

Gene expression pattern

OTX2 homeodomain protein binds a DNA element necessary for interphotoreceptor retinoid binding protein gene expression

Nicoletta Bobola^{a,*}, Paola Briata^b, Cristina Ilengo^b, Nadia Rosatto^d, Cheryl Craft^c,
Giorgio Corte^{a,b}, Roberto Ravazzolo^{a,d}

^aDepartment of Oncology, Biology and Genetics, University of Genova, Genoa, Italy

^bLaboratorio di Immunobiologia, I.S.T., Centro di Biotecnologie Avanzate, Largo R. Benzi 10, 16132 Genoa, Italy

^cMary D. Allen Laboratory for Vision Research, Doheny Eye Institute, Department of Cell and Neurobiology,
University of Southern California, School of Medicine, Los Angeles, CA 90033, USA

^dLaboratory of Molecular Genetics, G. Gaslini Institute, Largo G. Gaslini 5, 16148 Genoa, Italy

Received 20 July 1998; accepted 1 September 1998

Abstract

Transcription of the human interphotoreceptor retinoid binding protein (IRBP) gene is strictly tissue specific, being restricted to retinal photoreceptors and pinealocytes. We have previously demonstrated that a sequence named A element, in the IRBP promoter is essential for IRBP gene transcription *in vivo*. Here we demonstrate that the human homeodomain protein OTX2 is present in nuclear extracts of IRBP expressing cells and specifically interacts with the IRBP A promoter element *in vitro*. OTX2, as well as CRX, a homeodomain protein very similar to OTX2, activates the human IRBP promoter in co-transfection experiments. © 1998 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Gene; Promoter; Transcriptional regulation; HeLa; WERI-Rb1; Retinoblastoma; Gel retardation; Supershift; Immunohistochemistry; Transfection; Human; Vertebrate; *Drosophila*; Interphotoreceptor retinoid binding protein; IRBP; OTX2; CRX; Homeodomain; Bicoid; Eye; Retina; Photoreceptors; Development

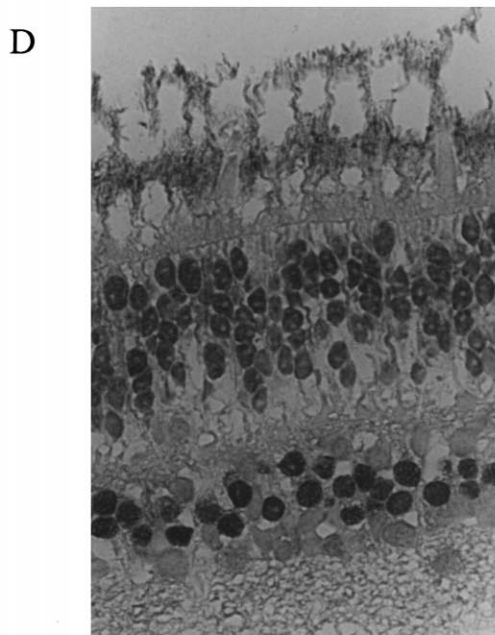
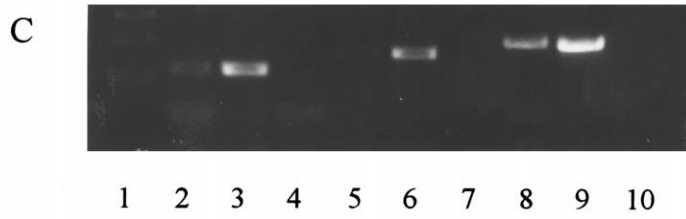
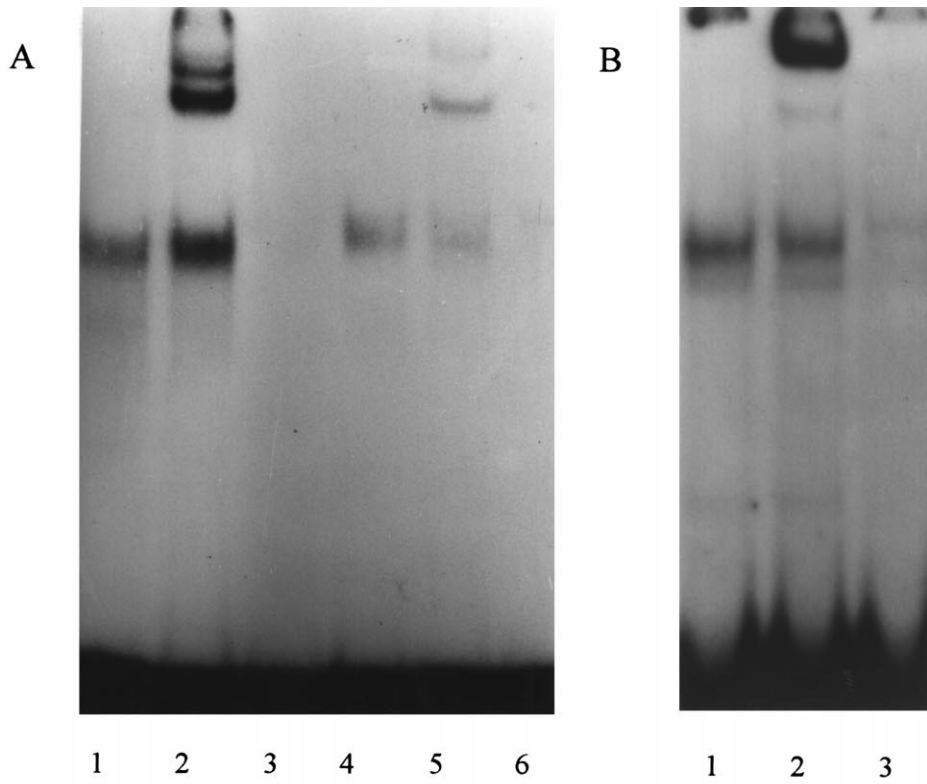
1. Introduction

