Thyroxine Affects Expression of KSPG-Related Genes, the Carbonic Anhydrase II Gene, and KS Sulfation in the Embryonic Chicken Cornea


PURPOSE. Opaque chick corneas become thin and transparent from embryonic day (E)9 to E20 of incubation. Thyroxine (T4) injected in ovo on E9 induces precocious transparency by E12. The present study was conducted to determine whether corneal cells differentially express genes for T4 regulation, keratan sulfate proteoglycan (KSPG) synthesis, crystallins, and endothelial cell ion transporters during transparency development and whether these expressions are altered when E9 embryos are treated with T4.

METHODS. E9 eggs received T4 or buffer; corneas were dissected on E12. Corneal transparency was measured digitally and thickness was determined from cryostat cross sections. mRNA expressions were determined by real-time PCR using cDNA synthesized from whole-cell RNA, cells expressing T4 receptor mRNAs assessed by in situ hybridization, and KS disaccharide sulfation measured by electrospray ionization tandem mass spectrometry (ESI-MS/MS).

RESULTS. All corneal layers expressed T4 receptor α (THRA) mRNA; keratocytes and endothelial cells expressed T4 receptor β (THRB) mRNA. During normal development, THRB expression increased 20-fold from E12 to E20; THRA expression remained constant. Expressions of most genes involved in KS synthesis increased from E9 to E16, and then decreased from E16 to E20. From E9 to E20, expressions of crystallin genes increased; T4/3-deiodinase DIII (DIO3) increased 10-fold; and sodium-potassium ATPase transporter (NCBE), sodium-bicarbonate transporter (NBC), and carbonic anhydride II (CA2) increased 5- to 10-fold. E9 T4 administration decreased corneal thickness by E12; increased DIO3, THRB, and CA2 expressions 5- to 20-fold; decreased KS5G core protein genes and galactose sulfotransferase CHST11 expressions 2-fold; and reduced KS-disulfated/monosulfated disaccharide (DSD/MSD) ratios.

CONCLUSIONS. Thyroxine modifies expressions of KSPG synthesis and carbonic anhydrase genes. (Invest Ophthalmol Vis Sci. 2006;47:120–132) DOI:10.1167/iovs.05-0806

Corneal transparency is determined in part by concentrations and patterns of sulfation of KSPGs in stromal extracellular matrix (ECM). KSPG interactions with stromal ECM collagen fibrils and water molecules, corneal epithelial and stromal cell crystallins, and the action of corneal endothelial cell membrane ion transporters that regulate stromal hydration. During chick embryogenesis, corneal epithelium is formed by embryonic day (E3), and a primary corneal stroma, consisting principally of collagen fibrils, is deposited by the corneal epithelium on its posterior surface.1 Neural crest-derived cells migrate across the posterior surface of the primary stroma to form the corneal endothelium from E3 to E5.2 Other neural crest-derived cells invade the primary stroma from E5 to E12,2 differentiate into keratocytes, and synthesize and secrete into the ECM collagens and KS- and chondroitin/dermatan-sulfate (CS/DS)-proteoglycans (PGs) to form a complex and highly ordered secondary corneal stroma.3,5 Epithelial cells and keratocytes synthesize corneal crystallins.6,7 The chick cornea reaches maximum thickness and opacity by E12 and then thins and achieves maximum transparency by E20.8 Regulation of this transition to transparency is not understood in mammals or birds. Moreover, transparency is difficult to measure in mice because of the thinness of their corneas and because of the great heterogeneity in corneal thicknesses in mice of different genetic backgrounds (Jester J, personal communication, May 2005). However, in the chick embryo, in ovo administration of T4 at E7 has been shown to accelerate clearing of the cornea by 2 to 3 days.9

Chick eggs contain maternally deposited, biologically inactive T4 and biologically active 3,5,3'-triiodothyronine (T3), primarily in the yolk, which are taken into the embryo during early (E4–E6) development.10 The developing thyroid gland begins to synthesize T4 by E9 to E10,10,11 plasma T4 and T3 levels begin to increase by E10,10 and concentrations of T4 and T3 increase steadily in the eye from E10 to E12 through E20.10 The thyroid hormone receptors TRα and TRβ bind both T4 and T3, and both TR-T4 and TR-T3 can act as agonists in cells.12 Receptor TRβ2 is expressed in the chick neural retina by E5,13 and TRα and TRβ are expressed in the whole eye from E9.14 T4, the primary secretion product of the chick thyroid gland,15 is converted to T3 by specific deiodinases T4D1 and T4DII, expressed by iodothyronine deiodinase type I (DIO1) and type II (DIO2) genes respectively, or to much less biologically active reverse T3 (rT3) by deiodinases T4DI and T4/3DIII, the product of the iodothyronine deiodinase type III (DIO3) gene.16 T3 is further inactivated by T4D1.16,17 In tissues, T4 is converted to T3 primarily by T4DII.17 T3 is inactivated principally by deiodinase T4/3DIII.18 Deiodinase activities vary markedly in tissue-specific patterns during chick embryogenesis,18 and T4 and T3 regulation of deiodinase expressions occurs at pre- and posttranscriptional and posttranslational levels.19 Nothing is known about the expressions of genes THRA, THRB, DIO1, DIO2, or DIO3 in developing chick corneas, or how their expressions change in response to precocious exposure to T4.

