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# Changes in Retinal and Choroidal Gene Expression during Development of Refractive Errors in Chicks

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**Purpose.** During growth, the retina analyzes the projected image to achieve a close match between eye length and focal length. Because the messengers released by retina and choroid are largely unknown, genes that are differently expressed in response to changes in the retinal image were identified. In addition, because glucagon may be important in the visual control of eye growth, the transcript levels of proglucagon were studied.

**Methods.** Reverse transcription–polymerase chain reaction differential display was used to identify genes that were differentially expressed in chick eyes that were deprived of sharp vision or treated with positive or negative lenses. Differences were analyzed through sequencing and database searches and confirmed by Northern blot analyses.

**Results.** Combining 40 and 33 arbitrary primers with 3 oligo-dT-primers, approximately 48% and 40% of the retinal and choroidal mRNAs were screened, respectively. Twelve differences were detected in retinal tissue and five in choroidal tissue after 6 to 24 hours of exposure to defocus. Only one of 10 sequenced products could be identified as cytochrome-*c* oxidase, subunit I. Northern blot analysis confirmed its twofold upregulation after positive lens wear and also changes in four other unknown genes. Finally, it was shown that retinal glucagon mRNA content increased after treatment with positive lenses.

**Conclusions.** Visual conditions that induce refractive errors produce changes in gene expression in retina and choroid within 1 day. In line with previous immunohistochemical data, it was found that the amount of glucagon mRNA was upregulated during wearing of positive lenses. (*Invest Ophthalmol Vis Sci.* 2000;41:1623–1628)

It has been demonstrated, for example in chicks[1] and rhesus monkeys,[2] that the eye can determine the average position of the image plane and can adjust its axial growth rates to tune the focal length to the eye length. Negative lenses, which place the image behind the retina, accelerate axial eye growth, whereas positive lenses slow it down. The biochemical messengers released by the retina to produce acceleration or inhibition, respectively, seem to be different, because a number of agents selectively influence only myopia development. Moreover, the time kinetics of how exposure to defocus is translated into growth are quite different for positive and negative lenses.[3] There is a striking response of the choroid if refractive errors are experimentally induced. When the image is placed in front of the retina, the choroid can thicken up to threefold within 1 day, which corresponds to a refractive change of 7 D in the chick.[4] With negative lenses or frosted goggles, the choroid becomes slightly thinner. These changes effectively contribute to the compensation of the imposed refractive errors in chicks.

Until now, the messengers released by the retina to induce these changes have been unknown. Some hints were provided by previous experiments. The required spatial processing is likely to be a function of amacrine cells, and recently it was possible to restrict the number of candidate amacrine cells and involved neuromodulators.[5] It was found that amacrine cells that were immunocytochemically double-stained, both by antibodies against glucagon and the immediate early gene *ZENK*, showed a sign of defocus-specific upregulation of *ZENK* with positive lenses and downregulation with negative lenses after only 30 minutes of treatment.[6] The neuropeptide glucagon acts in a paracrine fashion and could play an important role as a messenger for the inhibition of axial eye growth.

To perform a more general screening for possible retinal and choroidal messengers that are involved in signal transmission during myopia and hyperopia development, we initiated a differential display study. The mRNA expression pattern was analyzed after treatment with either lenses or frosted goggles for different exposure times. To date, after screening approximately 48% and 40% of the total gene repertoire in the retina and choroid, respectively, we have found 17 genes with altered expression in response to changes in visual experience. In addition, inspired by the described studies,[6] we have performed Northern blot analysis to quantify the amount of glucagon mRNA during treatment with defocusing lenses.

