Evidence of a tissue-restricting DNA regulatory element in the mouse IRBP promoter

J.H. Boatright[a,*], B.E. Knox[b], K.M. Jones[b], E. Stodulkova[a], H.T. Nguyen[a], S.A. Padove[a], D.E. Borst[c], J.M. Nickerson[a]

[a] *Emory Eye Center, Atlanta, GA, USA*
[b] *SUNY Health Science Center, Syracuse, NY, USA*
[c] *Uniformed Service University of Health Science, Bethesda, MD, USA*

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Abstract The expression of interphotoreceptor retinoid binding protein (IRBP) is limited to photoreceptor cells of the retina and pinealocytes of the pineal gland. We sought to define *cis*-elements of the mouse IRBP 5′ flanking region that are required for this restricted activity. In vitro transient transfections of retinoblastoma and neuroblastoma cells and in vivo experiments with transgenic *Xenopus laevis* indicate that −1783/+101 and −156/+101 IRBP gene fragments directed expression predominantly to the retina and pineal, with minor neuronal expression elsewhere. In contrast, a −70/+101 fragment was less restrictive in controlling expression, exhibiting activity not only in retina, but also in forebrain, hindbrain, spinal cord, and motor neurons innervating gills.

Key words: Interphotoreceptor retinoid binding protein; Transgenic *Xenopus laevis*; Transcription regulation; Promoter; Repressive element; Neuroblastoma; Retinoblastoma

1. Introduction

The expression of interphotoreceptor retinoid binding protein (IRBP), a large glycolipoprotein found abundantly in the interphotoreceptor matrix of many vertebrates [1–4], is limited to photoreceptor cells of the retina and pinealocytes of the pineal gland [5–7]. In exploring this tissue specificity, the *cis* regulatory fragment of the IRBP 5′ flanking region has been progressively defined through the work of several laboratories. A −1783 to +101 fragment (relative to transcription start) of the mouse IRBP gene drives reporter activity in transiently transfected embryonic chick retina cells, but not in brain cells or fibroblasts [8]. Liou et al. [5] found that a 1.3 kb human IRBP 5′ flanking region drove reporter gene activity in transgenic mice and that a promoter fragment spanning −156 to +19 conferred tissue specificity [9]. Bobola et al. [10] and Fei et al. [11] similarly found that −123 to +18 of the human IRBP gene drove reporter activity in transgenic mice, allowing expression exclusively in the retina and pineal gland [10]. They further found via DNase I footprinting that a −58 to −45 fragment, which is conserved across several species, bound protein complexes from retinoblastoma cells but not lymphoblasts or HeLa cells [10]. Similar results were obtained for the mouse promoter using cultured cells [12].

Several groups demonstrated that the conserved region contains *cis* elements that bind homeodomain *trans*-acting factors such as CRX, OTX2, and RX and that exogenous expression of these factors transactivates IRBP promoters in cultured cells [10,12–18]. As CRX is expressed exclusively in photoreceptors and pinealocytes with tightly regulated stage specificity [13,14], it may be that the homeobox consensus region mediates fully regulated IRBP expression. However, IRBP expression persists in CRX knock-out mice [19]. Though other homeodomain transcription factors can bind and transactivate the IRBP promoter [16,18,20,21] and thus may substitute for CRX in the CRX knock-out mouse [15], none are expressed exclusively in photoreceptor cells [22–25]. If the specificity of the IRBP promoter derives from the cell-specific expression of a transcription factor that binds it, then the homeobox consensus region may mediate neuron specificity, but not necessarily photoreceptor specificity.

In transient transfections of chick cell cultures, we found that the 70 bp mouse IRBP promoter, which contains the homeobox consensus region, was active in retina cells with photoreceptor or neuron morphologies, and in separate cultures of brain cells, but not in fibroblasts. Conversely, the promoter extending to 1783 bp was active only in retina cells with photoreceptor morphology [12]. Thus, though the 70 bp promoter fragment appears to allow high levels of promoter activity, it is not clear that it provides for fully regulated, cell-specific expression. In this study, we sought to define the *cis*-acting region of the mouse IRBP 5′ flanking region that is required for photoreceptor-restricted activity. Using in vitro transient transfection assays and in vivo experiments with transgenic *Xenopus laevis*, we found that elements 5′ to position −70 are required for photoreceptor-restricted reporter gene expression.

