Transcription of a quail gene expressed in embryonic retinal cells is shut off sharply at hatching

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ABSTRACT The avian neuroretina (NR) is part of the central nervous system and is composed of photoreceptors, neuronal cells, and Müller (glial) cells. These cells are derived from proliferating neuroectodermal precursors that differentiate after terminal mitosis and become organized in cell strata. Genes that are specifically expressed at the various stages of retinal development are presently unknown. We have isolated a quail (Coturnix coturnix japonica) cDNA clone, named QR1, encoding a 676-amino acid protein whose carboxyl-terminal portion shows significant similarity to that of the extracellular glycoprotein osteonectin/SPARC/BM40 and of the recently described SC1 protein. The QR1 cDNA identifies a mRNA detected in NR but not in other embryonic tissues examined. The levels of this mRNA are markedly reduced when nondividing NR cells are induced to proliferate by the v-src oncogene. QR1 expression in NR is limited to the middle portion of the inner nuclear layer, a localization that essentially corresponds to that of Müller cells. Transcription of QR1 takes place only during the late phase of retinal development and is shut off sharply at hatching. Signals that regulate this unique pattern of expression appear to originate within the NR, since the QR1 mRNA is transcribed in cultured NR cells and is shut off also in vitro at a time coinciding with hatching.

The neuroretina (NR) is part of the central nervous system formed by evagination of the neural tube and is composed of photoreceptors, horizontal, bipolar, amacrine, and ganglion neuronal cells, and Müller (glial) cells. Avian NR development proceeds through essentially three phases: proliferation of neuroectodermal precursor cells, lamination of cell strata, and differentiation of postmitotic cells (1, 2). Identification of genes whose expression correlates with each of these steps would greatly facilitate the molecular dissection of these processes.

We have shown that nondividing NR cells from avian embryos become transformed and acquire sustained growth capacity following in vitro infection with Rous sarcoma virus (RSV) (3). Proliferating NR cells can be established in permanent lines, including cells with neuronal properties (4). The v-src gene, which contains the genetic information for the transforming and tumorigenic properties of the virus (5), is also responsible for its mitogenic effect. This was demonstrated by isolating mutants that are temperature sensitive (ts) for their ability to induce NR cell proliferation (6, 7). We also described several mutants of RSV carrying point mutations or partial deletions in the v-src gene that induce NR cell multiplication in the absence of transformation (8–10). Therefore, NR cells infected with RSV mutants inducing various phenotypes constitute a potential model to study mechanisms that regulate growth or differentiation of these cells. We have used this experimental system to isolate cDNAs to mRNAs expressed in postmitotic NR cells and down-regulated when these cells are induced to proliferate by the v-src gene (11).

Here, we describe the nucleotide sequence§ and developmental regulation of a quail (Coturnix coturnix japonica) cDNA clone, named QR1, encoding a 676-amino acid protein whose carboxyl-terminal portion shows significant similarity to the homologous portions of the extracellular glycoprotein osteonectin/SPARC/BM40 (12–15) and of the recently described SC1 protein (16). The QR1 cDNA identifies a mRNA that displays two interesting properties: (i) it is detected in the NR but not in other embryonic tissues examined and is essentially expressed in Müller cells; (ii) transcription of QR1 takes place only during the late phase of NR development and disappears abruptly at hatching. Such a sharp arrest of gene transcription appears unique among vertebrate genes, transiently expressed during development. We also show that the QR1 mRNA is expressed in cultured NR cells and is shut off in vitro at a time coinciding with hatching.

MATERIALS AND METHODS

Cells and Viruses. NRs were dissected from 7- to 17-day quail embryos and from newly hatched quails as described (4). Quail NR (QNR) cultures were maintained in Eagle basal medium supplemented with 8% fetal calf serum. The Schmidt–Ruppin strain of RSV, subgroup A (SRA), was used as the wild-type virus. The isolation and characterization of RSV mutants tsNY68 (17) and PA101 (8) have been reported previously. Fujinami sarcoma virus (RAV-1) and MH2-PA200 (RAV-1) are two retroviruses containing the v-fps (18) and v-mil (19) oncogenes, respectively. QNR cells were infected as described (3).

