underlined and labeled. Arrow indicates the transcriptional start site.

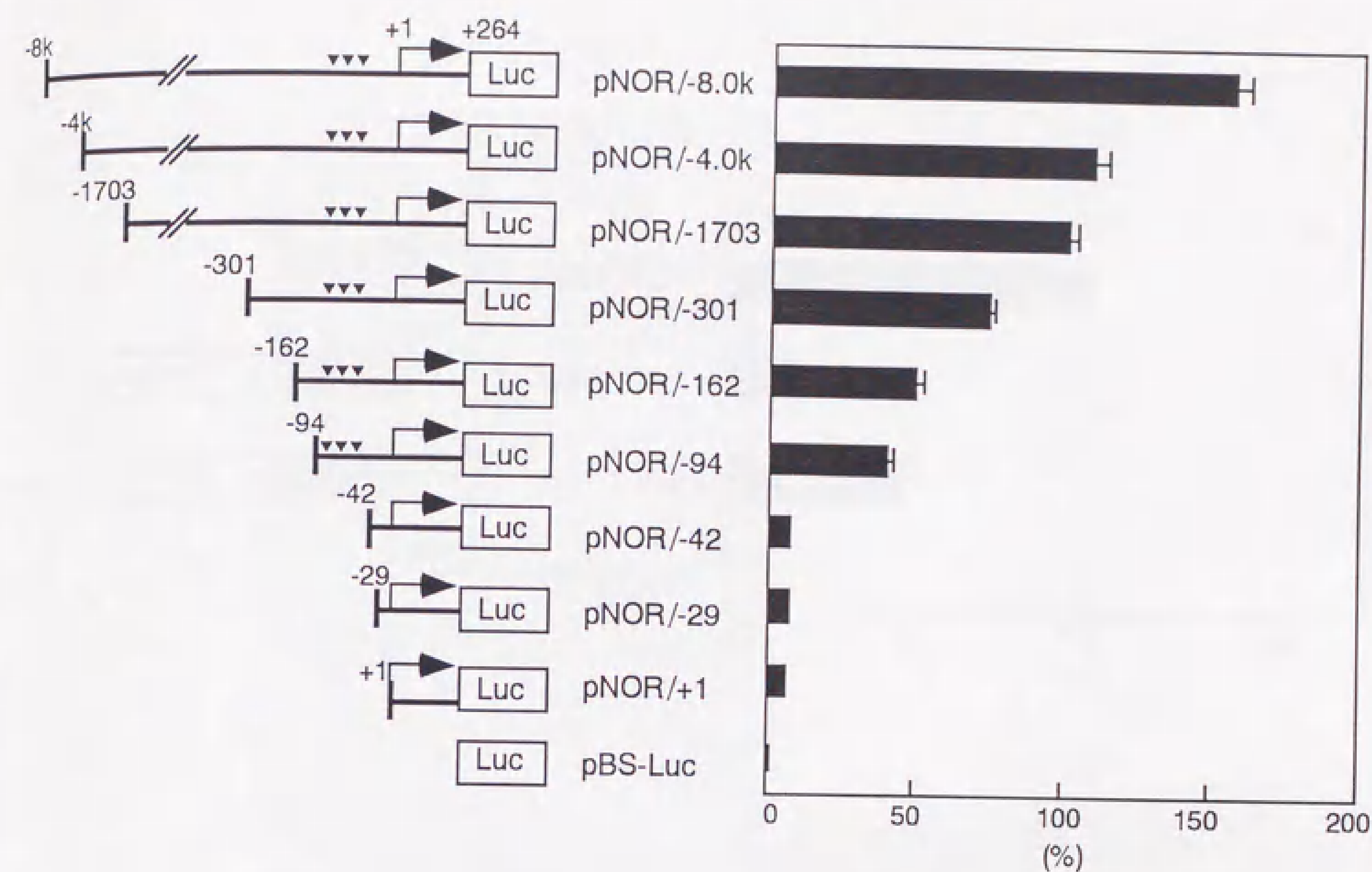

Fig. 6.5. The promoter activity of various deletion mutants of the NOR-1 5'-flanking region. Deletion constructs are numbered and named according to their length upstream of the transcriptional start site. Luc activity, after transiently transfected into L929 cells, was measured and normalized with β-galactosidase activity. Relative Luc activity of all deletion plasmids was calculated with pNOR/-1703 as 100%. Arrows and arrowheads indicate the transcriptional start sites of the gene, and positions of cAMP response element, respectively. Results were mean ± S.E.M. of at least four separate experiments.