Transcription of the human interphotoreceptor retinoid binding protein (IRBP) gene is strictly tissue specific, being restricted to retinal photoreceptors and pinealocytes (Bridges et al., 1986; Van Veen et al., 1986). We previously demonstrated that a sequence in the IRBP promoter, named A element and identical to the Bicoid binding site, is essential for IRBP transcription *in vivo* (Bobola et al., 1995). The homeodomain protein OTX2, homolog of the *Drosophila* orthodenticle (Finkelstein et al., 1990), is widely expressed during vertebrate embryo development (Simeone et al., 1992, 1993; Bally-Cuif et al., 1995). In particular, it is expressed in the developing neural retina and in differentiated retinal cell types, most notably in photoreceptor cells (Bovolenta et al., 1997) and its mRNA is present in the

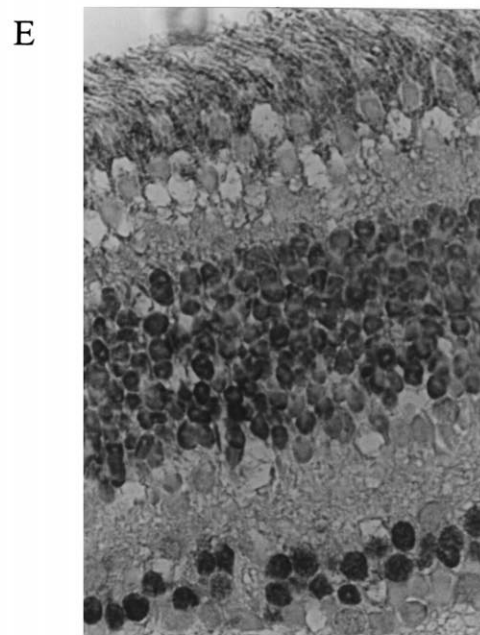
pineal recess of the mouse embryo (A. Simeone, personal communication). The fundamental role of OTX2 in eye formation is underscored by the microphthalmia or anophthalmia observed in nearly half of *Otx2*^{+/-} mice (Matsuo et al., 1995). Recombinant OTX2 binds with high affinity to the Bicoid target sequence (Gherzi et al., 1997). Here we demonstrate that OTX2 is present in nuclear extracts of IRBP expressing cells, specifically interacts with the IRBP A promoter element *in vitro* and, as well as CRX, a related homeoprotein, activates the human IRBP promoter in co-transfection experiments.