Isolation of QR1 cDNA. Poly(A)+ RNA (5 µg) prepared from 13-day embryonic QNR (ED13 QNR) was used to construct a cDNA library into the EcoRI site of the λgt10 phage vector (20) as described by Gubler and Hoffman (21). The library was differentially screened with 32P-labeled cDNA probes prepared from poly(A)+ RNAs from ED13 QNR and from QNR cells transformed with tsNY68 and grown at 37°C. Clones giving higher signals with the probe prepared from ED13 QNR than with the probe prepared from tsNY68-infected cells were selected and further purified as described (11).

Abbreviations: NR, neuroretina; QNR, quail NR; RSV, Rous sarcoma virus; SRA, Schmidt–Ruppin strain of RSV, subgroup A; ED, embryonic day; ts, temperature sensitive.

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†The sequence reported in this paper has been deposited in the GenBank database (accession no. M61908).
Northern (RNA) Blot Analysis and Nuclear Run-On Transcription Assay. Total RNA was isolated by using the guanidium thiocyanate/cesium chloride method (22). RNAs were denatured at 60°C in a formamide/formaldehyde mixture, fractionated by electrophoresis in 1% agarose/2.2 M formaldehyde gels (23), transferred to nitrocellulose filters in 20× SSC, and blocked (24) before hybridization to QR1 cDNA radioactively labeled by nick-translation under conditions described by Wahl et al. (25). Nuclear run-on experiments were done as described (11).

DNA Sequencing of QR1 cDNA. We sequenced several overlapping QR1 cDNA clones isolated from two ED13 QR1 cDNA libraries prepared by using oligo(dT) or random priming (21). Nucleotide sequence was determined on both strands after subcloning restriction fragments into M13mp18 or M13mp19 vectors, using the chain-termination method (26).

In Situ Hybridization and Immunocytochemistry. Preparation of RNA-labeled probes. An EcoRI internal cDNA fragment of 542 base pairs (bp) (nucleotides 913–1455 in the B region of QR1) was subcloned into the Bluescript plasmid vector and used to prepare high specific activity 35S-labeled RNA probes from sense and antisense strands (27). A limited alkaline hydrolysis of the probes generated fragments of 50–200 bp convenient for in situ hybridization. Unincorporated nucleotides were removed by Sephadex G-50 column filtration. After ethanol precipitation, the RNA pellet was dissolved in hybridization buffer. Concentration of the probe was adjusted to 50,000 cpm/μl.

Tissue preparation. Eyes were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS), sunk in 30% sucrose in PBS overnight, frozen in liquid nitrogen, and processed as described (28). ED7 QNR cultures were prepared on coverslips as described (29), fixed at indicated times in 4% paraformaldehyde in PBS, dehydrated through alcohol, and processed for in situ hybridization as described above.

Hybridization. Fifteen-micrometer cryostat sections and coverslips of QR1 cultured cells were hybridized with 35S-labeled RNA probes as described by Wilkinson et al. (27).

RESULTS
Isolation of QR1 cDNA. Experiments leading to the isolation of QR1 initially were designed to identify genes that might be involved in terminal mitosis or differentiation of NR cells. We assumed that transcription of such genes would be down-regulated when these nondividing cells are induced to proliferate by infection with RSV, as a consequence of the v-src oncogene expression. Therefore, we constructed a cDNA library from mRNAs of 13-day quail embryo NR (ED13 QNR). At this stage, the vast majority of NR cells have completed terminal mitosis and are undergoing differentiation (29). The library was screened by differential hybridization with radioactively labeled probes complementary to mRNAs either from ED13 QNR or from NR cells infected with tsNY68, an RSV mutant that induces proliferation and transformation of QNR cells at 37°C but not at 41°C. We isolated cDNA clones hybridizing to RNAs transcribed in ED13 QNR and significantly reduced in tsNY68-infected cells at 37°C (11). Several clones, isolated by this procedure,
hybridized to mRNAs expressed only in neural tissues. Among these, expression of the QR1 clone was restricted to the NR.

