

Isolation and Characterization of the Human CP49 Gene Promoter

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PURPOSE. This study focuses on the identification of regulatory elements that contribute to lens-specific expression of the human CP49 gene within the 5'-flanking DNA sequences.

METHODS. The DNA sequence upstream of the human CP49 coding region was subcloned as a set of 5' and 3' deletion series. The constructs were transfected into lens (N/N1003A) and nonlens (NIH3T3) cell lines and chicken primary lens cultures, to test for promoter activity and specificity. To further test the specificity, a portion of the 5' flanking DNA sequence was used to drive transgene expression in mice. The flanking DNA sequence was analyzed for potential transcription factor-binding sites.

RESULTS. The 5'-flanking DNA preferentially activated reporter gene expression in a lens-preferred manner when transfected into cultured cells. Transgene expression driven by the CP49 promoter region was lens specific. Analysis of the proximal promoter sequence revealed the presence of potential binding sites for the AP-1, AP-2, and OCT-1 transcription factors and the absence of TATA and CAAT boxes.

CONCLUSIONS. The sequence upstream of the CP49 gene possesses promoter activity and is able to drive lens-preferred expression in both transfection and transgenic experiments. Promoter activity is dependent on the presence of the proximal 300 bp directly upstream of the coding region. (*Invest Ophthalmol Vis Sci.* 2003;44:235-243) DOI:10.1167/iovs.02-0162

Ocular lens development commences with a thickening of the anterior surface ectoderm that establishes the boundary of the presumptive lens as the lens placode. Invagination of the thickened ectoderm creates the lens pit, which is then pinched off from the ectoderm as a hollow sphere of cells known as the lens vesicle. The epithelial cells at the posterior end of the lens vesicle undergo terminal differentiation to form primary lens fiber cells. During this process, the cells elongate to fill the lumen of the lens vesicle. The anterior portion of the lens is lined by a single layer of epithelial cells that remain mitotically active and undergo differentiation at the equator, or bow region, of the lens to continually add secondary fiber cells to the lens mass. In addition to cell elongation the differentiation process of lens epithelial cells to lens fiber cells involves the loss of the nucleus and other membrane-bound organelles and alterations in gene expression.¹

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Lens fiber cell formation results in the upregulation of a limited collection of genes that includes members of the soluble crystallin gene family, major intrinsic protein (MIP), and the core components of the lens fiber-specific beaded filament CP49 and CP115.² The CP49 and CP115 genes have been classified as divergent members of the intermediate filament family on the basis of conserved primary DNA sequence and gene structure.³⁻⁶ The expression of the beaded filament genes is unique to lens fiber cells, which allows the use of these genes as markers of differentiation. The CP49 and CP115 gene products are observed in lens fiber cells that are nearing full elongation.⁷ Mutations in the CP49 have been linked to cataract formation, suggesting a role for the beaded filament, which is critical to lens clarity.^{8,9}

The characterization of crystallin promoters has identified a wide variety of transcriptional regulators involved in establishing the specific spatial and temporal expression patterns of the individual crystallin genes. Of these transcription factors, only a few are used in a widespread manner. Consensus-binding sites for Pax, Sox, and Maf factors have been located within the promoter-enhancer regions of several crystallin genes and are integral in achieving the high levels of expression observed in vivo.¹⁰⁻¹³ The mutation or absence of any one of these factors in mice, accomplished through targeted gene deletion, results in abnormal eye and/or lens development, ranging from defective lens fiber cell differentiation to the complete absence of the lens.¹³⁻¹⁷ Although the regulation of crystallin gene expression has been well characterized, knowledge of the mechanism(s) governing the expression of noncrystallin lens fiber cell genes is limited. It is not known whether regulators present in crystallin promoters influence lens fiber cell gene expression as a whole and also take part in the regulation of expression of CP49, CP115, and MIP, or whether a distinct set of factors is involved outside of the crystallin gene family.

