NOR-2 (neuron-derived orphan receptor), a brain zinc finger protein, is highly induced during liver regeneration

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Abstract Zinc-finger proteins are involved in several cellular processes. Some of these proteins are implicated in the primary cellular response in regenerating liver and mitogen-stimulated cells. Using a rat cDNA brain library, we have isolated a clone designated NOR-2, encoding a protein containing two zinc-finger motifs and whose expression is highly induced during G0/G1 transition. We analysed the expression of NOR-2 mRNAs during early growth in regenerating liver and in both insulin-stimulated H4-II cells and pheochromocytoma-derived cell line PC12 treated by NGF. In these systems, there is an early, rapid and transient accumulation of NOR-2 mRNAs. The induction of NOR-2 mRNAs does not require de novo protein synthesis, since it is not prevented by cycloheximide treatment. Mobility shift assays show that NOR-2 protein binds to NBRE, a target sequence for r-NGFI-B family. Structurally, NOR-2 is closely related to the recently identified NOR-1 factor. Therefore, like NOR-1, NOR-2 belongs to the r-NGFI-B sub-family of nuclear receptors superfamily.

Key words: Immediate-early gene; Zinc-finger protein; r-NGFI-B family; Transition from G0 to G1 of the cell cycle; Liver regeneration

1. Introduction

The transition from quiescence into the G1 phase of the cell cycle is regulated through the input of extracellular signals. Indeed, multiple factors including circulating hormones, growth factors and nervous input, participate in the regulation of the primary cellular response. This response characterises, at least in part, the initiation of the G0 to G1 transition of the cell cycle. It also results in a transcriptional activation of the group of immediate-early response genes [1]. Basically, a transient and rapid activation of these genes occurs within the first few hours of cell activation and does not require de novo protein synthesis. Several immediate-early genes encoding secretory proteins, structural proteins such as actin and transcription factors such as Jun [2], Fos [3] and zinc-finger proteins [4] are probably involved in the regulation of expression of genes at the later point of the cell cycle (called delayed genes). As such, they may have a crucial role in the cell cycle progression and cell growth.

Several models of mitogen-stimulated cells have been used to study various aspects of the cell growth. However, liver regeneration provides one of the few models for analysis of cell growth in vivo. Indeed, partial hepatectomy synchronizes the majority of hepatocytes during the proliferating phase of the first mitosis. This model has also been used to analyse the expression of hepatic genes during different phases of the cell cycle. It has been shown that transferrin mRNA is detectable very late in the regenerating liver, and thus it belongs to the set of delayed genes of the cell cycle [5]. The transferrin gene is therefore probably regulated in part by immediate-early genes. Earlier studies allowed us to identify the 5′ regulatory regions involved in the expression of the transferrin gene in different tissues such as liver, brain and testis. Furthermore it was possible to characterize the specific combination of transcription factors which interact with these regions [6,7]. Such results emphasize the requirement for different combinations of transcription factors in different tissues to achieve specific expression. Amongst these factors the immediate-early gene are probably involved.

To further understand the regulation of the transferrin gene it was necessary to identify additional factors, unknown until now, involved in the tissue specific expression. To this end, we screened an adult rat brain cDNA library with a probe encoding the DNA binding domain of COUP-TF [8], a member of the nuclear receptor superfamilly. This screening resulted in the identification of a cDNA clone designated as NOR-2 (neuron-derived orphan receptor), since the structural analysis shows that it is similar to the members of the r-NGFI-B family [9], and particulary very closely related to the recently identified NOR-1 factor [10]. All of these factors belong to the nuclear receptor superfamily.

In this paper, we report the structure and characterization of NOR-2, a novel immediate-early gene. We show that NOR-2 mRNAs are highly and rapidly induced in the liver systems: both liver regeneration after partial hepatectomy and insulin-stimulated H4-II cells.

2. Materials and methods

2.1. cDNA library screening

An adult rat brain λZappII cDNA library was screened with a random labelled probe (megaprime kit, Amersham) encoding the DNA binding domain of COUP-TF. One partial cDNA clone was isolated and used for subsequent screening under high stringency (final wash: 0.1 SSPE, 0.1% SDS, 65°C for 20 min) to obtain full-length cDNA. Nucleotide sequencing of both strands was done by the dideoxy chain termination method using the T7 sequencing kit (Pharmacia Biotech).