**Nucleotide Sequence Analysis of QR1 cDNA.** The nucleotide sequence of a full-length cDNA was determined and is presented in Fig. 1. It contains a single long open reading frame encoding a putative protein of 676 amino acids, with a calculated molecular mass of 78.15 kDa. Analysis of the primary structure and comparison with sequence data bases indicated that the QR1 protein is composed of three regions. The carboxy-terminal portion (from amino acid 444 to the carboxy-terminal end) shows 71% identity to the 233 carboxy-terminal amino acids of the recently described SC1 protein, essentially expressed in mouse adult brain and heart (16), and 61% identity to the homologous portion of the ubiquitous extracellular glycoprotein osteonectin/SPARC/BM40 (12–15) (Fig. 2). All three proteins contain a calcium-binding loop of "E-F hand" structure in this region (31). The extreme amino-terminal portion (A region) is a hydrophobic stretch of 17 amino acids ending with a consensus sequence for a signal peptidase, characteristic of membrane-bound and secreted proteins (32). Between this signal peptidase and amino acid 443 (B region), the sequence of QR1 is rich in hydrophilic amino acids and shows no significant similarity with proteins already described. The presence of a putative signal peptide and the lack of an internal hydrophobic transmembrane domain suggest that the QR1 protein is a secreted protein.

**Down-Regulation of QR1 mRNA by p60<sup>src</sup> Correlates with Cell Division.** Proliferation and transformation of QRN cells infected with RSV depend on the continuous expression of a functional v-src protein. To determine whether transcription of QR1 mRNA was regulated by v-src protein activity, we examined the effects of p60<sup>src</sup> inactivation on QR1 gene expression. Therefore, we analyzed by Northern blotting RNAs extracted from ED13 QRN, from tsNY68-infected cells maintained at 37°C, and from duplicate cultures shifted to 41°C for 48 hr (Fig. 3A). The QR1 cDNA hybridized to a single mRNA species of about 3 kb in ED13 QRN. The steady-state level of this transcript was reduced by >200-fold in QRN cells transformed and induced to proliferate by tsNY68 virus at 37°C (Fig. 3A). Nuclear run-on experiments indicated that QR1 expression in RSV-infected cells was regulated at the transcriptional level (Fig. 4A). Transcription of QR1 mRNA resumed when these cells were transferred to 41°C and reached a level comparable to that of ED13 QRN (Fig. 3A). To determine whether the down-regulation of QR1 mRNA was correlated with cell transformation or proliferation, we examined expression of this gene in QRN cells infected with the conditional mutant PA101 (Fig. 3A). Like tsNY68, this mutant virus induces QRN cell proliferation at 37°C but not at 41°C. However, in contrast to tsNY68, PA101 does not morphologically transform QRN cells at 37°C (6). We found that the QR1 mRNA was regulated similarly in QRN cells infected with PA101 virus.

Therefore, down-regulation of QR1 expression appeared to be correlated primarily with cell division. We also found that QR1 expression was down-regulated in QRN cells induced to proliferate by two other oncogenes: v-fps and v-mil, encoding a tyrosine and a serine/threonine protein kinase, respectively (34, 35) (Fig. 4B).

**Tissue Specificity and Developmental Regulation of QR1 Expression.** We investigated the presence of QR1 mRNA in various tissues from 13-day quail embryos and in cultured fibroblasts (Fig. 3B). Expression of this gene exhibited restricted tissue specificity, since its mRNA was detected in QRN but not in other ED13 tissues examined. To determine the cellular localization of QR1 expression, we hybridized 35S-labeled RNA probes to eye sections prepared at ED14. Hybridization signals were strong and clearly localized to the middle portion of the inner nuclear layer. No hybridization was observed outside this region (Fig. 5).
over background was observed in other areas (Fig. 5A2). This localization corresponds essentially to that of the somas of Müller cells. Indeed, when parallel sections were labeled with monoclonal antibody 7G4, shown to specifically stain Müller cells of chicken embryo NR (30), the somas of these cells were visible at the same position as that of QRF1 hybridization signals (Fig. 5A3).

We next analyzed QRF1 expression in QNR dissected at various intervals during development and in newly hatched quails (Fig. 3C). QRF1 mRNA was not detected in ED7 and ED8 QNR, which still contain proliferating undifferentiated cells. Thereafter, the steady-state levels of QRF1 mRNA progressively increased between the 9th and 14th day of development (Fig. 3C) and remained stable until hatching, which takes place at ED17 (data not shown). Strikingly, QRF1 mRNA was no longer detectable in QNR dissected 1 and 3 days after hatching, indicating that expression of this gene was restricted to the late phase of NR development (Fig. 3C).