2. Materials and methods

2.1. Cell culture, transient transfections, and CAT assays

Cell lines were obtained from the American Type Culture Collec-

*Corresponding author. Department of Ophthalmology, Emory University, Room B5511, 1365B Clifton Road, NE, Atlanta, GA 30322, USA. Fax: (1)-404-778 2231.
E-mail address: jboatri@emory.edu (J.H. Boatright).

Abbreviations: CAT, chloramphenicol acetyltransferase; EGFP, enhanced green fluorescent protein; IRBP, interphotoreceptor retinoid binding protein; NRSE, neuron-restrictive silencing element; NRSF, neuron-restrictive silencing factor; HEK 293, 293 human embryonic kidney cells

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tion (ATCC, Manassas, VA, USA) and established as per ATCC instructions. Cells were maintained at 37°C in 5% CO_2. WERI-Rb1 retinoblastoma (ATCC HTB-169) and CATH.a neuroblastoma (ATCC CRL-11179) cells were grown in RPMI 1640 medium (Mediatech, Herdon, VA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT, USA), 100 U/ml penicillin G, 100 μg/ml streptomycin (Gibco-BRL, Grand Island, NY, USA) and 1 mM HEPES (Gibco-BRL), with renewal every 3–4 days and 1:4 splitting weekly. Neuro2a neuroblastoma cells (ATCC CCL-131) and 293 human embryonic kidney cells (HEK 293; ATCC CRL-1573) were grown in Eagle's minimum essential medium (ATCC) supplemented with 10% fetal bovine serum (Hyclone), 100 U/ml penicillin G and 100 μg/ml streptomycin (Gibco-BRL), with renewal every 3–4 days and 1:4 splitting weekly.

All cells were plated at 5×10^5 cells per 35 mm culture well in 1 ml of growth medium and transfected within 24 h. WERI retinoblastoma and human kidney 293 cells were transfected with Superfect (Qiagen, Valencia, CA, USA) as per the manufacturer's instructions: 1 μg of plasmid DNA was added to 1 μl Superfect in a total of 100 μl of OptiMem medium (Gibco-BRL). This solution was added dropwise to a well of cells. CATH.a and Neuro2a neuroblastoma cells were transfected using Effectene (Qiagen) as per the manufacturer's instructions: 16 μl Enhancer and 50 μl of Effectene were added to 2 μg of plasmid DNA previously diluted into 100 μl of EC buffer. This solution was added dropwise to a well of cells.

Following a 48 h incubation at 37°C in 5% CO_2, cells were harvested and assayed for chloramphenicol acetyltransferase (CAT) activity as previously described [8]. Briefly, cell cytoplasm solutes were mixed with tritiated acetyl CoA and chloramphenicol and added to an organic scintillation liquid. CAT activity was measured as the accumulation of [^{3}H]acetylchloramphenicol over time on an LS6500 scintillation counter (Beckman Instruments). CAT activity is reported as CAT enzyme units$\times10^{-3}$. Each transfection group had a sample size of three to six per experiment. Each experiment was repeated at least three times. Data shown are representative.

2.2. Transgenic Xenopus laevis

These studies adhered to the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research. IRBP-enhanced green fluorescent protein (EGFP) plasmids were linearized using *Xho*I and transgenic *Xenopus laevis* embryos were produced as described [26]. Promoter/reporter gene fragments were excised and used to produce transgenic *Xenopus* tadpoles by restriction enzyme-mediated integration. Embryos were examined every day for EGFP expression and those that exhibited non-mosaic fluorescence above that from the yolk were studied further. Non-fluorescent siblings served as controls. Typically, about 30% of the tadpoles express EGFP and have integrated the plasmid. The distribution of EGFP was evaluated in vivo and in frozen ocular sections by conventional fluorescence microscopy and by confocal microscopy.