2.2. Cells culture and treatment conditions

H4-II cells were grown in COON's F-12 modified medium (Seromed)

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Abbreviations: NOR-2, neuron-derived orphan receptor; NGF, nerve growth factor; NGFI-B, nerve growth factor inducible-B; NBRE, NGFI-B reponse element; COUP-TF, chicken ovalbumin upstream promoter-transcription factor.

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supplemented with 5% fetal calf serum (Gibco-BRL). PC12 cells were grown in Dulbecco's modified Eagle medium (Gibco-BRL) containing 10% horse serum (Boehringer Mannheim) and 5% fetal calf serum.

To produce quiescence, the medium was changed to serum free medium for 48 h. Following serum privation, H4-II cells were treated with insulin (10^{-8} M, Sigma) and cycloheximide (10 μg/μl, Sigma). PC12 cells were treated with NGF (50 ng/ml, Sigma).

2.3. Regenerating liver after partial hepatectomy

Male Wistar rats (2 months old, 150–220 g) were anesthesized with ether and subjected to 70% partial hepatectomy [11]. Animals were allowed to recover for different times prior to decapitation and isolation of the remaining liver lobes.

2.4. RNA preparation and analyses

Poly $(A)^+$ RNA were prepared according to the Dynabeads kit (Dynal) from cells or tissues (brain and liver). Northern blots analyses were performed with 1.5 μg of Poly $(A)^+$ RNA after separated by electrophoresis on a 1% agarose, 0.6% formaldehyde, MOPS denaturing gel and capillary transfer on hybond N^+ membranes (Amersham). The NOR-2 probe corresponds to the *Nru*I–*Bss*HII fragment, which included nucleotides 765–1051. Standard hybridization and wash conditions were used according to the manufacturer. The amount of RNA was normalized by hybridization with the GAPDH probe (data not shown).

2.5. In vitro transcription/translation

In vitro transcription/translation of NOR-2 was performed with the TNT T7 coupled reticulocyte system (Promega, Madison, WI, USA). The experiment was carried out either in the presence of [^{35}S]methionine and run on a 12.5% (SDS)-polyacrylamide gel or cold methionine for use in mobility shift assay. We obtained a major band at about 48 kDa corresponding to a protein translated from the predicted initiation codon (487 nucleotides) (data not shown).

2.6. Mobility shift assay

The conditions of mobility shift assay was adapted from [12]. The binding reactions were performed with 5 μl of reticulocyte lysate. After 20 min of incubation at room temperature, the samples were loaded on a 6% acrylamide gel. For the competition experiments, 100 ng of cold oligonucleotide were added to the binding reactions.

The sequences of synthetic oligonucleotides used are:
NBRE: 5′ GAGTTTTAAAAGGTCATGCTCAATTT 3′
non-specific oligonucleotide from the melanotransferrin gene [13]:
5′ CAGCGTCACGTGGAGTTTTGCCAAAGGA 3′

3. Results

3.1. Isolation and sequence of NOR-2 cDNA

The complete cDNA encoding NOR-2 was isolated from an adult rat brain λZAPII cDNA library. The library was screened with a ^{32}P-labeled cDNA probe corresponding to the DNA binding domain of COUP-TF. One partial cDNA clone was isolated and used for subsequent screening to obtain full-length cDNA. This last cDNA was designated as NOR-2. The size of the cDNA insert is 4,275 nucleotides (nt) and contains a major open reading frame of 1,290 nt starting with an initiation methionine at 487 nt. Nevertheless, the ATG codon at this position had a poor flanking sequence for translation initiation according to the Kozak consensus sequence. The 5′ untranslated region of the NOR-2 cDNA contains several stop codons indicating that the whole coding region has been cloned. The open reading frame is followed by a 3′ untranslated region in which the ATTTA motif was found seven times. This element is known to confer mRNA instability, a property of many immediate-early genes [14]. No canonical AATAAAA has been found, instead an ATTAAA motif was localized 40 nucleotides before the poly(A) tail [15] (Fig. 1). However, we cannot rule out the possibility that the NOR-2 cDNA lacked a poly (A) tail.

