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 (E9) 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 alpha (THRA) mRNA; keratocytes and endothelial cells expressed T4 receptor beta (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 III (DIO3) increased 10-fold; and sodium-potassium ATPase transporter (ATP1A1), sodium-bicarbonate transporter (NBC), and carbonic anhydrase 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 KS disulfate 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

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In embryonic chick corneas, genes encoding KSPG small leucine-rich repeat (SLRP) core proteins lumican (LUM), keratan (KERA), and mimecan (OGN) are expressed by stromal and endothelial cells during development. The SLRP proteins are hypothesized to wrap around ECM collagen fibrils, regulating their diameter and assembly. 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. 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, trimming the tree, and then adding to one mannose residue N-acetylglucosamine and then alternating residues of galactose and N-acetylglucosamine to assemble a chain of repeating disaccharides. 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, 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’-phosphateadenosine 5’-phosphosulfate (PAPS), the sulfate donor used by KS glycosaminoglycan sulfotransferases. Corneal PAPS synthesis peaks at E16, then declines and concentration of disulfated disaccharides (DS D) plus monosulfated disaccharides (MSD) in corneal KS chains peaks at E8, E14, and E20, whereas the DSD/MSD ratio peaks at E10, declines by 40% by E14, and then declines below 1 after hatching.

Corneal epithelial cells and stromal keratocytes also express crystallins that contribute to corneal transparency. Crystallins are intracellular, water-soluble, frequently metabolic proteins and are often species-specific. 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 δ-crystallin, products of arginosuccinate lyase I and II (ASL1 and ASL2) genes respectively, and α-enolase, product of the ENOL1 gene.

Embryonic chick cornea thickness, specific hydration, and sodium ion concentration decrease from E12 to E19 as transparency increases. A sodium/potassium (Na+/K+) ATPase transporter, product of the sodium/potassium ATPase α-1 gene (ATP1A1) and cytochemically localized to basolateral membranes in mammalian corneal endothelial cells, has been hypothesized to establish a Na+/K+-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. 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, a product of the CA4 gene. Chloride ions (Cl−) are also essential for pump activity. Basolateral Cl−/HCO3− anion exchanger AE2, a product of AE2, has been implicated in Cl− fluxes in cells.

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, decrease their thickness and increase their potassium content, and increase their concentrations of APS and PAPS 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 NaH2PO4, 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 (PBS): 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 NaH2PO4, 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. 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, Fairfield, NJ) at −24°C. Sections were mounted on slides (SuperFrost Plus; Fisher Scientific, Pittsburgh, PA), refixed 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 Cybernet-
ics, 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 mea-
surements 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), refixed for 20 minutes in PBS
containing 4% paraformaldehyde, rinsed in 3X 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
(ImmEdge; Vector Laboratories, Burlingame, CA), slides were rehy-
drated through an EtOH series, and in situ hybridization was performed
as described previously.70,71 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 photo-
dgraphed with 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 TRo-
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 and stored at −70°C, were pooled in groups of 20 or more,
pulverized in a stainless steel pulverizer (Biopulverizer; BioSpec Prod-
ucts, Inc., Bartlesville, OK) that had been prechilled in liquid nitrogen,
transferred immediately to lysis buffer from a kit (RNasey Protect;
Qiagen, Valencia, CA) containing mercaptoethanol at room tempera-
ture, further homogenized using a rotor (Pro 200; Pro Scientific, Ox-
ford, CT) at maximum speed for 1 minute, and stored at −70°C.
Whole-cell RNA was isolated according to the manufacturer’s protocol
for tissues containing abundant connective tissue, including proteinase
K digestion and column DNase digestion and stored at −70°C. cDNA
was synthesized from 1 μg whole-cell RNA using a cDNA synthesis
protocol (iScript; Bio-Rad, Hercules, CA) and stored at −20°C. Se-
quences for genes of interest were obtained from GenBank (GI) or
from the BBRC ChickEST Database.46 PCR primers for real-time PCR
were designed using software (Designer 31 Molecular Beacons Design;
Sigma-Aldrich) to amplify fragments between 80 and 150 base pairs in
length, and are listed in Table 1. Gene names conform to guidelines
established by the Second International Workshop on Poultry Genome
primer set generates only one amplified band with chick cornea cDNA,
and is between 90% and 110% efficient when analyzed over 5-fold
dilutions of both its own amplified fragment and E14 cDNA. Each PCR
primer set was cloned and sequenced to confirm its identity. Housekeeping
gene glyceraldehyde-3-phosphate dehydrogenase (GAPD) was chosen
for normalization of all gene expressions. For real-time PCR, cDNA
dilutions (X) were adjusted so that the cycle threshold (Ct) for GAPD
was between 16 and 18. All comparative real-time PCR reaction series
consisted of duplicates for 1X and 10X cDNA dilutions for each PCR
primer pair. PCR primer efficiencies for this 10-fold cDNA dilution
were between 90% and 110%.