In this study, the 5'-flanking and intronic DNA sequences of the human CP49 gene have been analyzed for potential regulatory elements. The transfection of cell lines and primary lens cultures and the production of transgenic animals were performed to test the proximal promoter for contributions to the cell type and differentiation-stage-specific expression pattern observed for the human CP49 gene. The results of these experiments suggest that the 5'-flanking DNA proximal to the human coding sequence confers lens-preferred expression of reporter genes. Sequence analysis of the 5'-flanking region does not identify consensus binding sites for transcription factors used in the regulation of crystallin genes, suggesting the possibility of separate control mechanism(s) for crystallin and noncrystallin gene regulation within the lens fiber cell.

METHODS

Generation of Human CP49 Transfection Constructs

5'-Deletion Series. DNA sequences flanking the 5' end of the human CP49 gene were obtained from the screening of a human chromosome 3 *Hind*III λ -phage library. The isolated clone contained approximately 3 kb of upstream sequence, along with a portion of the

coding sequence, and was shuttled into the pCR II vector by using PCR primers within the human CP49 rod 1B region and a primer flanking the insertion site of the phage. The entire portion of the 5'-flanking DNA was cloned into the pGL2-Basic vector (Promega, Madison, WI) as two restriction fragments, which resulted in the -3183/-19 clone, relative to the translation start site. The 5' end of the sequence was excised as a 2.8-kb *KpnI-BclI* fragment from the pCRII clone. The 3' end of the flanking sequence was subcloned into the pSP72 vector (Promega) as a blunted *BamI* fragment that spans the -911/-19 region of the upstream sequence. The proximal upstream sequence was then excised as a *BclI-HindIII* fragment and ligated, along with the 5' end fragment, into the pGL2-Basic vector that had been cut with *KpnI* and *HindIII*. Subsequent deletion constructs were produced through restriction enzyme digestion of this parent clone or through PCR amplification of the 5'-flanking region. The integrity of the DNA sequence for PCR-amplified regions was confirmed by sequencing at the University of California Davis Division of Biological Sciences automated sequencing facility.

3'-Deletion Series. Oligonucleotide primers were obtained to manufacture a series of 3' deletion constructs through PCR. Each 3' deletion PCR primer was designed with a *HindIII* restriction site at the 5' end. The 5' oligonucleotide primer used in the production of the 3' deletion series spanned the -382/-362 region of the 5'-flanking sequence and possessed a *XbaI* restriction site at its 5' end. All the PCR reactions were cut with *XbaI* and *HindIII* and cloned into pGL2-Basic, cut with the same enzymes. Once again, DNA sequencing was used to confirm integrity of the inserted sequences generated by PCR.

Mouse CP49 Transfection Constructs. A P1 clone containing the coding region of the mouse CP49 gene and 5'-flanking sequence was obtained from Incyte Genomics (St. Louis, MO), through the screening of a mouse genomic P1 library, with CP49 exon I used as a probe. The 5'-flanking sequence of the CP49 gene was sequenced, and oligonucleotides were produced to generate the two constructs. The 5' primer used spans the region of the flanking sequence from -924 to -903, and the 3' primers correspond to the -38/-15 and -300/-277 portions of the upstream sequence. The primers possessed an additional 5' sequence containing restriction enzyme sites to aid in subcloning (*XbaI* for the 5' primer and *HindIII* for each of the 3' primers). PCR amplification yielded two fragments that encompass the -924/-15 and -924/-277 flanking DNA sequences that were then cloned into the pGL2-Basic vector. Both clones were sequenced to verify that the amplified fragments were not altered.

Transfection Protocol

N/N1003A Cells. The nontransformed rabbit lens epithelial cell line was maintained by culturing in Eagle's minimal essential medium (EMEM; Sigma, St. Louis, MO) and 8% rabbit serum (Sigma) in a 37.0°C, 6.0% CO₂, and humidified environment. Cells were seeded in 12-well plates (Falcon, Franklin Lakes, NJ) and grown to 60% to 80% confluence, typically within 24 to 30 hours, before the transfection was begun. Both serum and antibiotics were absent from the medium at the time of transfection. One hundred nanograms of pRL-TK (Promega), the internal standard, and 250 ng of each transfection construct was added to 50 μ L of EMEM with nonessential amino acids. DNA condensing reagent (2 μ L; Invitrogen, Carlsbad, CA) was then added to the diluted DNA, mixed, and incubated at room temperature for 15 minutes. Transfection reagent (2.5 μ L; Lipofectamine; Invitrogen) was added to 50 μ L of EMEM with nonessential amino acids. The transfection solution was then mixed with the DNA/Plus reagent solution and incubated at room temperature for 15 minutes. The cells were washed with 1 mL of EMEM with nonessential amino acids, and the medium was aspirated and replaced with 400 μ L of fresh medium. The cells were covered with the DNA-transfection reagent mixture (100 μ L) and incubated for 3 hours, at which time, 500 μ L of fresh growth medium (EMEM supplemented with nonessential amino acids and 16% rabbit serum) was added to each well. At 24 hours after transfection, the medium was aspirated and replaced with 1 mL of EMEM complete with