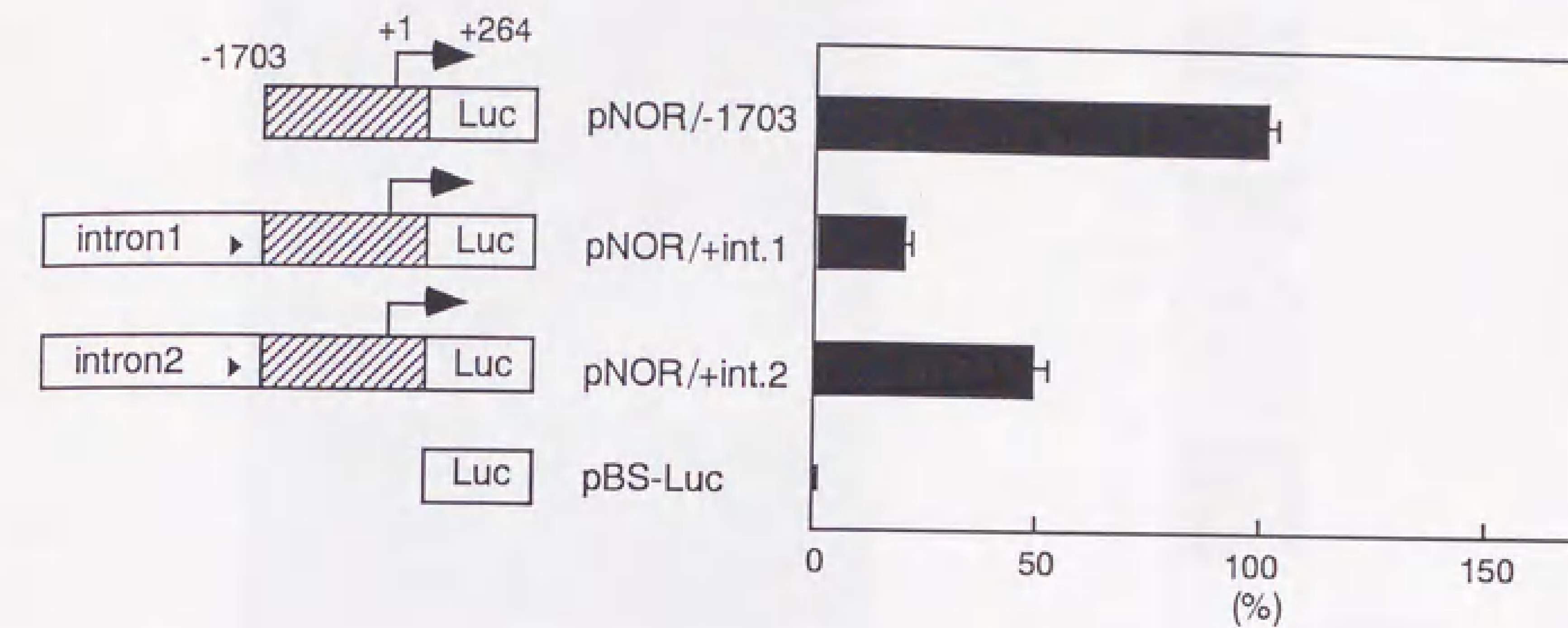

Fig. 6.6. Suppression of Luc activities in the NOR-1 promoter by the introns. The structure of intron-promoter chimeric constructs and their activities are shown. Luc activity, after transiently transfected into L929 cells, was measured and normalized with β-galactosidase activity. Relative Luc activity of all plasmids was calculated with pNOR/-1703 as 100%. The NOR-1 5'-flanking region (from -1703 to +264) is indicated by shadowed boxes. Arrowheads and arrows indicate the orientations of introns and the transcriptional start sites of NOR-1, respectively. Results were mean $\pm$ S.E.M. of at least four separate experiments.