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In embryonic chick corneas, genes encoding KSPG small leucine-rich repeat (SLRP) core proteins lumican (LUM), keratocan (KERA), and mimecan (OGN) are expressed by stromal and endothelial cells during development.\textsuperscript{20–24} The SLRP proteins are hypothesized to wrap around ECM collagen fibrils, regulating their diameter and assembly.\textsuperscript{25,26} Nonuniformly sulfated KS chains, extending from convex surfaces of collagen-bound KS SLRPs, are thought to regulate spacing between collagen fibrils, influence stromal hydration, and facilitate corneal transparency.\textsuperscript{4,5,27,28} Addition of sulfated polylactosamine KS side chains to SLRP proteins in keratocytes is a complex process of co- and posttranslational modifications involving transfer of a mannose tree to specific asparagine residues in core protein backbones,\textsuperscript{29} trimming the tree, and then adding to one mannose residue first N-acetylglucosamine and then alternating residues of galactose and N-acetylglucosamine to assemble a chain of repeating disaccharides.\textsuperscript{30,31} Sulfation of KS lactosamines occurs as they are added to the end of the growing chain, or, for galactoses also later, after they have been incorporated into the chain.\textsuperscript{32} Creating nonsulfated, monosulfated, or disulfated disaccharide “hot spots” along the KS chain. Each step requires specific synthetases or transferases, some cornea-specific. Inorganic sulfate must also be transported into cells and incorporated into 3’-phosphoadenosine-5’-phosphosulfate (PAPS), the sulfate donor used by KS glycosaminoglycan sulfotransferases.\textsuperscript{32} Corneal PAPS synthesis peaks at E16, then declines, and concentration of KS glycosaminoglycan sulfotransferases.\textsuperscript{32} Coreen PAPS synthesis peaks at E16, then declines, and concentration of KS glycosaminoglycan sulfotransferases.\textsuperscript{32} Conenal PAPS synthesis peaks at E16, then declines, and concentration of disulfated disaccharides (DS) plus monosulfated disaccharides (MSD) in corneal KS chains peaks at E8, E14, and E20, whereas the DS/MSD ratio peaks at E10, declines by 40% by E14, and then declines below 1 after hatching.\textsuperscript{33}

Corneal epithelial cells and stromal keratocytes also express crystallins that contribute to corneal transparency.\textsuperscript{6,7,35} Crystallins are intracellular, water-soluble, frequently metabolic proteins and are often species-specific.\textsuperscript{6,36} E18 chick corneal crystallins include cyclophilin, product of the peptidylprolyl isomerase B gene (PPIB), α-glutamate sulfotransferase, product of the GSTA gene, δ-crystallin, and ω-crystallin, products of argininosuccinate lyase I and II (ASL1 and ASL2) genes respectively, and α-enolase, product of the ENOL1 gene.\textsuperscript{6}

Embryonic chick cornea thickness,\textsuperscript{37} specific hydration,\textsuperscript{9} and sodium ion concentration\textsuperscript{37} decrease from E12 to E19 as transparency increases. A sodium/potassium (Na+/K+) ATPase transporter, product of the sodium/potassium ATPase α-1 gene (ATPIA1) and cytochemically localized to basolateral membranes in mammalian corneal endothelial cells,\textsuperscript{38,39} has been hypothesized to establish a Na+-based osmotic gradient, providing a “pump” by which water is continuously moved out of the stroma to maintain correct corneal thickness for maximum transparency. However, there is no net movement of Na+ ions from stroma to anterior chamber across the corneal endothelium.\textsuperscript{40} Instead, bicarbonate ions (HCO₃⁻) traverse endothelial cells from stroma to anterior chamber, via a basolateral Na⁺/HCO₃⁻ cotransporter, a product of the NBC gene; apical membrane HCO₃⁻ channels, products of AE genes; intracellular carbonic anhydrase II, a product of the CA2 gene; and outer apical membrane-linked carbonic anhydrase IV,\textsuperscript{41} a product of the CA4 gene. Chloride ions (Cl⁻) are also essential for pump activity.\textsuperscript{42} Basolateral Cl⁻/HCO₃⁻ anion exchanger AE2, a product of AE2, has been implicated in Cl⁻ fluxes in cells.\textsuperscript{43}

Little is known about how expressions of genes for enzymes for corneal KSPG synthesis, corneal crystallins, or hydration-related ion transporters and ion generators change as the chick cornea becomes transparent. In ovo treatment of E7 to E12 chick embryos for 2 to 3 days with T4 causes their corneas to lose water of hydration,\textsuperscript{9} decrease their thickness and increase their potassium content,\textsuperscript{22} and increase their concentrations of APS and PAPS\textsuperscript{14} compared with controls. However, nothing is known about what genes might be involved in any of these T4-induced changes. To determine how T4 may contribute to transparency in the chick cornea, we examined the expressions of some of the genes for transparency-implicated proteins through the development of corneal transparency, stimulated precocious onset of transparency by in ovo treatment of E9 embryos with T4, and examined the changes in these gene expressions and in KS sulfation patterns in E12 corneas.

**Materials and Methods**

**Embryo Culture and Cornea Isolation**

Fertile White Leghorn chicken eggs were transferred to an incubator on E0 for incubation at 38°C. For transparency measurements, corneas from embryos of desired ages and/or treatment protocols were dissected into sterile saline G (Sal G: 137 mM NaCl, 2.7 mM KCl, 4.3 mM NaHPO₄, 1.4 mM KH₂PO₄, 6.1 mM glucose, 0.6 mM MgSO₄, and 0.1 mM CaCl₂ [pH 7.4]), trimmed close to the scleral rim, placed in a 35-mm culture dish with the epithelial surface resting on the dish bottom, and photographed at 12× magnification with a Wild dissecting microscope (Leica, Deerfield, IL) and a digital camera (Coolpix 995; Nikon, Tokyo, Japan) in black and white mode, with light transmitted from a source placed below the glass microscope stage. For in situ hybridization, corneas were transferred into 0.1 M phosphate-buffered saline (CPBS: 50% 23 parts 0.2 M NaH₂PO₄ plus 77 parts 0.2 M Na₂HPO₄/50% H₂O [pH 7.3]) on ice, fixed overnight at 4°C in CPBS containing 4% paraformaldehyde, washed in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM NaHPO₄, and 1.4 mM KH₂PO₄ [pH 7.3]), dehydrated through a methanol series, and stored at −20°C in 100% MeOH until hybridization. For KS MSD and DSD determinations, the scleral rim was cut away, and corneas were transferred to PBS and analyzed by mass spectrometry, as described previously.\textsuperscript{34} For RNA isolation, the scleral rim was cut away, and corneas were quick frozen in liquid nitrogen, and stored at −70°C until used. For osmotic pressure experiments, corneas were transferred into 0.658 M NaCl or PBS+8M sucrose, incubated for the stated times, photographed as just described, fixed for 20 minutes at room temperature in 0.658 M NaCl or PBS+8 M sucrose containing 4% paraformaldehyde, washed in 0.658 M NaCl or PBS+8 M sucrose, quick frozen in OCT compound (VWR; Sakura Finetek, Torrance, CA) on cryostat chucks, and sectioned at 10 μm with a cryostat (OTF; Hacker-Bright, Fairford, NJ) at −24°C. Sections were mounted on slides (SuperFrost Plus; Fisher Scientific, Pittsburgh, PA), fixed for 20 minutes in PBS containing 4% paraformaldehyde, rinsed in 3× PBS, dehydrated through an EOH series, air dried, and photographed under a compound microscope. Images of whole corneas were analyzed for transparency using image analysis software (Image Pro; Media Cybernetics, Inc., Silver Spring, MD) as described in the next section. Section images were analyzed for corneal or stromal thickness with NIH Image software (available by ftp at zippy.nimh.nih.gov) or at http://rsb.info.nih.gov/nih-image, developed by Wayne Rasband, National Institutes of Health, Bethesda, MD).

