

Expression and functional analysis of Nr2e3, a photoreceptor-specific nuclear receptor, suggest common mechanisms in retinal development between avians and mammals

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Abstract The photoreceptor-specific nuclear receptor (PNR; Nr2e3) is a transcription factor important for retinal development. We report here the identification and expression analysis of the avian Nr2e3. *Nr2e3* mRNA is expressed in the photoreceptor layer of the neural retina during early stages of chick embryogenesis. Its temporal expression is distinct from that of a related nuclear receptor, Tlx. Chick Nr2e3 recognizes and binds to the same target DNA sequence as its vertebrate orthologs. Functional assays revealed that chick Nr2e3 acts as a transcriptional repressor. Our results suggest that Nr2e3 plays a common role in retinal development in vertebrates.

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The sequence reported in this paper has been deposited in the GenBank database [accession no. AY053465 (chicken Nr2e3)].

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Introduction

Transcription factor proteins regulate gene expression by binding to specific DNA sequences in regulatory regions of the gene. Nuclear receptors have a unique modular structure, with a characteristic ligand-binding domain in addition to their DNA-binding domain (Mangelsdorf et al. 1995). They are receptors for lipophilic molecules such as steroid and thyroid hormones, vitamins A and D, and at the same time, they function as transcription factors. Many nuclear receptors are known to regulate cell fate determination. However, ligands have not been identified for a substantial number of nuclear receptors and members of this class are called orphan receptors (Benoit et al. 2006).

Photoreceptor-specific nuclear receptor (PNR; Nr2e3) is one of these orphan receptors, whose expression is restricted almost exclusively to the photoreceptor layer of the neural retina (Kobayashi et al. 1999; Chen et al. 2005; Kitambi and Hauptmann 2007; Martinez-De Luna and El-Hodiri 2007; McIlvain and Knox 2007). Mutations of this gene have been implicated in several forms of retinal disorder (Gerber et al. 2000 and references in Chen et al. 2005). Detailed analyses of its function have been conducted using various model vertebrate systems and suggested that Nr2e3 is important in the development and maintenance of rod photoreceptor cells (Chen et al. 2005; McIlvain and Knox 2007; Oh et al. 2008).

Chick embryos have long been a favorite model for performing functional analyses of transcription factor proteins. It allows observation of retinal development in

vivo with relative ease and the availability of gene transfer methods such as electroporation is an additional advantage (Stern 2005). Identification of the chick *Nr2e3* gene is, thus, a useful tool to further these studies.

We used a cDNA library from embryonic day 8 (E8) chick retina to identify an avian *Nr2e3* clone. The mRNA expression of this chick *Nr2e3* was found to be restricted in the neural retina. We analyzed its expression during a developmental time course along with a related transcription factor, *Tlx*. Structurally, the chick *Nr2e3* shares an asparagine substitution in the P box of its DNA-binding domain with its vertebrate orthologs in contrast to the acidic amino acid residue present in other nuclear receptor family members. Accordingly, the *Nr2e3* proteins show the same DNA-binding preferences, suggesting that they regulate a similar set of target genes. Transcription assays further confirmed that chick *Nr2e3* function as a transcriptional repressor.

Materials and methods

RNA extraction

Fertilized chicken eggs were obtained from Okada Farm (Nara, Japan) and incubated at 35°C for up to 14 days. The embryos were harvested, tissues were dissected, and total RNA was extracted using ISOGEN (Nippon Gene, Toyama, Japan) following the manufacturer's protocol.