Sulfation of KS
After dissection and photographing for transparency determination,
corneas were 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) and digitally photographed under a compound microscope.
Section area or volume was subsequently computed using NIH Image
software. Sections were then circled with a hydrophobic pen
(ImmEdge; Vector Laboratories), fixed with 100% MeOH for 20 min-
utes at room temperature, air dried at room temperature, and analyzed
for KS MSD and DSD by ESI-MS/MS, as described previously.54

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.46 Despite some data scattering at various incubation ages, increases of approx-
imately 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 syn-
thesis of KSPGs, genes for corneal crystallins, genes for thyrox-
ine receptors and endothelial cell ion transport, and genes for thyroxine deiodinators. Although not related to corneal trans-
parency, 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 KSPG core protein genes LUM, KERA, and OGN (Fig. 2A) were about half
that of GAPD at E7, while the cornea was opaque and thick-
ening. 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 GAPD expression level. Expression of B4GAT4, which encodes the galactosyl transferase that adds galactose to
ends of growing KS chains, was approximately 200-fold lower than GAPD at E7, increased approximately 2-fold by E18,
deceased slightly during hatching, and then increased an addi-
tional 2-fold by 70 weeks after hatching (Fig. 2A). Genes for
SLC26, a protein sulfate transporter, and PAPS2, a PAPS syn-
thetase, 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 GAPD 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 hatch-
ing; and then increased expression another 2-fold by 70 weeks after hatching (Fig. 2A). Two KS sulfotransferases are used to
synthesize the corneal KSPGs: CHST1, which transfers SO4
−
groups to terminal or internal galactose residues on growing KS chains, and CHST6, which transfers SO4
−
groups to termi-
nal N-acetylgalcosamine residues on growing KS chains. CHST6 expression was approximately 20-fold less than GAPD
at E7, increased approximately 2-fold by E12, and maintained that level until E16 (Fig. 2A). In contrast, CHST1 expression

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Table 1. Real-Time PCR Primers for Cornea Transparency-Related Genes

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<th>Symbol</th>
<th>Gene</th>
<th>Primer Pair</th>
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| **GAPD** | Glyceraldehyde-3-phosphate dehydrogenase Gl:4105595 | F: 5'-GCCGAAAAGGGAACCTAGGTA-3'  
R: 5'-GCACTCGATCTGGCCCATT-3' |