**QRF1 mRNA Is Shut Off Abruptly at Hatching.** To precisely determine the time of loss of QRF1 expression, we hybridized 35S-labeled RNA probes to eye sections prepared at short intervals around hatching (ED17). Nine hours before hatching, the signal levels and localization were identical to those of ED14 (Fig. 5B1). Six hours before hatching, the intensity of hybridization signals was markedly reduced (Fig. 5B2). Finally, no signal was detected in sections prepared from quails in the process of hatching (Fig. 5B3). The disappearance of QRF1 expression at hatching, within such a short interval of time, appears to be unique among developmentally regulated vertebrate genes.

**Signals That Regulate Expression of QRF1 mRNA Originate Within the NR.** We investigated whether expression and loss of the QRF1 message are controlled by regulatory signals originating within the NR or whether they depend on extraocular signals. Therefore, cultures were prepared from ED7 QNR, which do not express the QRF1 gene, and examined by in situ hybridization at daily intervals thereafter. The QRF1 transcript was detected after 2 days and became maximal 4–6 days later. Signals were visible in areas composed of flat Müller-like cells without neurons (Fig. 5B4). Hybridization signals were still present on the 10th day of culture. They then significantly decreased within 12 hr—that is, at a time that corresponded to hatching (Fig. 5B5).

**DISCUSSION**

We have identified in quail NR cells a gene, QRF1, that displays two particularly interesting properties: a restricted tissue specificity and a unique pattern of expression during development. QRF1 was expressed in the NR and was not detected in other embryonic tissues examined. Moreover, transcription of this gene appears to be limited to a subpopulation of NR cells, since it was observed only in the middle portion of the inner nuclear layer. This localization and the immunofluorescent labeling with monoclonal antibody 7G4 suggest that QRF1 is expressed essentially in Müller cells. The sharp arrest of QRF1 expression at the time of hatching obeys regulatory mechanisms that have not been described in studies of developmentally regulated vertebrate genes. The signal(s) responsible for expression of the QRF1 message must
be present within the NR, since this transcript appears in cultures of pure NR cells. Furthermore, in vitro expression of QR1 in flat Müller-like cells does not seem to require close contact with neurons. The marked decrease of QR1 mRNA levels in cultured NR cells, at a time that corresponds to prehatching, indicates that the shut down of QR1 expression does not require the structural organization of the NR and is not modulated by an extraretinal signal.

What may be the function of the QR1 gene product? The presence of a signal peptide and of several glycosylation sites suggests that the QR1 protein is a secreted glycoprotein. Moreover, the partial sequence similarity with the carboxy-terminal portions of two proteins, SCI and osteonectin/SPARC/BM40, suggests that QR1 is an additional member of a family of extracellular proteins that differ greatly in their tissue specificity of expression. SCI is detected in different types of neurons of the adult mouse brain and in the heart (16). Osteonectin/SPARC/BM40 was originally isolated as an extracellular protein in bones, where it could play a role in regulation of mineralization (12, 36, 37). It is also expressed in a wide variety of other tissues where it could have a different role (38–40). High levels of osteonectin/SPARC/BM40 expression are correlated with the rapid synthesis and assembly of basal membranes, during embryonic development in the parietal endothelium or during growth and repair of adult endothelial and epithelial tissues (13, 40). QR1 transient expression, at the developmental stage where a basal lamina is formed at the vitreal surface of the retina (41) and where the NR becomes organized in alternating neuronal somas (nuclear) and synapses (plexiform) layers, suggests that the QR1 protein may play a role during this lamination process. Therefore, it is tempting to speculate that this protein, like osteonectin/SPARC/BM40, induces sufficient cellular and tissue plasticity to allow final positioning of neurons, neurite outgrowth, and synapses formation during the late stage of NR morphogenesis. This expression would no longer be required when formation of the NR is achieved. The amino-terminal domain of QR1, which lacks significant similarity with other proteins, may be essential in determining retinal-specific protein interactions, whereas the carboxyl-terminal domain may share common molecular functions with osteonectin/SPARC/BM40 and SCI.

Transcription of the QR1 gene, in QNR cells infected by mutants with ts mitogenic capacity, is reversibly controlled by the v-src gene and is seen only in non-dividing cells. Down-regulation of this gene is also a target for the mitogenic effect of two other oncopgenes: v-fps and v-mil. This in vitro experimental model should prove useful in investigating the regulatory mechanisms of QR1 expression and studying their interactions with oncogene products.

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