**In Ovo Administration of T4**

Eggs were incubated vertically from E0, so that the air chamber formed beneath the broader end of the egg. On E9, 100 μL Sal G containing 5 or 2.5 μg T4 (Sigma-Aldrich, St. Louis, MO), 5 μg streptomycin, and 5 units penicillin were injected onto the inner shell membrane at the bottom of the air chamber. Control eggs were injected with 100 μL Sal G containing 5 μg streptomycin and 5 units penicillin. Injected eggs were further incubated vertically at 40% to 45% relative humidity, 38°C, for 3 days. Then, their corneas were dissected for transparency measurements, determination of KS sulfation, or RNA isolation.

**Transparency Determination**

Each cornea was placed epithelial side down in a dish of Sal G and three digital images were recorded, one with a wire mesh screen...
placed between the microscope stage and the dish (focus on the screen), one with no screen between the dish and the microscope stage (focus on the endothelial surface of the cornea), and one with no cornea in the dish and no screen under the dish at the same focal level as the endothelial surface of the cornea. Corneal transparency was determined using image-analysis software (Image Pro; Media Cybernetics, Inc.) by placing the screenless cornea digital image over the Sal G-alone digital image, selecting an area of interest in the cornea center, performing a background correction, converting the corrected image to a 16-bit gray scale, and allowing the software to construct a histogram of the 16-bit image and calculate mean and SD for the intensity of light that passed through the cornea. Transparency measurements are given as actual pixels contained in the corrected image bitmap.

In Situ Hybridization
Corneas stored at −20°C in 100% MeOH were brought to room temperature, rehydrated through a MeOH series to PBS, quick frozen in OCT compound (VWR; Sakura Finetek) and sectioned at 10 μm using a cryostat (OTF; Hacker-Bright) at −24°C. Sections were mounted on slides (SuperFrost Plus; Fisher Scientific), fixed for 20 minutes in PBS containing 4% paraformaldehyde, rinsed in 3× PBS, dehydrated through an EtOH series, air dried, and stored in desiccated boxes at −20°C. For hybridization, slides were brought to room temperature in desiccated boxes, sections were circled with a hydrophobic pen (ImmmEdge; Vector Laboratories, Burlingame, CA), slides were rehydrated through an EtOH series, and in situ hybridization was performed as described previously. 70-74 Staining was stopped by washing in pH 5.5 PBS. Slides were rinsed in pH 7.5 PBS, mounted in 70% glycerol/30%PBS, viewed with a microscope (Diaphot 300; Nikon) and photographed with a 35-mm color slide film (Fujichrome T64 Type II; Fuji, Tokyo, Japan). Sense probe controls for THRA (data not shown) and THRB did not hybridize with cornea sections. To confirm probe fidelity, probes were hybridized with E7 retinas and found to identify TRα- and TRβ-specific regions identified by Sjoberg et al. 15 (data not shown). Section images were digitized with a scanner (Coolscan 4000; Nikon).

Real-Time PCR
For each data point, at least three separate RNA isolations, cDNA syntheses, and real-time-PCR reactions were performed. Corneas of appropriate ages or treatments, previously quick frozen in liquid nitrogen, were homogenized by grinding with a stainless steel pulverizer (Biopulverizer; BioSpec Products, Inc., Bartlesville, OK) that had been prechilled in liquid nitrogen, pulverized in a stainless steel pulverizer (Biopulverizer; BioSpec Products, Inc., Bartlesville, OK) at maximum speed for 1 minute, and stored at 20°C. For hybridization, slides were brought to room temperature in desiccated boxes, sections were circled with a hydrophobic pen (ImmmEdge; Vector Laboratories), fixed with 100% MeOH for 20 minutes at room temperature, air dried at room temperature, and analyzed for KS MSD and DSD by ESI-MS/MS, as described previously. 34

RESULTS
Expressions of Genes Related to Corneal Transparency Change during Development
Digital conversions of transmitted light confirmed that the chick corneas were opaque during early development from E5 to E12, and increased the amount of light they transmitted linearly from E12 through E20 (Fig. 1), in agreement with previous reports by Coulombre and Coulombre. 8 Despite some data scattering at various incubation ages, increases of approximately 2500 pixels of transmitted light per day occurred as the chick embryonic corneas matured.

Some of the genes related to development of corneal transparency may be grouped into four categories: genes for synthesis of KS PGs, genes for corneal crystallins, genes for thyroxine receptors and endothelial cell ion transport, and genes for thyroxine deiodinators. Although not related to corneal transparency, several genes known to be regulated by thyroxine in other tissues were assessed and included in the final category. Real-time PCR analysis revealed that expressions of KS PG core protein genes LUM, KERA, and OGN (Fig. 2A) were about half that of GAPDH at E7, while the cornea was opaque and thickening. LUM expression increased approximately 7-fold, while KERA and OGN expressions increased approximately 2-fold by E16, and then all three expressions decreased approximately 2-fold by E20. After hatching, OGN expression decreased an additional 7-fold, while LUM and KERA expressions stabilized at about the GAPDH expression level. Expression of B4GT7, which encodes the galactosyltransferase that adds galactose to ends of growing KS chains, was approximately 200-fold lower than GAPDH at E7, increased approximately 2-fold by E18, decreased slightly during hatching, and then increased an additional 2-fold by 70 weeks after hatching (Fig. 2A). Genes for SGC26, a protein sulfate transporter, and PAPSS2, a PAPS synthetase, both of which are involved in synthesizing sulfate donor PAPS from which SO4− groups are transferred to KS chains, were expressed from 250- to 1000-fold less than GAPDH at E7, but then increased 2- to 7-fold by E16, as accumulation of extracellular highly sulfated KS spread across the cornea; decreased briefly; resumed higher expression through hatching; and then increased expression another 2-fold by 70 weeks after hatching (Fig. 2A). Two KS sulfotransferases are used to synthesize the corneal KS PGs: CHST1, which transfers SO4− groups to terminal or internal galactose residues on growing KS chains, and CHST6, which transfers SO4− groups to terminal N-acetylgalactosamine residues on growing KS chains. CHST6 expression was approximately 20-fold less than GAPDH at E7, increased approximately 2-fold by E12, and maintained that level until E16 (Fig. 2A). In contrast, CHST1 expression
Corneal crystallin genes

