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A developmentally regulated basic–leucine zipper–like gene and its expression in embryonic retina and lens

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ABSTRACT **Transcriptional regulators play important roles in the control of key developmental events. We have identified *sw3-3*, a likely candidate for such a function, in tissues of the eye and other neural organs. It encodes a basic–leucine zipper-like protein, in which two leucine zipper motifs flank a basic domain. The latter contains helix-disturbing amino acids such as glycine and proline, at positions occupied by conserved asparagine and alanine residues (respectively) in "conventional" basic–leucine zipper proteins. *sw3-3* is widely expressed at early embryonic stages in the lens, retina, and other neural tissues and is down-regulated thereafter with a spatial and temporal pattern that correlates with the cessation of mitotic activity and the onset of cell migration and differentiation.**

Development of the vertebrate retina involves the transformation of the simple, apparently homogeneous neuroepithelium present at early embryonic stages into the adult tissue consisting of several different cell types distributed in an orderly, stereotyped laminar pattern. The developmental history of each differentiated neuronal cell in the adult retina includes a series of mitotic divisions, a terminal mitosis (which defines the time of "cell birth"), cell migration to one of the retinal layers, and expression of cell type-specific differentiated properties (1, 2). Considerable progress has been made in recent years in the investigation of these developmental transitions, but the underlying molecular mechanisms remain poorly understood.

Transcriptional factors are gaining increasing recognition as molecules likely to play a fundamental role in the regulation of cell differentiation and other important events in neural development (3–5). Members of several of the 12 families of transcription factors described so far (3) have been found to be expressed in the embryonic retina and neural tube in a developmentally regulated manner (6–14). Further studies appear necessary to elucidate the functional significance of these factors and to identify others that may also be involved as positive or negative regulators in the development of the retina and other neural organs.

The tissue that we have chosen for the investigation of these issues is the embryonic chicken neural retina. Its development has been thoroughly described (15), and adequate *in vitro* systems are available for its experimental analysis (16, 17). We report here the characterization of *sw3-3*,† a gene that encodes an additional member of the basic–leucine zipper (b-zip) family of transcription factors, characterized by the presence of a basic domain and a heptad repeat of leucine over a region of 30–40 residues (18–21). Sw3-3 shows several distinct molecular features, including the presence of *two* leucine zipper motifs that flank the basic domain and the presence in the latter of helix-disturbing amino acids. Its temporal and spacial pattern of expression suggests that *sw3-3* may be involved in the transition from cell proliferation to cell differentiation not only in the retina but also in the lens and in other regions of the central nervous system.

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MATERIALS AND METHODS

Synthesis of First-Strand cDNAs. White Leghorn chicken embryo retinas were dissected free of other eye tissues at different developmental stages. Total retinal RNA was isolated by using the acid/guanidinium thiocyanate/phenol/chloroform method of Chomczynski and Sacchi (22). First-strand cDNA synthesis was primed with RACE 1 oligonucleotide ($GATGGATCCTGCAGAAGCT_{17}$) and catalyzed by Moloney murine leukemia virus reverse transcriptase. Aliquots of 11 μl of total RNA were heated at 65°C for 5 min, chilled on ice, and mixed with 8 μl of 5× reverse transcription buffer (BRL), 16 μl of 2.5 mM dNTPs, 2 μl of 0.1 M dithiothreitol, 0.5 μl of RNasin (40 units/μl; Promega), 0.2 μl of bovine serum albumin (20 mg/ml), 2 μl of Moloney murine leukemia virus reverse transcriptase (200 units/μl; BRL), and 1 μl of RACE 1 (100 ng/μl). The reaction was incubated at 41°C for 2 hr and then diluted with 0.5–1 ml of 10 mM Tris/1 mM EDTA, pH 7.5.

