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Six3-mediated auto repression and eye development requires its interaction with members of the Groucho-related family of co-repressors

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SUMMARY

Recent findings suggest that Six3, a member of the evolutionarily conserved So/Six homeodomain family, plays an important role in vertebrate visual system development. However, little is known about the molecular mechanisms by which this function is accomplished. Although several members of the So/Six gene family interact with members of the eyes absent (Eya) gene family and function as transcriptional activators, Six3 does not interact with any known member of the Eya family. Here, we report that Grg4 and Grg5, mouse counterparts of the *Drosophila* transcriptional co-repressor Groucho, interact with mouse Six3 and its closely related member Six6, which may also be involved in vertebrate eye development. The specificity of the interaction was validated by co-immunoprecipitation of Six3 and Grg4 complexes from cell lines. We also show that the interaction between Six3 and Grg5 requires the Q domain of Grg5 and a conserved phenylalanine residue present in an eh1-like motif located in the Six domain of Six3. The pattern of *Grg5* expression in the mouse ventral forebrain and developing optic vesicles overlapped that previously reported for *Six3* and *Six6*. Using PCR, we identified a specific DNA motif that is bound by Six3 and we demonstrated that Six3 acts as a potent transcriptional repressor upon its interaction with Groucho-related members. We also demonstrated that this interaction is required for Six3 auto repression. The biological significance of this interaction in the retina and lens was assessed by overexpression experiments using either wild type full-length *Six3* cDNA or a mutated form of this gene in which the interaction with Groucho proteins was disrupted. Overexpression of wild type Six3 by *in vivo* retroviral infection of newborn rat retinae led to an altered photoreceptor phenotype, while the in ovo electroporation of chicken embryos resulted in failure of lens placode invagination and production of δ-crystallin-negative cells within the placode. These specific alterations were not seen when the mutated form of *Six3* cDNA was used in similar experimental approaches, indicating that Six3 interaction with Groucho proteins plays an essential role in vertebrate eye development.

Key words: Six3, Groucho, Transcriptional repression, Retina, Mouse, Eye, Homeobox

INTRODUCTION

The mouse *Six3* gene was originally isolated on the basis of its homology with the *Drosophila sine oculis* (*so*) gene (Oliver et al., 1995a). Members of the So/Six gene family encode proteins that have a conserved *Six* domain (SD) and a homeodomain (HD). To date, six members of this family (*Six1-Six6*) have been identified in mammals (Boucher et al., 1995; Oliver et al., 1995a; Oliver et al., 1995b; Kawakami et al., 1996a; Kawakami et al., 1996b; Heath et al., 1997; Toy et al., 1998) and three (*so*, *optix* and *Dsix4*) have been identified in *Drosophila* (Cheyette et al., 1994; Serikaku et al., 1994; Toy et al., 1998; Seo et al., 1999). On the basis of phylogenetic analysis, the vertebrate Six gene family has been divided into the three subclasses: *Six1*/*Six2*, *Six4*/*Six5* and *Six3*/*Six6* (Jean et al., 1999; Seo et al., 1999; Seimiya and Gehring, 2000). By the same criteria, *Drosophila so* was included in the *Six1/Six2* subclass, *optix* in the *Six3/Six6* and *Dsix4* in the *Six4/Six5* (Jean et al., 1999; Seo et al., 1999; Seimiya and Gehring, 2000).

Six3 and *Six6* are the only members of the *Six* gene family expressed during the early stages of visual system development (Oliver et al., 1995a; Jean et al., 1999; Lopez-Rios et al., 1999; Toy et al., 1999). In the anterior neuroectoderm of mice, *Six3* is expressed as early as embryonic day (E) 7.5 (Lagutin et al., 2001). *Six3* expression subsequently persists in the developing ventral forebrain, optic vesicles, retina, lens placode and pituitary gland (Oliver et al., 1995a; Lagutin et al., 2001).

The theory that *Six3* activity is required during eye formation was supported by the induction of ectopic optic vesicle-like structures or lenses upon *Six3* misexpression in transgenic fish

(Oliver et al., 1996; Loosli et al., 1999) and in transgenic mouse embryos (Lagutin et al., 2001). Furthermore, mutations in the human *SIX3* gene are associated with holoprosencephaly type 2, a severe forebrain malformation that in some of its most severe forms includes cyclopia (Wallis et al., 1999). Although it is clear from these studies that *Six3* plays an important role during forebrain patterning, not much is yet known regarding possible functional roles of *Six3* in the specification or differentiation of individual cell types in the retina or lens during development.

The suggested roles of *Six3* and *Six6* during development of the vertebrate visual system are reminiscent of the roles of their *Drosophila* counterparts, *optix* and *so*. In *Drosophila*, loss of *so* function leads to extensive death of eye progenitor cells, which results in the absence of eyes or in eyes of reduced size (Cheyette et al., 1994; Serikaku et al., 1994; Pignoni et al., 1997). Misexpression of *so* and *eyes absent* (*eya*) (Pignoni et al., 1997) or of *optix* alone (Seimiya and Gehring, 2000) can induce ectopic eye formation in flies. In *Drosophila* eye development, eya physically interacts with So (Pignoni et al., 1997) but not with optix (Seimiya and Gehring, 2000). Mammalian homologs of *Drosophila eya* genes (*eya1-4*) have been cloned and found to be expressed in various tissues during mouse embryonic development (Xu et al., 1997; Borsani et al., 1999). Similar to their fly counterparts, Six1 and Six4 can interact with Eya proteins (Heanue et al., 1999; Ohto et al., 1999); however, Eya proteins do not interact with Six3 (Heanue et al., 1999; Ohto et al., 1999; Seimiya and Gehring, 2000) (C. C. Z. and G. O., unpublished).

To gain further information regarding the functional roles of Six3 during mammalian development, we searched for Six3-interacting proteins. Using a yeast two-hybrid system, we identified the transcriptional co-repressor Grg5 (Aes – Mouse Genome Informatics) as an interacting partner of mouse Six3. This finding is consistent with that of Kobayashi et al. (Kobayashi et al., 2001), who reported that in zebrafish Six3 functions as a transcriptional repressor by interacting with Grg3. Groucho-related proteins (Grg in mouse) are the vertebrate counterparts of *Drosophila* Groucho (Gro) (Mallo et al., 1993; Koop et al., 1996). Grg proteins interact with many different transcription factors and function as transcriptional co-repressors (Choi et al., 1999; Eberhard et al., 2000; Jimenez et al., 1997; Jimenez et al., 1999; Ren et al., 1999; Roose et al., 1998). Our study further demonstrates that the interaction between Six3 and the Grg family of co-repressors is required for Six3 transcriptional auto repression and that this interaction is also relevant in vivo during vertebrate eye development.

MATERIALS AND METHODS

Plasmids

The plasmid pc97-*Six3* was generated by digesting mouse *Six3* cDNA with *Nco*I and *Bst*EII, and subcloning the filled-in fragment into the *Sma*I site of pPC97-cyh2 (Life Technologies). *Six3* deletion constructs for the yeast two-hybrid assay were made using endogenous restriction sites within *Six3*. Full-length *Six3* cDNA was cloned into the *Eco*RI site of KS pBluescript vector so that transcription of *Six3* cDNA was controlled by the T7 promoter. Full-length mouse *Grg5* was released from pc86-Grg5 by *Not*I digestion and cloned into the *Not*I site of pGEX-6P-1 to generate the pGEX-6P-1-Grg5 expression plasmid. Similarly, mouse Six3 and Grg4 (Tle4 – Mouse Genome Informatics) cDNAs were cloned into pGEX-6P-1 and pGEX-4T-2 respectively to generate the pGEX-6P-1-Six3 and pGEX-4T-2-Grg4 expression plasmids. A *Not*I (filled-in)-*Hin*dIII *Grg5* cDNA fragment was cloned into *Sma*I-*Hin*dIII-digested pSP72 vector for in situ hybridization. Full-length mouse *Grg5* cDNA was released from pGEX-6P-1-Grg5 by digestion with *Bam*HI and *Hin*dIII and subcloned into PM2 expression vector digested with the same enzymes. Full-length *Grg5* cDNA was subcloned into the filled-in *Eco*RI site of pFlex-EB vector (Hollenbach et al., 1999). Mouse *Grg4* was amplified by RT-PCR and the product was cloned into pFlex-EB expression vector. A 1.4-kb *Nco*I fragment containing the mouse *Six3* promoter was cloned into the *Nco*I site of pG5 luc vector (Promega). The resulting plasmid, Six3pro-luc, contained four putative Six3-binding sites in the *Six3* promoter and four Gal4 upstream activating sequences (UAS) that were upstream of *Six3* promoter. All of the plasmids were sequenced to determine that they had been accurately constructed.

Yeast two-hybrid screening

A ProQuest yeast two-hybrid system was used to screen an E10.5 mouse cDNA library (Life Technologies) by following procedures described by the manufacturer.

RNA collection and RT-PCR

The RNeasy Total RNA System (Qiagen) was used to isolate total RNA from eye tissue dissected from E10.5 and E11.5 mouse embryos. Trizol (Life Technologies) was used to extract total RNA from NIH3T3 mouse embryonic fibroblasts and human kidney 293T cells. Reverse transcription was performed by using a First Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech). The following *Grg5* primers were included in the reaction mixtures for PCR: 5′-CAGCTCCAGGCTCACCAG-3′ (sense) and 5′-GCTCGAGCTAA-TCCGACTTCTC-3′ (antisense).

In situ hybridization

A *Grg5* antisense probe labeled with digoxigenin (Roche Molecular Biochemicals) was synthesized by using Sp6 RNA polymerase and 1 μg of *Bgl*II-digested pSP72-Grg5Δ*Hin*dIII as a template. Digoxigenin-labeled *Grg5* sense probe was synthesized by using T7 RNA polymerase in *Hin*dIII-digested-plasmid as a template.

Cryosections were hybridized with digoxigenin-labeled *Grg5* sense or antisense probes overnight at 70°C. The signal was visualized by using nitroblue tetrazolium (NBT)-5-bromo-4-chloro-3-indolyl phosphate (BCIP) reagent (Roche Molecular Biochemicals).

GST pull-down assay

BL21 cells (Stratagene) that were transformed with pGEX-6P-1, pGEX-6P-1-Grg5, pGEX-4T-2-Grg4 or pGEX-6P-1-Six3 were grown in the presence of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 hours at either 37°C or 30°C. The induced proteins were purified by incubation with pre-swelled glutathione-Sepharose 4B beads (Sigma) in NETN buffer (20 mM Tris, pH 8.0; 100 mM NaCl; 1 mM EDTA; 0.5% Nonidet P-40) at 4°C. GST pull-down assay was performed by incubating in vitro translated [^{35}S]methionine-labeled protein with either glutathione-Sepharose-bound GST or GST fusion proteins in the binding buffer (10 mM Tris, pH 7.6; 50 mM NaCl; 5 mM EDTA; 1% Triton-X 100; protease inhibitor) at 4°C for 1 hour. After incubation, the beads were washed three times with 1 ml binding buffer and boiled in 2×SDS sample buffer (0.1 M dithiothreitol). The eluted binding proteins were loaded on a 12% SDS-acrylamide gel and visualized by autoradiographic analysis.