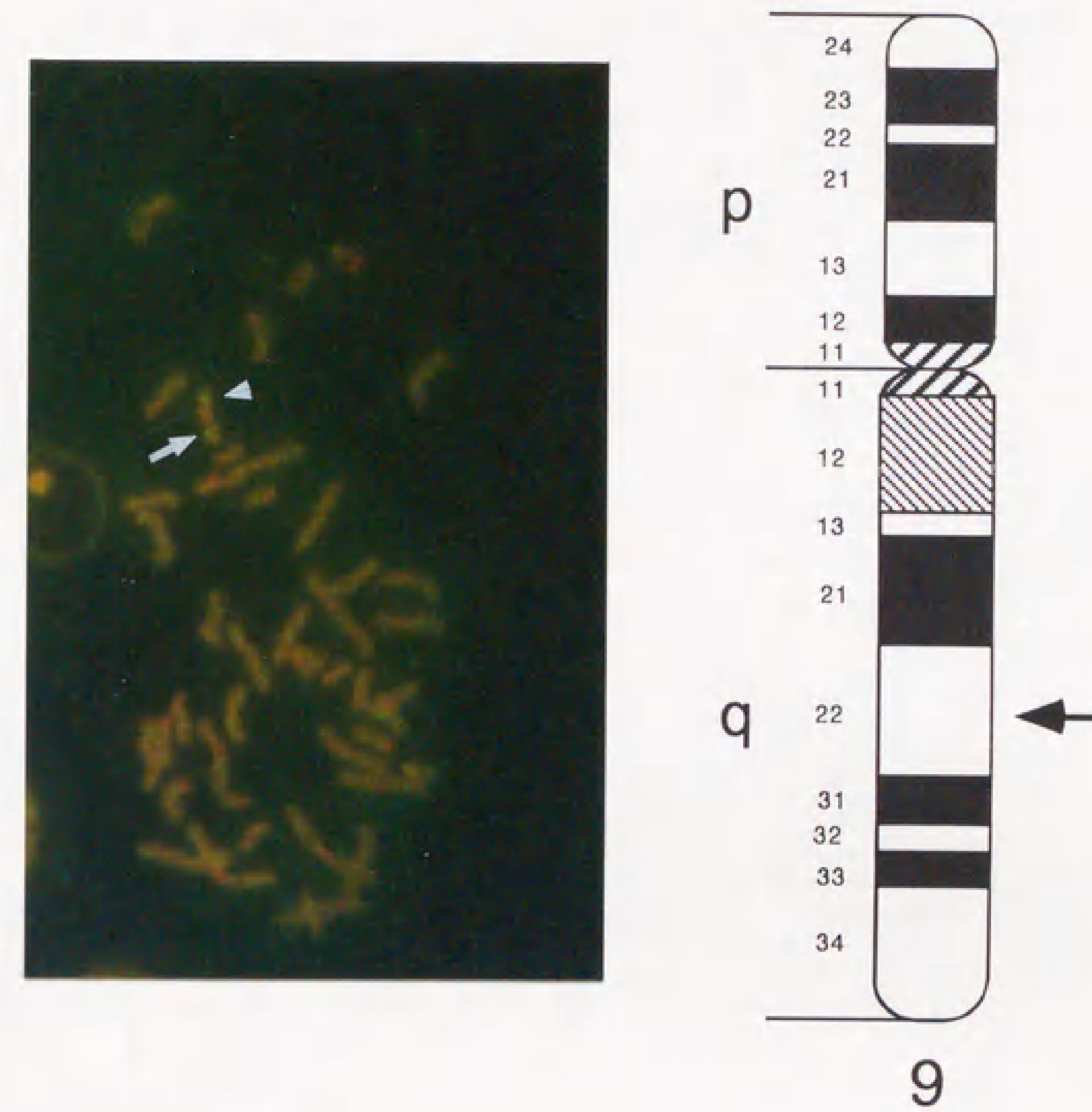

Fig. 6.7. Chromosomal localization of the hNOR-1 gene to chromosome 9q22. Fluorescence in situ hybridization was performed on metaphase chromosomes counterstained with propidium iodide. A labeled hNOR-1 genomic clone (#EN3) was cohybridized with a genomic clone from the p16 locus, which was assigned to 9p21. Specific labeling was detected on 9p21 (arrowhead) as well as 9q22 (arrow).

CHAPTER 7

General discussion

NOR-1 belongs to the family of Nur77/NGFI-B

Hormones, such as glucocorticoids, mineralocorticoids, estrogen, progesterone, androgens, retinoic acids, thyroid hormone and vitamin D_3 are known to activate the transcription of specific genes through a receptor-mediated mechanism. The receptors for these compounds, in contrast to cell-surface receptors, comprise a superfamily of intracellular proteins that directly modulate transcription by an interaction with their target genes (Evans, 1988). Binding to their respective ligands, which enter the cell by diffusion, increases affinity for specific enhancer-like DNA sequence elements associated with the target genes, resulting in activation or repression of transcription. The amino-terminal domain, which is considered to be responsible in part for modulating gene expression, exhibits little sequence similarity among the different receptors. On the contrary, the central DNA-binding domain is highly conserved.

I identified a novel nuclear receptor gene, designated as NOR-1, from both rat and human fetal brains by using RT-PCR amplification with primers designed from conserved sequences among the superfamily. NOR-1 contains all structural elements of steroid receptors: amino terminus fulfilling the criteria of a transactivation domain, a Cys^2-Cys^2 zinc-finger domain including a P box and a D box, which have been shown to be critical for DNA binding properties of steroid receptors, and a ligand binding domain (Evans, 1988; Beato, 1989; O'Malley, 1990). A leucine-zipper motif, however, was identified in the ligand binding domain, a feature usually not observed with steroid receptors. This motif has been shown previously to participate in protein-protein interactions (Landschulz, Johnson & McKnight, 1988; Kouzarides & Ziff, 1989). Interestingly, the zinc-finger and the ligand-binding domains of NOR-1 are highly homologous to other identified orphan receptors NGFI-B (in rat; also called Nur77 in mouse, and TR3 in human) and RNR-1 (in rat; also called NURR1 in mouse, and NOT

in human) in which the leucine-zipper motif is also conserved. In addition, a motif located N-terminal of the putative ligand-binding domain, which was identified to be essential for DNA recognition (A-box; Wilson, Paulsen *et al.*, 1992) is also evident in NOR-1, NGFI-B and RNR-1. Comparing the sequence and structure of NOR-1, NGFI-B and RNR-1 with the steroid receptors, it is apparent that they form a closely related subgroup within the orphan receptors, but most of the receptors belonging to the superfamily are less related. Thus, mNur77/rNGFI-B/hTR3 and mNURR1/rRNR-1/hNOT as well as rNOR-1/hNOR-1 constitute a subfamily of the steroid/thyroid receptor superfamily. These receptors might function as a modulator of gene expression upon binding to the enhancer-like DNA sequence elements (5'-AAAAGGTCA-3') associated with target genes (Davis, Hazel *et al.*, 1993; Scearce, Laz *et al.*, 1993).

The relationship among the zinc finger domains of NOR-1 and other nuclear receptors is depicted in the dendogram of Fig. 7.1. The zinc finger regions of NOR-1, NGFI-B and RNR-1 are more closely related to each other than to other nuclear receptors. Recent studies have shown that a number of nuclear receptor-based signaling systems are comprised of more than one receptor [*e. g.*, Rev-Erb, COUP, RXR and ERR (Cooney, Tsai *et al.*, 1992; Giguère, Yang *et al.*, 1988; Retnakaran, Flock & Giguère, 1994; Schoorlemmer, van Puijenbroek *et al.*, 1994; Tran, Zhang *et al.*, 1992)]. It should be noted that the Nur77/NGFI-B family belongs to those nuclear receptor families.

The nuclear receptor superfamily has been generally divided into four classes based on their dimerization and DNA-binding properties (*cf.*, chapter 1). Class I receptors include the known steroid hormone receptors, which function as homodimers and bind to DNA half-sites organized as inverted repeats. Class II receptors heterodimerize with RXR and characteristically bind to direct repeats. Class III receptors bind primarily to

direct repeats as homodimers. Class IV receptors typically bind to extended core sites as monomers. Thus, NOR-1, Nur77/NGFI-B and RNR-1 fall into class IV categories.