Identification of the chicken *Nr2e3* ortholog

Using total RNA extracted from the neural retina of E8 chick embryos, mRNA was purified with Oligotex™-dT30 <Super> Kit (Roche). A lambda phage cDNA library was constructed using the Lambda System for cDNA Cloning (Life Technologies) and Gigapack III Gold Packaging extract (Stratagene). A polymerase chain reaction fragment corresponding to full-length human *Nr2e3* (amino acids 8 to poly(A)+ region) was amplified from a human PNR (*Nr2e3*) plasmid construct using primers NMO70 (5'-TCTGATGAGCTCCACAGTGGCTGC-3') and T3 (5'-GCAATTAACCCTCACTAAAGGG-3') (Kobayashi et al. 1999). The DNA fragment was gel-purified, labeled with alpha-³²P-dCTP using Megaprime DNA Labeling System (Amersham Pharmacia) and used as a probe to screen 0.8 × 10⁵ independent clones in the E8 retinal library using an ExAssist helper phage (Stratagene). Twenty-seven positive clones were identified and further analyzed in a second screening using the same probe. Fifteen strongly positive clones were selected for DNA sequencing and three potential chicken *Nr2e3* clones were identified. The most 5' end, which corresponded to the region between the DNA- and ligand-binding domain of the potential chicken

Nr2e3 was amplified using primers KMO40 (5'-TAGA CAGCATTGAGCTGGACGCCGA-3') and KMO22 (5'-CGTGCTGAGGTCTCGTAGACGTT-3') and another screening was performed. Independent clones of 2.0 × 10⁵ were screened and nine positive clones were identified. After a second screening using the same probe, three positive clones were identified and DNA sequence was determined using an ALF express sequencing machine (Amersham Pharmacia).

Northern blotting and in situ hybridization

A 0.75-kb cDNA fragment of chicken *Nr2e3* [nucleotides 177–915] was amplified using primers KMO6 (5'-CCAGTGGACAAGGCACACCGCAAC-3') and KMO22 and labeled with digoxigenin (Boehringer; for *Nr2e3* probe), or amplified using KMOcT5 (5'-ATCCAGGCTT TACAGGAGGTTGTGG-3') and KMOcT3 (5'-TCCG AAACGACAGGGTTGTGTAGG-3') and labeled with alpha-³²P-dCTP (for *Tlx* probe). Gel electrophoresis, membrane transfer, and hybridization conditions were as described (Kobayashi et al. 1999). A nylon membrane blot containing 10 µg of each total RNA was prepared. The 18S and 28S rRNA on the membrane was visualized by 5% methylene blue in 10% acetic acid and used as loading control. After hybridization with the *Nr2e3* probe, the signals were detected using an ECL Detection System (Amersham Pharmacia). Subsequently, the blot was rehybridized with a *Tlx* probe and the signal was visualized on X-ray film. In situ hybridization of frozen tissue sections was performed as described (Kobayashi et al. 1999) using a 0.75-kb digoxigenin-labeled antisense RNA probe.

DNA-binding assays

The chick *Nr2e3* protein was translated from a pCMX c*Nr2e3* expression vector (Kobayashi et al. 1999) using T7 RNA polymerase and a TNT® coupled rabbit reticulocyte in vitro translation kit (Promega). Probe-labeling and gel shift assay was performed as described (Kobayashi et al. 1999). The sequences of double-stranded probes used for the assays are indicated in the figure with 5'-agct hangovers.

Transactivation assays

Expression plasmids for the GAL4–*Nr2e3* fusion protein were obtained by ligating cDNA fragments encoding chick (Arg 92 to stop codon) or human (Met 113 to stop codon) *Nr2e3* ligand-binding domain into a cytomegalovirus promoter-driven plasmid pCMX-GAL4 (Gerber et al. 2000). CV-1 cells were used for transfection assays in 12-well tissue culture plates by calcium phosphate precipitation (Umesono and Evans 1989). Cells were maintained in

Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (BCS). Transfection mixtures contained 100 ng of receptor expression plasmid, 250 ng of MH100×4-tk-luc reporter plasmid and 500 ng of pCMX-βGAL as control for transfection efficiency. Cells were exposed to the precipitate for 6–8 h, washed, and treated with phenol red-free DMEM, 10% charcoal–resin-treated BCS, before harvesting and assaying for luciferase and β-galactosidase activity. All points were performed in triplicate and repeated at least twice in independent experiments with variations of less than 10%.