Cell culture, transfection, chloramphenicol acetyl transferase (CAT) and luciferase assays

NIH3T3 and 293T cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (FBS),

antibiotics and glutamine. The retinoblastoma cell line Y79 was cultured in RPMI medium supplemented with 10% FBS, antibiotics and glutamine. One day before transfection, 2×10^5 cells were plated in each well of a six-well plate. Using the transfection reagent FuGENE 6 (Roche), we transfected cells with 0.1 μg of the expression plasmids Six3, Grg5, Grg4 or Groucho together with 1 μg of the reporter plasmids Six3pro-luc or Gal4 UAS-TK-CAT (Hollenbach et al., 1999). Secreted alkaline phosphatase (SEAP) plasmid (0.1 μg) (Hollenbach et al., 1999) was used as an internal control to normalize transfection efficiency. CAT activity was measured with the Quan-T-CAT assay system (Amersham Life Science), and the luciferase assay was performed as described previously (Zhu et al., 1999). Each experiment was repeated at least three times.

Immunoprecipitation (IP) and western blot analysis

NIH3T3 cells were transfected with either a CMV-based Six3 expression plasmid alone or with Six3 expression plasmid together with either FLAG-Grg4 expression plasmid or Flag-Groucho plasmid. Cells were lysed with a solution of 50 mM Hepes (pH 7.0), 1% NP-40, and proteinase inhibitors. Six3 was immunoprecipitated together with Flag-Grg4 by using a mouse monoclonal anti-Flag antibody (Sigma) in binding buffer (120 mM NaCl, 1% NP-40, 50 mM Tris, pH 8.0). After four washes with the binding buffer, precipitated Six3 was subjected to SDS-PAGE and blotted onto nitrocellulose membrane. The membrane was then incubated with a rabbit anti-mouse Six3 antibody (1:2000 dilution) (Lagutin et al., 2001).

Identification of the DNA sequence bound by Six3

The method originally described by Inaba et al. (Inaba et al., 1994) was followed for this purpose. GST and GST-Six3 proteins were used to identify DNA sequences bound by Six3. Oligonucleotides (75 mer) with the following sequence were synthesized: CGCGGATCCTGC-AGCTCGAGN$_{30}$GTCGACAAGCTTCTAGAGCA. Oligonucleotides were amplified by PCR. The PCR products were mixed with glutathione-Sepharose-bound GST and GST-Six3 proteins in binding buffer (25 mM Hepes, pH 7.5; 100 mM KCl; 1 mM EDTA; 10 mM $MgCl_2$; 0.1% NP-40; 5% glycerol; and 1 mM DTT) supplemented with 0.6 μg/μl poly(dI-dC). After incubation and washing, bound oligonucleotides were recovered and amplified again by PCR. The PCR products were used for a second round of selection. After the sixth round of selection, the PCR products were cloned into pGEM-T-Easy (Promega), and 24 clones were subjected to sequencing.

Electrophoretic mobility shift assay (EMSA)

Klenow enzyme was used to end-label the double-stranded DNA fragments with [α-^{32}P] dCTP. The labeled probes were incubated with GST, GST-Six3 or Six3 protein purified after cleavage of the induced GST-Six3 protein in binding buffer (25 mM Hepes, pH 7.5; 100 mM KCl; 1 mM EDTA; 10 mM $MgCl_2$; 0.1% NP-40; 5% glycerol; and 1 mM DTT) supplemented with 0.6 μg/μl poly(dI-dC). The DNA-protein complex was resolved in 5% non-denaturing protein gel. Electrophoresis was done at 110 V at room temperature for several hours. The gel was dried, and the protein-DNA complexes were visualized by autoradiography.

Replication-incompetent retroviral vectors and in vivo lineage analysis

The replication-incompetent retroviral vectors used for this study have been described previously (Dyer and Cepko, 2001; Dyer and Cepko, 2000). In pLIA-E^{Six3}, the full-length mouse *Six3*-coding region is upstream of an internal ribosome entry site (IRES) and the human placental alkaline phosphatase reporter gene (PLAP) (Fig. 9A). The vector pLIA-E^{Six3F88E} encodes mouse Six3 containing a single amino acid substitution (F88E) that abolishes the interaction between Six3 and Grg family proteins.

Retroviral stocks were prepared by transiently transfecting the plasmid constructs pLIA-E^{Six3} and pLIA-E^{Six3F88E} into a 293T ecotropic producer cell line (Phoenix-E) by calcium phosphate co-precipitation as previously described (Cepko, 1997). Supernatant containing the viral particles was harvested 48 hours after transfection, and the viral titer was determined by using NIH3T3 cells. In vivo lineage analysis was performed as described previously (Fields-Berry et al., 1992; Turner and Cepko, 1987).

In ovo electroporation of chicken embryos

cDNAs encoding *Six3*, *Six3*$_{F88E}$ and *Grg5* were inserted into the pFlex-EB vector and head ectoderm of stage 10 chicken embryos was electroporated using 6 μg/μl total concentration of plasmid DNAs, as described in Kamachi et al. (Kamachi et al., 2001). Electroporated embryos were processed for whole-mount in situ hybridization and sections were observed under Nomarski optics.

RESULTS

Grg proteins are Six3-interacting partners in mouse

To gain insight into Six3 functions during murine embryonic development, we used the yeast two-hybrid system to identify putative Six3-interacting proteins. A Gal4-DNA-binding domain (Gal4 –DB)/Six3 fusion protein (Fig. 1A) was used as bait in the screening of a Gal4 activation domain (Gal4-AD)-tagged E10.5 mouse cDNA expression library. The Six3 bait used in this study (pc97-Six3) included the two highly conserved domains (amino acids 1-326) of Six3, the Six domain (SD) in the N terminus and the adjacent homeodomain (HD) in the C terminus (Fig. 1A). In the initial screening, two of 1.2 million clones encoded a protein that specifically interacted with Six3 but not with the unrelated protein Raf1 or

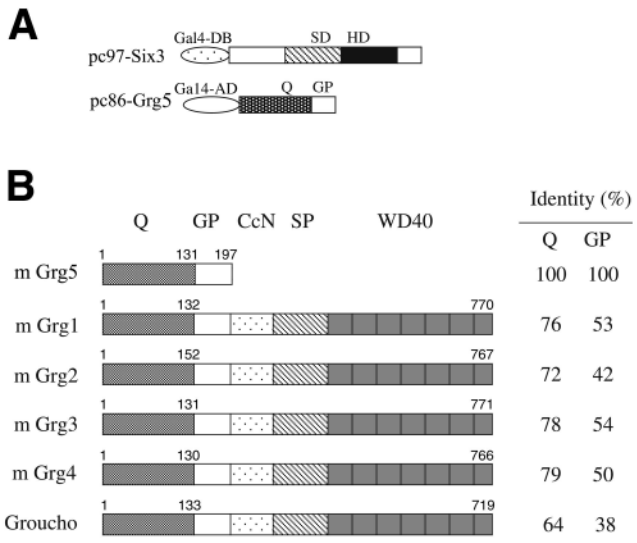

Fig. 1. Six3 and members of the Groucho family of corepressors. (A) The pc97-Six3 and pc86-Grg5 constructs used in the yeast transformation assay. Gal4-DB, Gal4 DNA-binding domain; SD, Six domain; HD, homeodomain; Gal4-AD, Gal4 activation domain; Q, glutamine-rich domain; GP, glycine and proline-rich domain. (B) Comparison of murine Grg family members with *Drosophila* Groucho protein. Mouse Grg5 lacks the CcN domain (potential phosphorylation sites for casein kinase II and cdc2), SP domain (serine- and proline-rich) and WD40 repeat domain (40 amino acid repeats separated by tryptophan and aspartic acid), but it contains the Q and GP domains. The Q domain of mouse Grg5 shares as much as 64% amino acid identity with *Drosophila* Groucho.

the pc97 vector containing only the Gal4-DB (data not shown). Sequence analysis identified one of the Gal4-AD in-frame fusion proteins as the 197 amino acid full-length Grg5, one of the murine counterparts of *Drosophila* Groucho, also known as Grg (Fig. 1A) (Mallo et al., 1993). Grg5 is localized in the nucleus but does not bind directly to DNA (Mallo et al., 1995a; Mallo et al., 1995b), a finding that suggests that Grg5 may be a transcriptional co-factor. This finding was in agreement with a recent report that showed that zebrafish Six3 can interact with Grg3 (Kobayashi et al., 2001).

The Groucho-related transcriptional repressors consist of several highly homologous proteins (Mallo et al., 1993; Leon and Lobe, 1997; Fisher and Caudy, 1998; Molenaar et al., 2000) (Fig. 1B). One of the most conserved domains is the Q domain, which is a glutamine-rich domain that is located in the N terminus and is involved in protein dimerization (Pinto and Lobe, 1996). Another domain, the WD-40 domain, is related to an amino acid motif present in G-protein β-subunits and is found in the C-terminal region (Mallo et al., 1993). Three less conserved domains are present in most Groucho gene products; the CcN domain, which contains a putative nuclear localization signal and phosphorylation sites for casein kinase II and the kinase cdc2; the GP domain, which is rich in glycine and proline residues; and SP domain, which is rich in serine or proline (Mallo et al., 1993) (Fig. 1B).

Direct interaction between Grg proteins and Six proteins

We performed an in vitro binding assay to confirm the Six3-Grg5 interaction that we identified in yeast cells. For this assay, we fused the full-length mouse Grg5 cDNA to the GST gene in an expression plasmid. The [^{35}S]-labeled Six3 protein interacted with the GST-Grg5 fusion protein but failed to interact with the GST protein alone (Fig. 2A). The specificity of the binding was corroborated by the failure of the unrelated homeodomain protein Pax4 to bind to GST-Grg5 (data not shown). These results confirmed the interactions between Six3 and Grg5 that was initially identified in yeast cells and suggested that this interaction is specific. Similar GST pull-down experiments were performed to examine the interaction between Grg5 and the murine Six/So family members Six6 and Six2, and *Drosophila* sine oculis (So). Six6 and *Drosophila* So, like Six3, interacted with Grg5; however, Six2 did not (Fig. 2A). We extended these initial experiments and demonstrated that mouse Grg4 and fly Groucho proteins can also interact strongly with mouse Six3 and Six6 (Fig. 2B). Although much weaker, interactions between mouse Grg4 and fly optix were also detected (Fig. 2B). These results suggest that mammalian Six3 can directly interact with members of the Groucho family in vitro.