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<th>Gene</th>
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<td>Lumican: GI:212280</td>
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<td>Keratocan: GI:2570518</td>
<td>R: 5'-TGGTCTTATGTTGAGGATCTGAA-3'</td>
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<td>OGN</td>
<td>Mimecan: GI:4455120</td>
<td>F: 5'-TTTGTATATGTTGAGGATCTGAA-3'</td>
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Thyroxine receptors and genes involved in endothelial ion transport

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<td>ATPIA1</td>
<td>Sodium/potassium ATPase alpha 1: GI:114372</td>
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<td>AE2</td>
<td>Anion Exchanger AE2-1: GI:1305444</td>
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<td>CA2</td>
<td>Carbonic Anhydrase II: GI:115454</td>
<td>F: 5'-ATGTGCGGTCTCCAGTGCTAA-3'</td>
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<td>CA4</td>
<td>Carbonic Anhydrase IV: BBSRC: 053186.2</td>
<td>R: 5'-TCTTGATGCGGTCTCCAGTGCTAA-3'</td>
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Thyroxine deiodinases and genes regulated by thyroxine in other tissues

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<td>DIO2</td>
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<td>DIO3</td>
<td>Iodothyronine Deiodinase type III: GI:6225571</td>
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<td>ME1</td>
<td>Malic Enzyme: GI:4538537</td>
<td>R: 5'-GTCGGAGATCCTGAGAGGAGG-3'</td>
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<td>TRIP15</td>
<td>Thyroid hormone receptor interactor: thyroid hormone receptor binding protein: Allen: GI:50800409</td>
<td>R: 5'-AATCGGAGATCCTGAGAGGAGG-3'</td>
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(2A) was approximately 250-fold lower than GAPD at E7, but increased approximately 4-fold by E9 and another 4-fold from E9 to E16, as ECM accumulation of highly sulfated KSPGs spread across the corneal stroma. Both CHST1 and CHST6 expressions decreased slightly from E16 to E20 and then increased again after hatching.
Genes whose protein products have been identified as cytosolic crystallins in late embryonic chick corneal epithelial cells include \textit{PPIB}, \textit{GSTA}, \textit{ENOL1}, \textit{ASL1}, and \textit{ASL2}. In the embryonic cornea, \textit{ENOL1} was the most highly expressed, beginning at approximately 7.5-fold lower expression than \textit{GAPD} at E7 and increasing to the same level as \textit{GAPD} by E20 (Fig. 2B). \textit{PPIB} expression fluctuated between 7.5 and 10-fold lower than \textit{GAPD} throughout development (Fig. 2B). In contrast, \textit{GSTA} expression was approximately 100-fold lower than \textit{GAPD} at E7, but increased 10-fold by E12 to about the same level of expression as \textit{PPIB}, and maintained that level through development (Fig. 2B). \textit{ASL1} and \textit{ASL2} are paralogues, but the protein product of \textit{ASL1} is enzymatically inactive, whereas the product of \textit{ASL2} is enzymatically active. In the developing cornea, expressions of their mRNAs were 4000- and 2000-fold lower than \textit{GAPD} at E7, respectively, with \textit{ASL2} expression approximately 2-fold higher than that of \textit{ASL1}. Their expressions increased in parallel approximately 2-fold by E14. After E14, \textit{ASL1} expression remained relatively stable at the E14 level, whereas \textit{ASL2} expression continued to increase another 7.5-fold by E20 and an additional 2-fold after 4 weeks, so that by 70 weeks after hatching \textit{ASL2} expression was approximately 200 times greater than \textit{ASL1} expression (Fig. 2B).

Conical expressions of thyroxine receptor \(\alpha\) and \(\beta\) genes were very different. \textit{THRA} expression was approximately 100-fold lower than \textit{GAPD} at E7 and remained constant at that level throughout corneal development (Fig. 2C). \textit{THRB} expression, however, initially was almost 10,000-fold lower than \textit{GAPD} from E7 to E12, but increased 20-fold from E12 through E20 (Fig. 2C), specifically during the time when corneal transparency developed. Translocations of \(\text{Na}^+\), \(\text{K}^+\), \(\text{Cl}^-\), and \(\text{HCO}_3^-\) ions across the corneal endothelium are thought to be performed by a \(\text{Na}^+/\text{K}^+\) ATP transporter, a \(\text{Na}^+/\text{HCO}_3^-\) cotransporter, and the anion exchanger \textit{2-1}, products of \textit{ATP1A1}, \textit{NBC}, and \textit{AE2}, respectively. Inside endothelial cells, carbonic anhydrase II, a product of \textit{CA2}, generates bicarbonate ions for the \(\text{Na}^+/\text{HCO}_3^-\) cotransporter and \textit{AE2}, and on the anterior chamber side of the endothelium, transmembrane carbonic anhydrase IV, a product of \textit{CA4}, contributes to \(\text{HCO}_3^-\) balance. Expression of \textit{ATP1A1} was 20-fold lower than \textit{GAPD} at E7, decreased 5-fold by E9, increased 5-fold by E16, and maintained that expression in adult corneas. \textit{NBC} expression, initially approximately 500-fold below \textit{GAPD} at E7, increased approximately 10-fold through development and into adult corneas (Fig. 2C). In contrast, \textit{AE2} expression fluctuated \(\sim\)100-fold lower than \textit{GAPD} throughout development and increased approximately 2-fold by 70 weeks after hatching (Fig. 2C). Of the genes for enzymes that generate \(\text{HCO}_3^-\), initial expression of \textit{CA2} was approximately 1000- to 2000-fold lower than \textit{GAPD} until E12, but then increased approximately 7.5-fold by E18 while transparency was increasing, whereas \textit{CA4} expression fluctuated at approximately 10,000-fold lower than \textit{GAPD} until after hatching, and then increased 10-fold (Fig. 2C).