rabbit serum, nonessential amino acids, and gentamicin. The cells were harvested at 48 hours after transfection through addition of 250 μ L of 1 \times passive lysis buffer from the a dual luciferase kit (Promega) and incubation for 15 minutes at room temperature. Lysates were cleared of particulate debris and stored at -80°C until analyzed.

NIH3T3 Cells. The cell line was maintained by culturing in Dulbecco's minimum essential medium (DMEM; Invitrogen) supplemented with 25 mM HEPES (Invitrogen), 10% bovine calf serum (HyClone, Logan, UT), and penicillin-streptomycin (Invitrogen). The cell line was cultured in the same environment as described earlier. The protocol for transfection of the NIH3T3 cell line was the same as that used for the N/N1003A cell line except that DMEM with HEPES was used and 3 μ L of Plus reagent and 3 μ L of transfection reagent replaced the volumes used in the N/N1003A protocol. At 3 hours, 500 μ L of growth medium (DMEM with 20% bovine calf serum and 50 mM HEPES) was added, and, at 24 hours after transfection, the medium was replaced with 1 mL complete DMEM as described earlier.

Embryonic Chicken Lens Epithelial Cell Cultures. Lenses were harvested from 14-day-old chicken embryos and prepared for culturing as described by Menko et al.¹⁸ Once isolated, the cells were cultured in medium 199 (Sigma) supplemented with 10% fetal calf serum (Sigma), cultured to confluence in a T-75 flask (Falcon, Franklin Lakes, NJ) coated with rat-tail collagen (Sigma), trypsinized, and seeded onto collagen-coated 12-well plates. The cells were grown until the proper confluence, 60% to 80%, was reached (36-48 hours), at which time the transfection was started. The protocol followed the same outline as that for NIH3T3 cells, with medium 199 replacing DMEM. Antibiotics and antifungal reagents were absent only for the initial 3-hour incubation at the start of the transfection. These reagents were then added with the growth medium. Two microliters of Plus reagent and transfection reagent were used for the transfection of the chicken lens epithelial cells.

Analysis of Transfected Cells. The lysates were assayed for each transfection experiment using the dual luciferase kit (Promega) and a luminometer (DyneX ML3000; Thermo Labsystems, Chantilly, VA). Twenty microliters of cell lysate was pipetted into individual wells of a flat-bottomed, 96-well microtiter plate (Microplate I; Thermo Labsystems). Luciferase assay reagent (100 μ L, LARI; Promega) was dispensed into a well, and the luciferase activity allowed to integrate over a 10-second period. After the wells had been assayed, 100 μ L of buffer (Stop n Glo; Promega) was dispensed into each well, and the luciferase activity from the internal standard pRL-TK was measured over a 10-second interval. Each construct within an experiment was transfected in triplicate. The summarized data are the result of at least three separate transfection experiments. Data were normalized within each experiment for each cell line and were used only when the internal standard levels were similar between the two cell lines, suggesting that the transfection efficiency across the experiments was similar and that differences in activity were not due to a significant disparity in transfection efficiency. The data from the luciferase experiments were then compared with the activity of the promoterless and enhancerless pGL2-Basic vector and expressed as multiples of increase over the activity of the pGL2-Basic vector.