2.3. Plasmid construction

The CAT plasmid vectors used were based on the promoterless CAT vector pBLCAT3 (GenBank accession number X64409). The plasmid pSV40-CAT contained the SV40 promoter and enhancer regions (pCAT Control; Promega, Madison, WI, USA). The experimental plasmid pIRBP1783-CAT was constructed by inserting into pBLCAT3 a *Bam*HI fragment from −1783 to +101 (relative to transcription start) of the murine IRBP sequence (GenBank accession number M32734). A nested set of deletions based on this plasmid was prepared by the *Exo*III/mung bean nuclease method. The fragments generated had 5′ ends corresponding to −156 and −70 of the murine IRBP gene and are referred to by those numbers.

The EGFP plasmids used in producing transgenic *Xenopus* were based on the promoterless pEGFP-1 reporter vector (Clontech Laboratories, Palo Alto, CA, USA; GenBank accession number U55761), which served as negative control. Experimental plasmids pIRBP1783-EGFP and pIRBP156-EGFP were constructed by inserting a *Bam*HI fragment of the murine IRBP gene (bases −1783 and −156 to +101 relative to transcription start) into pEGFP-1. The plasmid pIRBP70-EGFP was constructed by inserting a *Hin*dIII/*Bam*HI fragment from the murine IRBP gene (−70 to +101 relative to transcription start) into pEGFP-1.

2.4. Statistical analyses

Numerical data are means of groups ± S.D. Statistical differences were determined by simple analysis of variance followed by Newman-Keuls post hoc testing. *P* values and sample size per experiment are given in figure legends.