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## METHODS

### Animals and Experimental Procedures

White leghorn chickens were raised under a 12–12-hour light–dark cycle. Their treatment was in accordance with the ARVO Statement for Care and Use of Animals in Ophthalmic and Vision Research. Ten- to 14-day-old chicks were unilaterally treated for different periods (4 hours, 6 hours, 1 day, and 1 week) with a −7-D lens, a +7-D lens, or a frosted goggle that acts as a low-pass filter on the spatial frequency spectrum but also reduces contrast over a wide range of spatial frequencies and produces deprivation myopia.[7] The contralateral eye served as the control. Because both eyes had different visual exposure, differences in their fundal gene expression can be attributed to their individual treatment. The observed changes are triggered by differences in the spatial features of the image, because no significant difference in retinal image brightness with lenses of refractive powers with different sign is present. Four chicks were used for each treatment. Tissue preparations were performed between 1 and 2 PM to exclude possible influence of diurnal factors.

### Isolation of Total RNA

Animals were killed by an overdose of ether, and eyes were immediately enucleated. The retina and choroid were carefully removed from the posterior segment and cooled quickly. Contamination by RPE cells could be reduced to a minimum, because retinal and RPE genes could be separately measured (Bitzer and Feldkaemper, unpublished data, 1999) in a parallel study. Total RNA was extracted (RNeasy Mini Kit; Qiagen, Hilden, Germany) and digested with DNase-I (Boehringer–Mannheim, Mannheim, Germany).

### Differential Display Analysis

The mRNA differential display technique[8] (DD-RT-PCR) was performed with some modifications using a kit (RNAimage; GeneHunter, Nashville, TN). Three one-base-anchored $HT_{11}N$ primers (H is AAGCTT; N is G, A, C) were used to subdivide the mRNA population. Reverse transcription was performed according to the manufacturer's instructions, except that the reaction contained 400 ng total RNA. During PCR reactions, different arbitrary 13mer primers (HAP) were used in combination with the appropriate $HT_{11}N$ primer.

The PCR products were separated on 6% sequencing gels at 45 W. A film (GelBond PAG; FMC BioProducts, Rockland, ME) was used to support the gels. Afterwards, nucleic acids were stained by using an improved silver staining method.[9]

### Reamplification and Sequencing

Bands of differing intensity between treated and untreated eyes were excised, resuspended, and purified. Reamplification occurred under the same conditions as during the first PCR, except that dNTP concentration was reduced to 2.5 $\mu$M. Reaction products were checked by gel electrophoresis and directly sequenced using a fluorescence sequencer and the sequencing kit and protocol (model 310 sequencer with DNA BigDye kit; Perkin–Elmer Applied Biosystems, Weiterstadt, Germany). Sequences were analyzed using the BLAST and FASTA network services (Geniusnet, Heidelberg, Germany). Amplimers were cloned and sequenced afterward, if direct sequencing failed.

### Cloning and Identification of Differential Display Bands

For cloning, the purified PCR amplimer was polished, ligated into a vector (pCR-Script Amp SK (+); Stratagene, Amsterdam, The Netherlands), and used to transform Epicurian coli XL-1 blue MRF′ Kan supercompetent cells. Inserts were sized by colony PCR. Amplimers that showed the correct length were automatically sequenced. If more than one sequence was obtained, the most abundant one was used for further studies.

### Probe Preparation for Northern Blot Analysis

For each amplimer that had been sequenced without cloning, a specific forward primer was designed, according to the sequence information: The antisense primer was composed of a leader sequence, the consensus T3 sequence and the gene-specific sequence as previously described in detail.[10] The resultant PCR product was purified and digoxigenin (DIG) was incorporated during transcription, by using T3 polymerase. Focusing on differentially expressed genes that were cloned before sequencing, DIG-labeled riboprobes were prepared by in vitro transcription using T7 polymerase (DIG RNA labeling kit; Boehringer–Mannheim). Primers for the cytochrome-*c* oxidase probe were complementary to sense nucleotides +7313 through +7331 and antisense nucleotides +7681 through +7671 of the mitochondrial genome sequence (EMBL: X52392). A 51mer oligonucleotide (5′-GAT GTG GTA GCC GTT TCT CAG GCT CCC TCT CCG GAA TCG AAC CCT GAT TCC-3′) was end labeled to generate the 18S-rRNA probe (DIG-oligonucleotide 3′-end labeling kit, Boehringer–Mannheim). Primers for glucagon amplification were complementary to sense nucleotides +47 through +69 of the 5′ untranslated region and antisense nucleotides +427 through +417 of the pancreatic pre- proglucagon coding sequence (EMBL:Y07539) plus leader and T3 consensus sequence.