3.2. Structural characteristics of NOR-2 cDNA and homology to the r-NGFI-B family

The first analysis of the predicted NOR-2 protein sequence revealed that this factor exhibits similiarities in sequence and structure to the nuclear receptor superfamily. Indeed, NOR-2 contains two zinc-fingers of the C2C2 type, as do of the members of this family [16]. While we finished the sequencing of NOR-2, a second computer analysis showed that this factor was identical, from ATG (at 487 nt) until 1,746 nt, to NOR-1 which has been very recently identified [10]. In NOR-2 the stop codon was found at 1,777 nt, (i.e. 31 nt from 1,746 nt) whereas the stop codon in NOR-1 was found 627nt from the divergent point between these two proteins. Furthermore, NOR-2 and NOR-1 differ completely in both 5′ and 3′ non coding regions (Fig. 2A).

It is therefore indisputable that NOR-2 is very closely related to NOR-1. Like NOR-1, NOR-2 belongs specifically to the r-NGFI-B [9] family showing in particular high homology with both r-NGFI-B and r-RNR-1 [17] factors in the DNA binding domain (91% and 97% respectively). More precisely, in the P and A box regions, NOR-2 shows 100% homology to the NGFI-B and RNR-1. These two regions have been identified as crucial in DNA recognition. It has been shown that the A box is required for recognition of the two adenine-thymidine bases pairs at the 5′ end of the NGFI-B response element (NBRE) [18,19]. Concerning the D box, which is involved in the orientation of dimeric receptors [20], NOR-2 again shows high homology with the other three members of the sub-family (Fig. 2B). In contrast, NOR-2 does not contain the carboxy-terminal region, which corresponds to the ligand binding domain. NOR-2, as well as NOR-1, shows a high variability in the N-terminal region compared to the other three related proteins. Several studies concerning the other factors of this family, indicated that the N-terminal region is involved in the transactivation activity [21,22].

3.3. Induction of NOR-2 in liver proliferating cells and in PC 12 cells stimulated by NGF

In order to analyse the induction of NOR-2 expression during G0/G1 transition of the cell cycle we used three different models: an in vivo model consisting of liver regeneration after partial hepatectomy, and both insulin stimulated H4-II hepatoma cell line and pheochromocytoma-derived cell line PC12 treated by NGF, as in vitro systems. Northern blot analysis revealed the presence of several mRNAs species corresponding to NOR-2 in all types of cells and tissues that have been studied. NOR-2 mRNAs (6 kb, 5.6 kb, 2.9 kb, 2.4 kb) were only barely detectable in adult liver tissue and were rapidly induced in regenerating liver, after only 15 min. They reached a maximum level 30 min after partial hepatectomy. Thereafter the mRNAs decreased and reached resting levels 16 h after hepatectomy (Fig. 3A). NOR-2 mRNAs were barely detectable at all time points following sham surgery (data not shown).

The same species of NOR-2 mRNAs detected in regenerating liver, were also expressed at a high levels in insulin-treated H4-II cells 3 hours after stimulation (Fig. 3B). Superinduction of NOR-2 mRNAs in the presence of cycloheximide (CHX) indicated that NOR-2 gene does not require de novo protein synthesis and thus defined NOR-2 as an immediate-early gene. NOR-2 mRNAs are induced to a lesser extent in NGF-treated PC12 cells (Fig. 3C). It should be noted that all these experi-

Fig. 1. Nucleotide sequence of NOR-2. The ATG flanked by the Kozak consensus sequence and TGA are underlined. In the 3′ untranslated region, the seven ATTTA motifs are indicated in italic and underlined. The motif ATTAAA which could be a polyadenylation signal is also indicated.

