Characterization of a chicken retinoid X receptor- γ gene promoter and identification of sequences that direct expression in retinal cells

Clara AMEIXA and Paul M. BRICKELL¹

Molecular Haematology Unit, Institute of Child Health, 30 Guilford Street, London WC1N 1EH, U.K.

Development of the cellular complexity of the vertebrate neural retina relies on an intricate interplay between extracellular signals and intracellular factors. In particular, transcription factors play a key role in determining the competence of cells to respond to extracellular signals. We have previously shown that, in the developing chick neural retina, expression of the retinoid X receptor- γ (RXR- γ 2) nuclear receptor gene is restricted to photoreceptors. To characterize the mechanisms that regulate expression of this gene in the neural retina, we isolated a chicken RXR- γ genomic clone containing the RXR- γ 2 promoter and mapped the transcription initiation site by means of ribonuclease protection. We analysed promoter activity by transient transfection of luciferase reporter gene constructs into cultured cells isolated from embryonic-chick neural retina or facial mesenchyme, which does not normally express detectable RXR- γ 2 transcripts. The DNA fragment lying between nucleotides –657 and +37 with respect to the transcription initiation site had

basal promoter activity in both cell types. The fragment lying between nucleotides –1198 and –991 directed 10–20-fold higher levels of luciferase activity in neural retina cells, but only basal levels in facial mesenchyme cells. This 208 bp fragment also enhanced the activity of the simian-virus-40 promoter, when placed upstream in either orientation. Electrophoretic-mobility-shift assays using this 208 bp fragment demonstrated the formation of four neural retina-specific protein–DNA complexes. These results indicate that regulation of RXR- γ 2 transcription in the developing chick neural retina involves the binding of one or more neural retina-specific protein factors to an enhancer element located approx. 1 kbp upstream of the transcription initiation site.

Key words: electrophoretic-mobility-shift assay, gene expression, luciferase, retinoic acid, transcription.

INTRODUCTION

The vertebrate neural retina contains a limited number of different cell types that are generated in a well-characterized temporal order during embryonic development in order to create a laminated structure [1]. At the inner surface of the neural retina is the ganglion cell layer, followed by the inner nuclear layer containing ganglion cells, amacrine cells, horizontal cells and bipolar cells, and the outer nuclear layer containing photoreceptor cells [1]. A range of evidence shows that all of these retinal cell types are derived from a single uncommitted retinal precursor [2], and a number of extracellular signalling molecules have been shown to influence cell fate [3]. For example, there is evidence that, in rodents, thyroid hormone promotes cone photoreceptor fate [4], retinoic acid promotes rod photoreceptor fate [5], transforming growth factor- β 3 promotes amacrine cell fate [6] and Sonic Hedgehog (Shh) promotes all photoreceptor fates [7]. The competence of precursor cells to respond to particular extracellular signals is thought to be defined by their expression of a temporally changing complement of transcription factors [3,8]. For example, expression of the basic helix–loop–helix transcription factor Mash-1 promotes the generation of all late-appearing cell types [9,10], whereas expression of the homoeobox-containing Crx transcription factor promotes rod photoreceptor fate and inhibits generation of amacrine cells [11].

We have previously shown that the retinoid X receptor- γ (RXR- γ 2) transcription factor is expressed in the developing chick retina [12]. RXR- γ is a member of the RXR subfamily of steroid/thyroid hormone nuclear receptors and is involved in regulating the transcription of target genes in response to retinoic acid, thyroid hormone, vitamin D and peroxisome proliferators [13]. The chicken RXR- γ gene gives rise to two mRNA species encoding two protein isoforms (RXR- γ 1 and RXR- γ 2) that differ at their N-termini [14]. The structure of the chicken RXR- γ gene suggests that the RXR- γ 1 and RXR- γ 2 mRNAs arise from two different promoters, and expression data indicate that these promoters may have different levels of activity in different tissues [14]. RXR- γ 1 mRNA is found in embryonic and adult liver [14], whereas RXR- γ 2 is expressed in the developing neural retina and in migrating neural-crest cells and their progeny in peripheral nervous tissue [15,16]. In cranial neural-crest cells, RXR- γ 2 expression is maintained during differentiation into peripheral nervous tissue, but is switched off during differentiation into facial mesenchyme [15,16]. In the neural retina, RXR- γ 2 mRNA is restricted to photoreceptor cells and is one of the earliest known markers of photoreceptors [12]. The role of RXR- γ 2 in photoreceptor development is unclear, but it may be involved in regulating expression of genes important in photoreceptor function, such as visinin and opsins [12].