PCR. First-strand cDNAs from different stages of chicken embryo retinal development were used as templates for PCR screening of developmentally regulated genes. The oligonucleotides used during the initial PCR that led to the discovery of *sw3-3* were RACE 2 (similar to RACE 1 except that it lacks the oligo(T) tail; i.e., GATGGATCCTGCAGAAGC) and a degenerate "POU" oligonucleotide [AATGCGAATTCA-(A/G)(A/T)(C/G)NACNAT(A/C/T)(A/T)(C/G)N(A/C) GNTT(C/T)GA] (23). The PCR thermal profile used with these nonhomologous oligonucleotides was 1 cycle of 3 min at 95°C; 5 cycles of 1 min at 37°C, 3 min at 72°C, and 1 min at 94°C; 30 cycles of 1 min at 55°C, 3 min at 72°C, and 1 min at 94°C; a final annealing of 1 min at 55°C; and extension at 72°C for 5 min. Cloning and sequencing the PCR products generated by this reaction led to the discovery of *sw3-3* (see *Results*). A pair of homologous primers was then synthesized based on the *sw3-3* sequence corresponding to amino acids 42–48 and 338–344 (see Fig. 1). These oligonucleotides were used for a new round of PCR amplifications using chicken embryo retina cDNAs as templates. The PCR thermal profile was 1 cycle of 3 min at 95°C; either 25 cycles (*sw3-3*) or 17 cycles (ribosomal protein S17; rps17) of 30 sec at 55°C and 30 sec at 95°C; final annealing of 1 min at 55°C; and extension at 72°C for 5 min. A "hot start" protocol was used for all PCR amplifications.

Northern Hybridization. Total RNA was isolated from chicken embryo at different developmental stages (22). Equal

Abbreviations: b-zip, basic–leucine zipper; E, embryonic day; P, posthatching day; b-HLH, basic–helix–loop–helix; rps17, ribosomal protein S17.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. U04821).

amounts (20 μg) of total RNA were separated on 1% agarose gels in the presence of formaldehyde and transferred to a nylon membrane (DuPont) as described by the manufacturer. ^{32}P-labeled DNA probes were synthesized with gel-purified *sw3-3* (≈1.1 kb) and β-actin (1.9 kb; ref. 24) inserts by random priming using a DNA labeling kit (Boehringer Mannheim). Hybridization was at 68°C in the presence of 6× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0), 2× Denhardt's solution, and 0.5% SDS. Final posthybridization wash was at 65°C with 0.2× SSC/0.1% SDS.

***In Situ* mRNA Hybridization with Digoxigenin-Labeled RNA Probes.** The 1086-bp fragment of *sw3-3* was subcloned into pGEM-3Z and -4Z for the synthesis of digoxigenin-labeled sense and antisense RNA probes using T7 RNA polymerase and the Genius kit (Boehringer Mannheim). Probe concentrations were calculated by dot blots using labeled standards included in the Genius kit.

Chicken embryo tissues were fixed for 1–2 hr at 4°C in 4% paraformaldehyde in phosphate-buffered saline (PBS; 137 mM NaCl/8 mM Na_2HPO_4/2.6 mM KH_2PO_4, pH 7.4). After two PBS washes, tissues were infiltrated overnight with 20% (wt/vol) sucrose in PBS at 4°C, followed by a 2:1 mixture of 20% sucrose and Tissue-Tek OCT (Miles) for 30 min. Specimens were then frozen in liquid nitrogen and stored at −70°C. Cryostat sections, 14 μm in thickness, were mounted on 3-aminopropyltriethoxysilane-coated slides, postfixed for 5 min with 4% paraformaldehyde in PBS, and washed twice with PBS. The sections were then acetylated for 10 min (0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0), washed twice with PBS, and treated with 50% formamide/2× SSC for 30 min at 42°C. They were then overlaid with 25–70 μl of hybridization solution [50% (vol/vol) formamide, 5× SSC, 2% blocking agent (Boehringer Mannheim), heparin at 50 μg/ml, 0.1% Tween 20, yeast tRNA at 250 μg/ml] containing a 1-kb, digoxigenin-labeled *sw3-3* sense or antisense RNA probe (400 ng/ml), synthesized with the Genius RNA labeling kit (Boehringer Mannheim). After covering the sections with a coverslip, hybridization was carried out overnight at 55–60°C in a moist chamber. After floating the coverslips off the slides in 2× SSC, the sections were sequentially washed in 50% formamide in 2× SSC (1 hr at 45°C), 2× SSC (two rinses), RNase A (20 μg/ml in 2× SSC; 30 min at 37°C), PBS (two rinses), and Genius buffer 1 (0.1 M maleic acid, pH 7.5/0.15 M NaCl) plus 0.1% Tween 20 at 45°C for 1 hr. Immunodetection was carried out as recommended by the manufacturer (Boehringer Mannheim).