**Genes involved in synthesis of corneal KSPGs**

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<th>Symbol</th>
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| **BAG7** | UDP-Gal/betaGlCNac beta 1,4-galactosyltransferase, polypeptide 4: GI: 46019432 | F: 5'-CTCTCAGCTGAGAAGAAGTGGAA-3'  
R: 5'-GGTACAGCATGGCCTGCTAC-3' |
| **CHST1** | Carbohydrate (keratan sulfate Gal-6) sulfotransferase 1: BBSRC:345163.1 | F: 5'-CCCCAAATGACAGTCAGAC-3'  
R: 5'-CTTACAAAGCAGCCAGCAG-3' |
| **CHST6** | Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase: corneal KS N-acetylglucosamine 6-sulfotransferase: Gl:41631977 | F: 5'-GGTAATACGAGGGATGCTGTT-3'  
R: 5'-ACGGGCTAATGCTTTGCTGGATACTA-3' |
| **LUM** | Lumican: GI:212280 | F: 5'-CTCAGACCTTAAGCTTCAATCAACT-3'  
R: 5'-ATCAGAGAATTTGAGATCGGTT-3' |
| **KERA** | Keratocan: Gl:2570518 | F: 5'-GCAAGGAAATATTGGAGATCTGGTT-3'  
R: 5'-TTGCTGATATGAGTGTGTTGAA-3' |
| **OGN** | Mimecan: GI:2145120 | F: 5'-AGAGTGGAACAAATCTGTTCTGGTGA-3'  
R: 5'-GCTTAAACACAGCAGGACAGC-3' |
| **PAPSS2** | 3'-Phosphoadenosine 5'-phosphosulfate synthase 2: PAPS synthetase-2: APS kinase SK2: Gl:50749263 | F: 5'-ACAGGGATTTGAGACTACAGA-3'  
R: 5'-AGCTCTCTTAACTGTGCGCAGC-3' |
| **SLC26** | Solute carrier family 26 (sulfate transporter): diastrophic dysplasia protein DTDST-SO4 transporter: BBSRC:346067.2 | F: 5'-TCGATACGCCCTGGAGACT-3'  
R: 5'-GTTGCTGATCTGAGTCGATAC-3' |

**Corneal crystallin genes**

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| **ASL1** | Argininosuccinate Lyase 1: delta crystallin 1: GI:211678 | F: 5'-GGATCACTGTCTGCTCTGCC-3'  
R: 5'-ACCTGATGCTCAGTCTGCTTCA-3' |
| **ASL2** | Argininosuccinate Lyase II: delta crystallin II: BBSRC:050981.1 | F: 5'-ACCTGATGCTCAGTCTGCTTCA-3'  
R: 5'-TTAAGGCTCAACCTCAGTCTGCTT-3' |
| **ENOL1** | alpha Enolase 5'-transferase: Gl:4415623 | F: 5'-TTAGAGAAATATTGGAGATCTGGTT-3'  
R: 5'-GTTAAGGAAATATTGGAGATCTGGTT-3' |
| **GSTA** | alpha Glutathione S-transferase: BBSRC:2145120 | F: 5'-GGATCACTGTCTGCTCTGCC-3'  
R: 5'-ACCTGATGCTCAGTCTGCTTCA-3' |
| **PPIB** | Peptidylprolyl isomerase B: Cyclophilin B: Gl:212648 | F: 5'-GGATCACTGTCTGCTCTGCC-3'  
R: 5'-ACCTGATGCTCAGTCTGCTTCA-3' |

**Thyroid receptors and genes involved in endothelial ion transport**

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<th>Symbol</th>
<th>Gene</th>
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| **ATP1A1** | Sodium/potassium ATPase alpha 1: Gl:114372 | F: 5'-CTCTGCCCTTACTGCTGCTAAC-3'  
R: 5'-AGTGCCGCGTCTCAGTCTGCTTCA-3' |
| **AE2** | Anion Exchanger AE2-1: GI:1305444 | F: 5'-ATCGCTGCTGCTCTGCTTCA-3'  
R: 5'-GAAAGTCGCTGATAGAAGGATAC-3' |
| **CA2** | Carbonic Anhydrase II: Gl:115454 | F: 5'-AGCGCTGCTGCTCTGCTTCA-3'  
R: 5'-ATCCGCCAGCTGGAAGAACAG-3' |
| **CA4** | Carbonic Anhydrase IV: BBSRC: 053186.2 | F: 5'-CTCTGCCCTTACTGCTGCTAAC-3'  
R: 5'-AAAGCCGCAACCTTGAATGAG-3' |
| **NBT** | Sodium Bicarbonate Cotransporter: BBSRC:346067.2 | F: 5'-GGTAGACTTCCTGGACGGACG-3'  
R: 5'-GAAAGTCGCTGATAGAAGGATAC-3' |
| **THRA** | Thyroid Hormone Receptor alpha: Gl:63177 | F: 5'-GCTGTAGCTCCTCAGTCTGCTTCA-3'  
R: 5'-GAGTGGTGGTCTCTTCAAAGA-3' |
| **THR8** | Thyroid Hormone Receptor beta: Gl:63820 | F: 5'-TGTGTGAGGTAGTCTGCTTCA-3'  
R: 5'-TTCTCTGTGTTTCCTCATGACG-3' |