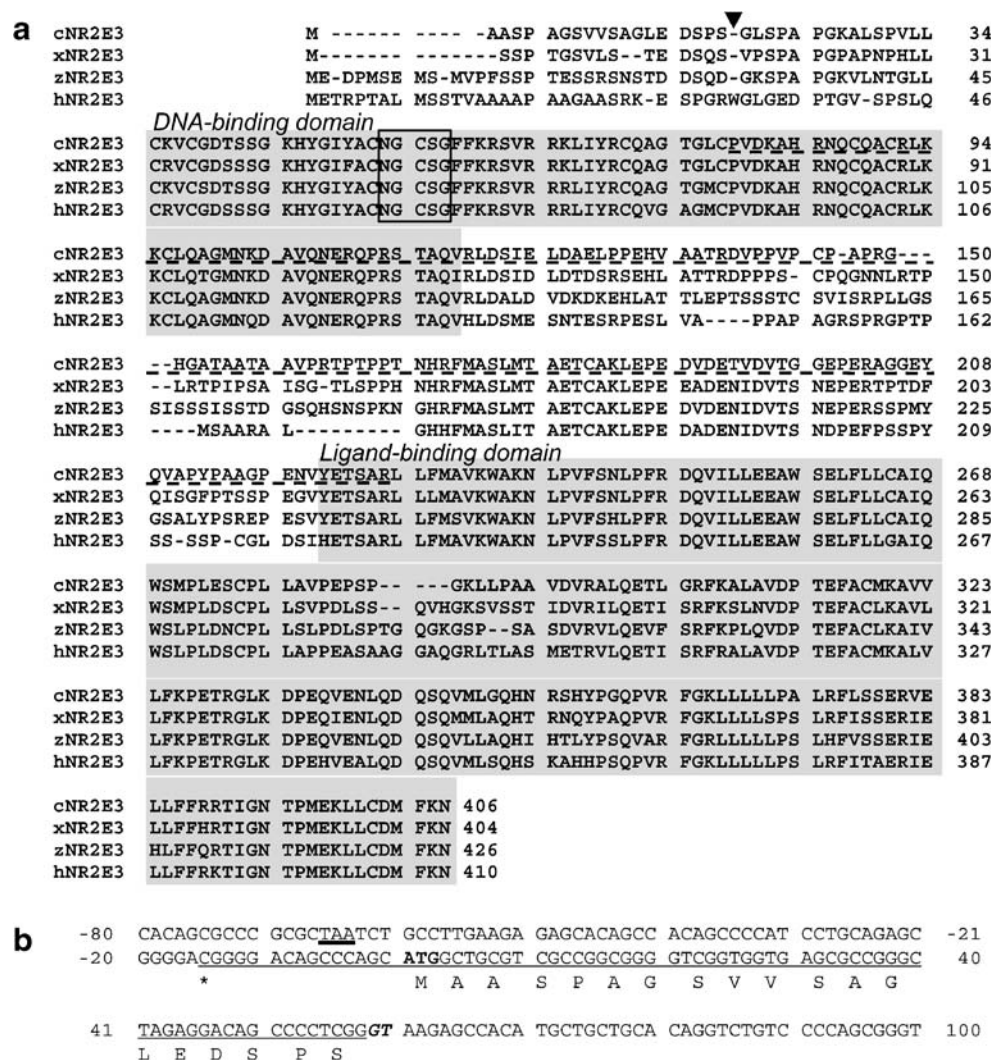
Results and discussion

Identification of the chicken *Nr2e3* gene

In vertebrates including humans and mice, the *Nr2e3* gene is known to be exclusively expressed in the neural retina (Kobayashi et al. 1999; Chen et al. 2005; Kitambi and

Hauptmann 2007; Martinez-De Luna and El-Hodiri 2007; McIlvain and Knox 2007). Therefore, to identify a chick *Nr2e3* cDNA, we constructed a phage cDNA library from E8 chick neural retina. A total of 0.3×10^6 independent clones were screened and six clones that showed a high homology to mammalian *Nr2e3* were identified. The deduced amino acid sequence of chick *Nr2e3* showed 70% homology to its mammalian counterparts, with even more similarity to the amphibian *Nr2e3* (Fig. 1a; Kobayashi et al. 1999; Chen et al. 2005; Martinez-De Luna and El-Hodiri 2007). Three other clones, which showed relatively high hybridization signals to the *Nr2e3* probe, were found to encode *Nr2e1* (*Tlx*) and *Nr2f* (*COUP-TF*) family genes, which are most closely related to *Nr2e3* among the nuclear receptor superfamily (Benoit et al. 2006). Translation of the *Nr2e3* protein is assumed to initiate from the first ATG codon found in the cloned mRNA because an in-frame stop codon (TAA) was found 80 bp upstream of the initiation codon in *Gallus gallus* whole genome shotgun sequence pqd72h09.b1 (accession number 140842551; Fig. 1b). In