Since RXR- γ 2 is one of the earliest known transcription factors to be expressed in developing photoreceptor cells, it is

Abbreviations used: DTT, dithiothreitol; HBS, Hepes-buffered saline; RXR- γ , retinoid X receptor- γ ; SV40, simian virus 40.

¹ To whom correspondence should be addressed (e-mail p.brickell@ich.ucl.ac.uk).

The nucleotide sequence of the DNA fragment shown in Figure 1 has been deposited in the EMBL, GenBank®, DDBJ and GSDB Nucleotide Sequence Database under the accession number AJ239067.

important to characterize the mechanism of its highly restricted pattern of expression. To approach this, we isolated a chicken RXR- γ cosmid genomic clone containing the RXR- γ 2 promoter and analysed the promoter region by means of luciferase-reporter-gene assays in dissociated embryonic-chick cells and electrophoretic-mobility-shift assays using extracts of embryonic-chick tissues.

MATERIALS AND METHODS

Chicken embryos

Fertilized White Leghorn chicken eggs were obtained from Poyndon Farm, Waltham Cross, Herts., U.K., and incubated at $38 \pm 1^\circ\text{C}$. Embryos were assigned to stages as described by Hamburger and Hamilton [17].

Isolation of a chicken RXR- γ cosmid genomic clone

Cosmid clone clc3 was isolated from a chicken genomic library constructed in cosmid vector pWE15 (Stratagene, La Jolla, CA, U.S.A.) by hybridization screening [18] using the radiolabelled insert of chicken RXR- γ cDNA clone pR2.1 [15] as a probe.

DNA sequencing

Dideoxy sequencing was performed on denatured double-stranded DNA templates using Sequenase Version 2.0 (USB, Cleveland, OH, U.S.A.), according to the manufacturer's instructions. Oligonucleotide primers were custom-synthesized by Genosys Biotechnologies (Cambridge, U.K.). Nucleotide sequences were analysed using MacVector 6.0.1 software (Oxford Molecular Ltd., Oxford, U.K.).

Ribonuclease protection assays

Eyes were removed from 7-day-old chick-embryos and washed in sterile PBS. Total RNA was extracted using RNeasy B (Biogenesis Ltd., Poole, Dorset, U.K.) according to the manufacturer's instructions. ^{32}P -labelled RNA probes were prepared as described previously [15] using the template shown in Figure 1 below, and ribonuclease protection assays were performed using RNase ONE (Promega, Madison, WI, U.S.A.), as described previously [14].

Reporter plasmids

Luciferase reporter plasmid vectors pGL3-Basic and pGL3-Promoter were obtained from Promega (Southampton, U.K.). Plasmid vector pCH110, containing an *Escherichia coli lacZ* reporter gene was obtained from Clontech, Palo Alto, CA, U.S.A.

Cell culture

Chick-embryo tissues were dissected into sterile PBS, plated out in 35 mm-diameter tissue-culture dishes at 5×10^5 cells/ml in appropriate serum-containing medium, and cultured for 20–24 h at 37°C in a humidified incubator in an atmosphere containing 5–7% CO_2 , before transfection. Neural retina cells from stage-26 chick-embryos were cultured as described by Adler [19] in Dulbecco's Modified Eagle's medium supplemented with 10% (v/v) heat-inactivated foetal bovine serum and 100 units/ml of penicillin and streptomycin (Gibco BRL, Paisley, Renfrewshire, Scotland, U.K.). Facial mesenchyme cells from stage-26 chick-embryos were cultured as described by Wedden et al. [20], except that the medium used was Nutrient Mixture F-12 supplemented

with L-glutamine, 10% (v/v) heat-inactivated horse serum and 100 units/ml of penicillin and streptomycin.

Transient transfection and reporter gene assay

For each set of three 35 mm-diameter culture dishes of cells to be transfected, a mixture was prepared containing 5 μg of reporter plasmid DNA, 5 μg of pCH110 DNA as a control for transfection efficiency and 37 μl of 2 M CaCl_2 in a total volume of 300 μl . This was added dropwise to 300 μl of 2 $\times$ Hepes-buffered saline (HBS), comprising 280 mM NaCl, 10 mM KCl, 1.5 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 12 mM dextrose, 50 mM Hepes, pH 7.05. A 200 μl portion of this mixture was added dropwise to each 35 mm-diameter dish, giving three replicates for each transfection mixture. The cells were incubated for 6–8 h at 37°C in a humidified incubator in an atmosphere of 5–7% CO_2 , after which the medium was discarded and the cells washed with serum-free media, before adding fresh complete medium. Cells were cultured for a further 48 h, after which time the medium was discarded and the cells harvested for reporter gene assays.