Nuclear extracts of Sf9 cells producing OTX2, WERI-Rb1 retinoblastoma cells (which express the IRBP gene) and bovine retina were incubated with a labeled oligonucleotide containing the A element in gel retardation experiments. All nuclear extracts formed a retarded complex with the same electrophoretic mobility which was abolished by the monoclonal antibody α OTX2 (Fig. 1A,B).

* Corresponding author.



polyclonal antiserum



adsorbed
polyclonal antiserum

Recombinant OTX2 is then capable of forming a complex with the A sequence similar to the one formed by the factor present in nuclear extracts of cells which express IRBP. However, the identification of this factor as OTX2 cannot be conclusive, because a recently discovered homolog of OTX2 (86% homology in the homeodomain), CRX, has been shown to bind the A sequence and to have an expression pattern superimposable to that of IRBP (Chen et al., 1997; Freund et al., 1997; Furukawa et al., 1997). It was then possible that the α OTX2 could recognize CRX, in addition to or instead of OTX2, in the complex observed in gel retardation.

The antibody indeed reacted with HeLa cells transfected with an expression vector containing Crx cDNA, probably through an epitope of the conserved homeodomain. To remove reactivity against the homeodomain, a rabbit polyclonal antiserum adsorbed with the highly related recombinant OTX1 protein (pa- α OTX2) was prepared and found negative for staining of Crx transfected cells. In the gel retardation assays, pa- α OTX2 modified the complex formed between the A sequence and all three nuclear extracts by forming a supershifted band (Fig. 1A,B).

Although OTX2 expression during eye development had been extensively investigated (Matsuo et al., 1995; Bovolenta et al., 1997), no data were available about its expression in the adult retina. PCR experiments demonstrated the presence of both Otx2 and Crx cDNAs in libraries obtained from human adult retina and Y-79 retinoblastoma cell line (Fig. 1C), where IRBP is also expressed. The expression of OTX2 in adult retinal cells was further investigated in sections of adult retina. The pa- α OTX2 stained, though less strongly than the polyclonal antiserum that reacts also with CRX, nuclei of photoreceptor cells and nuclei of cells in the inner nuclear layer, which, according to previous observation in the chicken retina (Bovolenta et al., 1997), might be horizontal cells (Fig. 1D,E).

The first 123 bp of the IRBP promoter are very specific in regulating the transcription of the gene since, when fused to a *lacZ* reporter gene, they restrict expression of the transgene to the retina and pineal gland. The same promoter fragment, fused to the luciferase reporter gene (120 IRBP/LUC) shows thousand fold higher luciferase activity when transfected in cells which express the IRBP gene (WERI-Rb1) compared to cells which do not express IRBP (HeLa, Fig. 2A), suggesting that the strictly retina-specific IRBP promoter is activated by a tissue-specific factor(s) present in this cell type. We tested if expression of OTX2 in HeLa cells (unable to express IRBP) would lead to an activation of the 120 IRBP/LUC construct, by measuring luciferase activ-