Fig. 1 **a** Sequence alignment of chicken, *Xenopus*, zebrafish, and human *Nr2e3*. DNA- and ligand-binding domains are shaded. The position of the first intron and the P box are indicated by an arrowhead and a box. The dashed underline indicates the region used as a probe for northern and in situ hybridization analyses. **b** Primary structure of the 5' region of the chicken *Nr2e3* gene. The sequence found in cDNA clone(s) is underlined with the 5' end indicated by asterisk. The first ATG is in bold, with an in-frame upstream stop codon (bold-underlined). The splice donor sequence (GT) is shown in italics



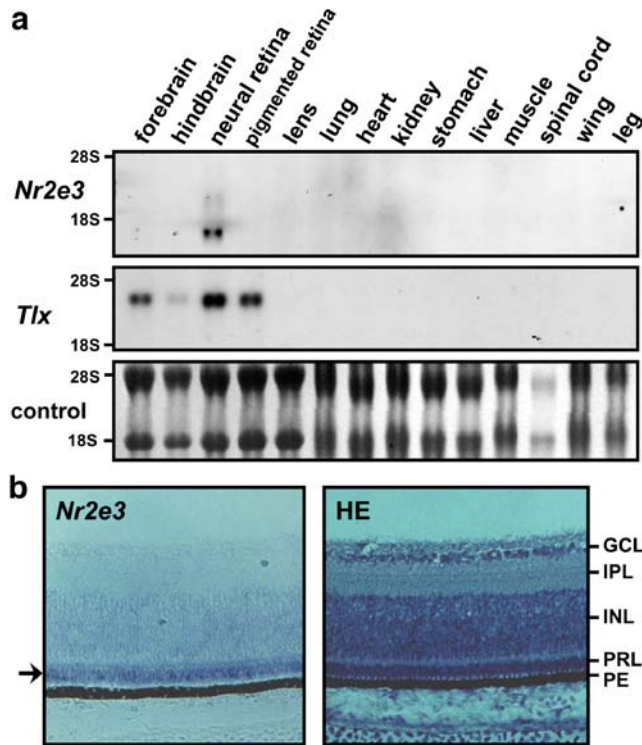


Fig. 2 Expression of *Nr2e3* mRNA in chicken. **a** Northern hybridization analyses of mRNA from E8 chick embryo tissues/organs using the chicken *Nr2e3* or *Tlx* cDNA as probes. All lanes contain 10 μg of total RNA. For *Nr2e3*, strong signal was detected around 1.7 kb and relatively weak signal of 2.6 kb. The 18S and 28S rRNA were used as a loading control. **b** In situ hybridization of E14 chick embryo retina sections with digoxigenin-labeled *Nr2e3* RNA probe. The arrow indicates *Nr2e3* signal in the photoreceptor layer. An adjacent section was stained with hematoxylin–eosin (HE) to help visualize different cell layers. GCL Ganglion cell layer, IPL inner plexiform layer, INL inner nuclear layer, PRL photoreceptor layer, PE pigment epithelium

addition, a putative splice donor sequence (gt) for the first intron is found at the same position where the first intron is located in the zebrafish *Nr2e3* gene (Chen et al. 2005).