Generation of the Transgenic Construct p β gal-Basic hCP49 -1859/-19. The -1859/-19 region of the 5'-flanking DNA sequence was shuttled from the pGL2-Basic vector into the p β gal-Basic vector (Clontech, Palo Alto, CA). The p β gal-Basic h49 -1859/-19 was then cut with *XbaI* and *BamHI* and the fragment gel purified. The inserted fragment contained all the h49 5'-flanking sequence and was ligated upstream of the coding region for the β -galactosidase gene along with an SV40 intron and polyA signal 3' to the coding sequence. The fragment was microinjected into the fertilized pronuclei of a B6CBA mouse. Sixty-seven of the 250 injected pronuclei survived to term. Genomic DNA from the 67 mice was isolated by using a kit (DNeasy; Qiagen, Valencia, CA). A primer from the coding region of the β -galactosidase gene, within the p β Gal-Basic vector corresponding to bases 228 to 252 of the vector sequence and a primer

from the 5'-flanking DNA sequence of the human CP49 gene spanning the -227/-207 region were used in the PCR screen. PCR analysis confirmed the presence of the transgene within the genomic DNA of seven animals (three males and four females). The seven founder mice were then bred to a BL6 background. Offspring from the seven founder B6CBA transgenic mice and the BL6 mice were genotyped by PCR and subsequently analyzed for expression of the β -galactosidase reporter gene. All procedures involving animals were performed in compliance with the ARVO Statement for the use of Animals in Ophthalmic and Vision Research.

Tissue Lysate Preparation from Transgenic Animals.

Samples were taken from several tissues and prepared in the following manner. The tissue samples were first rinsed with ice cold $1 \times$ PBS three times. A 1-mL aliquot of cell lysis buffer (100 mM potassium phosphate [pH 7.8], 0.2% Triton X-100, and 1 mM dithiothreitol [DTT]) was added to the tissue sample, and the samples were homogenized. The homogenate was shaken for 1 minute in lysis buffer after homogenization and subsequently centrifuged for 5 minutes at 13,000 rpm, to clear cellular debris. The supernatant was aspirated and total protein concentration was established by bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL).

β -Galactosidase activity from the tissue lysates was measured with a luminescent β -gal kit (Clontech). Thirty-five micrograms of total protein for each sample was pipetted into the wells of a flat-bottomed, 96-well microtiter plate. Two hundred microliters of reaction buffer and reaction substrate (Clontech) mixture was added to each sample and mixed. The reaction was then incubated for 1 hour at room temperature and assayed on a luminometer (Dynex ML3000; Thermo Labsystems) for a 10-second interval. The β -galactosidase levels were the mean results of at least three independent assays for each transgenic line.

Immunohistochemical Analysis of Transgenic Animals. The eyes of transgenic and wild-type animals were harvested and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer at 4°C overnight. The tissue was then embedded in paraffin and sectioned for immunostaining. After deparaffinization and rehydration, the sections were treated for 15 minutes, at room temperature, with pepsin (Sigma), at a concentration of 1 mg/mL in 2.8% glacial acetic acid, followed by subsequent treatment with 3% H₂O₂. The tissue was then incubated in 10% normal goat serum (Invitrogen) for 30 minutes. The primary antibody, a rabbit polyclonal antibody to the bacterial β -galactosidase gene (Novus Biochemicals, Littleton, CO), was diluted 1:500 in PBS and the sections were exposed to the antibody solution for 90 minutes. After a wash in PBS, the sections were incubated with the secondary antibody solution, biotinylated goat anti-rabbit IgG (Zymed, Orange, CA), and then exposed to streptavidin-peroxidase (Zymed) for 15 minutes. Reactivity was visualized by a 3-minute incubation with 3,3-diaminobenzidine (DAB; Sigma). The sections were then counterstained in hematoxylin (Sigma), dehydrated, and coverslipped. For the preadsorbed primary antibody solution, an excess of purified bacterial β -galactosidase (Calbiochem, La Jolla, CA) was added to the diluted β -galactosidase antibody. The solution was then cleared by centrifugation.