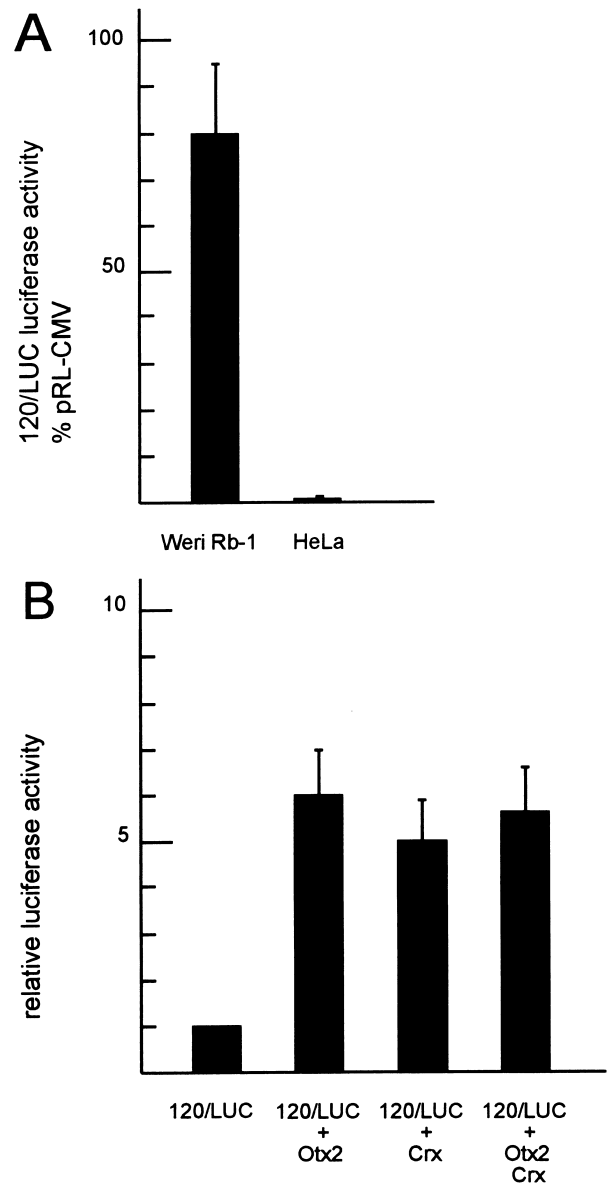


Fig. 2. 123 bp IRBP promoter activity in transiently transfected cells. (A) *Renilla* luciferase activity of 120 IRBP/LUC in HeLa and WERI-Rb1 cell lines, expressed as percent of pRL-CMV measured in the same cell lines (mean \pm SEM). (B) Co-transfections in HeLa cells of 120 IRBP/LUC with pCMV-OTX2, pCMV-CRX or both, expressed as fold stimulation (mean \pm SEM) of luciferase activity relative to the values obtained co-transfecting an empty CMV vector.

ity in co-transfection experiments (Fig. 2B). Expression of OTX2 from the CMV promoter increased luciferase activity by five to seven times and a comparable level of activation was observed in the presence of CRX (four to five times, in accordance with Chen et al., 1997). Co-expression of both

Fig. 1. OTX2 binds a sequence necessary for IRBP gene transcription and is present in cells that express IRBP. (A) Nuclear extracts from Sf9-OTX2 (1–3) and WERI-Rb1 cells (4–6) were mixed with a labeled double-stranded oligonucleotide containing the A element in the absence (1,4) or in the presence of the polyclonal pa- α OTX2 (2,5) or the monoclonal α OTX2 (3,6). (B) Nuclear extract from bovine retina (1–3), in the absence (1), or in the presence of pa- α OTX2 (2), or α OTX2 (3). (C) PCR on cDNA libraries from adult human retina (2,5,8) and Y-79 retinoblastoma cells (3,6,9) using Otx2 (2,3), Crx (5,6) or G3PD (8,9) specific primers. Molecular weight marker (1) and negative controls (4,7,10) are shown. (D,E) Immunohistochemical staining of sections from human retina with non-adsorbed polyclonal anti-OTX2 and adsorbed pa- α OX2.

CRX and OTX2 in the same cells did not increase the IRBP promoter activity observed in the presence of either OTX2 or CRX, demonstrating that the two proteins do not act synergistically on the IRBP promoter.

So far very few genes are known to be regulated by otd/OTX proteins (Gherzi et al., 1997). The IRBP gene offers a good model to study how transcriptional activation can be achieved in vivo in the presence of these proteins and may help to understand the relationship between CRX and OTX2 in the retina. Furthermore, the mechanism of activation of a retina-specific gene such as IRBP could shed light on the steps of gene expression which result in the definition of the mature photoreceptor cell phenotype.

2. Methods

2.1. Plasmid constructions and PCR

120 IRBP/LUC contains the human IRBP promoter fragment from –123 to +18 into the *Hind*III site of the pRL null plasmid (Promega). The expression construct pCMV-OTX2 is described elsewhere (Gherzi et al., 1997). pCMV-CRX contains the bovine Crx cDNA cloned in the *Eco*RI site of pCDNA3 expression vector (Invitrogen, San Diego, CA). pRL-CMV (Promega) contains the *Renilla* luciferase driven by the CMV immediate-early enhancer promoter region.