RESULTS AND DISCUSSION

We synthesized first-strand cDNA by reverse transcription of RNA isolated from chicken retinas at daily intervals between embryonic day 5 (E5) and posthatching day 5 (P5). The cDNAs were used as templates for PCR amplification of candidate genes. We describe a PCR product obtained using a RACE 2 oligonucleotide and a degenerate oligonucleotide corresponding to the POU family of transcription factors (see *Materials and Methods*). After observing that the intensity of PCR products on agarose gels decreased as embryos became older, we cloned them into pUC19 plasmids and sequenced them from both ends. The discovery of *sw3-3* was serendipitous, since it had no sequence homology with POU and was actually amplified with RACE 2 at both its 5′ and 3′ ends.

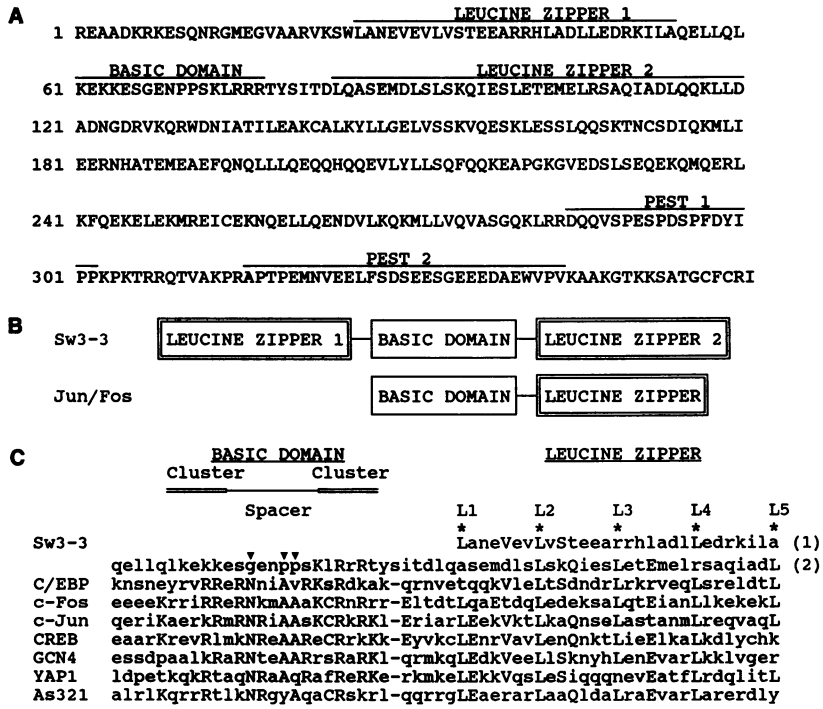

FIG. 1. (*A*) Deduced amino acid sequence corresponding to a 1086-bp fragment of *sw3-3*. The basic domain, the two leucine zippers that flank it, and the two PEST sequences are indicated. (*B*) Schematic illustration of the organization of the basic domain and leucine zipper motifs in Sw3-3 and in Jun/Fos, members of the b-zip family. (*C*) Alignment of the amino acid sequences of the basic and leucine zipper domains of Sw3-3 with those of other b-zip proteins. A dash indicates that a gap was introduced in the sequences to maximize homology. Amino acids that are present in four or more sequences are in uppercase letters. The basic domain of Sw3-3 resembles those of other b-zip proteins in terms of the presence of two clusters of basic amino acid in this region. Note, however, that Sw3-3 contains helix-disturbing residues (glycine and proline) in the spacer between them, at positions occupied by conserved asparagine and alanine in other b-zip proteins (arrowheads). Asterisks L1–L5 indicate the position of leucine residues in canonical leucine zippers. Both leucine zippers in Sw3-3 show replacements of leucine by alanine near the basic domain and by arginine elsewhere. While the latter is not expected to affect significantly the function of the leucine zipper (19, 20), the former could shorten the Sw3-3 leucine zippers, with a concomitant lengthening of the "neck" between the basic domain and the leucine zipper. Note also the many sequence differences between leucine zippers 1 and 2, respectively, located upstream and downstream from the basic domain in Sw3-3. Sequences of C/EBP, c-Fos, c-Jun, CREB, GCN4, and YAP1 are from ref. 24, and the sequence of As321 is from ref. 25.