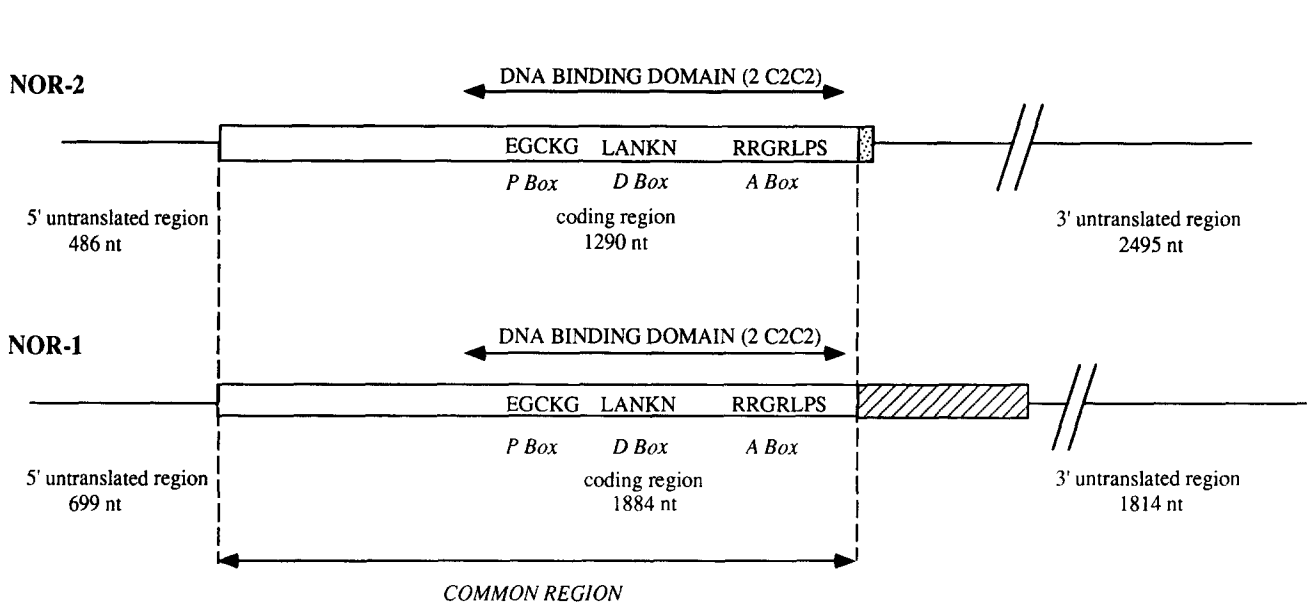
ments were carried out using polyadenylated RNAs, indicating that NOR-2 is expressed at very low levels. Its expression analysis in adult tissues (heart, spleen, lung, liver, skeletal muscle, kidney, testis) from polyadenylated RNAs, shows a weak basal expression (data not shown). In contrast, in the brain both 6Kb and 5.6 kb transcripts are more abundant (Fig. 3D).

3.4. *In vitro translation and characterization of DNA binding elements of NOR-2*

In vitro translated NOR-2 protein was used to test the binding of the NOR-2 to an NGFI-B response element (NBRE) [18–19]. This motif has been identified as an r-NGFI-B/m-Nur77 and r-RNR-1/m-Nurr1 response element. The predicted molecular mass of 47 kDa of NOR-2 was confirmed by SDS-polyacrylamide gel electrophoresis using the translated in vitro protein (data not shown). Reticulocyte lysate has endogenous binding activity to the NBRE oligonucleotide. It is however possible to separate this activity from the specific binding activity of NOR-2 (Fig. 4). The mobility shift assay shows that NOR-2 binds specifically to NBRE response element. Excess of unlabeled oligonucleotide (100-fold) reduced significantly this binding and no competition was observed when a non specific oligonucleotide was used (Fig. 4, lane 2 and lane 4). Thus, as expected, NOR-2 binds to the NBRE motif, like the other three members of the r-NGFI-B family.

4. Discussion

We have identified NOR-2, a novel member of the orphan steroid receptor superfamily. We demonstrated that the NOR-2



B

NGFI-B	EGCKG	LANKD	RRGRLPS
RNR-1	EGCKG	LANKN	RRGRLPS
	P Box	*D Box*	*A Box*

Fig. 2. (A) Schematic representation of full-length NOR-2 cDNA. Comparison with NOR-1 cDNA. The boxes and represent the divergent sequence between the coding region of the two cDNA. (B) Comparison between the P, D and A boxes of NOR-2 and NOR-1 with that of NGFI-B and Nurr1.

mRNAs are rapidly and highly induced in liver regeneration following partial hepatectomy, as well as in both insulin-stimulated H4-II hepatoma cell line and pheochromocytoma-derived cell line PC12 treated by NGF. This early and transient NOR-2 mRNAs expression occurs during the G0 to G1 transition when the cells re-enter the cell cycle. Furthermore, the fact that this expression was increased in the presence of cycloheximide indicated that the NOR-2 gene does not require de novo protein synthesis. All of these characteristics allowed us to define NOR-2 as an immediate-early gene.