**Thyroxine deiodinases and genes regulated by thyroxine in other tissues**

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<th>Symbol</th>
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| **ACHE** | Acetylcholinesterase: Gl:623031 | F: 5'-CTCAGCCACAGCGCTGCTT-3'  
R: 5'-GTAAGAACTCCTCGAGGCTGGA-3' |
| **DIO1** | Iodothyronine Deidinase type I: Gl:2440060 | F: 5'-CAGCCTGGTGGCCCATCATT-3'  
R: 5'-AACAGTGATAGTGTTGTGATTCT-3' |
| **DIO2** | Iodothyronine Deidinase type II: GI:51316530 | F: 5'-AAGAGCGGAGAATACGAGGAG-3'  
R: 5'-GCGAACTCGGATGGAAAGAAC-3' |
| **DIO3** | Iodothyronine Deidinase type III: Gl:6225571 | F: 5'-ACGGGCTCTAGTGCTAGC-3'  
R: 5'-GGTGTTTTTCTGCTGCTGCTAAC-3' |
| **ME1** | Malic Enzyme: Gl:4538537 | F: 5'-CTGAGAGTTCTGCTGAGGATTG-3'  
R: 5'-ATCTGCGAAGCAGTGAGG-3' |
| **TRIP15** | Thyroid hormone receptor interactor: thyroid hormone receptor binding protein: Alien: Gl:50800409 | F: 5'-GTCGAGATGAGGAGGACATC-3'  
R: 5'-AATGCGCTTACTGCTGCTGCTT-3' |

(Fig. 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 KSPG 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 PPIB, GSTA, ENOL1, ASL1, and ASL2. In the embryonic cornea, ENOL1 was the most highly expressed, beginning at approximately 7.5-fold lower expression than GAPD at E7 and increasing to the same level as GAPD by E20 (Fig. 2B). PPIB expression fluctuated between 7.5 and 10-fold lower than GAPD throughout development (Fig. 2B). In contrast, GSTA expression was approximately 100-fold lower than GAPD at E7, but increased 10-fold by E12 to about the same level of expression as PPIB, and maintained that level throughout development (Fig. 2B). ASL1 and ASL2 are paralogues, but the protein product of ASL1 is enzymatically inactive, whereas the product of ASL2 is enzymatically active. In the developing cornea, expressions of their mRNAs were 4000- and 2000-fold lower than GAPD at E7, respectively, with ASL2 expression approximately 2-fold higher than that of ASL1. Their expression increased in parallel approximately 2-fold by E14. After E14, ASL1 expression remained relatively stable at the E14 level, whereas 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 ASL2 expression was approximately 200 times greater than ASL1 expression (Fig. 2B).