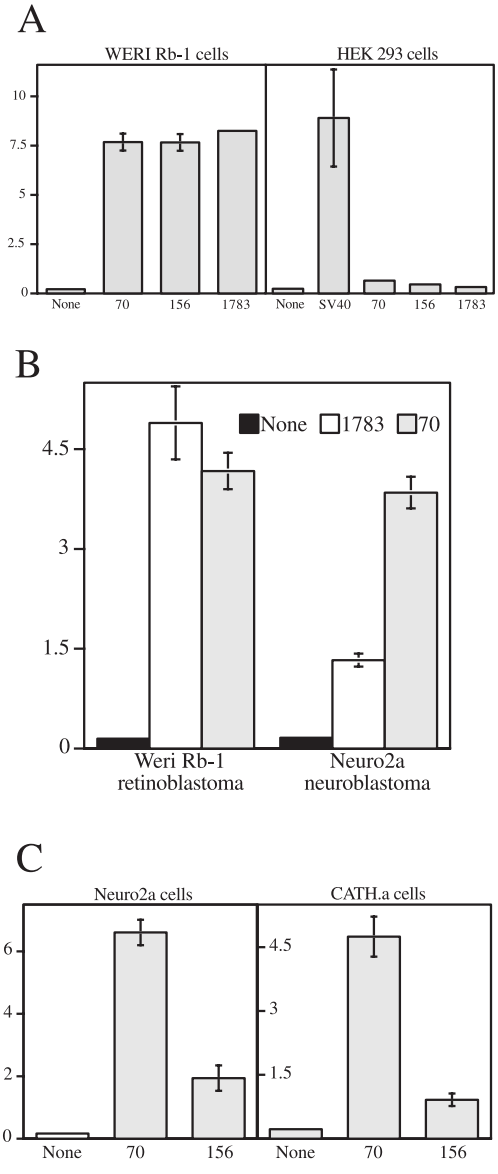

Fig. 1. Effect of truncation on IRBP promoter activity in expressing and non-expressing cell lines. Human WERI-Rb1 retinoblastoma cells, mouse CATH.a or Neuro2a neuroblastoma cells, and HEK 293 cells were transiently transfected with CAT reporter constructs containing IRBP gene fragments (−1783, −156, and −70 to +101, relative to transcription start; '1783', '156', and '70', respectively), an SV40 promoter ('SV40'), or no promoter ('None'; pSVOAT-CAT). CAT activity, as measured by radioenzymatic assay, was used as an index of promoter activity. Ordinate values are 10^{-3} CAT activity units. Error bars represent S.D.; some S.D.s were too small for bars to be seen. A: The three IRBP promoters are active in retinoblastoma cells (left panel, $n=5$/group, $P<0.05$ vs. 'None'). Conversely, in HEK 293 cells, only the SV40 promoter was active (right panel, $P<0.05$, $n=3$/group). B: Though they were equally active in retinoblastoma cells, the −1783/+101 promoter was much less active in neuroblastoma cells than the −70/+101 promoter ($P<0.05$, $n=4$–5/group). C: The −156/+101 promoter was much less active in neuroblastoma cells than the −70/+101 promoter ($P<0.05$; $n=6$/group).

3. Results

3.1. IRBP promoter activity in cultured cells

In transient transfections of HEK 293 cells, the SV40 promoter was active, but not the IRBP promoters (Fig. 1A). In transient transfections of WERI-Rb1 retinoblastoma cells, pIRBP70-CAT, pIRBP156-CAT, and pIRBP1783-CAT were active to similar levels (Fig. 1A,B). In contrast, in transiently transfected Neuro2a or CATH.a neuroblastoma cells, pIRBP70-CAT consistently produced activity higher than pIRBP1783-CAT (Fig. 1B) or pIRBP156-CAT (Fig. 1C). These data suggest that an element between −156 and −70 of the mouse IRBP promoter is required for tissue-restricted activity in the relatively artificial environment of immortalized, cultured cells.

3.2. IRBP promoter activity in transgenic X. laevis

For each construct, several expressing transgenic frogs were produced. For pIRBP1783-EGFP, 10 animals were EGFP-positive and examined at the dissecting microscope. Four were sectioned. For pIRBP156-EGFP, 24 of 40 animals were EGFP-positive at 2 weeks. Three were raised to adulthood. Five were sectioned and examined by confocal microscopy. For pIRBP70-EGFP, 19 of 56 animals were EGFP-positive at 2 weeks. Four were raised to adulthood. Five were sectioned and examined by confocal microscopy. EGFP expression levels varied with a construct group, estimated at about fivefold based upon observations with the dissecting microscope, most likely reflecting varying number of transgenes in each animal. However, there was little variation in spatial expression pattern for each construct. Typical embryos are shown in the figures.

Similar to results in transiently transfected cells, transgenic *Xenopus* expressing EGFP under the control of the various IRBP promoter fragments showed marked differences in tissue and cell type expression. Transgenic animals containing the EGFP gene linked to either the −1783/+101 or −156/+101 IRBP promoter fragments showed fluorescence only in eyes and possibly pineal gland (Fig. 2A,B), with more variable and much lower expression levels in neurons in the ventral head region and forebrain. In retina, the −156/+101 promoter fragment drove EGFP expression almost exclusively in the photoreceptor cell layer (Fig. 2B). In contrast, the −70/+101 promoter fragment drove EGFP expression not only in retina, but also in forebrain, hindbrain, spinal cord, and motor neurons innervating gills (Fig. 2C). These data suggest that an element between −156 and −70 of the IRBP promoter is required to restrict expression in a tissue-specific fashion in vivo.

4. Discussion

We previously found that pIRBP1783-CAT and pIRBP70-CAT produced high levels of CAT expression in transiently transfected primary cultures of embryonic chick retina cells [8,12]. pIRBP70-CAT was additionally active in primary cultures of embryonic chick brain cells and also cells with neuron-like morphologies in retina cell cultures, suggesting that an element between −1783 and −70 is required to restrict promoter activity to photoreceptor cells [8,12]. In the present study, we find that this differential activity extends to species beyond chicken. Additionally, the location of the putative