### Northern Blot Analysis

Differences in gene expression were confirmed by Northern blot analyses. One microgram of RNA was run on 1.2% formaldehyde-agarose gels, blotted overnight onto a positively charged nylon membrane (Boehringer–Mannheim) and UV cross-linked. Blots were hybridized for 16 hours with 100 ng/ml DIG-labeled probe at 68°C (cytochrome-*c* oxidase), 61°C (18S-rRNA), or 53°C (all other probes). Afterwards, Northern blots were washed two times for 10 minutes each with prewarmed 2× SSC/0.1% sodium dodecyl sulfate (SDS) at the respective hybridization temperature. This was followed by two 15-minute washing steps with 0.1× SSC/0.1% SDS (cytochrome-*c* oxidase, 18S-rRNA) or 0.3× SSC/0.1% SDS (all other probes). The highest possible stringency was tested in advance. Chemiluminescence detection was performed using disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclodecan}-4-yl (CSPD), Boehringer–Mannheim) as substrate. Blots were exposed to x-ray film (Curix HT1; AGFA, Leverkusen, Germany), stripped, and reprobed with an 18S-rRNA probe to control for gel loading.

Bands were quantified by digitization with a scanner. Using NIH Image software (National Institutes of Health, Be-

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thesda, MD), bands were analyzed and pixel intensity calculated as an arbitrary value. The ratio of the intensity of the band of the probe to the band intensity of 18S rRNA revealed the normalized probe mRNA level. These normalized mRNA levels were compared using the appropriate Student's *t*-test. Moreover, band intensities (probe/18S-rRNA) were calculated as a percentage of control levels (treated eye/control eye) for each animal, because this allowed a better comparison of different blots. The average percentages and SDs are given in the text. Absolute values could not be calculated using chemiluminescence detection and exposure to x-ray film, because the film has a limited linear range.

## Results and Discussion

As seen in Figure 1, most bands remained unchanged with changes in visual exposure.

The arrows in Figure 1 mark a possibly differentially expressed gene that was downregulated in three of four subjects in the retina after goggling for 4 hours. Its sequence was determined (Table 1, fragment 7) but did not show homology to any known genes. For analysis of retinal and choroidal tissue, 40 or 33 arbitrary primers were combined with the three different anchor primers, respectively. Given that 60 bands per primer combination were obtained on average and assuming that approximately 15,000 genes are expressed per cell, approximately 48% (retina) and 40% (choroid) of the total gene repertoire was screened. As shown in Table 1, DD-RT-PCR revealed 12 differences in gene expression in the retina and 5 in the choroid. In the cases in which genes were downregulated during treatment, gene fragments were excised from control lanes. To date, 10 of these bands have been sequenced.

Surprisingly low numbers of genes were affected by visual conditions that determine ocular elongation. Nevertheless, the screen proved to be a powerful tool, because the differential regulation could be confirmed in all five investigated cases (see discussion later). We analyzed changes in gene expression mainly 6 hours to 1 day after treatment. At this time, signals should be expected that trigger initial steps of the cascade. In chicken eyes, imposed refractive errors are compensated within a few days. Our time window for sampling was probably too late to screen for changes in immediate early gene expression, because previous studies showed focus-dependent changes in expression of *ZENK* after only 30 minutes.[6] In situ hybridization studies will be performed in the future to investigate the cellular distribution of differentially expressed genes.

### Analyses of Nonidentified Transcripts

Fragments 5 and 7, derived from retina, and 13 and 15, derived from choroid (Table 1), were tested by Northern blot analysis to confirm changes in gene expression. The eyes with normal vision did not show significant changes in the expression pattern, no matter how the contralateral eye was treated.