Conical expressions of thyroxine receptor α and β genes were very different. THR4 expression was approximately 100-fold lower than GAPD at E7 and remained constant at that level throughout corneal development (Fig. 2C). THR2 expression, however, initially was almost 10,000-fold lower than 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 Na⁺, K⁺, Cl⁻, and HCO₃⁻ ions across the corneal endothelium are thought to be performed by a Na⁺/K⁺ ATP transporter, a Na⁺/HCO₃⁻ cotransporter, and the anion exchanger 2-1, products of ATP1A1, NBC, and AE2, respectively. Inside endothelial cells, carbonic anhydrase II, a product of CA2, generates bicarbonate ions for the Na⁺/HCO₃⁻ cotransporter and AE2, and on the anterior chamber side of the endothelium, transmembrane carbonic anhydrase IV, a product of CA4, contributes to HCO₃⁻ balance. Expression of ATP1A1 was 20-fold lower than GAPD at E7, decreased 5-fold by E9, increased 5-fold by E16, and maintained that expression in adult corneas. NBC expression, initially approximately 500-fold below GAPD at E7, increased approximately 10-fold through development and into adult corneas (Fig. 2C). In contrast, AE2 expression fluctuated ~100-fold lower than GAPD throughout development and increased approximately 2-fold by 70 weeks after hatching (Fig. 2C). Of the genes for enzymes that generate HCO₃⁻, initial expression of CA2 was approximately 1000- to 2000-fold lower than GAPD until E12, but then increased approximately 7.5-fold by E18. While transparency was increasing, whereas CA4 expression fluctuated at approximately 10,000-fold lower than GAPD until after hatching, and then increased 10-fold (Fig. 2C).

Corneal E7 to E9 expression of DI02, which encodes T4-activating T4DII, was significantly higher than other deiodinases, at approximately 200-fold lower than 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 DIO1, which encodes T4-inactivating T4D1, was approximately 10,000-fold lower than 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 DIO3, which encodes T4/5-deactivating T4/5DIII, was even lower than that of 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). ACHE,48 whose product, acetylcholinesterase, increased slowly in specific activities in the chick cornea, increased to E10, then declined 6-fold by hatching,49 TRIP15, whose product, ALLENI, functions as a corepressor with thyroid hormone receptor50; and ME1,51 whose product is malic enzyme, are all known to be regulated, directly or indirectly, by thyroxine in other tissues. In chick cornea, TRIP15 expression was highest, fluctuating ~75-fold below GAPD throughout development, and then increasing approximately 5-fold after hatching (Fig. 2D). Similarly, ME1 expression fluctuated ~750-fold lower than GAPD throughout development, then increased 2-fold after hatching (Fig. 2D). In contrast, initial ACHE expression was approximately 20,000-fold lower than 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 GAPD after hatching (Fig. 2D).

**Cellular Expression of Genes for Thyroxine Receptors-α and -β**

Longer RNA probes that recognize either THR3 or THR2 transcripts were generated with the primer sets shown in Table 2. In situ hybridization revealed that THR3 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, THR2 expression was much lower throughout the cornea, with transcripts detected most strongly in the endothelium (Figs. 3C, 3D; arrowheads).

**Effect of T4 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...
embryos 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

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

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<td></td>
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</table>
corneas, or (3) T4-treated corneas less transparent than control corneas less than 3000 pixels more transparent than control pixels more transparent than control corneas, (2) T4-treated the time of isolation: (1) T4-treated corneas greater than 3000 into three groups based on their transparency measurements at 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

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 (arrow). (B) THRB was expressed by corneal endothelial cells (arrowheads) and stromal keratocytes. (C) THRB sense control. (D) Enlarged view of corneal endothelium expressing 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.