Conical E7 to E9 expression of \textit{DIO2}, which encodes \(T^4\)-activating \textit{T4DII}, was significantly higher than other deiodinases, at approximately 200-fold lower than \textit{GAPD}, spiked 5-fold at E12 just before transparency began to develop, actually declined to the E9 level by E18 as transparency increased, spiked 2-fold just before hatching, and declined by 4 weeks (Fig. 2D). In contrast, expression of \textit{DIO1}, which encodes \(T^4\)-inactivating \textit{T4DIII}, was approximately 10,000-fold lower than \textit{GAPD} at E7, remained at that level throughout corneal development, spiked 2-fold at E20, and remained high after hatching (Fig. 2D). E7 to E9 expression of \textit{DIO3}, which encodes \(T^4/5\)-deactivating \textit{T4/5DIII}, was even lower than that of \textit{DIO1}, increased approximately 10-fold from E9 to E16, declined at E18, increased at E20, and continued increasing after hatching to 20-fold higher than its E9 level by 4 weeks (Fig. 2D). \textit{ACHE}, whose product, acetylcholinesterase, increased 60-fold in specific activity in the chick cornea from E7 to E18, then declined 6-fold by hatching, \textit{TRIP15}, whose product, \textit{ALIEN}, functions as a corepressor with thyroid hormone receptor \(\alpha\), and \textit{ME1}, whose product is malic enzyme, are all known to be regulated, directly or indirectly, by thyroxine in other tissues. In chick cornea, \textit{TRIP15} expression was highest, fluctuating \(\sim\)75-fold below \textit{GAPD} throughout development, and then increasing approximately 5-fold after hatching (Fig. 2D). Similarly, \textit{ME1} expression fluctuated \(\sim\)750-fold lower than \textit{GAPD} throughout development, then increased 2-fold after hatching (Fig. 2D). In contrast, initial \textit{ACHE} expression was approximately 20,000-fold lower than \textit{GAPD} from E7 to E9, abruptly increased 7.5-fold by E12, maintained that level through E18, then fell to 100,000-fold lower than \textit{GAPD} after hatching (Fig. 2D).

Cellular Expression of Genes for Thyroxine Receptors-\(\alpha\) and -\(\beta\)

Longer RNA probes that recognize either \textit{THRA} or \textit{THRB} transcripts were generated with the primer sets shown in Table 2. In situ hybridization revealed that \textit{THRA} was expressed strongly in all E18 cornea cell layers, with strongest expression in the endothelium (Figs. 3A, 3B; arrowheads), and significant expression in stromal keratocytes and in the basal layers of the epithelium (Fig. 3A, arrows; 3B). In contrast, and consistent with the real-time PCR thyroid hormone receptor expression results, \textit{THRB} expression was much lower throughout the cornea, with transcripts detected most strongly in the endothelium (Figs. 3C, 3D; arrowheads).

Effect of \(T^4\) on Corneal Transparency

The relationship between digital quantification of transparency and visual assessment of transparency can be seen in Figure 4. Opaque corneas dissected from Sal G-injected E12 control...
Figure 2. Corneal transparency-related gene expressions change during embryonic development and early adulthood. Whole cell RNA was isolated from pools of corneas of specific ages. cDNAs were synthesized from each pool, and the abundance of mRNA of the genes of interest were determined with real-time PCR. Results were normalized to GAPD expression. (A) Genes involved in synthesis of KS PGs: LUM, KERA, OGN, B4GAT4, SLC26, PAPSS2, CHST1, and CHST6. (B) Corneal crystallins: PP1B, GSTA, ENOL1, ASL1 and ASL2. (C) Thyroxine receptors and genes involved in endothelial ion transport: THRA, THRβ, ATP1A1, NBC, AE2, CA2, and CA4. (D) Thyroxine deiodinases and some genes regulated by thyroxine in other tissues: DIO1, DIO2, DIO3, ACHE, TRIP15, and ME1. Gene names are listed in Table 1. Error bars, SD.

Table 2. PCR Primers for TRα and TRβ In Situ Hybridization Probes

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<th>Gene</th>
<th>Size (bp)</th>
<th>Primer Pair</th>
</tr>
</thead>
<tbody>
<tr>
<td>THRA: GI:63177</td>
<td>909</td>
<td>F: 5′-GTGAAAGAGGAGCATGTCAGG-3′&lt;br&gt;R: 5′-CTTATCAGCCAGCTGAGCC-3′</td>
</tr>
<tr>
<td>THRβ: GI:63820</td>
<td>857</td>
<td>F: 5′-ATGGAAGAGGAGCATGTCACG-3′&lt;br&gt;R: 5′-CAACCTTCATGTGAGGAAGGC-3′</td>
</tr>
</tbody>
</table>

Embyos shown in Figures 4A–D transmitted ~15,000 pixels of light, based on a 16-bit gray scale, whereas visibly more transparent E12 corneas from T4-treated embryos shown in Figures 4E–H transmitted ~21,000 pixels. Thus, in ovo administration of 5 μg T4 to E9 eggs resulted in E12 corneas that were, on average, more transparent than corneas from control eggs, both visually (Figs. 4A–H) and when light transmission was quantified from digital images of intact corneas (Fig. 4I). Because curved corneas are photographed with their convex sides down, the number of grids seen through the corneas varied somewhat with the angle at which they lay when photographed. However, there was no evidence of significant change in cornea diameter as the result of T4 treatment. In sharp contrast, all corneal stromas from T4-treated embryos were thinner than any corneal stromas from Sal G-injected control embryos by E12 (Fig. 4J).