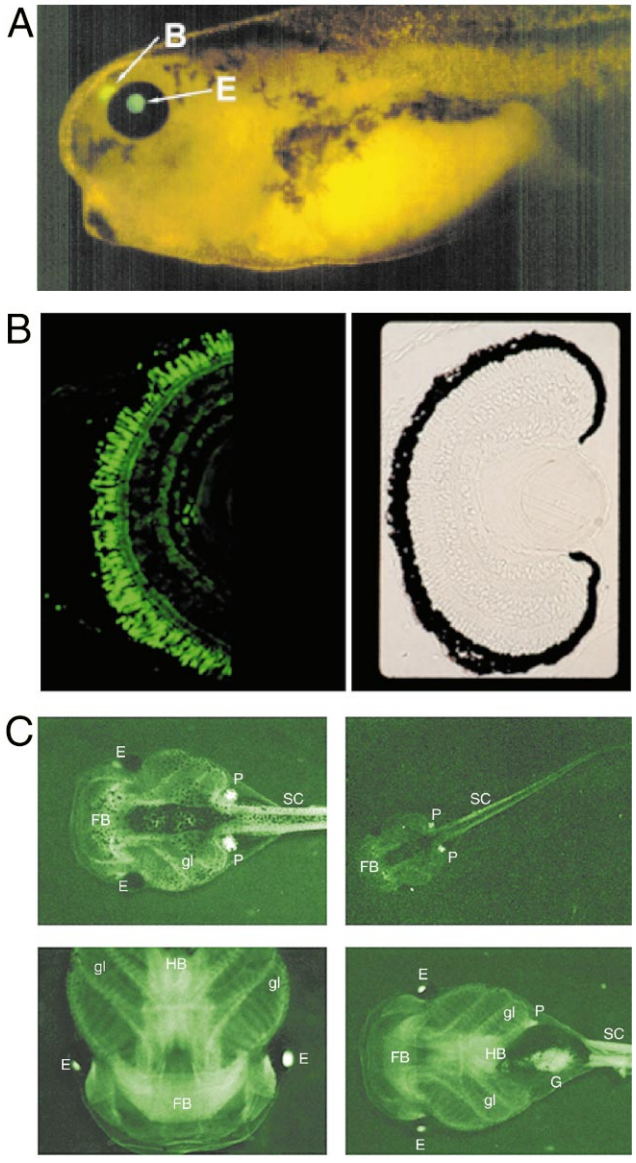

Fig. 2. Effect of truncation on IRBP promoter activity in EGFP-transgenic *X. laevis* tadpoles. The −1783/+101, −156/+101, or −70/+101 IRBP promoters were inserted upstream of the EGFP gene and the resulting promoter/EGFP fragment was used to produce transgenic *Xenopus* tadpoles by restriction enzyme-mediated integration. Fluorescence was used as an index of promoter activity. A: A pIRBP1783-EGFP transgenic tadpole, 5 days old. EGFP was expressed in the eye (E) and possibly pineal gland of the brain (B). B: Radial cross section through the central retina of an eye from a pIRBP156-EGFP transgenic tadpole, 3 weeks old (fluorescence microscopy in left panel, differential interference contrast image in right panel). The rod and cone photoreceptor cells of the outer retina and possibly a few other neuronal cells of the inner retina expressed EGFP. C: Several views of a pIRBP70-EGFP transgenic tadpole, 3–4 weeks old. Many types of neuronal structures, including the eyes, brain, motor neurons, and spinal cord, expressed EGFP. Top panels: Dorsal views showing the eye, forebrain, motor neurons innervating gills, and spinal cord express EGFP. The hindbrain is partially obscured by skin pigmentation (see lower right panel). Note that as reported previously [26] the pronephros autofluoresces in control animals (data not shown). Lower panels: Ventral views showing EGFP expression in the same structures and also the hindbrain. Note that the reflection of the illuminating beam from the gut lining in the lower right panel gives a false signal. E = eye, G = gut, FB = forebrain, HB = hindbrain, SC = spinal cord, gl = gills, and P = pronephros.

restriction element has been further defined. All three mouse IRBP 5′ flanking regions (−1783/+101, −156/+101, and −70/+101) provide high promoter activity in a human cell line and in vivo in *Xenopus*. All three activities appear to be restricted to neuronal or sensory cells. However, only the activities of the longer promoter fragments (−1783/+101 or −156/+101) appear to be additionally restricted to photoreceptors and pineal cells, mimicking the endogenous expression of IRBP. These data indicate that a −70 to +101 bp fragment of the mouse IRBP gene, which contains functional *cis* elements that bind CRX, OTX2, and RX [11–14,16,18,20,21], can act as a strong promoter, but silencing elements between −70 and −156 are required for cell-restricted expression.