during the time course of these experiments, parallel incubation control corneas were transferred into fresh isotonic PBS for each experiment, and their transparencies and thickness are shown next to experimental transparencies and thickness for each time interval and each type of hypertonic solution in Figure 5. In 0.658 M NaCl, average corneal thickness decreased slightly after 5 minutes and by 40% after 30 minutes, compared with incubation controls maintained in isotonic saline during the same period. However, transparency decreased approximately 40% after 5 minutes and remained at that basal level after 30 minutes in corneas in 0.658 M NaCl, compared both with their initial t = 0 transparencies and with incubation control transparencies. Because Na⁺ and Cl⁻ ions are thought to be involved in maintaining corneal hydration balance in vivo and therefore might exert other unintended effects on corneal cell homeostasis, the same experiment was repeated with hypertonic nonpermeable solute 0.8 M sucrose in isotonic PBS (Fig. 5B). In 0.8 M sucrose solutions, corneal thickness decreased 25% after 5 minutes and approximately 40% after 30 minutes compared with incubation controls. Concomitantly, transparency decreased approximately 26% after 5 minutes and approximately 40% after 30 minutes in 0.8 M sucrose compared with their initial t = 0 transparency, as well as with incubation control transparencies. In both experimental regimens, corneas incubated in hypertonic solutions were thinner than both the freshly isolated t = 0 controls and their corresponding incubation controls by both 5 and 30 minutes, but they were also much less transparent than either the t = 0 or the incubation controls. Therefore, simply becoming thinner is not sufficient to make chick corneas more transparent.

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 differences 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 transparencies were slightly more than or slightly less than the control transparencies. In contrast, LUM, KERA, and OGN expressions were reduced by approximately 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 expression 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 significantly 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 deiodinases T4D1 and T4D1, were not altered significantly in T4-treated corneas, nor were expressions of ACHÉ, TRIP15, or ME1, genes known to be directly regulated by thyroxine in other tissues.

Injections of 2.5 μg T4 on E9 also were conducted. Changes in gene expressions elicited by 2.5 μg T4 compared with the Sal G–injected controls were compared with changes elicited by 5 μg T4 in corneas that became more transparent after treatment. For the genes most affected by T4, these changes were compared with the changes in gene expressions shown in Figure 2 that occurred from E9 to E12, before the onset of transparency, and from E16 to E20, as transparency was culminating (Fig. 6B). Increases in DIO3 expression and reductions in LUM, KERA, OGN, and CHST1 expressions were dose dependent. THRB and CA2 expressions were not signifi- cantly different at 2.5 μg T4 than at 5 μg T4. For DIO3 both 2.5 and 5 μg T4 from E9 to E12 elicited much greater increases in mRNA expression than occurs naturally from E9 to E12 or from E16 to E20. Changes in THRB, LUM, KERA, OGN, and CHST1 expressions elicited 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 culminating, 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 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, and 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 SL26a1, and chick ME, and also within intrinsic enhancers, such as chick CA2. Also acting at the level of translation, T3 has been reported to stimulate ACH activity by stabilizing ACH 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, quick frozen in OCT using liquid nitrogen, and sectioned. Sections were photographed under a compound microscope. Stromal thicknesses were measured from digital images of the sections. All E12 corneas from T4-treated embryos were thinner than any E12 corneas from control embryos, although some corneas from T4-treated embryos were less transparent than some corneas from control embryos.

**FIGURE 4.** E9 in ovo T4 administration increases corneal transparency by E12 and causes corneas to become thinner. Five micrograms of T4 was injected in ovo on E9. Control embryos received Sal G in ovo on E9. Corneas were dissected on E12, placed in a dish, and photographed digitally using transmitted light. Images were analyzed on 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\(^+/\)HCO\(_3^-\) and Cl\(^-\)/HCO\(_3^-\) transport.

DIO3 expression both increased significantly in parallel with natural corneal transition to transparency and was highly

![Figure 5](http://www.journal.com/figures/figure5.png)

**Figure 5.** Decrease in corneal thickness is not sufficient to increase corneal transparency. (A) E12 corneas were dissected into 0.137 M NaCl (isotonic saline), photographed using transmitted light, and moved into 0.658 M NaCl (hypertonic solution) or 0.137 M NaCl (incubation control) and incubated for 5 or 30 minutes. The 5-minute corneas were rephotographed at 5 minutes, fixed in their incubation solution, sectioned, and measured for corneal thickness. The 30-minute corneas were rephotographed after 5 minutes, rephotographed again after 30 minutes, fixed in their incubation solution, sectioned, and measured for corneal thickness. Corneas incubated in hypertonic saline were thinner than their incubation controls after both 5 and 30 minutes, but they were also much less transparent. (B) The same protocol was repeated using 0.8 M sucrose to make the hypertonic solutions. Corneas incubated in hypertonic solutions were much thinner than their incubation controls by both 5 and 30 minutes, but they were also much less transparent. Error bars, SD.