To determine whether simply becoming thinner is all that is required to make E12 corneas more transparent, E12 corneas were dissected into isotonic PBS (0.137 M NaCl), immediately photographed for transparency determination, and then transferred either into PBS solutions with hypertonic NaCl concentration (0.658 M NaCl; Fig. 5A) or into PBS containing hypertonic 0.8 M sucrose (Fig. 5B). To monitor for possible changes resulting solely from in vitro maintenance of corneas during...
corneas, or (3) T4-treated corneas less transparent than control
pixels more transparent than control corneas, (2) T4-treated
into three groups based on their transparency measurements at
dissected from Sal-G-injected control embryos, were sorted
bation controls. Therefore, simply becoming thinner is not
also much less transparent than either the
incubation controls by both 5 and 30 minutes, but they were
25% after 5 minutes and approximately 40% after 30 minutes
compared with incubation controls. Concomitantly, transpar-
decreased approximately 26% after 5 minutes and ap-
pressed by in ovo administration of 5 μg T4 from E9 to E12
were in the same directions (increases or decreases)
and of the same magnitudes as the changes in expressions that
occurred from E16 to E20, while transparency was culminat-
ing, and not in the directions or magnitudes that normally
occurred from E9 to E12. In contrast, CA2 expression was
strongly stimulated by in ovo administration of 5 μg T4 from E9
to E12, even though normal changes in its expression from E16
to E20 were negative and minimal.

Effect of T4 on Expressions of Transparency-Related Genes
Corneas from embryos treated in ovo on E9 with 5 μg T4 and
harvested on E12, and from their companion control corneas
dissected from Sal-G-injected control embryos, were sorted
into three groups based on their transparency measurements at
the time of isolation: (1) T4-treated corneas greater than 3000
pixels more transparent than control corneas, (2) T4-treated
corneas less than 3000 pixels more transparent than control
corneas, or (3) T4-treated corneas less transparent than control
corneas. mRNA expressions of transparency-related genes
were assessed by real-time PCR for each group, the difference
between the Ct in the T4-treated corneas compared with the
control corneas was calculated for each gene, and the differ-
ences were normalized to the change in GAPD expression. As
can be seen in Figure 6A, the strongest stimulatory effect of T4
was on DIO3 expression, which increased approximately 50-
fold in those T4-treated corneas that were greatly or slightly
more transparent than controls, and 5- to 7-fold in those T4-
treated corneas that were less transparent than controls. THRB
and CA2 expressions increased approximately 2-fold in T4-
treated corneas, which were much more transparent than
controls, but 5- to 7-fold in T4-treated corneas with transpar-
encies only slightly different from controls, regardless of
whether the T4-treated transparency were slightly more than
or slightly less than the control transparencies. In contrast,
LUM, KERA, and OGN expressions were reduced by approxi-
ately 2-fold in T4-treated corneas that were greatly or slightly
more transparent than controls, and, for OGN, also in T4-
treated corneas less transparent than controls. CHST1 expres-
sion was reduced 2- to 5-fold in corneas that were greatly or
slightly more transparent than controls, but unchanged in
corneas that were less transparent than controls. Expressions
of other genes related to corneal transparency were not signifi-
cantly altered by precocious T4 treatment, and are listed in
Table 3. Of interest, in the cornea, expressions of THRA, the
gene for the more ubiquitously expressed thyroxine receptor,
and DIO1 and DIO2, genes that encode T4-activating deoidi-
nases T4DI and T4DII, were not altered significantly in T4-
treated corneas, nor were expressions of ACHE, TRIP15, or
ME1, genes known to be directly regulated by thyroxine in
other tissues.

Figure 3. Localization of THRA and THRB expression in corneal
cells. In situ hybridization was performed on sections of E18 corneas
using labeled probes for THRA and THRB. (A) THRA was expressed by
corneal endothelial cells (arrowheads), stromal keratocytes, and cells
in the basal layer of the epithelium (arrows). (B) THRB was expressed
by corneal endothelial cells (arrowheads) and stromal keratocytes. (C)
THRB sense control. (D) Enlarged view of corneal endothelium ex-
pressing THRA. (E) Enlarged view of corneal endothelium expressing
THRB. (F) Enlarged view of corneal endothelium stained with the
THRB sense probe. Ep, epithelium; St, stroma; En, endothelium. Scale
bar: (A–C) 50 μm; (D–F) 12.5 μm.

Effect of T4 on KSPG Sulfation
During chick corneal development the DSD/MSD ratio of KS
disaccharides peaked on E10 and then decreased 40% by E14,
whereas the concentration of (MSD+DSD) KS disaccharides on
E10 increased by E14, as transparency began to increase.34
In the present study, in ovo administration of 5 μg T4 at E9
resulted in a very significant decrease in the DSD/MSD ratio in
the E12 corneas of T4-treated embryos compared with Sal
G-injected controls (Fig. 7). In addition, there were some
increases in the concentration of MSD+DSD in the corneas of
T4-treated embryos compared with the corneas of the Sal G-injected controls, although there is overlap in the error bars between the corneas of T4-treated embryos and the controls (Fig. 7). Thus, precocious exposure to T4 caused the KS disaccharide sulfation characteristics of E12 corneas to resemble more closely those of more transparent, thinner normal E14 corneas, compared with controls.

**Discussion**

The developing chick cornea is ideal for investigating factors that regulate corneal transparency because arrangements of its molecular components affect its transparency, changes in both its thickness and its transparency can be measured, and at least one agent, T4, has been identified that induces increased transparency at young ages. Our digital imaging system is sufficiently sensitive to document an almost linear increase in light transmitted through the chick cornea as it develops from opaque on E12 to transparent by E20. Thyroxine functions as a transcription regulator, binding to nuclear receptors TRα and TRβ. TRα and TRβ bind DNA thyroxine receptor response elements (TREs) and recruit either corepressor or coactivator (when bound by T3) complexes that subsequently repress or activate target gene transcription. TREs have been identified in the promoters of thyroxine-responsive genes such as human DIO1, human and Xenopus THRB, mouse SLC26a1, and chick ME. Also within intronic enhancers, such as chick CA2. Also acting at the level of translation, T3 has been reported to stimulate ACHE activity by stabilizing ACHE mRNA. In addition, T4 can act at the cell membrane by nongenomic mechanisms to regulate gene product activity, as it may do in regulating DIO2.

In this study in precociously transparent corneas of T4-treated chick embryos, stromas were thinner; expressions of DIO3, THRB, and CA2 were increased; and expressions of LUM, KERA, OGN, and CHST1 were decreased. We do not yet know whether any of these corneal thyroxine-sensitive genes were directly regulated by thyroid hormone/thyroid hormone receptor complex interactions with nuclear TREs, or indeed, except for CA2, whether any of these chick genes have TREs in their promoters or enhancers. Of the three DIOs, only human, but not rodent, DIO1 has been shown to have TREs in its promoter. Our study lasted several days, and so primary corneal targets of thyroxine may have been stimulated early, and then products from those genes may have induced or repressed the genes whose responses we observed.