To determine whether Grg and Six3 proteins also interact in the milieu of mammalian cells, a Six3 expression plasmid was transfected into NIH3T3 cells either with or without FLAG-Grg5 or FLAG-Grg4 expression plasmids. In co-immunoprecipitation assays, an anti-Flag antibody co-

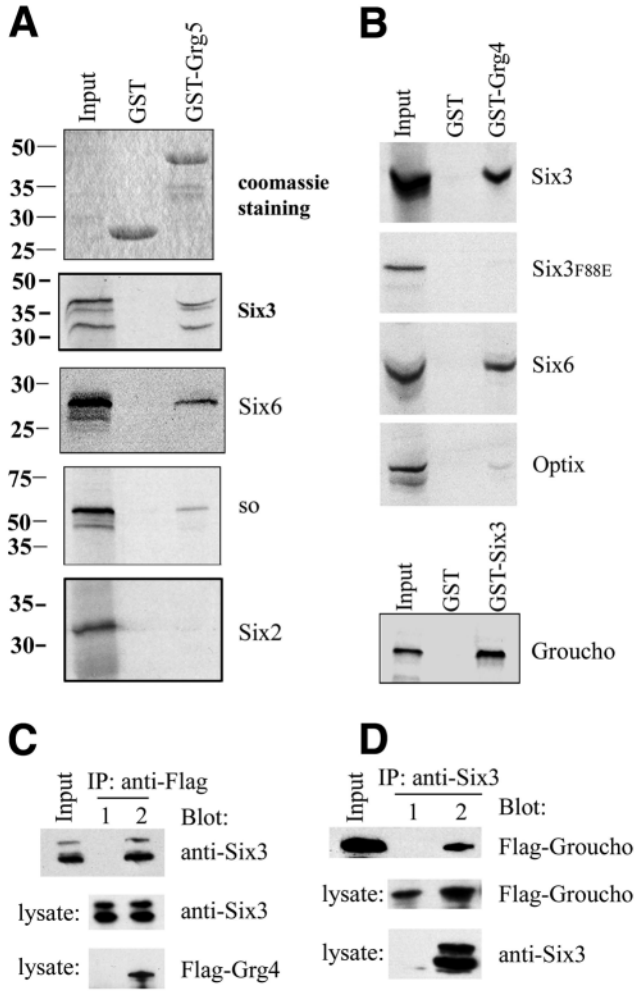

Fig. 2. Binding of Six proteins to Grg proteins in vitro and in vivo. (A) Six family proteins bind to Grg5 in vitro. Coomassie staining of a gel containing GST (0.2 μg) and GST-Grg5 fusion proteins (0.2 μg) used in the GST pull-down experiments is shown on the top row. The input lane shows 10% of the total protein used in the GST pull-down experiments. Full-length Six3 migrated as a 37 kDa protein. The faster migrating bands may have been shorter forms of Six3 that originated from internal translation start sites in the *Six3* transcript. (B) Six family proteins interact in vitro with mouse Grg4 and with *Drosophila* Groucho. Input lane shows 10% of the total [^{35}S]methionine-labeled proteins used in the GST pull-down experiments. Specifically, mouse Six3 and Six6 protein bound to GST-Grg4 fusion protein (GST-Grg4 lane) but not to GST alone (GST lane). *Drosophila* Optix bound only weakly to GST-Grg4 fusion protein, whereas Six3$_{F88E}$ mutant protein did not. Fly Groucho strongly bound to mouse Six3 (lower panel). (C) Mouse Six3 bound to Grg4 in mammalian cells. NIH3T3 cells were transfected with either Six3 expression vector alone (lane 1) or Six3 expression vector and Flag-tagged Grg4 (lane 2). Co-immunoprecipitation (IP) was performed with anti-Flag antibody, and precipitated Six3 was detected with rabbit anti-mouse Six3 antibody (lane 2). Input lane shows 10% of the total protein used in the IP experiment. Six3 and Flag-Grg4 proteins in the crude cell lysate underwent western blot analysis. (D) Fly Groucho immunoprecipitated with mouse Six3. NIH3T3 cells were transfected with either Flag-tagged Groucho expression construct alone (lane 1) or Flag-tagged Groucho expression construct and Six3 expression vector (lane 2). Immunoprecipitation was carried out with anti-Six3 antibody. The precipitated Flag-tagged Groucho protein underwent western blot analysis with anti-Flag antibody. The input lane shows 10% of the total cell lysate used in the IP experiment. Flag-Groucho and Six3 in the crude cell lysate was subjected to western blot analysis.

precipitated approximately 10% of the total Six3 from FLAG-Grg4-transfected cells (Fig. 2C, lane 2); no detectable amount of Six3 was precipitated from cells that were not transfected with FLAG-Grg4 plasmid (Fig. 2C, lane 1), nor from cells transfected with FLAG-Grg5 (data not shown). The failure to precipitate Six3 together with Grg5 is likely to be due to lower binding affinities between these two proteins under these experimental conditions. In a similar experiment, Flag-Groucho fusion protein was specifically co-immunoprecipitated by an anti-Six3 antibody (Fig. 2D, lane 2). Taken together, the results of the in vitro binding assays and the transfection experiments demonstrated that Grg proteins interact specifically with proteins of the Six family both in vitro and in mammalian cells.

The eh1-like motif in the Six domain interacts with the Q domain of Grg proteins

To identify the specific domains of Six3 required for the interaction with Grg5, we generated a series of Six3 deletion constructs and analyzed them in the Gal4 yeast two-hybrid system (Fig. 3B). The protein-protein binding affinity was determined by the growth rate of the transformed yeast cells on histidine and 3-amino-1,2,4-triazole (3AT)-selective and uracil-selective plates. We found that the N terminus and most of the six domain (SD) ($Six3_{1\text{-}183}$) were sufficient to mediate specific interactions with Grg5 (Fig. 3B); no interaction with Grg5 was detected when we used a construct encoding the C terminus of the SD, the homeodomain (HD) and the C-terminal region of the Six3 protein ($Six3_{184\text{-}333}$) (Fig. 3B). Protein encoded by the deletion construct $Six3_{1\text{-}120}$ interacted with Grg5; however, deletion construct $Six3_{184\text{-}333}$ failed to interact with Grg5 (Fig. 3B). Protein expressed from the construct $Six3_{73\text{-}229}$ also interacted with Grg5 (Fig. 3B); however, no interaction was observed when we used the deletion construct $Six3_{121\text{-}183}$ (Fig. 3B). Generation of additional deletion constructs identified the region encoded by $Six3_{73\text{-}120}$ as sufficient to mediate interaction with Grg5 (Fig. 3B).

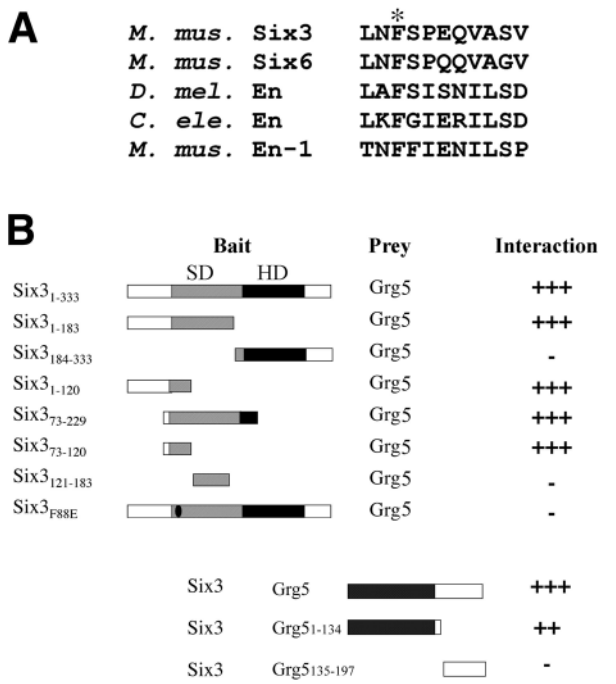

Fig. 3. Mapping of the interaction domains of Six3 and Grg5 using a yeast two-hybrid assay. (A) Alignment of eh1-like motif identified in mouse Six3 and Six6 with the corresponding eh1 motifs present in the *Drosophila*, *C. elegans* and mouse engrailed protein.
(B) Mapping of the interaction domains by using a yeast two-hybrid assay. The strength of the interaction between each pair of proteins was reflected by the growth rate of the transformants on both uracil-selective and histidine-selective plates. The N terminus and SD ($Six3_{1\text{-}183}$) of Six3 bound to Grg5 similarly to the full-length Six3. Removal of amino acids 1-183 ($Six3_{184\text{-}333}$) abolished the interaction with Grg5. Binding to Grg5 was restored when the construct $Six3_{1\text{-}120}$ was used. $Six3_{73\text{-}120}$ also interacted strongly with Grg5; however, $Six3_{121\text{-}183}$ did not interact with Grg5. Construct $Six3_{F88E}$, including a point mutation at position 88 of the eh1-like motif of Six3 (phenylalanine was replaced by glutamic acid), abolished the interaction with Grg5. The fragment containing the Q domain and four amino acids of the SP domain of Grg5 ($Grg5_{1\text{-}134}$) interacted with Six3, whereas the fragment containing the C terminus of Grg5 ($Grg5_{135\text{-}197}$) did not.

A sequence comparison of the region identified as sufficient to interact with Grg5 revealed the presence of an eh1-like motif (Fig. 3A). The amino acid sequence of this motif is highly similar to those of eh1 motifs previously identified in engrailed, goosecoid and Pax5 proteins, which mediate interactions with the Groucho family of corepressors (Smith and Jaynes, 1996; Jimenez et al., 1997; Jimenez et al., 1999; Eberhard et al., 2000). To determine whether the eh1-like motif identified in mouse Six3 can also mediate the interaction with Grg5, we replaced the highly conserved phenylalanine at position 88 of Six3 (Fig. 3A) by glutamic acid ($Six3_{F88E}$). This mutation has been demonstrated to abolish the interaction between homeodomain transcription factors and the Grg family of corepressors (Jimenez et al., 1999; Eberhard et al., 2000). As expected, the interaction of Six3 and Grg5 proteins in the yeast two-hybrid assay (Fig. 3B), and of Six3 and Grg4 in the GST pull-down experiments (Fig. 2B) was disrupted when using the $Six3_{F88E}$ expression construct. This conserved phenylalanine was also identified by Kobayashi et al. (Kobayashi et al., 2001) as one of the residues that mediate the interaction between zebrafish Six3 and Grg3. In addition, they also identified another eh1-like motif in the SD of zebrafish Six3 as mediating this protein-protein interaction with Grg3. However, in our assay, the construct $Six3_{121\text{-}183}$ harboring the second eh1-like motif reported by Kobayashi et al. (Kobayashi et al., 2001) failed to interact with Grg5 (Fig. 3B).

To map the Grg5 domain that interacts with Six3, we made two deletion constructs: pc86-$Grg5_{1\text{-}134}$, which encoded the region containing the Q domain, and pc86-$Grg5_{135\text{-}197}$, which encoded the portion containing the GP domain. The Q domain interacted with Six3, whereas the GP alone did not (Fig. 3B); this finding was confirmed by results of a GST pull-down experiment (data not shown). Taken together, our results demonstrate that the eh1-like motif located in the N terminus of the SD of Six3 interacts with the Q domain of Grg5.