Nur77 was originally identified as an immediate early gene rapidly activated by serum stimulation of quiescent fibroblasts (cell cycle reentry; Lau & Nathans, 1985; Lau & Nathans, 1987). It is also activated by such diverse signals as membrane depolarization and NGF treatment in the PC12 cells, and by chemically induced seizure in hippocampal neurons (Hazel, Misra *et al.*, 1991; Nathans, Lau *et al.*, 1988; Watson & Milbrandt, 1989). The expression of Nur77 is also induced during anti-CD3-induced apoptosis in T cell hybridomas. In addition, the expression of a dominant negative Nur77 or Nur77 antisense transcripts prevents activation-induced apoptosis of T cells (Liu, Smith *et al.*, 1994; Woronicz, Calnan *et al.*, 1994). These facts indicate that Nur77 might act in the control of activation-induced apoptosis of thymocytes and T cell hybridomas. NURR1 also belongs to the immediate-early genes. It was isolated from a mouse brain cDNA library by homology to the DNA binding domain of another orphan nuclear receptor, chicken ovalbumin upstream promoter transcription factor (COUP-TF), using low stringency hybridization conditions (Law, Conneely *et al.*, 1992). NURR1 is inducible by membrane depolarization in PC12 cells, and its mRNA is present at elevated levels in the brain. RNR-1, the rat homolog of NURR1, was identified in a differential screening for genes expressed after partial hepatectomy (Scearce, Laz *et al.*, 1993). This gene is not expressed in quiescent liver but is rapidly induced following partial hepatectomy and is specific to hepatic growth.

In the present study, NOR-1 was identified in primary cultured rat forebrain cells undergoing apoptosis, and its mRNA was detected abundantly in fetal brain and weakly in adult brain, with marginal expression in other normal adult tissues. In addition, the

tissue distribution of human NOR-1 also showed predominant and marginal expression in the fetal and adult brain, respectively. This expression profile was similar to that of rat NOR-1, indicating that NOR-1 may be involved in the molecular mechanisms regulating neural development. However, human NOR-1 mRNA was also expressed abundantly in skeletal muscles, suggesting additional role in biological process.

Although there are many similarities among NOR-1, NGFI-B and RNR-1, the tissue-specific expression of the respective genes suggests that the encoded proteins may have different functions. Moreover, the effective periods of the three members of the family are different in certain stages of the biological processes, for example during neural development, G1 phase of regeneration, or apoptosis. The multiplicity of its signaling system presumably reflects the crucial role played by those nuclear receptors in development and homeostasis.

NOR-1 is one of the immediate-early genes

After stimulation by growth factors, intracellular messengers activate specific genes. Some of these are induced very rapidly, and their expression is then greatly diminished within a few hours. Maruyama *et al.* (1995) have shown that NOR-1 behave as a typical early response gene, since its mRNA induction, by treatment with forskolin or TPA, proceeds in the absence of *de novo* protein synthesis. Within the steroid/thyroid receptor superfamily, this characteristic is specifically observed upon the members of the Nur77/NGFI-B family. It has been well established that c-jun, c-fos, c-myc, krox-20 and -24, EgrI and II, and NGFI-A and -B are typical early response genes (Greenberg, Hermanowski & Ziff, 1986; Lamp, Waamsley *et al.*, 1988; Chavier, Zerial *et al.*, 1988; Chavvier, Janssen-Timmen *et al.*, 1989; Curran, Bravo & Muller, 1985; Milbrandt, 1987; Sukhatme, Cao *et al.*, 1988; Lemaire, Revelant *et al.*, 1988). Their products are

believed to bind to specific DNA sequences and regulate the expression of other genes, creating a cascade leading to cell division. Early response genes can be separated into two groups: one with a zinc finger motif and the other without it. C-jun, c-fos, and c-myc do not have zinc fingers, but they probably bind other proteins regulating transcription through a leucine zipper (Landschultz, Johnson & Macknight, 1988). Another group of early response gene (Krox-20, EgrI and II, and NGFI-A) contain the zinc finger motif. NOR-1, NGFI-B, and Nur77 also have zinc fingers, but are slightly different from the previous group. The zinc finger of the Krox-like proteins is characterized by three repeated Cys^2-His^2 fingers, while the NOR-1 group has two repeated Cys^2-Cys^2 fingers. This suggests that the Nur77/NGFI-B family may be classified as a novel group within the immediate-early genes.

Possible physiological function of NOR-1

To clarify the physiological role of NOR-1 in fetal forebrains, the influence of antisense oligonucleotide to NOR-1 on primary cultured cells taken from rat fetal forebrains was examined in the present study. The antisense oligonucleotide to NOR-1 dramatically induced cellular morphological changes in primary cultured rat forebrain cells. Significant enhancement of cell migration, neurite extension, and formation of cellular aggregates was induced with only 24 h of antisense-treatment. Moreover, immunohistochemistry of MAP2 demonstrated that both the aggregates and processes, induced by antisense-treatment, were filled with neurites growing from neuronal cells. Since survival rates was not changed by the exposure to the antisense oligonucleotide, its effect might be specific to the cellular morphology. It seems, therefore, that antisense-treatment caused forebrain cells to undergo a morphological change that is considered to be a part of neuronal differentiation.

It is well known that formation of neuritic processes is influenced by a number of environmental factors including substrate adhesion (Letourneau, 1975) and concentration gradients of neurotrophic agents, such as NGF (Dichter, Tischler & Greene, 1977). Rydel and Greene (1988) have reported that cAMP could promote neurite outgrowth of rat sympathetic and sensory neurons independently of NGF-pathway. N18TG2 cells, derived from mouse neuroblastoma C1300 (Augsti-Tocco & Sato, 1969), are known to extend neurites in response to dibutyryl-cAMP or protein kinase inhibitor H-7 (Ono, Katayama *et al.*, 1991; Tsuda, Ono *et al.*, 1989). Furthermore, stimulating the N-methyl-D-aspartate receptor has a trophic effect on differentiating cerebellar granule cells (Baláz, Hack & Jorgensen, 1988; Moran & Patel, 1989). One possible explanation of the present results would be that NOR-1 is associated with those intracellular signal pathways transmitting the effects of inducers regulating neurite outgrowth. On the other hand, well-defined mitogenic proteins found in brain or peripheral tissues may also function as neurotrophic factors. For example, acidic and basic fibroblast growth factors (FGFs), epidermal growth factor (EGF), and insulin-like growth factors have been shown to enhance neurite outgrowth from either central or peripheral neurons *in vitro* and *in vivo* (Mill, Chao & Ishii, 1985; Morrison, Keating & Moskal, 1988; Unsicker, Reichert-Preibsch *et al.*, 1987; Walicke, 1988; Ferrari, Minozzi *et al.*, 1989). Another possibility is that the effects of antisense-treatment is mediated by neurotrophic agent(s). Moreover, the present result could not rule out the possibility that the forebrain neuronal cells are activated indirectly via an action on the glial elements of the forebrain cultures.