![Figure 6](http://www.journal.com/figures/figure6.png)

**Figure 6.** (A) Exposure to thyroxine in ovo from E9 to E12 stimulates increased expression of DIO3, THRB, and CA2, and decreased expression of LUM, KERA, OGN, and CHST1. Corneas from embryos exposed to 5 \(\mu\)g T4 or Sal G alone from E9 to E12 were dissected, analyzed for transparency, and then pooled in three groups based on their transparency, as shown in the illustration. Whole-cell RNA was extracted from each pool, and cDNA was synthesized. Gene expression in each pool was determined with real-time PCR, and results were normalized to GAPD expression. DIO3, THRB, and CA2 increase expression, whereas LUM, KERA, OGN, and CHST1 were reduced in expression. Error bars, SD. (B) Changes in gene expressions were determined in corneas treated with 2.5 \(\mu\)g T4 from E9 to E12. Shown are changes in gene expression from E9 to E12 taken from Figure 2; changes in gene expression in corneas from embryos treated with 2.5 \(\mu\)g T4; changes in gene expression in corneas made more transparent than the control by 5 \(\mu\)g T4, taken from (A); and changes in gene expression from E16 to E20, taken from Figure 2. THRB, LUM, KERA, OGN, and CHST1 demonstrate T4 stimulated changes in gene expression that were of the magnitude and in the direction of changes that normally occur in the expressions of these genes from E16 to E20. T4-induced changes in DIO3 and CA2 expressions were much greater than changes in their expressions under normal E16 to E20 conditions, and, for CA2, in the opposite direction. Error bars, SD.
**TABLE 3.** Cornea Transparency Gene Expressions Not Significantly Altered by T4

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<th>Gene</th>
<th>T4&gt;Cont C&lt;Cont</th>
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Cont, control; C<, cycle threshold; rel. relative.

**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 cornneas. Five micrograms T4 or Sal G alone was administered in ovo on E9; E12 cornneas were dissected, frozen, sectioned, and analyzed by ESI-MS/MS for amounts of KS DSD and MSD. Cornneas 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 corneal 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 TRβ1-T3, but not by TRα1-T3, in mouse liver and kidney,67 little is yet known about how T3 regulates expression but not other tissues.

**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 cornneas. Five micrograms T4 or Sal G alone was administered in ovo on E9; E12 cornneas were dissected, frozen, sectioned, and analyzed by ESI-MS/MS for amounts of KS DSD and MSD. Cornneas 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.
TRβ, and thus may be chick TRβ. TRβ1 and TRβ3 have not yet been reported in chick tissues. Our real-time PCR TR primers would amplify TRβ2 and -β3 and -β1 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 post-transcriptional 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 TRβ expression is accompanied by an increase in TRβ receptor protein.

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 T4, 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. 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 TRα in the absence of thyroid hormone, and with repressor sites. TRIP15 and TRH4 expression, 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 down-regulation. Expression of BAG7T4, a KS galactosyl transferase gene, increased only slightly during normal development, whereas SLC26 and PAPS2 mRNA expressions increased to 3- to 5-fold by E14, 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 some other genes in the PAPS synthetic pathway. Moreover, if a T4 is present in the promoter of chick SCL26, as in mouse SCL26, 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 PPBP and GSTA were high by E12 but did not change significantly thereafter. In contrast, the two δ-crystallin genes ASL1 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 ASL1 remained low. This is the reverse of expression patterns previously reported by Li et al. for ASL1 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 cornea expression of ASL2 175 times greater than expression of ASL1, 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 ACHE, TRIP15, and MEI are all regulated by thyroxine in some tissues. Expression of ACHE 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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