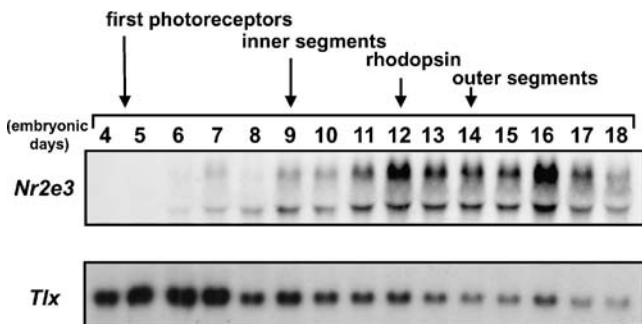


Fig. 3 Temporal expression of *Nr2e3* and *Tlx* mRNA in early retinal development. Northern hybridization analyses of mRNA from the neural retina of chick embryos at different stages of development using the chick *Nr2e3* or *Tlx* cDNA as a probe. All lanes contain 10 μg of total RNA. Approximate timing of events in eye development are indicated

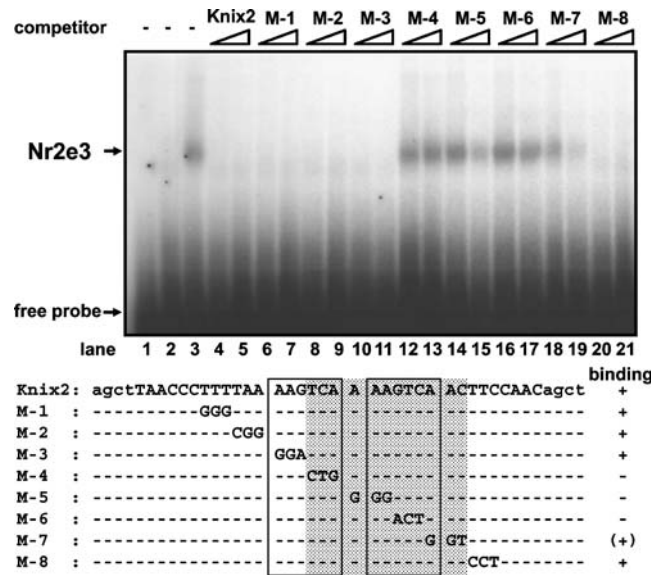


Fig. 4 In vitro DNA binding preference by *Nr2e3* protein. A gel mobility shift assay was performed. Lane 1 is probe only, and mock cell extract was added in lane 2. Lane 3 shows a specific protein–DNA complex formed when *Nr2e3* protein is added. Lanes 4–11 and 19–21 show that *Nr2e3* bound to labeled *Knix2* probe can be blocked by competition by addition of excess mutant cold probes shown below. Competitor probes are indicated above, with ten-fold excess added in lanes 4, 6, 8, 10, 12, 14, 16, 18, 20, and 50-fold excess in lanes 5, 7, 9, 11, 13, 15, 17, 19, 21. Shaded region indicates the nucleotide sequence shown to be important for recognition by *Nr2e3* protein. The tandem repeat of AAGTCA motifs in *Knix2* probe are boxed

Nr2e3 mRNA is expressed in photoreceptor cells of neural retina

Total RNA was extracted from different tissues of E8 chick embryos and the pattern of *Nr2e3* mRNA expression was analyzed. *Nr2e3* expression was observed only in the neural retina among the organs examined, while mRNA of the related *Tlx* (Yu et al. 2000) was also expressed in the brain and pigmented retina (Fig. 2a). Using RNA from E14 chick embryos, the restricted expression of *Nr2e3* in the neural retina was confirmed (data not shown). In situ hybridization of E14 chick retina sections revealed that *Nr2e3* expression is restricted to the photoreceptor layer of neural retina (Fig. 2b). These results confirm that the clone we identified

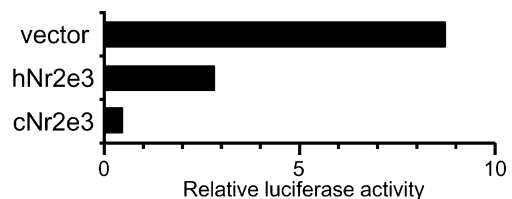


Fig. 5 *Nr2e3* protein represses transcription. Transient transfection assays in CV-1 cells show that the chick *Nr2e3* can repress the activity of a luciferase reporter gene to a greater extent than human *Nr2e3*

in chicken is indeed an ortholog of the mammalian *Nr2e3*. To elucidate the potential role of *Nr2e3* in retinal development, its mRNA expression was examined using RNA extracted from neural retina at different stages (Fig. 3). The expression of *Nr2e3* increased in the later stages of embryogenesis, while the expression of *Tlx* was the highest in the early stages (E5 to E7) and gradually declined (Fig. 3). Considering that *Nr2e3* is almost exclusively expressed in the photoreceptor layer, it is interesting that the expression of *Nr2e3* mRNA gradually increases as photoreceptor cells develop.