Initial thought as to the NOR-1 function was that its expression was needed for apoptosis on neuronal cells. Three features supported this suggestion: (i) NOR-1 amino acid sequence is most similar to Nur77, which is needed for apoptosis on T-cell hybridoma (Liu, Smith *et al.*, 1994; Woronicz, Calnan *et al.*, 1994). (ii) Nuclear

receptor exerts its biological effects by binding to a specific target element on genomic DNA. Both the Nur77 and NOR-1 proteins specifically bind to the same response-element (Chapter 3; Hazel, Nathans & Lau, 1988). (iii) NOR-1 mRNA is expressed in cultured forebrain cells that show the characteristic DNA ladder pattern of apoptosis (Chapter 3). To test this possibility, 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 (Chapter 4). These results indicate that the antisense oligonucleotide to NOR-1 has essentially no effect on apoptosis or survival in forebrain cells. The discrepancy between the antisense-treated cells and the functions postulated for NOR-1 probably reflects functional redundancy by closely related receptors (NGFI-B and RNR-1). Alternatively, the physiological role of NOR-1 might differ from those of the other Nur77 family members.

Bandoh *et al.* (1995) recently reported that NOR-1 mRNA is induced by various stimuli, including NGF, TPA and forskolin in various cultured cells including fibroblasts (NIH3T3 and L1), neuroblastoma cells (NB-OK-1, LA-N-1 and TGW), pheochromocytoma cells (PC12), glial cells (C_6) and endocrine cells (GH3, AtT-20 and Y-1). Therefore, NOR-1 may have an additional general role in cell growth and differentiation. In the result of Chapter 3, however, constitutive NOR-1 gene expression has been detected only in the brain tissue. This may reflect abundant stimulatory inputs to the neurons in the brain that induce NOR-1 gene expression, rather than neuron-specific expression of the NOR-1 gene. In these cases, NOR-1 may play a physiological role not only in neural tissues but also in the other tissues in response to various stimuli. In addition, since NOR-1 gene is defined as an immediate-early gene, it may function as a nuclear messenger of the signal transduction process and represent, along with the

products of other early-response genes, the primary effectors of the cellular response to extracellular environmental changes. Further studies are required to determine whether NOR-1 plays a role in mediating genomic responses to change in intracellular activity during development and homeostasis.

Features of chromosomal gene for human NOR-1

In the present study, I have cloned and characterized the chromosomal human NOR-1 gene. It was demonstrated in Chapter 5 that (i) the single copy more than 35 kb transcription unit consists of eight exons divided seven introns, (ii) conserved functional domains of the receptor protein are encoded by distinct exons, (iii) transcription initiation site is defined as a single nucleotide, and (iv) the gene for human NOR-1 is located on 9q, the long arm of chromosome 9.

According to the hypothesis of Gilbert (1978), division of the gene into exons is correlated with the proposed functional domains of the receptor molecule. Most strikingly, each of the two zinc fingers that have been postulated for the human NOR-1 DNA-binding domain is encoded by a distinct exon. The corresponding transactivating domain of the NOR-1 protein is encoded completely in a single exon (exon 3) as for several of the nuclear ligand receptor molecules. Another point of interest in the structure of the NOR-1 gene is the position of intron 3 which separates the exon 3 containing the first zinc finger from the second zinc finger. The position of this intron is identical to that of Nur77 and NGFI-B, but is different from those found in thyroid hormone, retinoic acid, progesterone, and estrogen receptors. It also suggests that NOR-1 and other members of the Nur77/NGFI-B family have evolved from a common ancestral gene.

As already mentioned, NOR-1 is an immediate early gene belonging to the steroid/thyroid receptor superfamily (Maruyama, Tsukada *et al.*, 1995). This

characteristic provides NOR-1 with the capacity to regulate a variety of cellular responses to extracellular stimuli, therefore it is important to understand how NOR-1 transcriptional activity is regulated. The study in Chapter 6 partly characterized the elements within the NOR-1 gene that are essential for its transcriptional activity. The promoter of the human NOR-1 gene appears to belong to a class of eukaryotic housekeeping gene promoters that lack TATA boxes and are exceptionally G+C rich (Dynan, 1986; McGrogan, Simonsen *et al.*, 1985; Velerio, Duyvesteyn *et al.*, 1985; Melton, Konecki, *et al.*, 1984). An important regulatory element in such genes may be the Sp1 motif whose core sequence is GGGCGG and is functional on either strand (Kadonaga, Jones & Tijan, 1986). The NOR-1 5'-flanking region also contains three copies of the Sp1 motifs. The lack of a perfect TATA box in the human NOR-1 promoter region is very similar to what was found in the promoter region of the progesterone receptor (Huckaby, Conneely *et al.*, 1987), androgen receptor (Mizokami, Yeh & Chang, 1994) and TR3 orphan receptor (Uemura, Mizokami & Chang, 1995; Chang, Saltzman *et al.*, 1993). The other similarity among these nuclear receptor gene promoter regions is the existence of highly G+C rich region. Promoters for some growth factor receptors and oncogenes also have these features: for example the epidermal growth factor receptor (Ishii, Xu *et al.*, 1985), NGF receptor (Sehgal, Patil & Chao, 1988), Ha-ras (Ishii, Merlino & Pastan, 1985), and N-myc (Kohl, Legouy *et al.*, 1986). These promoters are frequently associated with genes that are expressed at low levels in a non-tissue-specific manner.