Luciferase and β -galactosidase assays were performed using a β -galactosidase enzyme assay system and a luciferase assay system (Promega) according to the manufacturer's instructions. Values obtained for luciferase activity directed by test reporter plasmids were normalized for variation in transfection efficiency by dividing them by values obtained for β -galactosidase activity in the same culture dishes.

Electrophoretic-mobility-shift assay

Probes and competitors for use in electrophoretic-mobility-shift assays were made by PCR, using an upstream primer labelled at its 5' end with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and T4 polynucleotide kinase (Promega). Nuclear extracts were prepared using an adaptation of the method described by Schreiber et al. [21]. Briefly, chick-embryo tissue was dissected, washed in PBS and placed in approx. 2 vol. of lysis buffer, comprising 20 mM Hepes, pH 7.9, 140 mM NaCl, 1.5 mM MgCl_2 , 0.5% (v/v) Nonidet P40, 10 mM leupeptin (Sigma), 0.5 mg/ml aprotinin (Sigma), 1 mM dithiothreitol (DTT) and 0.05 mM PMSF. The tissue was cut into small pieces and the material vortex-mixed and placed on ice for 10 min. The mixture was centrifuged at 500 g for 5 min at 4°C , and the supernatant, containing cytoplasmic proteins, was discarded. The nuclear pellet was resuspended in approx. 2 vol. of ice-cold buffer C [20 mM Hepes (pH 7.9)/0.4 M NaCl/1 mM EDTA/1 mM DTT/1 mM PMSF] and rocked vigorously for 15 min at 4°C on a shaking platform. The mixture was then microcentrifuged for 5 min at 4°C , and aliquots of the supernatant were snap-frozen and stored at -70°C . The protein concentration was determined using a Bradford assay kit (Bio-Rad, Hemel Hempstead, Herts., U.K.).

Nuclear protein extract (1–5 μg) was incubated for 25 min on ice with approx. 8×10^3 c.p.m. of radiolabelled DNA probe in 30 μl of a buffer comprising 90 mM KCl, 10 mM Tris/HCl, pH 7.5, 1 mM DTT, 5% (v/v) glycerol and 2–10 mg of poly[dI-dC] (Sigma), and samples were analysed by electrophoresis in a neutral polyacrylamide gel.

RESULTS AND DISCUSSION

Analysis of the 5' flanking region of the chicken RXR- γ gene

To analyse the chicken RXR- γ 2 promoter, we first isolated a chicken genomic clone (clc3) by hybridization screening of a cosmid genomic library, using the radiolabelled insert of chicken RXR- γ cDNA clone pR2.1 [15] as a probe. The nucleotide

Figure 1 The 5' flanking region of the chicken RXR- γ gene

Restriction map of a 3666 bp *SmaI*–*SacI* fragment containing the 5' end of chicken RXR- γ 2 cDNA clone pR2.1. B, *Bst*1107I; E, *EcoRI*; E*, *EcoRI**; N, *NsiI*; S, *SacI*; Sm, *SmaI*; **, transcription initiation site; pRS1, fragment used as a template for synthesis of RNA probe for ribonuclease protection experiment.

sequence of a 3666 bp *SmaI*–*SacI* fragment containing the 5' end of pR2.1 was determined (EMBL Nucleotide Sequence Database accession number AJ239067) and computer analysis of this sequence resulted in the restriction map shown in Figure 1.

To identify a transcription initiation site, we performed ribonuclease protection using plasmid pRS1 (Figure 1) as a template for probe synthesis. As shown in Figure 2, left-hand panel, hybridization of the probe to total RNA isolated from 7-day-old chick-embryo eyes protected a probe fragment of 37 nt. No such fragment was protected by hybridization to total yeast RNA (Figure 2, left-hand panel). These data indicate that the chicken RXR- γ 2 promoter has a single transcription initiation site at the position shown in Figure 1 and Figure 2 (right-hand panel).

Inspection of the nucleotide sequence (Figure 2, right-hand panel) showed that there was no TATA box or TATA-like sequence in the vicinity of the transcription initiation site, although the region did contain other features characteristic of eukaryotic promoters, such as high overall GC content and high CpG dinucleotide content. A CCAAT box was located at –60 to –56, a typical location for CCAAT boxes in TATA-less promoters [22], but the region around the transcription initiation site did not conform to the Inr (initiator) consensus found in some TATA-less promoters [23].