Grg5 is expressed in the developing eye and forebrain of mouse embryos and colocalizes with Six3 in the nucleus

Mallo et al. (Mallo et al., 1993) detected *Grg5* transcripts in the yolk sac and ventral floor of the foregut and hindgut as early as E8.5. At E9.5, *Grg5* expression was observed in the heart,

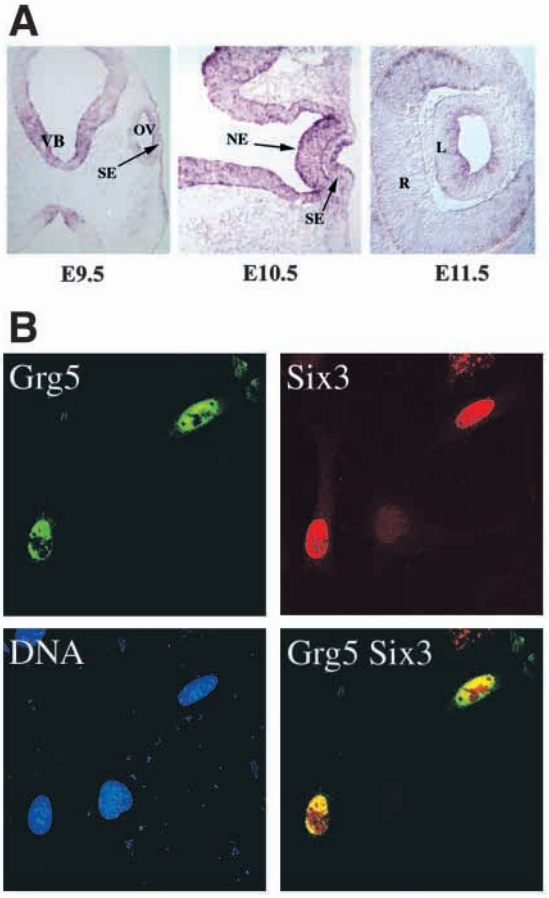

Fig. 4. Expression of *Grg5* in the developing mouse forebrain and eye tissue. (A) Digoxigenin-labeled *Grg5* antisense probe revealed expression of this gene in the ventral forebrain (VB), optic vesicle (OV) and surface ectoderm (SE) of E9.5 mouse embryos (left). A similar expression is also seen at E10.5 (middle) but is now more evident in the developing optic vesicle and invaginating surface ectoderm. At E11.5 (right) expression is seen in the retina (R) and lens (L). (B) Confocal images showing the colocalization of Grg5 and Six3 in the nuclei of transfected NIH3T3 cells. NIH3T3 cells were transfected with a Flag-tagged Grg5 and a CMV-based Six3 expression construct. Immunostaining was performed using mouse anti-Flag antibody and a rabbit anti-Six3 antibody; DNA was stained with TOTO-3.

liver primordium, gut, ventral portion of the spinal cord and floor of the brain. By midgestation, *Grg5* was ubiquitously expressed, and this expression continued through adulthood (Mallo et al., 1993). To determine whether early *Grg5* expression overlaps with that of *Six3* in the ventral forebrain and developing visual system, we performed an in situ hybridization experiment in E10.5 and E11.5 eye tissue and determined that *Grg5* is also expressed in the ventral forebrain and developing optic vesicles at E9.5 (Fig. 4A), a finding that is similar to those previously reported for *Six3* expression (Oliver et al., 1995a). Later, expression was detected in the optic stalk, neuroretina and lens (Fig. 4A). These results suggest that specific protein-protein interactions between Six3 and Grg5 can occur in vivo in any of the Six3-expressing tissues such as the ventral forebrain and developing visual system. Mallo et al. (Mallo et al., 1995a) reported that Grg5 is localized in the nucleus although it does not have an obvious nuclear localization signal. This could be explained by its interaction with a number of transcription factors that may help translocate Grg5 from the cytoplasm to the nucleus. Alternatively, Grg5 may have a nuclear localization signal-like sequence that can help the nuclear localization of Grg5. Similar results were obtained when we transfected NIH3T3 cells with a Flag-tagged Grg5 expression construct. Immunostaining of the transfected cells using an anti-Flag antibody corroborated the nuclear localization of Grg5 (Fig. 4B). Immunostaining of NIH3T3 cells transfected with a Six3 expression construct has also revealed the nuclear localization of Six3 (Fig. 4B).

Identification of the DNA sequence bound by Six3

Although previous studies have identified the DNA-binding motifs for the family members Six2 and Six4, the DNA-binding sequence of Six3 has remained elusive (Kawakami et al., 1996a; Spitz et al., 1998). To gain further insight into *Six3* function, we sought to identify the Six3-binding site by using a selection strategy involving random oligonucleotides (Inaba et al., 1994). After six rounds of selection using a GST-full length Six3 fusion protein, we identified a common ATTA core motif present in 19 of the 24 recovered random oligonucleotides. Each oligonucleotide was recovered once and their DNA sequence is aligned in Fig. 5A. Interestingly, the core ATTA sequence identified in this study is consistent with the core binding motif recognized by a majority of homeodomain proteins (Treisman et al., 1992), but is different from the one reportedly bound by Six2 and Six4 subfamily proteins (Kawakami et al., 1996a). It could be possible that under similar experimental conditions, Six2 and Six4 may also recognize the same DNA core motif identified here for Six3.

To confirm that Six3 can indeed bind to the oligonucleotides identified by the selection, EMSA was performed by using the recovered oligonucleotide listed first in Fig. 5A. The [α-^{32}P] dCTP-labeled oligonucleotide was incubated with either GST or GST-full length Six3 fusion protein, and the DNA-protein complex was subsequently competed with either nonradioactive normal oligonucleotide or nonradioactive mutated oligonucleotide (ATTA was changed to AGCA) (Fig. 5B). No specific complexes were observed when the reaction mixture contained labeled probe alone (Fig. 5B, lane 1) or GST and the labeled probe (Fig. 5B, lane 2). However, the GST-Six3 protein formed a specific complex with the labeled oligonucleotide (Fig. 5B, lane 3), and after a specific anti-Six3 antibody was added to the reaction mixture, the migration of the complex in the gel was supershifted (Fig. 5B, lane 4). We observed a titratable reduction of the formation of the GST-Six3-oligonucleotide complex upon competition with excess of nonradioactive normal oligonucleotide (Fig. 5B, lanes 5 and 6); however, complex formation was not inhibited by adding excess mutated oligonucleotide in which the core ATTA motif was changed to AGCA (Fig. 5B, lanes 7 and 8). This result demonstrated that the DNA sequence ATTA is the motif bound by Six3.

Six3 represses its own promoter activity through its interaction with Groucho-related corepressors

Sequence analysis revealed the presence of at least three clustered ATTA core motifs in the distal region of the *Six3* promoter (Fig. 6A). This sequence suggested that Six3 binds

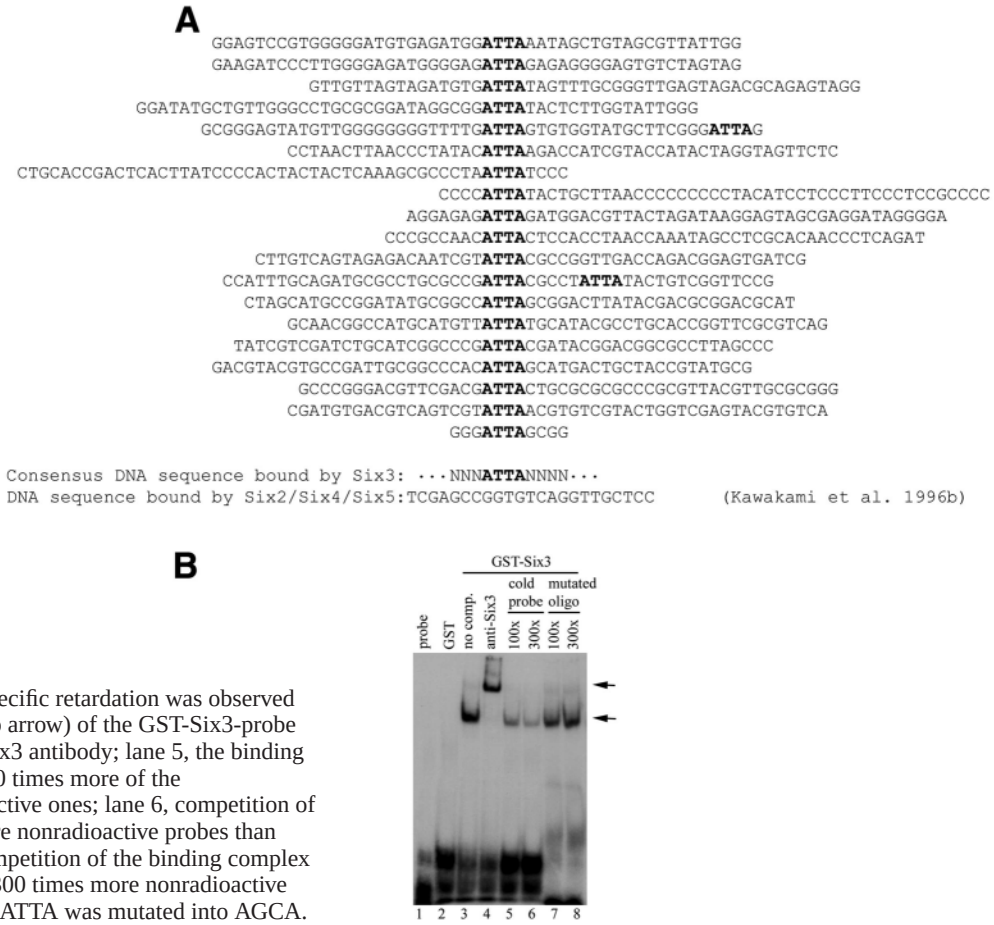

Fig. 5. Identification of the DNA sequence motif bound by Six3. (A) The sequence of the 19 oligonucleotides containing a core ATTA (bold) motif selected by the GST-Six3 fusion protein are aligned for comparison. The oligonucleotide at the top was used in the electrophoretic mobility shift assay (EMSA) experiments depicted below. The DNA sequence identified by Kawakami et al. (Kawakami et al., 1996b) as recognized by Six2, Six4 and Six5 is shown at the bottom. (B) Six3 bound specifically to the identified oligonucleotides in an EMSA. Double-stranded DNA of the first oligonucleotide represented in A was end-labeled with ^{32}P and used as a probe. Lane 1, ^{32}P-labeled probe alone; lane 2, GST and ^{32}P-labeled probe; lane 3, when the GST-Six3 fusion protein was combined with the ^{32}P-labeled probe, specific retardation was observed (bottom arrow); lane 4, a super-shift (top arrow) of the GST-Six3-probe complex was seen when using an anti-Six3 antibody; lane 5, the binding complex was competed when adding 100 times more of the nonradioactive probes than of the radioactive ones; lane 6, competition of the binding complex with 300 times more nonradioactive probes than radioactive probes; lanes 7 and 8, no competition of the binding complex was observed when using either 100 or 300 times more nonradioactive mutated probes, in which the core motif ATTA was mutated into AGCA.

to its own promoter and regulates its own transcription. To test this possibility, we performed an EMSA with DNA fragments representing these three promoter regions (Fig. 6C). Full-length Six3 protein was able to bind to the three different promoter fragments (Fig. 6B, lanes 2, 6 and 10). The binding specificity was reflected by the reduced amount of complex that formed when nonradioactive probes I, II or III were added (lanes 3, 7, and 11); specific binding complex of labeled probe and Six3 was not competed when the mutated nonradioactive oligonucleotides mut1, mut2 and mut3 in which each ATTA core sequence was changed to AGCA (lanes 4, 8 and 12) were added. The lower band present in lanes 2 and 4 (Fig. 6B, arrowhead) probably corresponds to a truncated form of Six3 protein bound to the probe. This complex can also be competed by nonradioactive probe I (lane 3), but not by mutated nonradioactive oligonucleotide mut1 (lane 4).