Excluding the RACE 2 sequences, the PCR-amplified 1086-bp *sw3-3* fragment contained a single open reading frame of 361 amino acid residues (Fig. 1*A*), which appeared to be part of a larger open reading frame (the original message was estimated to be 4.2 kb by Northern analysis, as shown in Fig. 2*B*). That this product is not a cloning artifact or the product of a noncontiguous ligation of two pieces of cDNA was supported by the results of PCR reactions using homologous oligonucleotides (see *Materials and Methods*) with chicken embryo retina cDNAs as templates. In all cases, these reactions yielded a PCR product of the size expected if the cloned *sw3-3* represented part of an individual cDNA (i.e., 909 bp; see Fig. 2*A*). *sw3-3* encodes a b-zip-like protein. Similarities with known members of the b-zip family of transcription factors (18–21) include the presence of a basic domain containing two clusters of basic amino acids separated by a spacer (Fig. 1*C*) and a leucine zipper motif immediately C-terminal to the basic domain (Fig. 1). A unique feature of *sw3-3*, however, is the presence of a *second* leucine zipper motif also adjacent to the basic domain but located on its N-terminal side (Fig. 1). These two leucine zipper motifs in *sw3-3* are similar, but not identical to each other. Another distinctive feature is the presence of helix-disturbing amino acids (one glycine and two proline residues) in the spacer between the two basic amino acid clusters in the basic domain of Sw3-3 (Fig. 1*C*, arrowheads). The corresponding positions are respectively occupied by highly conserved asparagine and alanine residues in "conventional" b-zip proteins (26), which bind DNA through an uninterrupted helix (27). Although proline is usually absent from conventional b-zip proteins, it has been found within the basic domain of some members of the basic helix-loop-helix (b-HLH) family of transcriptional factors such as hairy, enhancer of split, and HES-5 (refs. 9, 28, and 29; see also below). The Sw3-3 open reading frame also contains, near its C-terminal region, two PEST sequences flanked by basic amino acids. PEST sequences are rich in proline (P), glutamic acid (E), serine (S), and threonine (T) and are usually found in proteins with a half-life shorter than 2 hr (30) (Fig. 1*A*). This suggests that fast degradation of Sw3-3 protein may be important for some developmental events (see below). A search of the translated sequences from GenBank revealed no identities with the predicted amino acid sequence, suggesting that it is a previously uncharacterized protein. As shown in Fig. 1*C*, the 59-residue-long b-zip region of Sw3-3 has only 22% sequence identity with c-Jun, 17% with c-Fos, and 15% with c/EBP (24) and 10% with AS321, which resembles v-MAF and was found in the vertebrate retina and is expressed in a number of cell types (25).

The temporal down-regulation of *sw3-3* expression was first observed during the initial PCR screening using nonhomologous primers. This was subsequently verified using first-strand cDNA from chicken embryo retinas at different embryonic stages as templates for PCR reactions primed with homologous oligonucleotides corresponding to the 5′ and 3′ regions of the cloned *sw3-3* product (see *Materials and Methods*). This study showed a dramatic decrease in the intensity of PCR products after E7 (Fig. 2*A*). This decline appeared to reflect a decrease in *sw3-3* mRNA levels, since control PCR products generated from the same cDNA samples with oligonucleotides for rps17 did not show equivalent changes (Fig. 2*A*). The developmental decrease in *sw3-3* mRNA levels was also confirmed by Northern blot analysis using β-actin (24) as the internal standard (Fig. 2*B*).