RESULTS

Transcription Factor-Binding Sites within the CP49 Proximal Promoter

Potential transcription factor-binding sites within the 5'-flanking DNA sequence of the human CP49 gene were identified in searches of the TRANSFAC (<http://transfac.gbf.de/transfac/>, German Research Center for Biotechnology, Braunschweig, Germany), TFSEARCH (www.cbrc.jp/research/db/trsearch.html/, Computational Biology Research Center, Tokyo Japan), and Matinspector (<http://www.gsf.de/biodv/matinspector.html/>, National Research Center for Environment and Health, Neuherberg, Germany) databases (all provided in the public domain)

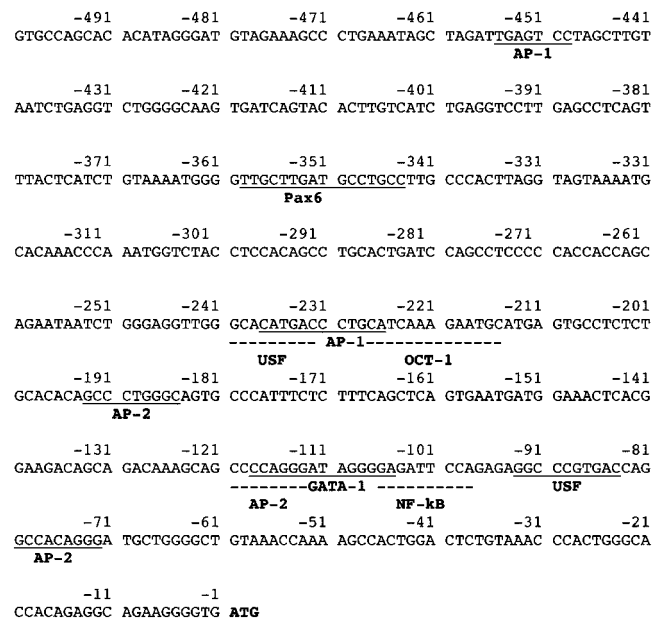


FIGURE 1. The proximal promoter sequence of the human CP49 gene with identified putative transcription factor binding sites, as determined by the TRANSFAC, TFSEARCH, and Matinspector databases. The start of the coding region (ATG) is in *bold*.

under default settings for vertebrates. The results of these searches have been compiled, and the putative sites for transcription factors are displayed in Figure 1.

Transfection of Cell Lines with Human CP49 5'-Flanking DNA Constructs

A set of 5'-deletion constructs (Fig. 2) were produced by restriction digestion or PCR, and each was transfected into both the rabbit lens epithelial cell line N/N1003A and the mouse embryonic fibroblast cell line NIH3T3. Figure 3A shows the transient transfection of lens and nonlens cell lines in an effort to assay for potential promoter activity and tissue specificity within the 5'-flanking DNA sequence of the human CP49 gene. The parent 5'-flanking construct, from which the deletion series was derived, contains the -3189/-19 sequence. Transfection of this construct in the N/N1003A cell line resulted in a relative 93.41-fold increase in luciferase activity over that of the promoterless and enhancerless pGL2-Basic vector. Sequential deletion of the 5' distal sequence did not yield a significant change in reporter gene expression. Each of the deletion constructs through -382/-19 exhibited luciferase activity similar to that of the larger -3189/-19 construct. Included in these transfection experiments were constructs containing the -911/-19 sequence in the negative orientation, in relation to the luciferase coding sequence and the -911/-277 construct in which 258 bp at the 3' end of the flanking DNA had been excised. The -911/-19 sequence in the negative orientation exhibited a reduced level of luciferase activity, 8.59-fold, compared with that of the 142.77-fold induction of reporter gene expression observed for the same sequence in the positive orientation. The deletion of the DNA sequence at the 3' end of the flanking region abolished promoter activity resulting in a relative luciferase activity of 5.51-fold. The retention of activated reporter gene expression in spite of truncation at the 5' end of the flanking DNA sequence suggests that the proximal sequence is important in promoter activity. This is further supported by the loss of promoter activity with truncation of the inserted flanking DNA at the 3'