NOR-2 shows some similarities in its expression to the r-NGFI-B and RNR-1 factors, both members of the 2-NGFI-B family. These genes are also induced during G0/G1 transtion, as the immediate-early genes. We show that like r-NGFI-B, NOR-2 was induced in PC12 cells treated by NGF and like RNR-1, NOR-2 was increased in the liver regenerating model. Despite these similarities, NOR-2 expression shows a notable difference with r-NGFI-B and RNR-1. For instance, unlike RNR-1 and r-NGFI-B, NOR-2 was accumulated at high level in insulin-stimulated H4 II hepatoma cell line. Furthermore, the pattern of NOR-2 expression shows the presence of several species of mRNAs (6 kb, 5.3 kb, 2.9 kb, 2.4 kb), whereas, with r-NGFI-B and RNR-1, only one messenger is detected. Moreover, apart from the brain, where a low accumulation of 6kb and 5.6 kb NOR-2 mRNAs was observed, its expression is very marginal. In constrast, r-NGFI-B is highly expressed in lung, brain and adrenal tissues. As far as RNR-1 is concerned, high expression is detected in the brain. Thus far, NOR-1, which has been recently identified, is the most closely related to NOR-2. Analysis of NOR-1 mRNA shows that it is expressed at high levels in cultured apoptotic neuronal cells and fetal brain. In addition, like NOR-2, NOR-1 is expressed at low level in adult brain.

The differences in tissue-specific expression pattern and also in the number of mRNAs species between NOR-2 and other members of the r-NGFI-B family, suggest that while they belong to the same family, they may accomplish different fonctions.

Several hypotheses could be made to explain the presence of different species of the NOR-2 messengers. For instance, this gene could fonction with several optional promoters. The optional use of these promoters could result in the synthesis of different types of NOR-2 mRNAs with different 5′ non-coding sequences. Genes with alternative use of promoters have been described and result in synthesis of transcripts with alternative 5′ exons. Such a phenomenon can result in the synthesis of different proteins as in the case of myosin light chain IF/3F [23] or of identical proteins as for aldolase A [24]. Secondly, alternative splicing and/or alternative polyadenylation sites could be at the origin of the different NOR-2 mRNAs.

The cDNA sequence analysis reveals that there is identity between the nucleotide sequences encoding the first 419 resi-

dues of NOR-2 and NOR-1. Furthermore, both 5′ and 3′ non-coding regions of the NOR-2 and NOR-1 are completely different. If these differences are not due to a cloning artifact, the same hypotheses which explain the presence of several species of NOR-2 mRNAs, could be advanced.

The analysis of the predicted NOR-2 protein sequence shows the high degree of homology in the DNA-binding domains between NOR-2, NGFI-B, and RNR-1. It also shows that the DNA-binding domain of NOR-2 is identical to that of NOR-1. We demonstrated by mobility shift assays that the NOR-2 protein binds specifically to NBRE, as predicted on the basis of the sequence homology. This result indicates that all of these factors could bind to very similar sequences in target genes, suggesting that they may be regulate similar genes. However, these factors exhibit strong divergence in the N-terminal region, the putative transactivation domain. It is possible that these differences are the reflection of their implication at different times during the G0/G1 transition of the cell cycle.

In conclusion, our results show that the NOR-2 expression in vitro and in vivo resemble the expression of immediate early transcription factors such as Jun and Fos. These proteins are thought to play a role in activating transcription of delayed genes, involved in later phases of the cell cycle. Further ongoing studies are being undertaken to identify target genes whose expression is regulated by NOR-2.