The finding that Six3 binds to its own promoter and that Six3 interacts with members of the Grg family of transcriptional corepressors suggested that Six3 may autorepress its own transcription by interacting with Grg proteins during murine embryonic development. The corepressor activity of different Groucho-family members, including Grg5 (Ren et al., 1999), has been widely demonstrated (Fisher and Caudy, 1998). To confirm this result, we transfected NIH3T3 cells with a Gal4 UAS reporter gene (Gal4 UAS-TK-CAT) and a Gal4-DB-Grg 5 fusion gene construct. When 1 μg of Gal4 UAS-TK-CAT reporter gene was transfected into mouse fibroblast NIH3T3 cells, high levels of CAT reporter activity were measured as a consequence of the constitutively active thymidine kinase (TK) promoter activity (Fig. 7A). This CAT baseline activity was repressed up to 80% of the control value in the presence of Gal4 DB-Grg5 (Fig. 7A). As a control, co-transfection of the Gal4-DB expression vector with the CAT reporter plasmid did not repress CAT activity (Fig. 7A). On the contrary, co-transfections of a construct containing Gal4-DB and VP16-fusion gene (Gal4-DB-VP16) together with the CAT reporter plasmid, resulted in a 60-fold activation of the reporter gene (data not shown). Altogether, these results demonstrated that Grg5 functions as a repressor when it is tethered to DNA, and that its repression activity resides in the Q domain (Gal4-DB-Grg5Q) (Fig. 7A).

In order to elucidate whether Six3 can repress its own promoter activity through its interaction with Grg proteins, similar DNA transfection and reporter gene assay were used in NIH3T3, human 293T kidney cells and human Y79 retinoblastoma cell lines. A Six3pro-luc reporter plasmid was constructed by inserting the 1.36 kb mouse *Six3* genomic fragment including the three clustered Six3 recognition sequences, a TATA box and the transcription start site of the Six3 promoter (Fig. 6A) upstream of a luciferase reporter gene in the pG5-luc vector. *Six3* promoter activity in these cell lines was demonstrated by the activation of the luciferase reporter gene (Fig. 7B). Co-transfection with 0.1 μg of CMV-Six3 (Six3) plasmid resulted in almost 60% repression of the reporter (Fig. 7B), whereas co-transfection of the same amount of either CMV empty vector or of CMV-Grg5 (Grg5) did not

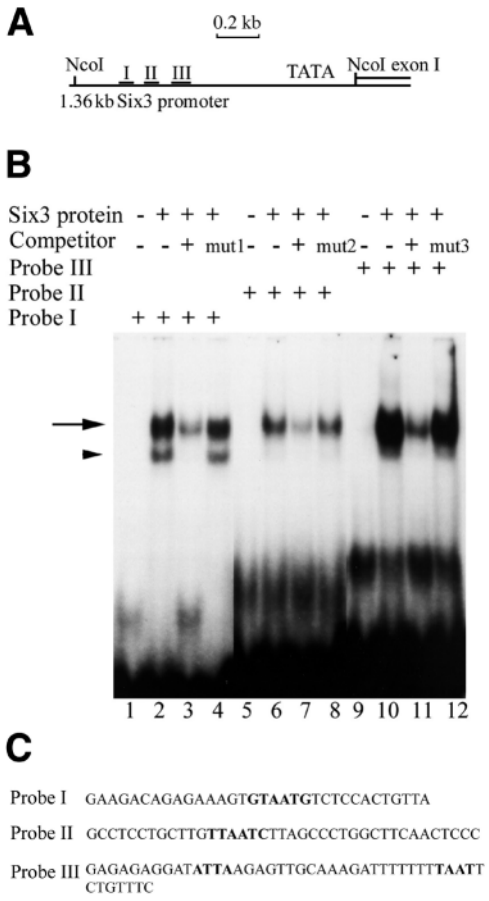

Fig. 6. Six3 binds to its own promoter. (A) The Six3 promoter region (GenBank Accession Number, AD487887). The putative Six3 recognition sequences identified in that genomic fragment are labeled I, II and III. (B) Bacterially expressed Six3 protein (full length) and ^{32}P-labeled DNA probes I, II and III were subjected to an electrophoretic mobility shift assay (EMSA). The upper band containing the Six3-bound DNA fragment is indicated (arrow). The binding specificity was determined by the ability of the complex to be competed by nonradioactive wild-type oligonucleotides (lanes 3, 7 and 11), but not by nonradioactive mutated oligonucleotides (lanes 4, 8 and 12). The lower band seen in lanes 2 and 4 (arrowhead) probably represents truncated form of Six3 protein bound to the probe. This band was also efficiently competed by the cold wild-type probe but not by the mutated form. (C) The sense strand sequences of the oligonucleotide probes I, II and III used in the EMSA are represented. The core sequence motif ATTA of probes I, II and III was mutated into AGCA in the mutated oligonucleotide probes mut1, mut2 and mut3.

show repression activity (Fig. 7B). However, co-transfection of 0.1 μg CMV-Grg5 together with 0.1 μg of CMV-Six3 enhanced the repression activity of Six3, resulting in almost 80% repression of the reporter activity (Fig. 7B). The repression activity measured for CMV-Six3 in the absence of overexpressed CMV-Grg5 could be due to the endogenous presence of Grg5, as determined by RT-PCR assay (data not shown), or of others as yet unidentified proteins in NIH3T3 cells. To show that the repression of the reporter gene is mediated through the Six3 recognition sequences identified in the *Six3* promoter region, a new reporter gene (ΔSix3pro-luc) was constructed by deleting the region containing the three Six3 recognition sequences. As shown in Fig. 7B, Six3 did not repress ΔSix3pro-luc reporter activity.

To map the repression domain of Six3, two additional constructs were generated by fusing the Gal4-DB with different regions of Six3; Gal4-DB-Six3N, encoding the Gal4-DB fused to amino acids 1 through 183 of Six3, and Gal4-DB-Six3C, which encoded the Gal4-DB fused to amino acids 184 through 333 of Six3 lacking the Grg5 interacting domain. NIH3T3 cells were transfected with 1 μg of a luciferase reporter gene (Gal4 UAS-TK-luc). As shown in Fig. 7C, co-transfection of either 0.1 μg of Gal4-DB-Six3N or 0.1 μg of Gal4-DB-Six3C was able to repress up to 50% of the reporter basal activity (Fig. 7C). However, while the Gal4-DB-Six3N, which harbors the Grg-interacting domain, responded to the presence of Grg5, the Gal4-DB-Six3C did not (Fig. 7C).

Mouse Grg4 has also been reported to function as a transcriptional co-repressor (Eberhard et al., 2000), and it was also shown to be expressed in the forebrain region (Koop et al., 1996), a pattern of expression similar to that of *Six3* (Oliver et al., 1995a). To determine whether Grg4 could also enhance Six3 repression activity, a similar co-transfection experiment was performed by using Grg4 and Six3. As expected, Six3 alone repressed transcription of the reporter gene up to 50% of the control value (Fig. 7D). Co-transfection with Grg4 resulted in further repression of the reporter activity (75%); Grg4 alone had no repression effect (Fig. 7D). In order to confirm that the repression activity measured for Six3 is mediated through its interaction with Groucho-related proteins, we included in the co-transfection assays the Six3$_{F88E}$ construct encoding the mutated version of Six3 unable to interact with Grg proteins. This mutated version of Six3 was not able to repress the activity of the reporter gene, or to respond to Grg4 (Fig. 7D). Taken together, our data suggest that Six3 is able to autorepress its own promoter activity, and that this repression function is mediated or enhanced through its interaction with members of the Grg family of co-repressors.

Six3 interaction with Grg proteins is functionally relevant during mammalian retina development

We have previously determined *Six3* mRNA expression in the developing retina and lens (Oliver et al., 1995a). To verify whether Six3 expression is maintained during later stages of retinal development, immunostaining of retinal sections was performed using a specific Six3 antibody (Lagutin et al., 2001). This analysis was carried out at five different stages of retina development (E14.5, E17.5, P0, P6 and adult). Similar to what was previously reported for the *Six3* mRNA (Oliver et al., 1995a), as early as E14.5, high levels of Six3 protein accumulate in the nuclei of a subset of cells in the inner neuroblastic layer (inbl) (Fig. 8A-C). The inner neuroblastic layer at this stage of development contains newly postmitotic cells that are differentiating to become amacrine and ganglion cells. Lower levels of Six3 expression were also detected in the nuclei of a subset of cells in the outer neuroblastic layer (onbl) (Fig. 8A-C). The onbl contains mitotic progenitor cells. A similar expression pattern was observed at later stages of development (E17.5, P0, P6) with high levels of Six3 protein in the nuclei of newly differentiating amacrine and ganglion cells, and lower levels of expression in a subset of mitotic progenitor cells (Fig. 8D-L). High levels of Six3 protein persist in the nuclei of a subset of amacrine cells in the adult retina