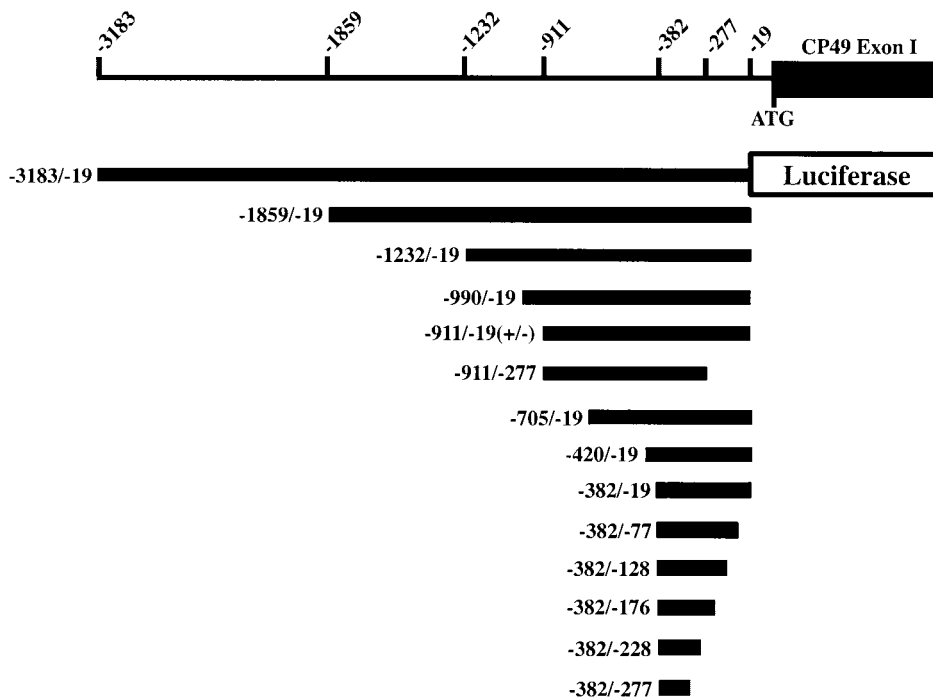


FIGURE 2. Illustration of the DNA sequences flanking the human CP49 coding region cloned upstream of the firefly luciferase gene. The list includes first the 5' and then the 3' deletion constructs, from *top* to *bottom*, that were used in the transfection experiments. The $-911/-19$ clone was inserted into the transfection vector, pGL2-Basic, in both the positive and negative orientations and is listed with a (+/-) in the figure.

end. The reduction of reporter gene expression observed for the $-911/-19$ sequence in the negative orientation suggests that the potential regulatory elements within the 5'-flanking sequence are dependent on proper orientation for optimal activation.

The same set of constructs were transfected into the NIH3T3 cell line to determine whether the 5'-flanking DNA sequence promotes reporter gene expression in a tissue-specific manner (Fig. 3A). The luciferase activity of all constructs was much lower than that observed within the lens epithelial cell line, peaking at 20.88-fold increase in activity in the $-420/-19$ construct. A previously characterized portion of the mouse α A-crystallin promoter, containing the $-364/+44$ sequence in relation to the transcriptional start site, was used as a positive control for promoter activity and lens fiber cell specificity.^{19,20} When transfected into the N/N1003A cell line, the crystallin promoter resulted in an increase in luciferase activity of 414.23-fold, which was reduced to 66.7-fold for the fragment in the negative orientation. A relative luciferase activity of 23.04-fold was observed in the mouse α A-crystallin promoter in the NIH3T3 cell line. The luciferase activities of the human CP49 flanking DNA were similar to that of the lens fiber-specific crystallin promoter, implying lens-preferred expression of the reporter gene.

Transfection of Cell Lines with Human CP49 3'-Deletion DNA Constructs

To localize further the sequence(s) responsible for promoter activity, a series of 3'-deletion (Fig. 2) constructs were transfected into the N/N1003A cell line. The observed activities are reported in Figure 3B. In these experiments, the $-382/-19$ construct was used to form a 3'-deletion series by sequential excision of the DNA sequence. The full-length $-382/-19$ sequence resulted in an increase in luciferase activity of 85.33-fold over pGL2-Basic. The deletion of 58 bp of 3' sequence did not affect promoter activity in a negative fashion, as shown by the 88.14-fold activation of reporter gene expression observed for the $-382/-77$ construct. Further deletion at the 3' end to create the $-382/-128$ construct lowered relative luciferase