This conclusion suggests that the restricted expression of endogenous IRBP in part requires the presence of a silencing mechanism in all cell types except photoreceptors or pinealocytes. Though this may appear unlikely, such regulatory mechanisms exist. The neuron-restrictive silencer factor (NRSF) represses transcription of over 20 neuronal genes in non-neuronal cells by binding to the neuron-restrictive silencer element (NRSE) [27,28]. In some instances, NRSF exists in neurons and stimulates transcription of neuron-specific genes, yet in non-neuronal cells, it inhibits the expression of those same genes [29]. Another neuron-restricting transcription mechanism, distinct from NRSF/NRSE, involves the SNOG consensus sequence, which regulates the neuron-restrictive expression of the genes SNAP-25, nNOS, and GAP-43 ('SNOG' derives from these gene names) [30,31]. SNOG does not bind a neuron-specific transcription factor. Rather, its presence in a gene is sufficient to block the transcription of that gene in non-neuronal cells that would otherwise be driven by a ubiquitous AP-1 element [31]. SNOG neuronal gene expression is additionally mediated in neurons by specific transcription factors.

Our data and those of other laboratories do not contradict the existence of similar transcription mechanisms that might be photoreceptor-specific and would require the presence of elements between −156 and −70. Several laboratories have reported that elements between −156 and −70 of the IRBP gene bind nuclear protein complexes from both retinal and non-retinal cells [10,12,16]. Additionally, an element from the human IRBP gene (−206 to −66 relative to transcription start) prevents transactivation of promoter/reporter constructs by exogenous OTX2 expression in transiently transfected HeLa cells [16]. That CRX-deficient mice express IRBP mRNA [15] suggests that non-CRX homeodomain factors can bind and activate the homeobox elements in the short IRBP promoter. Such a role has been suggested for OTX2 [16,18] and RX [20]. However, both of these factors are expressed in photoreceptor and non-photoreceptor cells [22–25]. Left unchecked, they should induce ectopic IRBP expression, suggesting that, since such expression does not occur, restrictive IRBP gene regulatory mechanisms are likely to exist. The exciting finding that MOK2 inhibits in vitro IRBP promoter activity [32] may prove useful in identifying potential suppressive mechanisms, though this is complicated by the observation that MOK2 is expressed endogenously in photoreceptors [32].