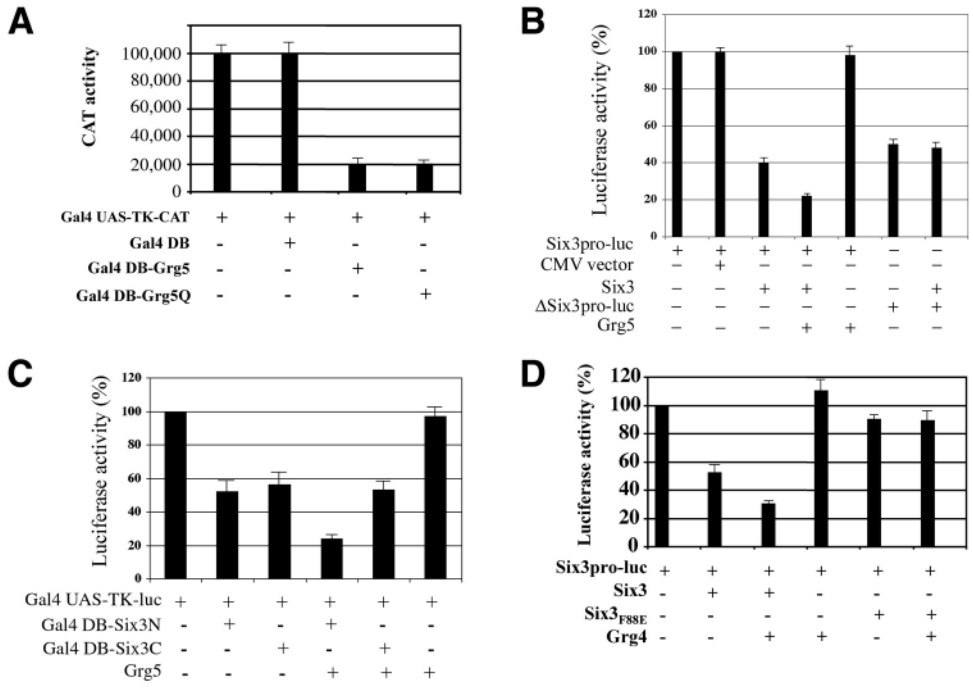

Fig. 7. Grg5 and Grg4 mediate Six3 autorepression. (A) The expression vector Gal4-DB has no effect on the Gal4 UAS-TK-CAT reporter gene, but co-transfection of Gal4-DB-Grg5 fusion gene expression plasmid (Gal4 DB-Grg5) resulted in about 80% repression of the basal activity of the CAT reporter gene. Similar repression activity was observed when using a construct containing the Gal4-DB fused to the Grg5 Q domain (Gal4 DB-Grg5Q). (B) Co-transfection of a CMV-based Six3 expression plasmid (Six3), together with the Six3pro-luc reporter gene into NIH3T3 cells led to about 60% repression of the activity of the luciferase reporter, whereas transfections using the CMV expression vector alone showed no repression of the activity of the reporter gene. Co-transfection of the CMV-based Grg5 (Grg5) and Six3 expression plasmids increased the Six3-mediated transcriptional repression to about 80%, while the use of the Grg5 expression plasmid alone had no effect on the activity of the reporter gene. No repression by Six3 was observed when co-transfecting the Six3 expression plasmid together with the ΔSix3pro-luc reporter gene in which the identified Six3 DNA recognition motifs I, II and III were removed. (C) Co-transfection of Gal4-DNA-binding domain (BD)-Six3$_{1\text{-}183}$ fusion gene expression plasmid (Gal4-DB-Six3N) with the Gal4 UAS-TK-luciferase reporter plasmid (Gal4 UAS-TK-luc) resulted in about 50% repression of the reporter activity in NIH3T3 cells. The Gal4-DNA binding domain and Six3$_{184\text{-}333}$ fusion gene expression construct (Gal4-DB-Six3C) had a similar repression effect on the reporter gene activity; however, only the plasmid Gal4-DB-Six3N containing the identified Grg-interacting domain was responsive to co-transfected Grg5 and repressed the activity of the reporter gene. The Gal4-DB-Six3C that did not include the Grg-interacting domain was not responsive to Grg5. (D) Grg4 enhances Six3-mediated autorepression in NIH3T3 cells. Co-transfection of Grg4 and Six3 expression constructs together with the Six3pro-luc reporter increased the repression activity of Six3. Unlike wild-type Six3, the construct containing the mutated Six3$_{F88E}$ in which interaction with Grg proteins was abolished, failed to repress Six3 promoter activity, and did not respond to Grg4.

(Fig. 8M,N) as determined by Pax6 colocalization (data not shown). In addition, lower levels of Six3 protein were detected in mature horizontal cells (Fig. 8M,N), as measured by calbindin colocalization (data not shown). Surprisingly, we also detected Six3 immunoreactivity in the cytoplasm of photoreceptors in the outer nuclear layer as indicated by the punctate pattern of staining seen in Fig. 8O. This expression pattern is consistent with the faint X-gal staining detected in photoreceptors of postnatal retina isolated from a generated *Six3* β-galactosidase knock-in mouse strain (O. V. L. and G. O., unpublished).

To determine whether the identified interaction between Six3 and Grg proteins was required during retina cell type differentiation, in vivo lineage analysis performed using three different replication incompetent retroviruses was performed. For these studies, we used a retroviral vector (pLIA-E) encoding the human placental alkaline phosphatase (PLAP) that allowed us to identify the morphology of the infected neurons and glia in the retina (Cepko, 1997). Retroviral stocks were generated from the plasmids containing the full-length *Six3* cDNA as well as the mutated *Six3*$_{F88E}$ (Fig. 9A) and injected into the left eyes of newborn (P0) rats. LIA-E (Fig. 9A) was injected into the contralateral eye to serve as an internal control. Three weeks later, the retinae were isolated, stained for PLAP expression, sectioned and clones of cells derived from individual retinal progenitor cells were reconstructed from adjacent sections. Analysis of over 350 clones revealed that the expression of Six3 interfered with normal photoreceptor differentiation; however, that of Six3$_{F88E}$ showed no effect on this process. Normally, rod photoreceptors have a cell body in the outer nuclear layer, a single process that extends toward the apical surface and connects to the outer-segment (Fig. 9B). Rod photoreceptors also send a single process toward the basal surface that ends in a terminus at the outer plexiform layer (opl). The rod termini displayed a characteristic morphology in Six3-infected clones (Fig. 9C); several clones (55/116, 44%) contained rod photoreceptors that lacked outer-segments (Fig. 9C and Table 1) and addition, these same cells had disrupted termini (Fig. 9C). Instead, when LIA-E or LIA-E^{F88E} were injected into the eyes of newborn rats, all of the rod photoreceptors exhibited well-formed outer-segments and normal termini (Fig. 9B and Table 1). Although the morphology of the photoreceptors was normal following Six3$_{F88E}$ expression, specific changes in the distribution of cell types among these clones were observed when compared with the retina infected with the control retrovirus LIA-E (Table 1). Nearly all of the clones (146/151, 97%) expressing Six3$_{F88E}$ contained only rods. This number was significantly higher than the one of the control retrovirus (LIA-E) in the contralateral eye (99/124, 79%, $P<0.05$). This increase in the proportion of clones containing only rods came at the expense of clones containing bipolar cells and Müller glia (Table 1). Clones of

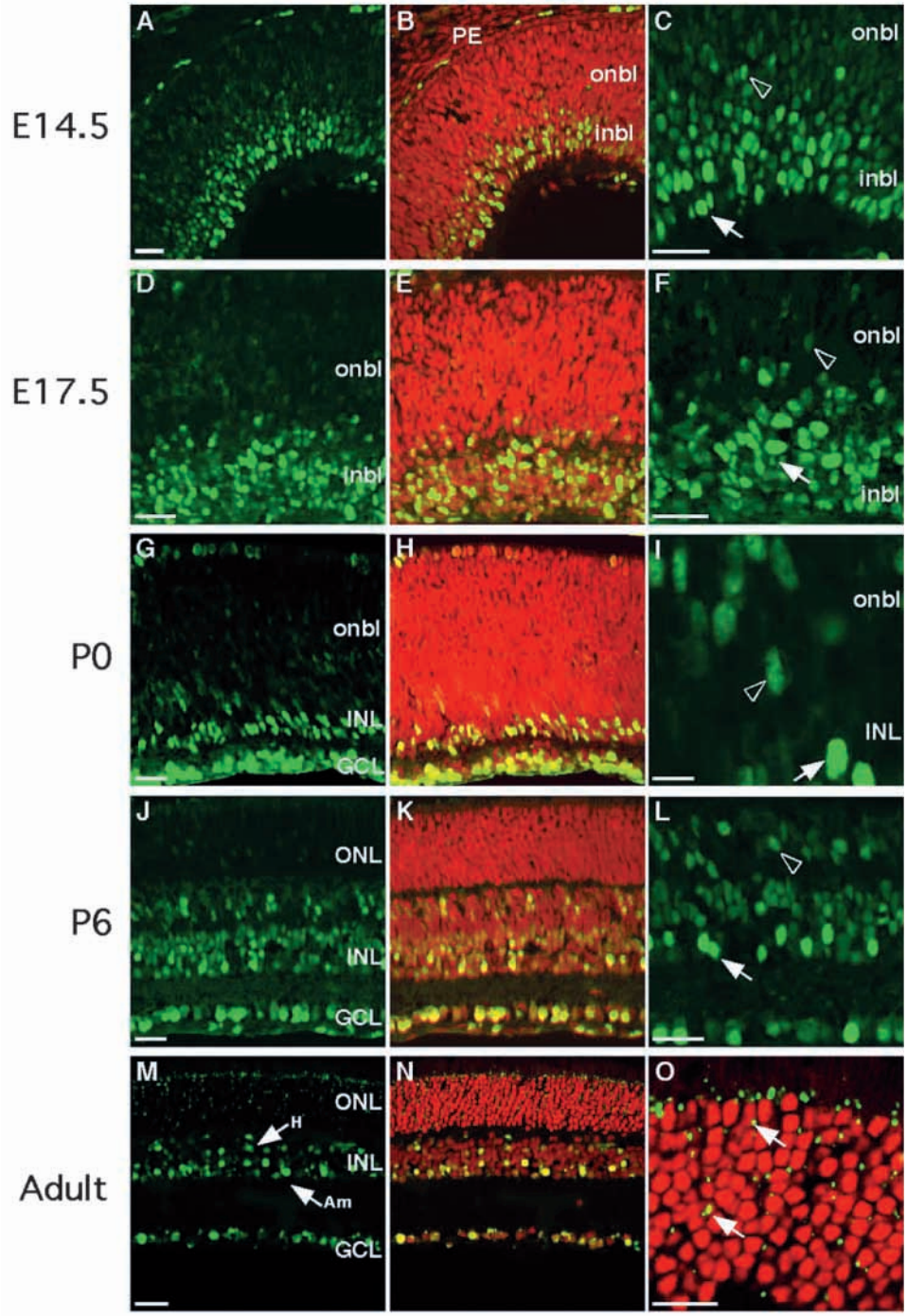

Fig. 8. Six3 expression in the developing mouse retina. (A-C) Six3 is expressed in the murine embryonic retina. Immunostaining of a cryosection of E14.5 retina revealed Six3 nuclear staining in the inner neuroblastic layer (inbl), where newly postmitotic cells are differentiating (arrow), and in the outer neuroblastic layer (onbl), containing mitotic progenitor cells (open arrowhead). The expression in onbl cells is generally lower compared with that in inbl cells at this stage. A similar pattern of expression was detected at E17.5 (D-F), P0 (G-I) and P6 (J-L). Immunostaining of a 3-week-old mouse retina (M-O) revealed nuclear staining in the inner nuclear layer (INL) and ganglion cell layer (GCL) (M, arrows) and a punctuated pattern in the photoreceptors found in the outer nuclear layer (ONL) (O, arrows). PE, pigmented epithelium; H, horizontal cell; Am, amacrine cell. Scale bars: 25 μm (low magnification) and 10 μm (high magnification) in A-H,J-O; 10 μm in I.

cells expressing Six3 also had fewer bipolar cells but they had the expected proportion of Müller glia (Table 1).

These results suggested that *Six3* plays a functional role during mammalian retinogenesis and that this activity is mediated through its interaction with Groucho family members.