Analysis of the contribution of elements that lie in the 5'-flanking region of the NOR-1 gene by transfection of deletions of reporter gene chimeric constructs has provided valuable information on the location and influence of proximal *cis*-acting regulatory elements. Sequential deletion analysis indicated that a minimal region exhibiting promoter activity is located between nt -94 and -42, where there are three

copies of cAMP response elements. Maruyama *et al.* (1995) have demonstrated that human NOR-1 gene is transiently induced by forskolin, an adenylate cyclase activating agent, in neuroblastoma cell line NB-OK-1. This finding is might be due to the motifs located immediately upstream of the transcriptional start site of NOR-1. The 5'-flanking region also contains two repeats of the AP1 recognition sequence, which confers TPA and epidermal growth factor inducibility on the c-fos gene. This finding is consistent with the previous observation that TPA induces NOR-1 expression in NB-OK-1 (Maruyama, Tsukada *et al.*, 1995).

NOR-1 expression was also induced by treatment of NGF in neuroblastoma cell line NB-OK-1 (Bandoh, Tsukada *et al.*, 1995). Two other genes that are transcriptionally activated by NGF, c-fos and NGFI-A, contain either an SRE (Triesman, 1986) or multiple CArG boxes (Minty & Kedes, 1986) in their 5'-flanking regions (Norman, Runswick *et al.*, 1988; Boulden & Sealy, 1992). I searched for similar elements in both the 5'-flanking and exon-intron sequences of NOR-1 but found nothing matching these consensus sequences. In NOR-1, these elements may lie in a novel location relative to the transcription site, or different regulatory factors may be responsible for the induction of this gene by NGF.

The human NOR-1 gene contains multiple components including several positive- and negative-acting elements in addition to the core promoter. Such a high level of structural complexity suggests a correspondingly high level of functional intricacy. Knowledge of the structural organization and promoter analyses of the NOR-1 gene will lead to a better understanding of the mechanisms of transcriptional regulation of the Nur77/NGFI-B family and will provide clues for genetic disorders involving this gene family.

Questions arise as to the NOR-1 gene

What is NOR-1's ligand? No ligand for the NOR-1 protein has been identified. Although the steroid/thyroid receptor superfamily was originally identified as a family of ligand-activated transcription factors, recent evidence suggests that ligand modulation may not always be required for the activity of all members of the superfamily. First, it has been shown that several orphan receptors are active transcription factors that do not require exogenously added ligand for their activity (Hazel, Nathans & Lau, 1988; Davis, Hazel & Lau, 1991; Lydon, Power & Conneely, 1992). Second, several members have been shown to be phosphoproteins that may be activated in the absence of ligand by phosphorylation (Denner, Weigel *et al.*, 1990) and by an intracellular signaling pathway initiated by at least one neurotransmitter (Power, Lydon *et al.*, 1991). In PC12 cells, Nur77 synthesized upon induction by NGF or KCl is differentially phosphorated (Hazel, Misra *et al.*, 1991). This observation suggests that phosphorylation might regulate the activity of Nur77, thus helping to specify the cellular responses to these stimulatory agents. The NOR-1 protein is also very rich in serines and threonines and contains numerous possible phosphorylation sites. It is most likely, therefore, that the activity of the NOR-1 protein is regulated by phosphorylation as that of Nur77. However, the following possibilities could not be excluded: (i) NOR-1 is another receptor for a known ligand, since retinoic acid and thyroid hormone bind multiple tissue-specific receptors; (ii) NOR-1 binds an intracellular molecule generated by growth factor, rather than by a ligand diffusing into the cell. It has been suggested that thyroid hormone-related and cholesterol-derived compounds, some of which are known to alter gene expression at the transcriptional level, may act through binding to unknown receptors. One such compound could be the ligand for NOR-1. Further studies are needed to determine whether the ligand for NOR-1 exists.

Is NOR-1 mRNA expressed as a single form? In 1995, Petropoulos *et al.* have found an mRNA that is closely related to NOR-1, and termed rat NOR-2, which does not contain the carboxyl-terminal region and was different sequences as in the 5' and 3' untranslated regions. Rat NOR-2 is expressed in regenerating liver and in both insulin-stimulated H4-U cells and pheochromocytoma cell line PC12 treated with NGF. A comparison of two mRNAs, NOR-1 and NOR-2, revealed that their 5' and 3' diverge points were identical to the boundary between the exons. This observation, taken together with the result of Chapter 5 that the NOR-1 gene is represented as a single copy in the haploid genome, strongly suggests that mRNA corresponding to the NOR-2 might be an isoform generated by alternative splicing.

The complexity of the organization and expression of the NOR-1 gene is reminiscent of that of other key developmental genes. This complexity probably provides the same purpose in all cases, (i) generating several regulatory factors from a single gene by using alternative splicing and multiple promoters and (ii) allowing the regulation of the stability and/or translation efficiency of the corresponding mRNAs by producing different 5' UTRs. However, more detailed studies are needed to identify the rationale for the existence of two (or more) NOR-1 isoforms.

Does the NOR-1 protein act alone? RXR has a pivotal role in the regulation of multiple hormonal pathways through heterodimerization with nuclear receptors, in addition to its role as a 9-*cis* retinoic acid receptor. Perlmann and Jansson (1995) have demonstrated using a two-hybrid system that both NGFI-B and NURR-1 can heterodimerize with RXR. These heterodimers bind selectively to a class of retinoic acid response elements composed of direct repeats spaced by 5 nucleotides. Since expression of both NGFI-B and NURR1 is rapidly induced by various growth factors, these

findings suggest a novel mechanism for convergence between the retinoid and growth factor signaling pathways. A comparison of amino acid sequences revealed that the putative dimerizing domain is also conserved between NOR-1, NGFI-B and NURR-1, indicating a possibility that NOR-1 can heterodimerize with RXR. In addition, a leucine-zipper motif, which participates in protein-protein interactions (Landschulz, Johnson & McKnight, 1988), was identified in the ligand-binding domain of NOR-1. Since transcription factors presumably interact with each other, it should be determined whether NOR-1 acts alone or cooperates with other agents.