Digoxigenin-labeled RNA probes were used for *in situ* hybridization of sections of the eye or the entire head. Antisense probes gave strong reddish-brown signals (Fig. 3 *A–E*), which were absent with control sense probes (Fig. 3*F*). In 2-day embryos (Fig. 3*A*), *sw3-3* was broadly expressed in the neuroepithelium of the neural tube and the optic cup; the external layer of the latter (future retinal pigment epithelium) appeared less intensely positive than its internal layer (future

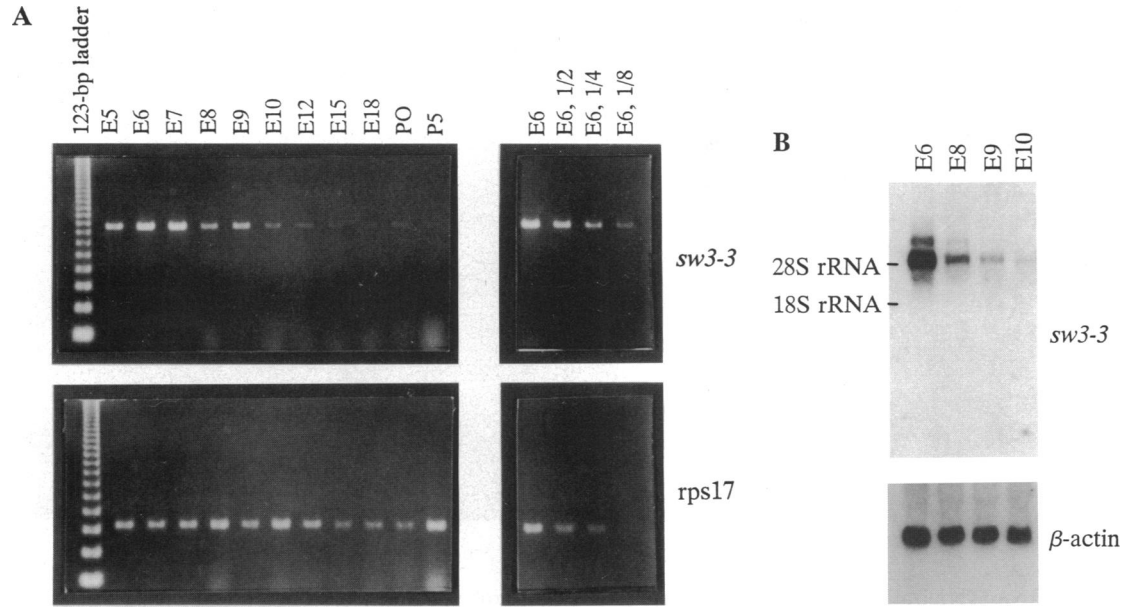

FIG. 2. (*A*) Agarose gel electrophoresis analysis of PCR products generated with first-strand cDNAs from E5 to P5 chicken retinas, using homologous primers for *sw3-3* and for rps17. The size of the *sw3-3* PCR product (909 bp) corresponds in all cases to that predicted based on the *sw3-3* sequence, thus ruling out the possibility that *sw3-3* could represent a cloning artifact. *sw3-3* appears developmentally down-regulated, with a marked decline after E7. No such changes are observed with rps17. RNA isolation, first-strand cDNA synthesis, and PCR conditions and primers are described in *Materials and Methods*. Dilution series of E6 cDNA (up to 8-fold) were included in the reactions to illustrate the changes in band intensity observed as a function of differences in the amount of template cDNA present in the reaction. (*B*) Northern hybridization of RNA isolated from embryonic chicken retinas at different stages, with ^{32}P-labeled *sw3-3* and β-actin probes. Equal amounts (20 μg) of total RNA were loaded in all lanes. The positions of the 28S and 18S ribosomal RNA are shown in the figure. The major band recognized by *sw3-3* probes has an apparent size corresponding to ≈4.2 kb. Minor bands of higher and lower apparent size could correspond to splice variants, multiple initiation sites, or, in the case of the larger band, to heterogeneous nuclear RNA.