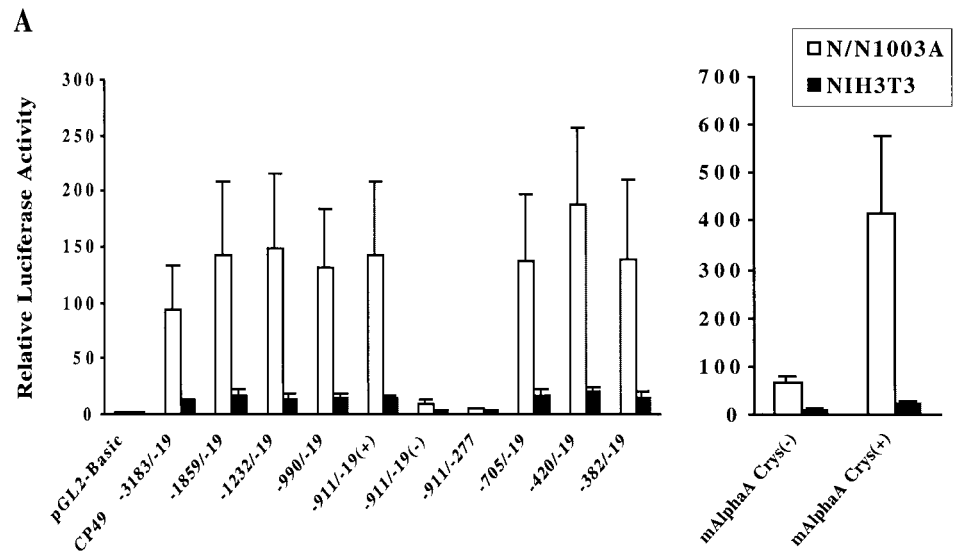
activity to 51.27-fold. Subsequent deletion of the flanking sequence reduced reporter gene expression to 1.644-fold observed for the $-382/-228$ construct. From these results, we concluded that the sequences between -176 and -19 are integral to the promoter activity of the human CP49 5'-flanking DNA sequence.

Transfection of Chicken Primary Lens Cultures

The chicken primary lens culture system is a common method for the characterization of mammalian promoter activity.^{21,22} The collection of CP49 5'- and 3'-deletion constructs were transfected into cultured chicken primary lens explants that have been similarly used in the characterization of other lens fiber cell promoters.^{19,23,24} As shown in Figure 4, the pattern of activity in previous transfections of the rabbit lens epithelial cell line was confirmed. The 5'-deletion constructs all showed increased levels of reporter gene expression, with the $-420/-19$ exhibiting the highest activity of 33.05-fold over the pGL2-Basic vector (Fig. 4A). Truncation of the 3' end in the $-911/-277$ showed a decrease in luciferase activity to 2.05-fold (Fig. 4A). The deletion of the 3' end beyond -176 of the 5'-flanking DNA sequence resulted in nominal reporter gene activity, as seen in the N/N1003A cell line transfection experiments with the CP49 3'-deletion series (Fig. 4B). This further supports the notion of the involvement of the proximal 5'-flanking DNA sequence in activating transcription of the reporter gene in transfection experiments.

Transfection of N/N1003A Cell Line with Mouse CP49 Promoter Constructs

The 5'-flanking DNA from the murine CP49 gene was isolated from a P2 clone and subcloned into the pGL2-Basic vector to analyze for potential regulatory elements. Two clones were produced: $-924/-15$ and $-924/-277$. The resultant luciferase activities of transfection into the N/N1003A cell line are shown in Figure 5. The presence of the $-924/-15$ sequence stimulated reporter gene expression, resulting in relative luciferase activity of 106.7-fold over pGL2-Basic. The $-924/-277$ construct, where the 3' end of the 5'-flanking sequence was