What genes are regulated by the NOR-1 protein? Steroid 21-hydroxylase (21-OHase, Cyp21) is one of the cytochrome P-450 enzymes that are required for steroid hormone biosynthesis (Miller, 1988). It is expressed in the adrenal cortex, which is the main source of both glucocorticoids and mineralocorticoids. The corticotropin (ACTH)-mediated increase in 21-OHase transcription is manifest only several hours after the stimulation and requires ongoing protein synthesis. These facts suggest that ACTH induces immediate-early genes which then increase 21-OHase transcription (John, John *et al.*, 1986). Previous analysis of the promoter region of the mouse 21-OHase gene demonstrated that only 330 nucleotides of the 5'-flanking region are required for cell-specific and hormonally inducible expression (Handler, Schimmer *et al.*, 1988). Specifically, the element at nt -65 contains 8 nt that correspond to the recognition sequence bound by Nur77/NGFI-B family (Wilson, Mouw *et al.*, 1993). Thus, there is a possibility that NOR-1, one of the immediate-early genes, might regulate the 21-OHase gene.

Furthermore, it has been reported that intracerebroventricular injection of corticotropin-releasing factor (CRF) increased the gene expression of both c-fos and

NGFI-B in the parvocellular division of the hypothalamic paraventricular nucleus (PVN) 30 min after the injection (Parkes, Rivest *et al.*, 1993). NGFI-B response element is present within the promoter region of the rat CRF gene (Wilson, Fahrner *et al.*, 1991). Since NOR-1 also binds to the NGFI-B response element, it is possible that Nur77 /NGFI-B family, including NOR-1, regulates the CRF expression.

In addition to 21-OHase and CRF, the promoters of genes encoding several steroidogenic enzymes, including cholesterol cytochrome P-450 side chain cleavage enzyme, 11 α-hydroxylase, 17 α-hydroxylase, and aromatase, contain sequence elements that resemble the NOR-1 binding site (Morohashi, Honda *et al.*, 1992; Rice, Mouw *et al.*, 1991), suggesting that NOR-1 or other members of the Nur77/NGFI-B family may also regulate these genes. However, potential functions of NOR-1 in fetal brain and skeletal muscle as well as in diverse cell types as neurons, glias, and fibroblasts remain enigmatic.

Is the NOR-1 gene associated with human diseases? Besides the physiological roles of the nuclear receptor family, several investigators have reported that there is a direct link between a specific human disease and the alteration of a nuclear receptor gene. For instance, the retinoic acid receptor RAR α and/or PML genes are frequently rearranged in acute promyelocytic leukemia (de Thé, Chomienne *et al.*, 1990). Hypocalcemic rickets is associated with point mutations in the human vitamin D receptor gene (Karl, von Wichert *et al.*, 1996; Hughes, Malloy *et al.*, 1988). Furthermore, an increased protein-coding CAG repeat within the androgen receptor gene appears to be the only type of mutation responsible for spinal and bulbar muscular atrophy (or Kennedy disease; Biancalana, Serville *et al.*, 1992; Ferlini, Patrosso *et al.*, 1995). These observations, taken together with the findings that the suppression of rNOR-1 transcripts

induces morphological changes in neurons, suggests that the NOR-1 gene might be associated with a 9q-linked human disease.

The specific chromosomal translocation t(9;22)(q22-31;q11-12) has been observed in the myxoid variant of human chondrosarcoma. Recently, Clark *et al.* (1996) revealed that the EWS gene located at chromosome band 22q12 becomes fused to the human NOR-1 gene in a skeletal myxoid chondrosarcoma. The chimeric EWS-NOR-1 gene encodes a EWS-NOR-1 fusion protein in which the C-terminal RNA-binding domain of EWS is replaced by the entire NOR-1 protein, comprising a long N-terminal domain, a central DNA-binding domain and a C-terminal ligand binding domain. This observation provides, after the PML/RAR α gene fusion, the second example of the oncogenic conversion of a nuclear receptor and the first example involving the orphan nuclear receptors. Analysis of the disturbance induced by the EWS-NOR-1 protein in the nuclear receptor network and their target genes may lead to new approaches for chondrosarcoma treatment.

Conclusion

The present dissertation described a discovery of a novel member of the steroid /thyroid receptor superfamily genes. The results in the present study suggested following assertions. (i) A novel orphan nuclear receptor, designated NOR-1, exists 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 predominantly in the fetal brain. (iv) The NOR-1 protein functions as transcription factor; upon binding to the enhancer-like response element (5'-AAAAGGTCA-3') on genome. (v) NOR-1 might be involved in the molecular mechanisms regulating neural differentiation. (vi) The human NOR-1

gene, which is over 35 kb in length, is located on chromosome 9q, and it is split into eight exons and seven introns. (vi) The 5'-flanking region of the gene is characteristic of housekeeping gene promoters. (vii) The members of the Nur77/NGFI-B family, including NOR-1, might evolved from a common ancestral gene. Since nuclear receptors are essential for signal transduction, further studies on the NOR-1 gene may provide valuable information for understanding the molecular basis of development, physiology and human diseases.