Human adult retina and Y-79 cDNA libraries were used for PCR amplification with *Otx2* specific primers (forward 5'-ACCTCAGTCCCAACCATGCC-3', reverse 5'-GATTGAGATGGCTGGTAACAGC-3'), Crx specific primers (forward 5'-AAGGCGGCACGTCCCAAG-3', reverse 5'-GCCTGATAGGGAGGTGG-3') and G3PD primers (forward 5'-CATGTGGCCATGAGTCC-3', reverse 5'-CAACTTTGGTATCGTGGAAGGA-3') as a control.

2.2. Cells and DNA transfections

Cells, cultured in standard conditions, were transfected with 1.7 μ g of pRL plasmids (either 120 IRBP/LUC or pRL-CMV) using Superfect (Qiagen), according to the manufacturer's protocol. For co-transfection 0.8 μ g of either pCMV-OTX2 or pRC CMV (Invitrogen, San Diego, CA) or pCMV-CRX expression vectors were added. Total cell extracts were assayed for luciferase activity, using the Dual Luciferase Kit (Promega). The data obtained represent the average \pm SEM of at least three independent experiments, each performed in duplicate.

Sf9 *Spodoptera frugiperda* cells expressing OTX2 (Sf9-OTX2) were obtained and cultured as described (Mallamaci et al., 1996; Gherzi et al., 1997).

2.3. Antibody, immunofluorescence and immunohistochemistry

Two different antibodies to human OTX2 were used: a

mouse monoclonal antibody (α OTX2, Gherzi et al., 1997) and a rabbit polyclonal antiserum (Mallamaci et al., 1996). Antibodies directed against the homeodomain were removed from the antiserum by adsorption with purified OTX1 recombinant protein, also recognized by the monoclonal α OTX2, and immobilized on CNBr-activated Sepharose 4B (Pharmacia).

Surgical specimens, removed from a patient affected by retinoblastoma, were fixed in 4% paraformaldehyde, embedded and cut as previously described (Mallamaci et al., 1996). The sections were then deparaffinized, rehydrated and boiled in a microwave four times for 4 min in 10 mM citric acid (pH 6). The immunohistochemistry was performed as described (Mallamaci et al., 1996) according to the streptavidin–biotin–horseradish peroxidase complex technique. The preadsorbed pa- α OTX2 antibody was diluted 1:300 in phosphate-buffered saline.

2.4. Preparation of nuclear extracts and gel retardation assay

Nuclear extracts were prepared as previously described for WERI-Rb1 cells (Dignam et al., 1983) and for Sf9-OTX2 cells (Gherzi et al., 1997). Bovine eyes were obtained from a local slaughter house, retinas were isolated and nuclear extracts prepared as described (Deryckere and Gannon, 1994).

Gel retardation experiments were performed as described (Bobola et al., 1995). Antibodies were added at a 1:100 final dilution and incubated for an additional 10 min with the binding mixture. The sequence of the double stranded A oligonucleotide is 5'-AGACAGGATTAAAGGCTTACTG-3' (upper strand).

Acknowledgements

We wish to thank Roberto Gherzi and Marco Musso for critical reading of the manuscript, Edoardo Boncinelli for helpful suggestions, Xue Mei Zhu, Francesca Giacomelli and Binoy Appukuttan for their invaluable support and Gianni Bruzzone for photographs. We thank Dr. Sandro Banfi, TIGEM, Milan, for kindly providing the human retina cDNA library. This work was supported by AIRC to R. R. and G.C., by the University of Genova local funds to R.R. and G.C. and by C.N.R.-Progetto Finalizzato Biotecnologie no. 97.01195.PF49 to R.R.