Lens morphogenesis requires Six3-Grg interactions

Early in development, *Six3* is expressed in the lens placode and in lens epithelium in more matured lenses (Oliver et al., 1995a; Bovolenta et al., 1998). To determine some of the functional roles of *Six3* in the lens, we electroporated the head ectoderm of stage 10 chicken embryos, a stage when lens induction is initiated, with plasmids expressing mouse *Six3* cDNA and GFP. Electroporated cells were traced by the expression of GFP and morphological development of the lenses was assessed by in situ hybridization using probes for δ-crystallin. Each experimental group comprised six individual embryos receiving the same plasmids; no differences were observed within the group.

Electroporation of *Six3* cDNA resulted in seriously perturbed lens morphogenesis. As determined by whole mount in situ hybridization, the shape of lens containing the δ-crystallin-expressing cells was irregular and contained isolated groups of δ-crystallin-expressing cells (Fig. 10B, top). Close inspection of these lenses revealed the presence of δ-crystallin-negative areas inside the lens cell mass (Fig. 10B, top, inset). Comparison with the distribution of GFP fluorescence indicated that those areas of δ-crystallin-negative cells corresponded with regions in which high levels of exogenous gene expression were accomplished. Histological sections of the electroporated embryos not only confirmed this finding, but also demonstrated that the invagination of the lens placode was strongly inhibited by the overexpression of Six3, a result that was never observed after electroporation with insert-free vectors (Fig. 10A). Both, the δ-crystallin-expressing cells and δ-crystallin-negative cells were found in contact within the placodal cell sheet and beside the retina tissue (Fig. 10B). The regions of the placode without δ-crystallin expression were

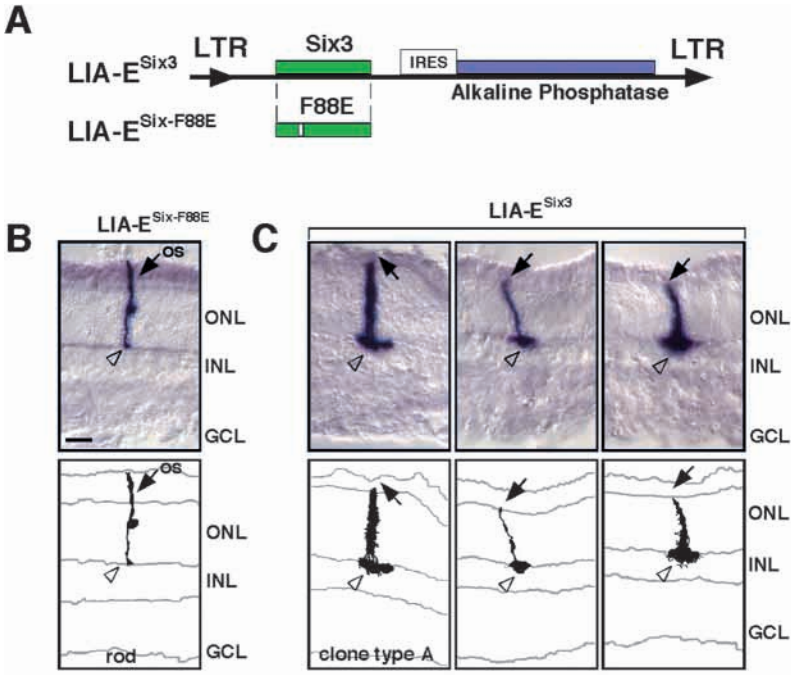

Fig. 9. Overexpression of Six3 and Six3$_{F88E}$ in the postnatal retina. (A) Postnatal day 0 (P0) retinal progenitor cells were infected with replication incompetent retroviral vectors carrying one of two different forms of the *Six3* cDNA upstream of an internal ribosome entry site (IRES) and a human placental alkaline phosphatase reporter gene. LIA-E^{Six3} contains the full-length mouse *Six3* cDNA. LIA-E^{SixF88E} contains the full-length *Six3* cDNA with the single amino acid substitution (F to E) at position 88. Each retroviral stock (0.5 μl) (LIA-E, LIA-E^{Six3} and LIA-E^{SixF88E}) was injected into the eyes of newborn rats. Three weeks later, the retinae were harvested, stained for alkaline phosphatase expression and sectioned. Clones of cells derived from individual retinal progenitor cells were scored for cell number and cell composition. (B) Normal morphology of photoreceptor cells in LIA-E^{SixF88E}-infected cells. (C) When the Six3 protein was overexpressed in the developing retinal progenitor cells, nearly 50% of the clones (see Table 1) exhibited an altered photoreceptor phenotype. For simplicity, we have designated this 'Clone Type A'. Processes were found in the outer nuclear layer similar to rod photoreceptor processes but the outer segments were absent (arrow) and the termini normally associated with rod photoreceptors were malformed (open arrowhead). The cell bodies in these clones tend to lie at the outer nuclear layer/inner nuclear layer boundary. OS, outer segment; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar: 25 μm.

thinner than those expressing δ-crystallin. Thus, δ-crystallin-expressing cells and δ-crystallin-negative cells segregated each other within the lens placode suggesting that differential cell adhesiveness between these cell populations. Co-electroporation of Grg5 with Six3 caused effects similar to those observed with Six3 alone (Fig. 10C). As shown in Fig. 4B, endogenous *Grg5* is expressed in the lens epithelial cells and this may be sufficient to mediate *Six3* function during lens development.

Inhibition of lens placode invagination, and presence of δ-crystallin-negative cells in the lens placode were not observed when similar experiments were carried out using the mutated version of *Six3* cDNA that carries the F88E amino acid mutation (Fig. 10D). Lens invagination proceeded comparable to the non-electroporated side of the same embryo and all lens cells expressed *δ-crystallin*.

These results indicated that *Six3* has important functional roles in lens mophogenesis and crystallin regulation, which are at least partially mediated by its interaction with the Groucho family of corepressors.

DISCUSSION

In this report we have demonstrated that members of the Groucho family of transcriptional corepressors interact with mouse Six3, that upon this interaction Six3 functions as a transcriptional repressor, and that this interaction is of biological relevance during retinal and lens morphogenesis. Furthermore, we have also shown that Six3 binds to its own promoter and negatively autoregulates its transcription. These findings provide additional insight regarding the molecular mechanisms by which Six3 functions during vertebrate embryonic development.

Table 1. In vivo lineage analysis using Six3 retroviruses

Retrovirus	Rod only*	Bipolar†	Müller‡	Amacrine	Clone A§	UINL	UONL
LIA-E	99/124 (79%)	16/124 (13%)	8/124 (6%)	1/124 (0.8%)	0/124 (0%)	0/124 (0%)	0/124 (0%)
LIA-E^{six3}	43/116 (37%)	2/116 (1.7%)	5/116 (4.3%)	1/116 (0.8%)	55/116 (47%)	3/116 (2.5%)	7/116 (6%)
LIA-E$^{Six-F88E}$	146/151 (97%)	2/151 (1.3%)	1/151 (0.7%)	0/151 (0%)	0/151 (0%)	2/151 (1.3%)	0/151 (0%)

*All data are presented as the number of clones within a given category (e.g. clones containing only rods) over the total number of clones scored, and their resulting ratio expressed as a percentage of the total clones. Rod photoreceptors are found in clones containing other cell types (bipolar cells for example), but for simplicity the clones containing only rods are presented in this column.

†All clones that contain a bipolar cell are presented here. This includes clones that contain just a single bipolar cell and those that also contain rod photoreceptors.

‡Owing to the extensive processes of Müller glia, it is difficult to identify other cell types in these clones. Therefore, this category represents clones that contain at least one Müller glial cell.

§This clone type exhibited a reproducible morphology (see Fig. 9C) that was characterized by processes in the outer nuclear layer, the absence of outer segments and processes spanning the outer plexiform layer. The cell body or cell bodies in these clones often resided at the outer plexiform layer. These clones may represent rod photoreceptors that could not appropriately differentiate.

UINL, unidentified inner nuclear layer cell; UONL, unidentified outer nuclear layer cell.

The interaction between members of the Six3 subfamily and Grg family members is evolutionarily conserved

In *Drosophila*, So and Dachshund (Dac) are capable of synergizing with eyes absent (eya) to promote ectopic eye formation; the proteins encoded by these three genes can form molecular complexes with one another (Pignoni et al., 1997; Shen and Mardon, 1997). Interestingly, the combination of transcriptional regulators required for eye formation in the fly (Eya, Ey, Dac and So) is also required for the genesis of other tissues during vertebrate embryonic development (e.g. Dach2, Pax3, Eya2 and Six1 are required for the formation of the somite and its skeletal muscle derivatives) (Heanue et al., 1999). It is important to mention that the proteins encoded by Six1, Six2, Six4 and Six5 not only share sequence similarity with one another but also interact with members of the Eya family (Ohto et al., 1999). Interestingly, this latter characteristic is not shared by Six3 (Ohto et al., 1999) (C. C. Z. and G. O., unpublished), a finding that suggests that at the functional level, this protein may differ from the rest of the family members.

We determined that members of the Groucho-related family of transcriptional corepressors interact strongly with Six3 and Six6; however, despite the similarities in the eh1-like motif identified in the Six/So family members, we found that the interactions of these family members with Grg proteins vary. On the basis of our work, we conclude that in case Grg proteins do interact with members of the other Six subfamilies (Six1 and Six4), then this interaction is rather weak and therefore was not detected in our experimental conditions. In fact, results of our GST pull-down experiments and yeast two-hybrid analyses failed to detect any specific interaction between Grg5 and Grg4 with Six2 and Six4. This result is in contrast with that reported by Kobayashi et al. (Kobayashi et al., 2001), who showed that all members of the Six family in zebrafish interacted with zebrafish Grg3. This discrepancy could be due to differences in the yeast two-hybrid systems used by Kobayashi et al. and us. Differences between the physical interactions of the Six proteins and the Grg family of corepressors can directly contribute to differences in their transcriptional properties. In our tissue culture experiments, we demonstrated that through their interaction with members of the Grg family of corepressors, Six3 and Six6 become strong transcriptional repressors. In addition, the ability of Six3 and Six6 to repress transcription largely depends on protein-protein interactions with Grg members; in Six3, replacement of the conserved phenylalanine at position 88 with glutamic acid prevented the interaction and eliminated the transcriptional repression activity of Six3. By overexpressing the Xoptx2-Engrailed chimeric repressor (*Xenopus* Six6), Zuber et al. (Zuber et al., 1999) demonstrated that Xoptx2 can function as a transcriptional repressor in *Xenopus* embryos. Using a similar approach, Kobayashi et al. (Kobayashi et al., 2001) showed that zebrafish Six3 acts as a transcriptional repressor in zebrafish embryos. We have demonstrated that mouse Six3 can bind its own promoter and negatively autoregulate its transcription through interaction with members of the Grg family. A similar transcriptional feedback loop was also identified for the homeobox gene *goosecoid* (Danilov et al., 1998). This Six3 autorepression activity probably reflects a direct feedback loop of Six3 regulation that operates only in certain tissues, during certain embryonic stages, or both. Loosli et al. (Loosli et al., 1999) have shown that injected mouse *Six3* mRNA induces ectopic expression of endogenous medaka *Six3*, a finding that