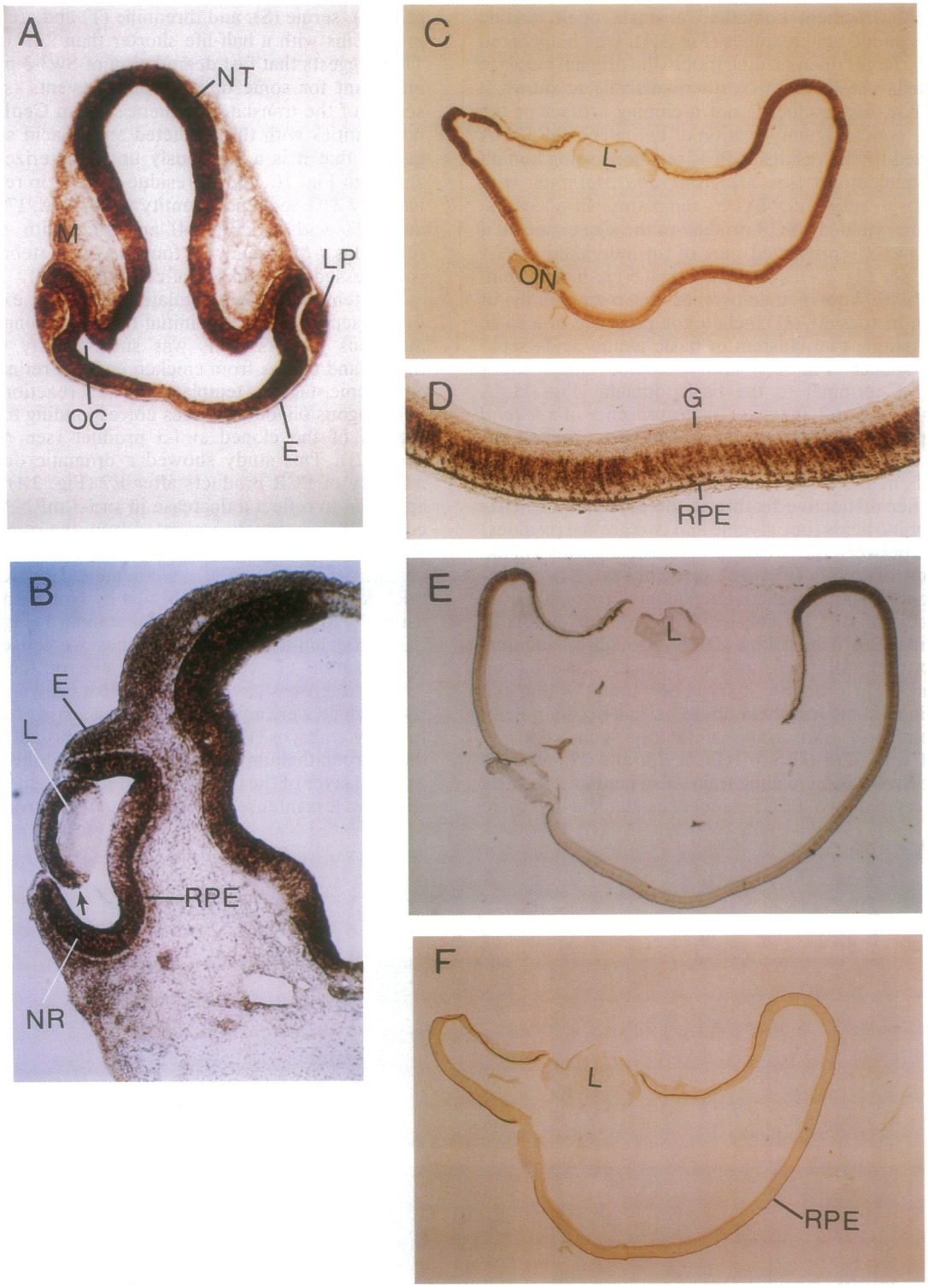

FIG. 3. *In situ* mRNA hybridization with digoxigenin-labeled *sw3-3* RNA probes. (*A*) E2. Note the strong *sw3-3* mRNA hybridization signal (in reddish-brown color) in the neuroepithelium of the neural tube (NT) and optic cup (OC), as well as in the lens placode (LP) and some mesenchymal cells (M). The general ectoderm (E) appears negative. (*B*) E3. The future neural retina (NR) shows a strong hybridization signal, but the external layer of the optic cup (future retinal pigment epithelium, RPE) is largely negative. In the lens (L) the anterior epithelial cells are positive, but the elongating lens fibers at the posterior pole are not; the transition zone at the lens equator is indicated by an arrow. (*C*) E8 retina. *sw3-3* expression remains stronger and more widespread toward the periphery than at the fundus (posterior pole), in the region adjacent to the optic nerve (ON). (*D*) Higher magnification of the fundal region in an E8 retina, showing that *sw3-3* mRNA-negative cells are present throughout the ganglion cell layer (G); negative cells are also scattered in other regions of the retina, but the bulk of the neuroepithelium remains positive. The dark appearance of the retinal pigment epithelium (RPE) in the photomicrograph corresponds to its endogenous pigment (see also *F*). (*E*) E9 retina. Note the presence of a gradient in *sw3-3* mRNA distribution from the fundus (which is largely negative) toward the periphery, which is positive. (*F*) E8 retina processed with a control (sense) RNA probe. No hybridization is detected. Note the endogenous pigmentation of the retinal pigment epithelium (RPE).