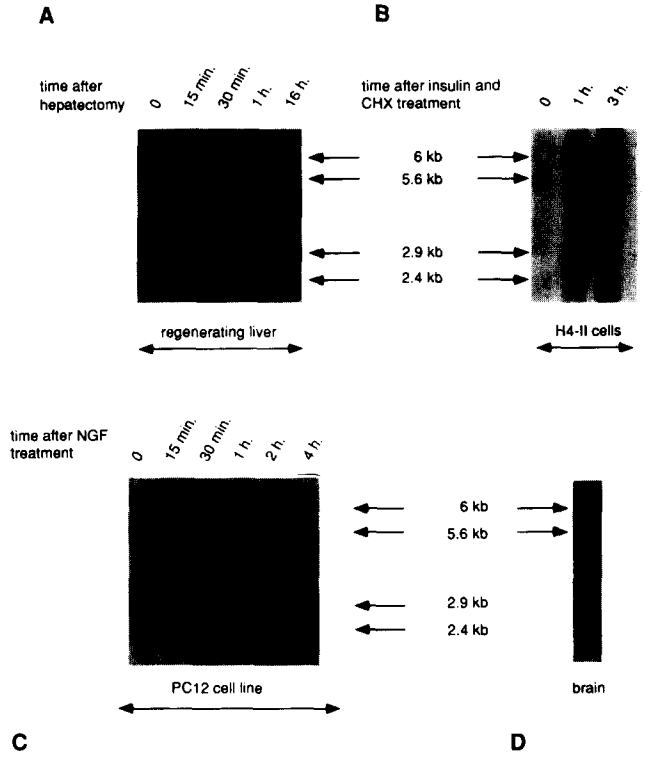


Fig. 3. Northern blot analysis of NOR-2 gene induction in regenerating liver, in various rat cell lines and in brain. (A) 0, Poly $(A)^+$ RNA (1.5 μg) was analysed from quiescent, normal liver, Poly $(A)^+$ RNA (1.5 μg) was analysed from regenerating liver (15 min, 30 min, 1 h and 16 h) after 70% hepatectomie. (B) Poly $(A)^+$ RNA (1.5 μg) was analysed from H4-II treated with insulin (10^{-8}M) and cycloheximide (10μg/ml) for various time, as indicated in the figure. (C) Poly $(A)^+$ RNA (1.5 μg) was analysed from PC12 cells treated with NGF (50 ng/ml) for various time as indicated. (D) Poly $(A)^+$ RNA (1.5 μg) was analysed from adult rat brain. The northern blots were exposed for 10 days.

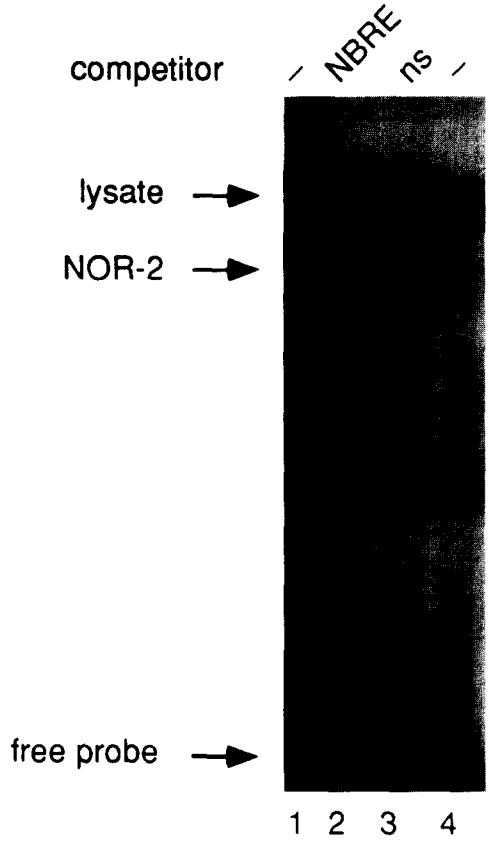


Fig. 4. NOR-2 binds specifically to the NGFI-B response element. NOR-2 cDNA was transcripted/translated in vitro with TNT T7 coupled reticulocyte lysate and bound to radiolabeled NBRE, and run on a 6% native acrylamide gel. Lane 1, reticulocyte lysate programmed with NOR-2 cDNA. Lane 2, reticulocyte lysate as in lane 1 with 100X cold NBRE oligonucleotide. Lane 3, reticulocyte lysate as in lane 1 with 100X cold non specific oligonucleotide (ns). Lane 4, unprogrammed reticulocyte lysate.

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