Thinning is the most consistent corneal response to precocious in ovo administration of T4. Of the five endothelial cell ion generation and transport genes that we examined, ATP1a1 and NBC expressions increase throughout corneal development, AE2 expression remains fairly constant, and CA4 expression remains very low until after hatching. Only CA2 expression both increased in parallel with the corneal transition to relative transparency of control corneas compared with corneas from T4-treated embryos. Transparency measurements are given in pixels contained in a 16-bit gray-scale image bit map. Control: n = 8 corneas; T4-treated: n = 10 corneas. Error bars, SD. (J) Five micrograms of T4 was injected in ovo on E9. Individual corneas were dissected into Sal G on E12, photographed digitally under transmitted light and analyzed by computer for the amount of light transmitted through the cornea. (A–D) Selected control corneas from eggs injected with Sal G only. (E–H) Selected corneas from eggs injected with T4. (I) Computer analysis of the
transparency and is significantly stimulated in response to T4. A TRE in the second intron of chick CA2 functions in repression and silencing of CA2 expression in chick erythrocytes, and binding of T3 to this intronic TRE-TR complex initiates CA2 transcription by replacing a TR-bound corepressor complex with a coactivator complex. Conceivably, in the cornea T3 could release TR-corepressor TRIP15/ALIEN from the CA2 enhancer TRE, and allow the TR-T3 complex to be occupied by a coactivator complex. Both cytosolic CA2 enzyme and membrane-bound CA4 enzyme are found in corneal endothelium. Our results suggest that both ATP1A1 and NBC ion transporters are important in initiating and maintaining corneal transparency. That only CA2 expression is significantly stimulated by T4 suggests that the efficacy of these pumps may be regulated in the cornea by ion availability, independent of changes in expressions of pump enzyme genes. Moreover, it has recently been reported that CA2 binding to the carboxyl terminus of AE2 potentiates the anion transport capacity of AE2. Thus, increased expression of CA2 could stimulate corneal thinning by increasing both Na+/HCO₃⁻ and Cl⁻ transport.

DIO3 expression both increased significantly in parallel with natural corneal transition to transparency and was highly
FIGURE 7. T4-treatment in ovo from E9 to E12 shifts total MSD + DSD and DSD/MSD ratios toward values that normally occur in more transparent corneas. Five micrograms T4 or Sal G alone was administered in ovo on E9; E12 corneas were dissected, frozen, sectioned, and analyzed by ESI-MS/MS for amounts of KS DSD and MSD. Corneas from embryos treated with T4 from E9 to E12 have significantly reduced DSD/MSD ratios and somewhat increased total MSD + DSD amounts compared with controls. Error bars, SD.

induced by in ovo injection of T4. In contrast, during cornea development DIO1 expression remained consistently low, and DIO2 expression was relatively high at E12, but declined as transparency increased. Neither gene responded to T4. Although DIO1, which has TREs in its promoter, is inducible by TRβ-T3, but not by TRα1-T3, in mouse liver and kidney,64 little is yet known about how T3 regulates DIO2 or DIO3 expression, or whether there are preferences for TRα or TRβ in these regulations. T3 can upregulate DIO2 mRNA expression in rat brown adipocytes,65 but the mechanism for this increase is not known. The very great stimulation of DIO3 expression in response to experimentally administrated T4 and the high constant expression of TRIP15 throughout development suggest that tightly controlling thyroxine regulated genes is important for corneal development.

**TABLE 3. Cornea Transparency Gene Expressions Not Significantly Altered by T4**

<table>
<thead>
<tr>
<th>Gene</th>
<th>C_t Change Rel. to GAPD</th>
<th>C_t Change Rel. to GAPD</th>
<th>C_t Change Rel. to GAPD</th>
<th>C_t Change Rel. to GAPD</th>
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<tr>
<td></td>
<td>SD</td>
<td>SD</td>
<td>SD</td>
<td>SD</td>
<td>SD</td>
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<tr>
<td>B4GT4</td>
<td>0.47 0.03</td>
<td>-0.86 0.04</td>
<td>0.05 0.15</td>
<td>-0.20 0.94</td>
<td>0.23 0.27</td>
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<tr>
<td>CHST6</td>
<td>-0.74 0.04</td>
<td>-1.12 0.04</td>
<td>-0.82 0.04</td>
<td>-0.93 0.27</td>
<td>-0.02 0.16</td>
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<td>PAPSS2</td>
<td>-0.94 0.06</td>
<td>-0.36 0.04</td>
<td>-0.28 0.08</td>
<td>-0.65 0.41</td>
<td>0.23 0.08</td>
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<tr>
<td>SL1C6</td>
<td>0.52 0.36</td>
<td>-0.51 0.06</td>
<td>-0.67 0.04</td>
<td>0.01 0.73</td>
<td>0.06 0.23</td>
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Corneal crystallin genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>C_t Change Rel. to GAPD</th>
<th>C_t Change Rel. to GAPD</th>
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<tr>
<td>ASL1</td>
<td>0.11 0.17</td>
<td>-0.01 0.09</td>
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<tr>
<td>ASL2</td>
<td>0.58 0.08</td>
<td>0.53 0.13</td>
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<tr>
<td>ENOL1</td>
<td>0.55 0.21</td>
<td>0.05 0.11</td>
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<td>GSTA</td>
<td>0.85 0.26</td>
<td>0.35 0.07</td>
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<tr>
<td>PPIB</td>
<td>-0.16 0.02</td>
<td>0.31 0.14</td>
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Thyroxine deiodinases and genes regulated by thyroxine in other tissues

<table>
<thead>
<tr>
<th>Gene</th>
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<tr>
<td>ACHRE</td>
<td>-0.68 0.20</td>
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<tr>
<td>DIO1</td>
<td>0.4 0.01</td>
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<tr>
<td>DIO2</td>
<td>-0.66 0.01</td>
</tr>
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<td>ME1</td>
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</tr>
<tr>
<td>TRP15</td>
<td>0.01 0.12</td>
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</table>

Cont, control; C_t, cycle threshold; rel. relative.
TRβ3, and thus may be chick TRβ3. TRβ1 and TRβ3 have not yet been reported in chick tissues. Our real-time PCR TR primers would amplify TRβ2 and TRβ and TRβ1 if it exists in chicks, but would not amplify TRβ3. Our TRβ in situ primers could detect TRβ1 to -3 and TRβ3. TRβ mRNA levels also sometimes do not reflect TRβ protein levels in tissues. Other posttranscriptional mechanisms may also control TRβ protein amounts. Frankton et al. recently cloned seven alternatively spliced 5’ untranslated regions (UTRs), identified five polyadenylation position elements in human TRβ1 mRNAs, and showed that all the 5’ UTRs strongly inhibited in vitro TRβ1 mRNA translation. It remains to be resolved which TRβ isoforms are expressed in chick embryonic corneas and whether increase in TRβ1 expression is accompanied by an increase in TRβ protein receptor.