NAME(GENES)	HORMONE	HALF-SITE	CONFIGURATION
GR	Cortisone	AGAACA	IR
MR	Aldosterone	AGAACA	IR
PR	Progesterone	AGAACA	IR
AR	Teststerone	AGAACA	IR
FXR	(Farnesoids)	AGGTCA	IR,DR
LXR α, β		RGKTCA	DR
VDR	Vitamin D3	RGKTCA	DR
xONR		RGKTCA	DR
MB67		RGKTCA	DR
TR α, β	Thyroid hormone	RGGTCA	IR,DR,ER
COUP α, β, γ		RGGTCA	DR,IR
RXR α, β, γ	9-cRA, Terpenoids	AGGTCA	DR
TR2-11 α, β		AGGTCA	DR
HNF-4		AGGTCA	DR
TLX		AAGTCA	NR
GCNF		TCAAGGTCA	DR
PPAR α, β. γ	(Eicosanoids)	AGGTCA	DR
RAR α, β, γ	Retinoic acid	AGTTCA	IR,DR,ER
Rev-Erb α, β		WAWNAGGTCA	NR,DR
ROR α, β, γ		WWCWRGGTCA	NR
NGFI-B		AAAGGTCA	NR
RNR-1		AAAGGTCA	NR
NOR-1 ⬅		AAAGGTCA	NR
FTZ-F1 α, β		YCAAGGYCR	NR
ERR α, β		TCAGGTCA	NR
ER	Estrogen	RGGTCA	IR

Fig. 7.1. Known members of the nuclear receptor superfamily in vertebrates. The dendrogram represents the relationship among known nuclear receptors prepared from a multiple sequence alignment (Feng & Doolittle, 1987). Amino acid sequences from the entire DNA-binding domain were aligned. Arrow indicates the position of NOR-1. Hormone activators given in parenthesis have not yet been proven to bind receptor. Hormone response elements are configured as direct repeats (DR), inverted repeats (IR), everted repeats (ER), or non repeats (NR) . A maximum of two GenBank/EMBL/DDBJ accession numbers (listed below) was allowed for each receptor type. Species designations are as follows: m, mouse; r, rat; h, human. NOR-1 (D38530. r; D78579, h); GR(M14053, r; X03225, h); MR (M36074, r; M16801, h); PR (L16922, r; M15716, h); AR (M20133, r; M21748, h); FXR (U09418, m; U18374, r); LXR α (U22662, h); LXR β (U14534, h); VDR (J04147, r; J03258, h); xONR (X75163, Xenopus); MB67 (L29623, h); TR α (M31174, r; M24899, h); TR β (J03933, r; M26747, h); COUP α (X12795, h); COUP β (X12794, h); COUP γ (M62760, h); RXR α (X52773, h); RXR β (M84820, h); RXR γ (S77808, r); TR2-11 α (M29960, h); TR2-11 β (L2753, r; L27586, h); HNF-4 (X57133, r; X76930, h); TLL (S77482, m); GCNF (U09563, m); PPAR α (M88592, r; S74349, h); PPAR β (L28116, m; U10375, h); PPAR γ (U09138, m; L40904, h); RAR α (S77802, r; X06614, h); RAR β (M60909, m; Y00291, h); RAR γ (S77804, r; M24857, h); Rev-Eeb α (M25804, r; M24898, h); Rev-Erb β (X82777, r; L31785, h); ROR α (U22437, m; L14611, h); ROR β (L14160, r; T26966, h); ROR γ (H33887. r; U16997, h); NGFI-B (U17254, r; L13740, h); RNR-1 (L08595, r; X75918, h); FTZ-F1 α (D42152, r; U32592, h); FTZ-F1 β (M81385, m); ERR α (X51416, h); ERR β (X51417, h); ER (X61098, r; M11457, h).

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  AUTHORS   Ohkura,N.
  JOURNAL   Unpublished (1995)
COMMENT     Submitted (14-Oct-1994) to DDBJ by:
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LOCUS       hNOR-1        3802 bp cDNA                            18-OCT-1996
DEFINITION  Homo sapiens of NOR-1 gene encoding NOR-1.
ACCESSION   D78579
KEYWORDS    NOR-1.
SOURCE      Homo sapiens Fetus Brain.
  ORGANISM  Homo sapiens
REFERENCE   1   (bases 1 to 3802)
  AUTHORS   Ohkura,N., Ito,M., Tsukada,T., Sasaki,K., Yamaguchi,K. and
            Miki,K.
  TITLE     Structure, mapping and expression of a human NOR-1 gene,
            the third member of the Nur77/NGFI-B family
  JOURNAL   Biochim. Biophys. Acta 1308, 205-214 (1996)
  STANDARD  full automatic
COMMENT
            Data kindly submitted in computer readable form by:
            Naganari Ohkura
            Institute of Biomedical Science
            TERUMO R&D Center
            1500 Inokuchi, Nakai
            Ashigarakami-gun Kanagawa 259-01
            Japan
            Phone:  0465-81-4103
            Fax:    0465-81-4194

FEATURES             Location/Qualifiers
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     exon            2365..>3802
                     /partial=(5'site:no, 3'site:yes)
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BASE COUNT     919 a   1141 c    912 g    830 t
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//
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LIST OF PUBLICATIONS CONCERNING THIS DISSERTATION

1. Ohkura, N., Hijikuro, M., Yamamoto, A. and Miki, K.

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

   Biochemical and Biophysical Research Communications **205**, 1959-1965 (1994).

2. Ohkura, N., Hijikurro, M. and Miki, K.

   Antisense oligonucleotide to NOR-1, a novel orphan receptor, induces migration and neurite extension of cultured forebrain cells.

   Molecular Brain Research **35**, 309-313 (1996).

3. Ohkura, N., Ito, M., Tsukada, T., Sasaki, K., Yamaguchi, K. and Miki, K.

   Structure, mapping and expression of a human NOR-1 gene, the third member of the Nur77/NGFI-B family.

   Biochimica et Biophysica Acta **1308**, 205-214 (1996).