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References

[1] Bunt-Milam, A.H. and Saari, J.C. (1983) J. Cell Biol. 97, 703–712.
[2] Carlson, A. and Bok, D. (1992) Biochemistry 31, 9056–9062.
[3] Okajima, T.-I.L., Pepperberg, D.R., Ripps, H., Wiggert, B. and Chader, G.J. (1989) Exp. Eye Res. 49, 629–644.
[4] Okajima, T.I., Pepperberg, D.R., Ripps, H., Wiggert, B. and Chader, G.J. (1990) Proc. Natl. Acad. Sci. USA 87, 6907–6911.
[5] Liou, G.I., Wang, M. and Matragoon, S. (1994) Dev. Biol. 161, 345–356.
[6] Porrello, K., Bhat, S.P. and Bok, D. (1991) J. Histochem. Cytochem. 39, 171–176.
[7] van Veen, T., Katial, A., Shinohara, T., Barrett, D.J., Wiggert, B., Chader, G.J. and Nickerson, J.M. (1986) FEBS Lett. 208, 133–137.
[8] Boatright, J.H., Buono, R., Bruno, J., Lang, R., Si, J.S., Shinohara, T., Peoples, J.W. and Nickerson, J.M. (1997) Exp. Eye Res. 64, 269–277.
[9] Liou, G.I., Matragoon, S., Overbeek, P.A. and Fei, Y. (1994) Biochem. Biophys. Res. Commun. 203, 1875–1881.
[10] Bobola, N., Hirsch, E., Albini, A., Altruda, F., Noonan, D. and Ravazzolo, R. (1995) J. Biol. Chem. 270, 1289–1294.
[11] Fei, Y., Matragoon, S., Smith, S.B., Overbeek, P.A., Chen, S., Zack, D.J. and Liou, G.I. (1999) J. Biochem. (Tokyo) 125, 1189–1199.
[12] Boatright, J.H., Borst, D.E., Peoples, J.W., Bruno, J., Edwards, C.L., Si, J.S. and Nickerson, J.M. (1997) Mol. Vis. 3, 15–24.
[13] Chen, S., Wang, Q.L., Nie, Z., Sun, H., Lennon, G., Copeland, N.G., Gilbert, D.J., Jenkins, N.A. and Zack, D.J. (1997) Neuron 19, 1017–1030.
[14] Furukawa, T., Morrow, E.M. and Cepko, C.L. (1997) Cell 91, 531–541.
[15] Livesey, F.J., Furukawa, T., Steffen, M.A., Church, G.M. and Cepko, C.L. (2000) Curr. Biol. 10, 301–310.
[16] Fong, S.L. and Fong, W.B. (1999) Curr. Eye Res. 18, 283–291.
[17] Wang, P.W., Eisenbart, J.D., Cordes, S.P., Barsh, G.S., Stoffel, M. and Le Beau, M.M. (1999) Genomics 59, 275–281.
[18] Bobola, N., Briata, P., Ilengo, C., Rosatto, N., Craft, C., Corte, G. and Ravazzolo, R. (1999) Mech. Dev. 82, 165–169.
[19] Chau, K.Y., Munshi, N., Keane-Myers, A., Cheung-Chau, K.W., Tai, A.K., Manfioletti, G., Dorey, C.K., Thanos, D., Zack, D.J. and Ono, S.J. (2000) J. Neurosci. 20, 7317–7324.
[20] Kimura, A., Singh, D., Wawrousek, E.F., Kikuchi, M., Nakamura, M. and Shinohara, T. (2000) J. Biol. Chem. 275, 1152–1160.
[21] Zhu, X. and Craft, C.M. (2000) Mol. Cell Biol. 20, 5216–5226.
[22] Briata, P., Ilengo, C., Bobola, N. and Corte, G. (1999) FEBS Lett. 445, 160–164.
[23] Nakano, T., Murata, T., Matsuo, I. and Aizawa, S. (2000) Biochem. Biophys. Res. Commun. 267, 64–70.
[24] Tomita, K., Ishibashi, M., Nakahara, K., Ang, S.L., Nakanishi, S., Guillemot, F. and Kageyama, R. (1996) Neuron 16, 723–734.
[25] Igarashi, K., Kataoka, K., Itoh, K., Hayashi, N., Nishizawa, M. and Yamamoto, M. (1994) Nature 367, 568–572.
[26] Knox, B.E., Schlueter, C., Sanger, B.M., Green, C.B. and Besharse, J.C. (1998) FEBS Lett. 423, 117–121.
[27] Mori, N., Schoenherr, C., Vandenbergh, D.J. and Anderson, D.J. (1992) Neuron 9, 45–54.
[28] Schoenherr, C.J., Paquette, A.J. and Anderson, D.J. (1996) Proc. Natl. Acad. Sci. USA 93, 9881–9886.
[29] Bessis, A., Champtiaux, N., Chatelin, L. and Changeux, J.P. (1997) Proc. Natl. Acad. Sci. USA 94, 5906–5911.
[30] Weber, J.R. and Skene, J.H. (1997) J. Neurosci. 17, 7583–7593.
[31] Weber, J.R. and Skene, J.H. (1998) J. Neurosci. 18, 5264–5274.
[32] Morrow, E.M., Furukawa, T., Lee, J.E. and Cepko, C.L. (1999) Development 126, 23–36.