DNA-binding specificity of Nr2e3

The first amino acid in the Nr2e3 P box of its DNA-binding domain (Umesono and Evans 1989), which is critical for recognition of target DNA sequence, is an asparagine (N) as is the case for other Nr2e3 orthologs (Fig. 1a; Kobayashi et al. 1999; Chen et al. 2005). This is a unique feature among nuclear receptors and it has been shown that mammalian and zebrafish Nr2e3 bind to a tandem repeat of AAGTCA motifs separated by one nucleotide (Kobayashi et al. 1999; Chen et al. 2005). To examine the binding specificity of chick Nr2e3 protein, a gel mobility shift assay was performed using a probe to which mammalian Nr2e3 protein was shown to bind. Chick Nr2e3 showed the same binding preferences with its mammalian counterpart (Fig. 4). Competition experiments further revealed that nucleotides important for recognition by mammalian Nr2e3 were also conserved in chick Nr2e3 and necessary for its target DNA specificity (Fig. 4).

Function of Nr2e3

In earlier studies, mammalian Nr2e3 was shown to repress transcription of its target genes (Gerber et al. 2000; Chen et al. 2005). To test the transcriptional activity of the chick Nr2e3, a plasmid coding for the GAL4 DNA-binding domain fused to the Nr2e3 ligand-binding domain was constructed and cotransfected with a reporter plasmid controlled by a promoter containing GAL4-binding sites (Fig. 5). Along with human Nr2e3, we confirmed that the chick Nr2e3 acts as a transcriptional repressor (Fig. 5).

The role of the *Nr2e3* gene in vertebrates has been widely analyzed (Gerber et al. 2000; Chen et al. 2005; McIlvain and Knox 2007; Oh et al. 2008) and suggests a common function for *Nr2e3* in those species. Recently, a *Nr2e3* ortholog was reported in the genome of the gray, short-tailed opossum (*Monodelphis domestica*) published as the first metatherian ('marsupial') species to be sequenced (GenBank accession number XM_001371384). In addition, putative Nr2e3 orthologs have been found in *Caenorhabditis elegans* (Kobayashi et al. 1999; Wightman et al. 2005)

and sea urchin *Strongylocentrotus purpuratus* (GenBank accession number XP_780706). The *C. elegans* counterpart, FAX-1, also contains an asparagine in its P box and has shown to be important for axon pathfinding and specification of neuron identities (Wightman et al. 2005). Considering the restricted expression of Nr2e3 in the vertebrate neural retina, it is interesting to investigate the function of Nr2e3 in those species that do not have apparent organ comparable to a vertebrate eye. Potential molecules that may modulate Nr2e3 function have also been reported, adding a tool to investigate the function of Nr2e3 (Wolkenberg et al. 2006).

Regarding factors that might control Nr2e3 expression, a Maf protein Nrl has been suggested for its regulation (Oh et al. 2008). Although Nrl is not found in chicken, another Maf protein, L-Maf has been found to be expressed in rod cells in the later stages (Ochi et al. 2004). It is possible that L-Maf or other Maf protein(s) may regulate Nr2e3 expression in chicken.

In conclusion, we succeeded in identifying a chicken ortholog of the *Nr2e3* gene, whose mRNA expression is found only in the photoreceptor layer of the neural retina. During chick development, *Nr2e3* expression was high in the later stages (E12 to E17) while the expression of closely related *Tlx* gene was high in the early stages (E5 to E7). The binding specificity of the avian Nr2e3 protein shares the same preferences as its vertebrate orthologs and also functions as a transcriptional repressor. Our results suggest that Nr2e3 has a common function in retinal development that is shared between avians and mammals.

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