In contrast, THRA is highly expressed throughout corneal development in endothelial, stroma keratocyte, and basal epithelial cells, an epithelial restriction also shown for other genes in the cornea, but embryonic corneal THRA expression is only minimally responsive to T4. In mammals, THRA primary transcript alternative splicing produces TRα2, which binds DNA but not T3 and thus antagonizes TRα T3 responsiveness. TRα2 is more highly expressed than TRα1 in most rat and mouse tissues, which may explain why rat brain shows high TRα mRNA expression, but no lyzate T3 binding. Alternative mouse THRA promoters produce truncated TRα1 and -α2, and TRα1 antagonizes TRα1’s T3 responsiveness. TRα1 mRNA has been demonstrated in E7 chick embryos. Our primers for real-time PCR and TRα in situ hybridization probe synthesis would have amplified TRα1 and -α2 mRNA, but not TRα1 and -α2. Also, TRIP15 is highly expressed in the chick embryonic cornea. Its protein product, ALIEN, interacts directly with TRs in the absence of thyroxine, and with repressor sites. High TRIP15 and THRA expressions, coupled with their lack of T4 responsiveness, suggest that perhaps TRα functions as a thyroxine target gene repressor in embryonic chick corneas.

LUM, KERA, and OGN expressions increased from E7 to E16 and then decreased from E16 to E20 as corneal transparency maximized, and early in ovo exposure to T4 decreased corneal expressions of these genes, suggesting that T4 could play a role in normal LUM, KERA, and OGN downregulation. Expression of BAG7/T4, a KS galactosyl transferase gene, increased only slightly during normal development, whereas SLC26 and PAPS2 expressions increased 2- to 7-fold by E16, as synthesis of PAPS reached its peak. However, in corneas from T4-treated embryos, these gene expressions were not significantly altered, suggesting that regulation of increased PAPS accumulation in T4-treated avian corneas occurs at other genes in the PAPS synthetic pathway. Moreover, if a T4RE is present in the promoter of chick SLC26, as in mouse SLC26, it is not functional in the chick cornea. During normal development, the DSD/MSD ratio of KS disaccharides peaked at E10, as expression of CHST1 increased more than 4-fold, before chick embryonic plasma levels of T4 and T3 began to increase. As transparency developed, expression of CHST1 declined from E16 to E20, T4 decreased expression of CHST1, the gene for a KS sulfotransferase that can add sulfate to both terminal and internal galactose moieties in the growing KS chain, and significantly lowered the DSD/MSD ratio of KS disaccharides. Of interest, T3 repressed expression of 2 sialyltransferases in rat liver, and chondroitin sulfate proteoglycan 2 in human skin fibroblasts. Our results suggest that thyroxine regulation of CHST1 expression may be important in decreasing the DSD/MSD ratio of KS disaccharides that accompanies normal transparency development. Expression of ENOL, the most highly expressed chick corneal crystallin, increased 2- to 5-fold during the development of transparency, whereas expressions of PPIB and GSTA were high by E12 but did not change significantly thereafter. In contrast, the two δ-crystallin genes ASLI and ASL2 were expressed at much lower levels during early development, but ASL2, which encodes the enzymatically active form of argininosuccinate lyase, continued to increase in expression, whereas expression of ASLI remained low. This is the reverse of expression patterns previously reported by Li et al. for ASLI and -2 in chick embryonic E10 and E20 corneas. Perhaps the competitive inhibition method used by Li et al. to quantitate mRNAs is not as accurate as real-time PCR when expression levels are low, and differences in expressions are small. After hatching, the study by Li et al. showed corneal expression of ASL2 175 times greater than expression of ASLI, in agreement with our data. Expressions of corneal crystallin genes were not changed significantly in response to T4 stimulation, suggesting that thyroxine does not play a significant role in regulating their expressions in the chick cornea.

Expressions of TRP2, MEI, and MEI are all regulated by thyroxine in some tissues. Expression of TRP2 increased markedly just before transition to transparency began and stayed high as the cornea becomes transparent, whereas expressions of TRIP15 and MEI did not change markedly during corneal development. T4 does not significantly alter the expressions of any of these genes in the E9 to E12 cornea, suggesting that cofactors necessary for thyroxine regulation of these genes may be absent in the cornea. This has, indeed, been shown for chick MEI regulation, which has five TRES in its promoter, and is sensitive to T3 stimulation in chick embryo hepatocytes, but not in chick embryo fibroblasts. Observation that two of the three genes most stimulated by T4 stimulation are highly expressed in the corneal endothelium suggests that endothelial cell function is critical in regulating corneal hydration and thickness, necessary for attaining and maintaining corneal transparency. However, embryonic chick corneal thickness does not decrease linearly during transition from opacity to transparency, but rather decreases from E10 to E14, and then increases from E14 to E20, while the stroma continues to decrease in specific hydration. Clearly, regulation of stromal thickness by itself is not sufficient to confer transparency, for, as demonstrated in this study, incubation in hypertonic saline or sucrose makes all treated corneas thinner, but decreases their transparency, relative both to their initial transparency and to the transparency of controls maintained in isotonic saline. We observed that treatment with T4 induced reduction in expressions of KSPG core protein genes and the galactose sulfotransferase gene and decreased the KS DSD/MSD ratio. These observations support the idea that KSPG regulation of collagen fibril diameter and spacing also are critical in bringing about an orderly transition to transparency and maintaining it once it has been achieved.

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**Effect of T4 on Corneal Transparency**

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