**Fragment 5.** Northern blot analysis of transcript 5 revealed one band of 2.2 kb (Fig. 2A). Band intensity increased, especially after short treatment periods with positive lenses in comparison to control levels (6 hours, 300% ± 99%; 1 day, 211% ± 130%, $n$ = 3). Goggle wear and negative lens treatment decreased mRNA levels slightly to 78% ± 29% and 94% ± 25%, respectively, after 6 hours of treatment (not shown, $n$ = 2) and to 74% ± 29% and 68% ± 57%, respectively ($n$ = 3) after 1 day.

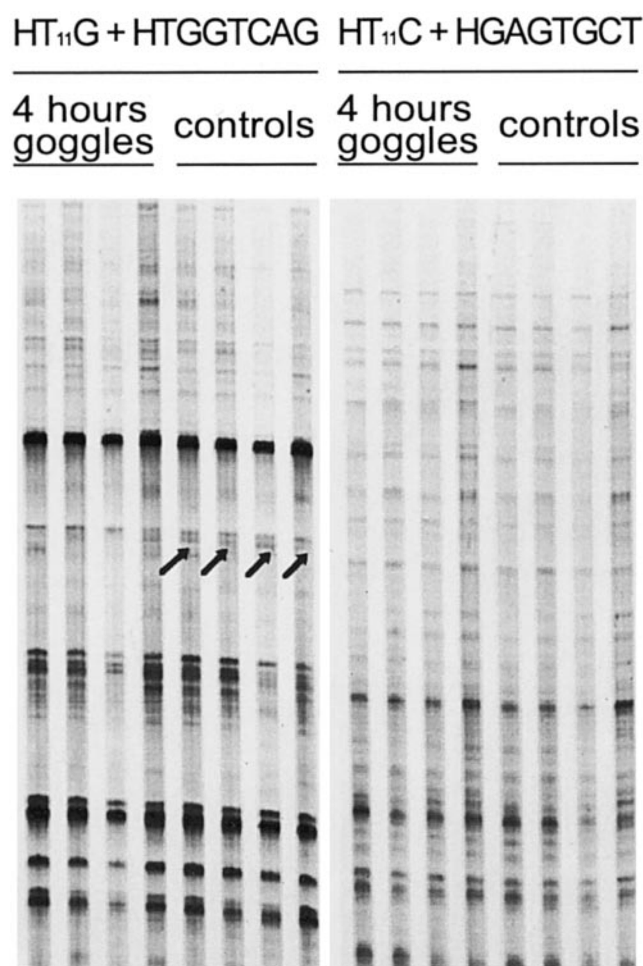


**Figure 1.** DD-RT-PCR using RNA isolated from retinas of eyes with normal vision (control retinas) and from retinas of the contralateral fellow eyes that were goggled for 4 hours. The silver-stained gel shows gene expression in four eyes with use of two primer combinations. *Arrows:* higher expression of the gene in the control retinas in three of the four subjects, with the primer combination $HT_{11}G$ and HTGGT-CAG. In one chick (*lanes 4* and *8*) expression of this band was high in the control and the treated retina. The approximate size of the band was 490 bp. No differences could be detected when the same RNAs were used for RT-PCR using the primer combination $HT_{11}C$ and HGAGTGCT. Changes in gene expression were not observed with the two primer sets in other areas of the gel.

**Fragment 7.** One prominent band of 1.9 to 2.1 kb was detected using probe 7. Band intensity was enhanced after 1 day of positive lens wear (146% ± 19%, $n$ = 3). One day of negative lens wear increased band intensity approximately twofold (232% ± 101%, $n$ = 3). In contrast, band intensity was decreased after 1 day of goggle wear (57% ± 18%, $n$ = 3).

**Fragment 13.** Hybridization with probe 13 showed one band of 2.0 kb (Fig. 2B). Positive and negative lenses did not affect its mRNA level (1 day of positive lens wear: 82% ± 10%, $n$ = 3; 1 day of negative lens wear: 83% ± 69%, $n$ = 2). Goggle wear led to a decrease of the transcript level down to 48% ± 7% of the control value ($n$ = 3).