References

- Bally-Cuif, L., Gulisano, M., Broccoli, V., Boncinelli, E., 1995. *c-otx2* is expressed in two different phases of gastrulation and is sensitive to retinoic acid treatment in chick embryo. *Mech. Dev.* 49, 49–63.
- Bobola, N., Hirsch, E., Albini, A., Altruda, F., Noonan, D., Ravazzolo, R.,

1995. A single cis-acting element in a short promoter segment of the gene encoding the interphotoreceptor retinoid-binding protein confers tissue-specific expression. *J. Biol. Chem.* 270, 1289–1294.
- Bovolenta, P., Mallamaci, A., Briata, P., Corte, G., Boncinelli, E., 1997. Implication of Otx2 in pigment epithelium determination and neural retina differentiation. *J. Neurosci.* 17, 4243–4252.
- Bridges, C.D.B., Landers, R.A., Fong, S.L., Liou, G.I., 1986. *Pineal and Retinal Relationship*. Academic Press, New York, pp. 383–400.
- Chen, S., Wang, Q.L., Nie, Z., Sun, H., Lennon, G., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., Zack, D.J., 1997. Crx, a novel Otx-like paired-homeodomain protein, binds to and transactivates photoreceptor cell-specific genes. *Neuron* 19, 1017–1030.
- Deryckere, F., Gannon, F., 1994. A one-hour miniprep technique for extraction of DNA-binding proteins from animal tissues. *BioTechnology* 16, 405.
- Dignam, J.D., Lebovitz, R.M., Roeder, R.G., 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* 11, 1475–1489.
- Finkelstein, R., Smouse, D., Capaci, T.M., Spradling, A.C., Perrion, N., 1990. The *orthodenticle* gene encodes a novel homeo domain protein involved in the development of the *Drosophila* nervous system and ocellar visual structures. *Genes Dev.* 4, 1516–1527.
- Freund, C.L., Gregory-Evans, C.Y., Furukawa, T., Papaioannou, M., Looser, J., Ploder, L., Bellingham, J., Ng, D., Herbrick, J.-A.S., Duncan, A., Scherer, S.W., Tsui, L.-C., Loutradis-Anagnostou, A., Jacobson, S.J., Cepko, C.L., Bhattacharya, S.S., McInnes, R., 1997. Cone-rod dystrophy due to mutations in a novel photoreceptor-specific homeobox gene (CRX) essential for the maintenance of the photoreceptor. *Cell* 91, 543–553.
- Furukawa, T., Morrow, E.M., Cepko, C., 1997. Crx, a novel otx-like homeobox gene, shows photoreceptor-specific expression and regulates photoreceptor differentiation. *Cell* 91, 531–541.
- Gherzi, R., Briata, P., Boncinelli, E., Ponassi, M., Querzé, G., Viti, F., Corte, G., Zardi, L., 1997. The human homeodomain Otx2 binds to the human tenascin-C promoter and trans-represses its activity in transfected cells. *DNA Cell Biol.* 16, 559–567.
- Mallamaci, A., Di Blas, E., Briata, P., Boncinelli, E., Corte, G., 1996. OTX2 homeoprotein in the developing central nervous system and migratory cells of the olfactory area. *Mech. Dev.* 58, 165–178.
- Matsuo, I., Kuratani, I., Kimura, C., Takeda, N., Aizana, S., 1995. Mouse *Otx2* functions in the formation and patterning of rostral head. *Genes Dev.* 9, 2646–2658.
- Simeone, A., Acampora, D., Gulisano, M., Stornaiuolo, A., Boncinelli, E., 1992. Nested expression domains of four homeobox in developing rostral brain. *Nature* 358, 687–690.
- Simeone, A., Acampora, D., Mallamaci, A., Stornaiuolo, A., D'Apice, M.R., Nigro, V., Boncinelli, E., 1993. A vertebrate gene related to *orthodenticle* contains a homeo-domain of the bicoid classes and demarcates anterior neuroectoderm in the gastrulating mouse embryo. *EMBO J.* 12, 2735–2747.
- Van Veen, T., Katial, A., Shinohara, T., Barret, D.J., Wiggert, B., Chader, G.J., Nickerson, J.M., 1986. Retinal photoreceptor neurons and pinealocytes accumulate mRNA for interphotoreceptor retinoid-binding protein (IRBP). *FEBS Lett.* 208, 133–137.