RXR- γ 2 promoter activity in cultured chick-embryo cells

To identify *cis*-acting DNA sequences that regulate RXR- γ 2 transcription in developing neural retina cells, we made a series of luciferase reporter constructs in plasmid vector pGL3-Basic.

As shown in Figure 3, top panel, these contained progressively restricted sequences upstream of the RXR- γ 2 transcription initiation site. The plasmids were transfected into cultured cells isolated from chick-embryo neural retina, along with the *E. coli lac Z* reporter plasmid pCH110, as a control for transfection efficiency. The plasmids were also transfected into cultured cells isolated from chick-embryo facial mesenchyme, which do not normally contain detectable RXR- γ 2 transcripts [16]. Transfection of chick-embryo neural retina and facial mesenchyme cells has not been described previously, but was readily achievable using a standard calcium phosphate co-precipitation protocol (Figure 3, middle panel).

As shown in Figure 3, middle panel, the fragment lying between nucleotides –657 and +37, with respect to the RXR- γ 2 transcription initiation site, had basal promoter activity of approximately equivalent strength in both neural retina and facial mesenchyme cells. The fragment lying between nucleotides –1198 and +37, with respect to the RXR- γ 2 transcription initiation site, directed 10–20-fold higher levels of luciferase activity than this in neural retina cells, but not in facial mesenchyme cells. This indicates the presence of neural-retina-specific regulatory sequences in the 208 bp fragment lying between nucleotides –1198 and –991. The nucleotide sequence of this fragment is shown in Figure 3, bottom panel.

Transcriptional activity of the 208 bp DNA fragment lying between –1198 and –991

To examine the possibility that the 208 bp fragment lying between –1198 and –991 had its own neural-retina-specific promoter activity, it was cloned into pGL3-Basic, upstream of the luciferase reporter gene, yielding plasmid p γ S (Figure 4, bottom panel). When transfected into cells from neural retina or facial mesenchyme, p γ S did not direct any luciferase expression above background (Figure 4, top panel), indicating that the 208 bp fragment does not possess detectable promoter activity.

To determine whether the same 208 bp fragment was able to enhance transcription from a heterologous promoter, it was placed in plasmid vector pGL3-Promoter, in either orientation, upstream of the simian-virus-40 (SV40) promoter (Figure 5, left-hand panel). As shown in Figure 5, right-hand panel, the 208 bp fragment caused a small but significant ($P < 0.01$) increase in SV40 promoter activity in neural retina cells when present in either orientation. In contrast, the same fragment caused a small decrease in SV40 promoter activity in facial mesenchyme cells (Figure 5, right-hand panel). This suggests that the 208 bp

Figure 2 Identification of a transcriptional start site by ribonuclease protection

Left panel: a 32 P-labelled RNA probe synthesized using plasmid pRS1 (Figure 1) as a template was hybridized with 18 μ g of total RNA isolated from 7-day-chick-embryo eye (lane 1) or from yeast (lane 2). The size of the protected fragment is indicated in nt. M, size markers. Right panel: nucleotide sequence of the region lying between nucleotides –90 and +30 with respect to the RXR- γ 2 transcription initiation site (indicated with an asterisk). The transcribed region is shown in **bold** type. The CCAAT box at –60 to –56 is underlined.

-1198
TACAAGGACT GGAGCCTCTC CCTAACACAA ACCCAGTGT TCCCCAAAAC GAGTTGCCTG

-1138
ATGCTTGCTT ATCTTGAGGG GGGGGGTTCT TTTGGAGGTG CTCGGTGTGC ATGTGTTGTG

-1078
CAGCACTAAG CACTCGCTAA CCCAGATCTG CTAAAAATCA ATAAGGTAAT CCACTTACAG

-1018 -991
CCTGGGACTG TGGCCTTTTCG AAGGAGCT

Figure 3 Reporter analysis of the RXR- γ 2 gene promoter

The reporter plasmids shown in the top panel were transfected into cultured cells isolated from chick-embryo neural retina or facial mesenchyme, along with control plasmid pCH110 (middle panel). Values for luciferase activity (mean $\pm$ S.D. of three independent determinations) were normalized for minor variations in transfection efficiency (see the Materials and methods section) and are expressed relative to the luciferase activity directed by the pGL3-Basic vector without any insert. Bottom panel: nucleotide sequence of the 208 bp fragment lying between nucleotides -1198 and -991 with respect to the RXR- γ 2 transcription initiation site.