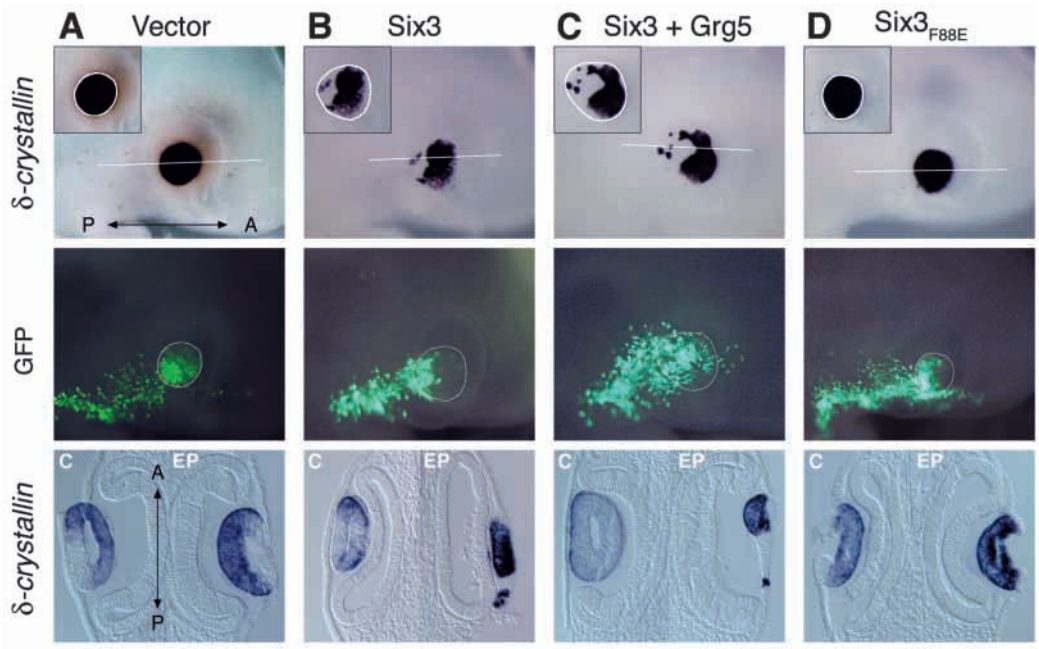

Fig. 10. Overexpression of Six3, but not of $Six3_{F88E}$ disrupts lens morphogenesis. Stage 10 chicken embryos were electroporated in ovo with the expression vectors indicated, together with a GFP expression vector around the head ectoderm. Twenty-four hours later, embryos were photographed for GFP fluorescence (second row), then fixed and hybridized for δ-crystallin mRNA (top row) and sectioned along the planes indicated in the top row (EP, third row). The shape of the lens tissue is demarcated by a white line for clarity. In the third row, the non-electroporated control side (left) of the same embryo is shown for reference, which usually bears lower hybridization signals because the side faced the bottom of the tubes during the hybridization process. A and P indicate anterior and posterior sides, respectively. (A) Control embryo electroporated with the insert-less expression vector. (B) After electroporation with *Six3*, δ-crystallin-expressing domains and δ-crystallin-negative domains were seen within the lens tissue (indicated by the line in the inset). Sections of the same embryo showed that the invagination of the lens placode was inhibited and confirmed the segregation of δ-crystallin-expressing and non-expressing domains within the placode. (C) Co-electroporation of *Six3* and *Grg5* caused essentially the same effect as *Six3* alone. (D) Electroporation with $Six3_{F88E}$ showed no effect.

suggests that in addition to a direct feedback loop, an indirect *Six3* autoregulation loop also operates during embryogenesis.

Because of the high sequence homology between mouse Grg proteins and *Drosophila* Groucho, we also investigated whether the Six3-Grg interaction is conserved with their *Drosophila* counterparts. We determined that murine Grg5 and Grg4 interact with *Drosophila* optix and So. We also showed that *Drosophila* Groucho interacts with Six3. These findings suggest that *Drosophila* So and optix may interact with Groucho during embryogenesis. *Drosophila* So functions as a transcriptional activator upon interaction with eya (Pignoni et al., 1997). Therefore, if *Drosophila* So interacts with Groucho in vivo, then it is conceivable that fly So can act as either a transcriptional activator or repressor, depending on the cell type and the availability and concentrations of eya or Groucho. Other transcription factors, such as *Drosophila* dorsal and mouse Pax5 act as either activators or repressors, depending on the concentrations of available co-factors (Dubnicoff et al., 1997; Eberhard et al., 2000). Interestingly, *Drosophila* optix does not interact with eya (Seimiya and Gehring, 2000); however, our findings suggest that optix may interact with Groucho to regulate eye development in *Drosophila*. It will be interesting to determine whether optix functions as a transcriptional repressor through its interaction with Groucho during visual system development in *Drosophila*.

The DNA motif bound by Six3 differs from that recognized by other family members

By using an approach involving PCR- and binding-site selection, we determined that Six3 binds to an ATTA core motif in the DNA. Surprisingly, this motif is similar to the classical DNA sequence recognized by homeoproteins, and it differs from the motif previously identified for Six2 and Six4 (Kawakami et al., 1996b). Wilson et al. (Wilson et al., 1993) showed that the *paired* type HD proteins bind either as homodimers or heterodimers to the palindromic sequence TAAT and ATTA, which are normally separated by two or three base pairs. The amino acid residue at position 50 of the HD is crucial for the binding specificity and recognition of the palindrome. A palindrome with a 2 bp spacing was present when serine was at position 50, whereas a 3 bp spacing was identified for HD proteins containing a lysine or glutamine at this position (Treisman et al., 1992). Similar to the vertebrate Otx1, Otx2 and goosecoid, Six3 contains a lysine at position 50. However, the binding site selection and promoter analysis that we used to characterize Six3 indicated that the entire palindromic sequence is not required for the binding of Six3 to DNA; half of the palindrome sequence is sufficient. Tucker and Wisdom (Tucker and Wisdom, 1999) reported that the HD protein Alx4, which also contains a lysine at position 50, not only binds to the palindromic sequence ATTA and TAAT but also binds to TAATC and TAATTT half-sites with high affinity. Although Six3 binds to these half-sites strongly, it may also bind to the whole palindromic DNA sequence.

Taken together, our findings pertaining to the biochemical characteristics of Six3 support the placement of Six3 and Six2/Six4 in two Six/So subfamilies. Six2 and Six4 subfamily members interact with Eya proteins but weakly or not at all with the Grg proteins. Instead, Six3 interacts strongly with Grg but not with Eya proteins. In addition, Six3 binds to a DNA sequence that differs from that bound by Six2 and Six4.

The role of the Groucho/Grg family of corepressors during development

The *Drosophila* Groucho counterparts identified in the mouse (Grg1-Grg5) (Mallo et al., 1993; Koop et al., 1996; Leon and Lobe, 1997) not only have similar amino acid sequences but also have overlapping expression patterns during embryogenesis (Leon and Lobe, 1997; Koop et al., 1996; Molenaar et al., 2000). Therefore, it is not surprising that mice nullizygous for *Grg5* are viable and exhibit only postnatal growth deficiencies (Mallo et al., 1995a). The function of *Grg5* during murine embryonic development can probably be compensated for by other members of this gene family, as suggested by Mallo et al. (Mallo et al., 1995a). This theory is supported by the fact that Grg5 and its related members often interact with the same transcription factors (Choi et al., 1999; Ren et al., 1999; Eberhard et al., 2000) and function as transcriptional corepressors.

In order to begin to address the biological in vivo significance of the identified Six3-Grg interaction, we first showed that Six3 is normally expressed during mouse retina development. We determined that as early as E14.5, high levels of Six3 protein accumulate in the nuclei of a subset of cells in the inner neuroblastic layer containing immature amacrine and ganglion cells. Lower levels of Six3 expression were also detected in the nuclei of a subset of progenitor cells in the outer neuroblastic layer. A similar expression pattern was observed at later stages of development. Interestingly, we also found that in contrast to the nuclear localization of Six3 in cells located in the inner nuclear layer, Six3 also appeared to be expressed in the cytoplasm of photoreceptors in the outer nuclear layer. As shown by Baas et al. (Baas et al., 2000), the subcellular localization of the homeodomain protein Otx2 is cell type specific and developmentally regulated in the mouse retina; in the postnatal eye, both the cellular and subcellular distribution of the Otx2 protein are cell type specific and it is present in the cytoplasm of rod photoreceptors. Therefore, it could be possible that something similar happens with Six3. However, further studies are still required to confirm that the observed punctate staining indeed corresponds to Six3 protein, and, if so, to determine whether in this cell type Six3 is localized in the cell body or the processes of photoreceptors.

Misexpression of wild-type Six3 using replication incompetent retroviruses resulted in a large number of rod photoreceptor clones (56%) that failed to differentiate properly. They lacked outer segments and exhibited defective rod photoreceptor termini. This type of clones were not observed when using the mutated form of Six3 ($Six3^{F88E}$) that cannot interact with Grg5, or the control (LIA-E) retrovirus. Misexpression of both Six3 and $Six3^{F88E}$ resulted in a reduction of the proportion of bipolar-containing clones but only $Six3^{F88E}$ reduced the proportion of Müller glia-containing clones. According to these results, the observed reduction of bipolar-containing clones does not require Six3-Grg interaction; however, the alterations on Müller glial cell fate specification and rod photoreceptor differentiation are dependent on this interaction. It is possible that additional alterations in other retinal cell types could also be observed in similar type of experiments performed prenatally. Our initial

studies suggest that the interaction between Six3 and Groucho family members is biologically relevant in the developing retina for the specification and differentiation of certain cell types. Detailed characterization of a generated Six3 knockout mouse strain (O. V. L. and G. O., unpublished) will be instrumental in the further pursuit of this functional characterization of Six3.

During lens development, *Six3* is expressed in the lens placode and in the epithelial cells of the lens (Oliver et al., 1995a; Bovolenta et al., 1998). Overexpression of Six3 in the lens-forming region of the head ectoderm at the stage of lens induction resulted in the inhibition of lens placode invagination and persistence of the morphologically placodal state. This result may imply that *Six3* activity is required during lens morphogenesis.

In addition to inhibit placode invagination, higher expression of *Six3* resulted in repression of δ-crystallin expression. It could be possible that this repression is directly mediated by the interaction of Six3 with an ATTA-core motif present in the δ-crystallin enhancer. Interestingly, we also found that δ-crystallin-expressing cells and negative cells do not mix and they segregate each other within the lens placode. This finding suggests that these cells differ in their cell adhesion properties and that *Six3* could also be involved in this morphogenetic regulation. All these *Six3*-dependent effects were lost when we used a mutated form of Six3 that abolished interaction with Grg proteins. This result confirmed that the interaction with the Groucho family of corepressors is essential for various regulatory activities of Six3 during lens development.

In summary, we explored the biological significance of the interaction between Six3 and Grg in tissue culture and in vivo by using retroviral infection and in ovo electroporation experiments. Our results indicate that the Grg-Six3 interaction is important for Six3-mediated in vivo transcriptional activities, at least during retina and lens development.

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