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**TABLE 1.** Analysis of cDNA Fragments Identified by Differential mRNA Display

| Band | Tissue | Forward Primer | Reverse Primer | Size (bp) | Treatment | Sequence Homology |
|---|---|---|---|---|---|---|
| 1 | Retina | HCCACGTA | $HT_{11}G$ | 300 | Control | No homologues |
| 2 | Retina | HTAGTCCA | $HT_{11}C$ | 190 | Control | |
| 3* | Retina | HCCACGTA | $HT_{11}A$ | 427 | 1 Day + 7-D lens | Cytochrome *c*-oxidase subunit I |
| 4 | Retina | HCGGCATA | $HT_{11}A$ | 350 | 4 Hours goggle | No homologues |
| 5* | Retina | HGACCTTT | $HT_{11}G$ | 360 | Control | No homologues |
| 6 | Retina | HGAGTGCT | $HT_{11}A$ | 380 | Control | No homologues |
| 7* | Retina | HTGGTCAG | $HT_{11}G$ | 490 | Control | No homologues |
| 8 | Retina | HCATTCCG | $HT_{11}G$ | 580 | 1 Day goggle | |
| 9 | Retina | HCGGGTAA | $HT_{11}G$ | 600 | 1 Day goggle | |
| 10 | Retina | HTCCTGGA | $HT_{11}G$ | 275 | Control | |
| 11 | Retina | HTGAGACT | $HT_{11}G$ | 640 | 1 Day goggle | No homologues |
| 12 | Retina | HCCACGTA | $HT_{11}G$ | 300 | Control | |
| 13* | Choroid | HCGAAATG | $HT_{11}C$ | 280 | 1 Day goggle | No homologues |
| 14 | Choroid | HTAGTCCA | $HT_{11}C$ | 350 | 1 Day − 7-D lens 4 hours goggles | No homologues |
| 15* | Choroid | HCCACGTA | $HT_{11}C$ | 400 | 1 Day goggle | No homologues |
| 16 | Choroid | HACGGGGT | $HT_{11}C$ | 320 | Control | |
| 17 | Choroid | HGCGGTGA | $HT_{11}C$ | 440 | 1 Day goggles | |

* Studied in more detail by Northern blot analysis.

**Fragment 15.** Probe 15 exposed two bands of approximately 1.9 kb and 4.4 kb. The mRNA level of probe 15 was unaffected for both bands by wearing positive lenses (109% ± 20% and 97% ± 58%, respectively, $n = 3$). After 1 day of goggle treatment, the intensity of the 1.9-kb band was decreased to 79% ± 40% and the 4.4-kb band intensity to 29% ± 14% ($n = 3$). Negative lens treatment for 1 day decreased intensity of both bands (1.9-kb band: 80% ± 23%; 4.4-kb band: 65% ± 30%, $n = 3$).