Figure 4 Test for promoter activity within the 208 bp fragment lying between nucleotides -1198 and -991

Plasmids p γ S (bottom panel), p γ 991 (Figure 3, top panel), p γ 1198 (Figure 3, top panel) or pGL3-Basic were transfected into cultured cells isolated from chick-embryo neural retina or facial mesenchyme, along with control plasmid pCH110. Values for luciferase activity (mean $\pm$ S.D. of three independent determinations) shown in the top panel were normalized for minor variations in transfection efficiency (see the Materials and methods section) and are expressed relative to the luciferase activity directed by the pGL3-Basic vector without any insert.

fragment has enhancer activity in neural retina cells and, possibly, suppressor activity in facial mesenchyme cells.

Protein factors binding to DNA sequences in the 208 bp fragment lying between -1198 and -991

To determine whether any retina-specific nuclear proteins could bind to the 208 bp fragment, we performed electrophoretic-mobility-shift assays in which the radiolabelled DNA fragment was incubated with nuclear protein extracts of neural retina or facial mesenchyme. At least six protein-DNA complexes were formed with both neural retina and facial mesenchyme nuclear protein extracts (Figure 6, complexes a-e and j). Four additional protein-DNA complexes were formed with neural retina nuclear protein extracts, but not with facial mesenchyme nuclear protein extracts (Figure 6, complexes f-i). Of these, complexes f and g were particularly abundant. Complexes h and i were

Figure 6 Binding of nuclear proteins to the 208 bp fragment

The 208 bp fragment lying between nucleotides -1198 and -991 was end-labelled and incubated with 5 μ g (tracks 1 and 3) or 1 μ g (tracks 2 and 4) of nuclear extract from neural retina (tracks 1 and 2) or facial mesenchyme (tracks 3 and 4). The probe, and complexes a-j, referred to in the text, are indicated with arrows.

not visible in all experiments. The formation of all complexes between radiolabelled probe and nuclear proteins was inhibited by incubation with excess unlabelled probe (Figure 7, lanes 1-3). The formation of complexes f and g was partially inhibited by incubation with an excess of an unlabelled DNA fragment corresponding to nucleotides -1107 to -991 (Figure 7, lanes 1, 4 and 5). Formation of all complexes except f and g was inhibited by incubation with an excess of an unlabelled DNA fragment corresponding to nucleotides -1198 to -1083 (Figure 7, tracks 1 and 6). These data suggest that the neural-retina-specific enhancer activity of the 208 bp fragment lying between nucleotides -1198 and -991 may be mediated by the binding to this region of one or more neural-retina-specific nuclear protein factors, giving rise to complexes f, g, h and i. They further suggest that formation of complexes f and g involves sequences lying between nucleotides -1107 and -991. Inspection of the nucleotide sequence of the 208 bp fragment for the consensus sequences of known transcription-factor-binding sites did not suggest any

Figure 5 Test for enhancer activity within the 208 bp fragment lying between nucleotides -1198 and -991

Plasmids pFor208 (left-hand panel), pRev208 (left-hand panel) or pGL3-Promoter were transfected into cultured cells isolated from chick-embryo neural retina or facial mesenchyme, along with control plasmid pCH110. Values for luciferase activity (mean $\pm$ S.D. of three independent determinations) shown in the right-hand panel were normalized for minor variations in transfection efficiency (see the Materials and methods section) and are expressed relative to the luciferase activity directed by pGL3-Promoter.

Figure 7 Competition for binding of nuclear proteins to the 208 bp fragment

The 208 bp fragment lying between nucleotides —1198 and —991 was end-labelled and incubated with 5 µg of nuclear extract from neural retina alone (lane 1), in the presence of unlabelled 208 bp fragment at 50-fold (lane 2) or 100-fold (lane 3) molar excess over probe, in the presence of unlabelled DNA fragment I at 50-fold (lane 4) or 100-fold (lane 5) molar excess over probe or in the presence of unlabelled DNA fragment II at 100-fold (lane 6) molar excess over probe. DNA fragment I corresponds to nucleotides —1107 to —991 (Figure 3, bottom panel). DNA fragment II corresponds to nucleotides —1198 to —1083 (Figure 3, bottom panel). The probe and complexes a–g and i, referred to in the text, are indicated with arrows. Low-abundance complexes h and j seen in Figure 6 are not visible in this experiment.

likely candidates for such factors. Identification of these will therefore require their purification and characterization.

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