neural retina). The lens placodes also showed strong hybridization signals, but little if any expression was observed in the adjacent general ectoderm or in the mesenchyme (except for a weak signal in mesenchymal cells adjacent to the ectoderm). By E3 (Fig. 3*B*), *sw3-3* expression was already minimal in the future retinal pigment epithelium; the future neural retina still showed a strong and fairly widespread positive signal, except near the posterior pole of the eye (Fig. 3*B*). In the E3 lens (Fig. 3*B*), *sw3-3* expression was restricted to the anterior epithelium, known to be a site of active cell proliferation. No hybridization signal was detectable in the posterior region of the lens, occupied by postmitotic cells, which are elongating and differentiating into fibers (31–33). There was a sharp demarcation between positive and negative territories at the lens equator, where the transition from cell proliferation to cell differentiation is known to occur (31–33). At E3, *sw3-3* expression continued to be high in the embryonic neural tube (although areas of decreased expression were seen in the roof and floor areas); the head mesenchyme showed a positive signal only in the area adjacent to the optic cup and in the region delimited by the neural tube, the ectoderm, and the optic cup.

Our analysis of *sw3-3* at later embryonic stages focused on the retina. Areas of decreased *sw3-3* expression appeared to spread from fundus to periphery during retinal development, following a pattern resembling the progression of neuronal birth described by using thymidine autoradiography (34, 35). The fundal region showed little *sw3-3* expression in the inner retina by E8 (Fig. 3 *C* and *D*), with the ganglion cell layer appearing completely negative and the rest of the neuroepithelium showing a mixture of negative and positive cells distributed in a radial columnar pattern (Fig. 3*D*). This radial columnar pattern has also been observed in studies of precursor cell lineages in developing retina and central nervous system (36–39). The fundus/periphery difference became even more evident by E9 (Fig. 3*E*), when *sw3-3* mRNA-positive cells appeared to be confined exclusively to the most peripheral region of the retina, adjacent to the ora serrata. An equivalent pattern (i.e., *sw3-3* expression in the neuroepithelium and its disappearance from cells migrating into the mantle layer) was observed in other regions of the central nervous system and will be described elsewhere.

CONCLUSIONS

We hypothesize that *sw3-3* encodes a transcriptional regulator whose disappearance may be involved in the transition from cell proliferation to a stable postmitotic status and the onset of cell differentiation in the retina, in other neural organs, and in the lens. Such a role is suggested by the pattern of *sw3-3* expression, by the presence in its sequence of a basic domain and leucine zipper motifs, reminiscent of those found in other DNA-binding proteins, by our immunocytochemical observations of the nuclear localization of the Sw3-3 protein (unpublished results), and by its two PEST motifs that indicate that Sw3-3 may have a short half-life. Interestingly, similar or related roles have been postulated for Id, a negative regulator of b-HLH transcriptional factors (40), ME1 and GE1, b-HLH factors that are expressed in neural tissues at early developmental stages in mice and chickens, respectively (14), and HES-5, a b-HLH factor that is distantly related to the *Drosophila* neural regulatory genes hairy and enhancer of split and is expressed in ventricular cells in the rat embryo neural tube (9). Some of the functional attributes of HES-5 and its *Drosophila* homologues appear to be related to the presence of a proline in place of an asparagine residue in their basic domains. Most of the evidence summarized above, therefore, suggests that *sw3-3* is likely to participate in the regulation of gene expression during development. Further experimental analysis, however, will be necessary to verify this hypothesis.

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