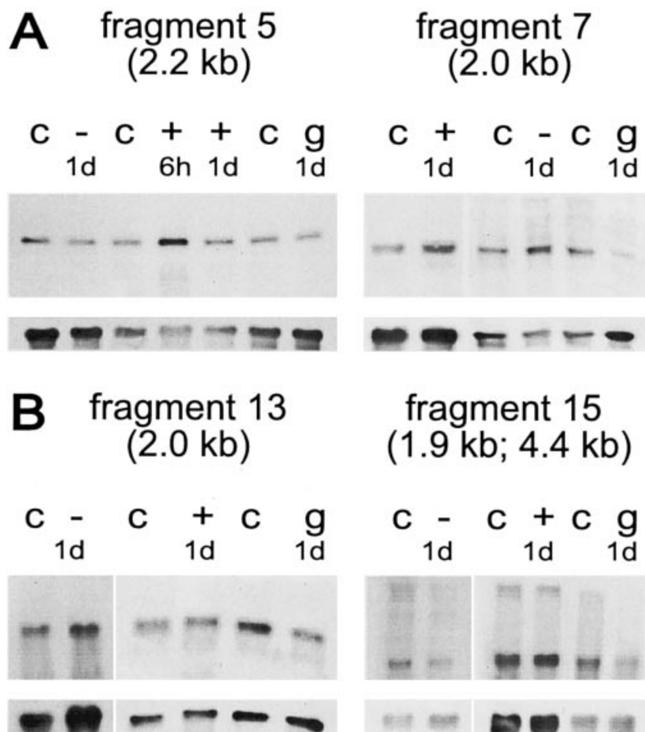


**FIGURE 2.** Confirmation of differential expression of nonidentified transcripts by Northern blot analyses. Chicks were unilaterally treated with +7-D lenses (+) for 6 hours (6h) or for 1 day (1d) and with −7-D lenses (−) or goggles (g) for 1 day. The contralateral eye served as control (c). To control for gel loading, the blots were stripped and reprobed with a probe for 18S-rRNA (*bottom lanes*). (**A**) Effects of lens and goggle wear on abundance of transcripts 5 and 7. RNA was derived from retina of treated chicks. (**B**) Effects of lens and goggle wear on abundance of transcripts 13 and 15. Differential expression of both genes was detected in the choroid.

Sequence analysis showed that only the reverse strand of probe 13 contained a complete open reading frame. Because the fragments 5, 7, 13, and 15 have not yet been assigned to known gene sequences, we cannot comment on their possible function(s). A search for longer homologous cDNA sequences is planned, because it is possible that these transcripts represent specific modulatory substances. At the least, transcript 5 changed after exposure to defocus in a signal-specific fashion. This requires complex and yet unknown image processing.

## Effects of Visual Exposure on Cytochrome-*c* Oxidase mRNA Levels

The only sequence that could be identified by database searches was 99.8% identical with base pair +7432 through +7854 of the chicken mitochondrial genome corresponding to cytochrome-*c* oxidase subunit I.[11] The hybridization signal was at the expected size for the full-length coding sequence (1545 bp, Fig. 3A). Treatment with positive lenses for 6 hours and 1 day increased mRNA levels significantly (1 day, $P = 0.018$, paired Student's *t*-test, $n = 10$) to 199% ± 140% and 176% ± 82%, respectively. This difference vanished after 1 week of positive lens treatment (80% ± 27%, $n = 3$). Negative lenses decreased relative cytochrome-*c* oxidase levels to 55% ± 32% ($n = 3$) after 6 hours and to 86% ± 56% ($n = 10$) after 1 day of treatment, but this effect did not achieve statistical significance. There was also a trend in goggle-treated eyes toward a decrease in cytochrome-*c* oxidase subunit I mRNA level (6 hours, 88% ± 40%, $n = 4$; 1 day, 76% ± 38%, $n = 9$; 1 week, 61% ± 27%, $n = 2$).

Cytochrome-*c* oxidase is an important energy delivering enzyme of the oxidative phosphorylation pathway. In the chicken retina, cytochrome-*c* oxidase (CO) antibodies specifically label convergent centrifugal axons, amacrine cells, and ganglion cells.[12] In the present study, a significant increase in

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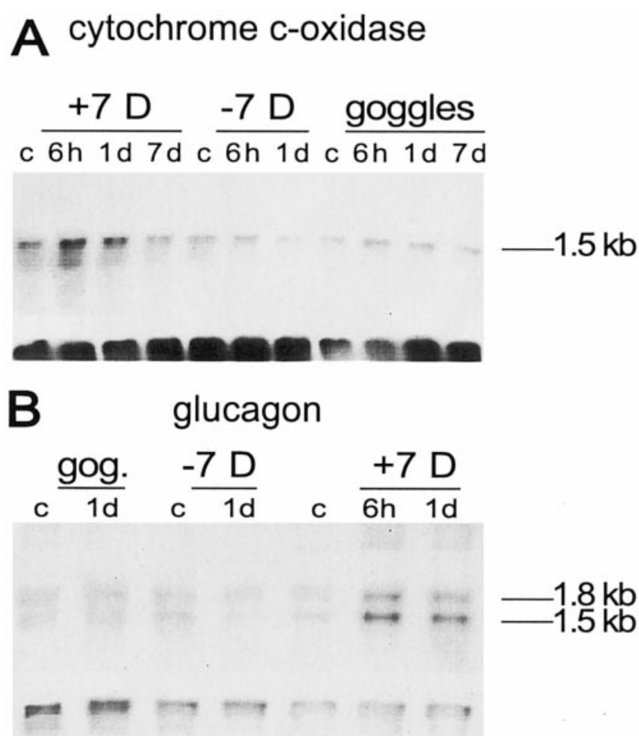


FIGURE 3. Increased retinal expression of cytochrome-*c* oxidase and proglucagon mRNA level after treatment with positive lenses. Chicks were unilaterally treated for different periods, with the contralateral eye serving as control. Total RNA (1 μg) was extracted from control eyes (c) positive lens–wearing (+7 D), negative lens–wearing (−7 D), and goggled chicks (goggles) after 6 hours (6h), 1 day (1d), or 7 days (7d) of treatment to determine the time course of gene expression. Northern blots were reprobed for 18S-rRNA (*bottom lanes*). (**A**) Effects of lens and goggle wear on abundance of cytochrome-*c* oxidase mRNA. (**B**) Effects of lens and goggle wear on abundance of proglucagon mRNA.

mRNA level was found during 6-hour and 1-day treatment with positive lenses. We did not measure CO activity, but because a close relationship between cytochrome-*c* oxidase subunit I mRNA level and CO activity was shown,[13] our results may indicate enhanced CO activity during development of hyperopia. The increased mRNA level during development of hyperopia may therefore indicate an increased metabolic requirement due to growth processes per se, or it may be due to changes in neuronal activity.

### Changes in Glucagon mRNA Expression Induced by Lens Wear

With a proglucagon probe, Northern blot analysis (Fig. 3B) showed bands with molecular weights varying between 1.8 and 2.0 kb, as well as a 1.5-kb band. Additionally, some blots showed a weaker band of 1 kb and of 4.6 kb. The 1.5-kb band corresponds to the expected size of the pre- proglucagon mRNA (1576 bp). Therefore, results concerning the intensity of the 1.5-kb band are given in detail. One day of positive lens wear increased glucagon mRNA levels significantly ($P = 0.045$, paired Student's *t*-test). After 6 hours of positive lens wear, the glucagon mRNA level was increased to 158% ± 27% ($n = 4$), and it was even higher after 1-day treatment with +7-D spectacles (197% ± 105%, $n = 8$). Glucagon levels reached control levels after 7 days of treatment with positive lenses (data not shown). There was a trend toward a decrease in the amount of glucagon mRNA during treatment with negative lenses for 6 hours (71% ± 13%, result not shown, $n = 3$) and 1 day (67% ± 32%, $n = 7$) that did not achieve statistical significance in comparison with the respective controls (1-day −7-D lens treatment versus control, $P = 0.067$, paired Student's *t*-test). Goggle wear did not influence glucagon mRNA level consistently (6 hours, 107% ± 15%, result not shown, $n = 3$; 1 day, 100% ± 33%, $n = 7$).

Although glucagon has been shown to act as a neurotransmitter/neuromodulator in the central nervous system,[14] its role in the retina is less clear. Treatment of chicken retina with glucagon increased the cyclic adenosine monophosphate (cAMP) level.[15] Other studies[16] in the turtle retina led to the conclusion that glucagonergic amacrine cell may provide OFF-modulation in interactions between the ON- and OFF-center visual pathways. From our Northern blot analysis studies, it can be concluded that glucagon is one promising candidate for a messenger carrying the sign of defocus information, because mRNA levels increased significantly after positive lens wear and showed at least a trend toward a decrease after negative lens wear ($P = 0.067$). To further strengthen the possible role of glucagon, we are currently conducting pharmacologic studies using glucagon antagonists and agonists.

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