B

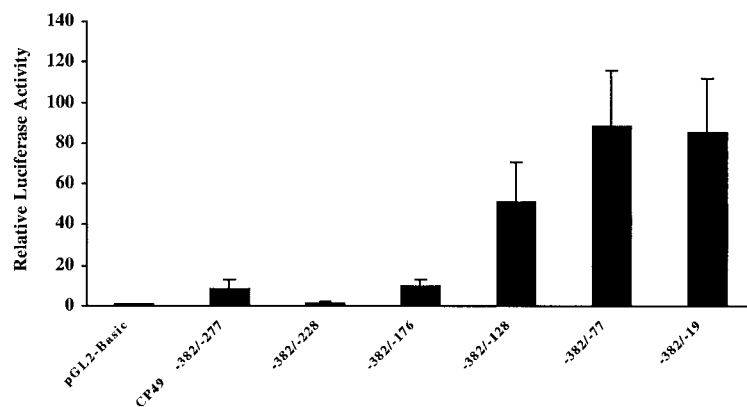


FIGURE 3. Analysis of the human CP49 proximal promoter region in transfection of the N/N1003A rabbit lens epithelial cell line and the NIH3T3 mouse fibroblast cell line. **(A)** Luciferase activity of the human CP49 5'-deletion series in the lens and nonlens cell lines. The mouse α -crystallin promoter was included as a positive control for lens-specific promoter activity. Note the separate axis for the 5'-deletion series and the positive control. **(B)** Luciferase activity of the human CP49 3'-deletion series in the N/N1003A cell line. All errors are expressed as SD.

removed, yielded an activity of 2.9-fold over pGL2-Basic. These results were similar to those observed for the human CP49 promoter. In both cases the truncation of the sequences proximal to the initiator methionine resulted in the abolishment of reporter gene activation.

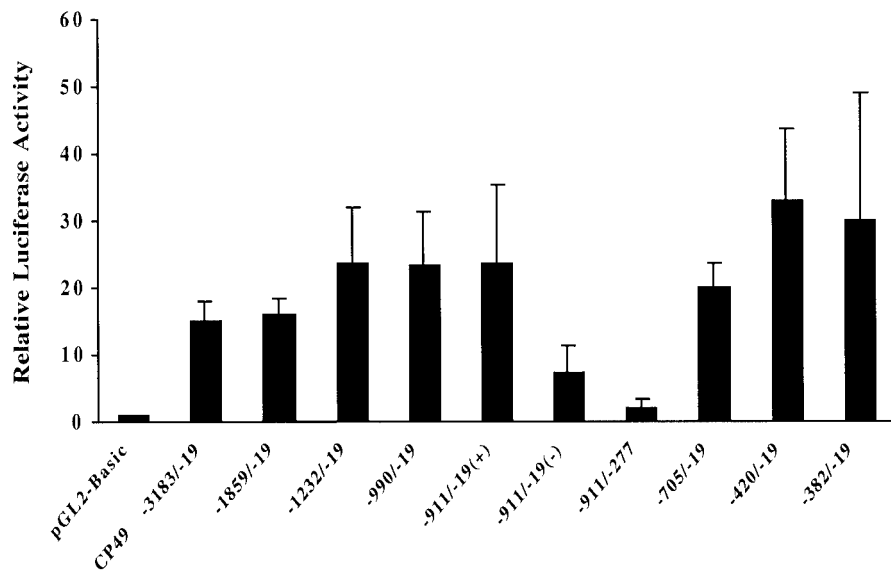
Analysis of Transgenic Animals

A transgene containing the $-1859/-19$ 5'-flanking DNA sequence of the human CP49 gene upstream of the bacterial β -galactosidase gene in the p β Gal-Basic vector (Promega) was injected into fertilized pronuclei in an effort to determine whether this segment of DNA is able to drive expression of the transgene in a lens-preferred manner. Genomic DNA of the implanted pronuclei was isolated and used to screen for the presence of the transgene. Seven animals carrying the transgene were identified. The offspring of these founder animals were used to analyze the expression pattern of the transgene. Samples were taken from several tissues to assess β -galactosidase activity in a luminescence assay and were compared with levels observed in wild-type mice. Of the tissues analyzed, increased levels of transgene expression were detected solely within the lens. Figure 6 shows the tissue analysis of the three lines that displayed increased levels of β -galactosidase activity. These results, along with those from the transfection experi-

ments, suggest that the 5'-flanking DNA sequence of the CP49 gene is able to establish lens-specific expression in vivo.

The expression pattern of the β -galactosidase transgene driven by the human CP49 promoter was addressed through immunohistochemical methods. Figure 7 displays the localization of transgene expression within the lenses of transgenic and wild-type mice. Figure 7A represents the resultant expression pattern in a transgenic animal. The reactivity, denoted by brown staining, was restricted to the terminally differentiated lens fiber cells and was not observed within the lens epithelium, similar to the endogenous pattern of CP49 gene expression. In Figure 7B, the addition of bacterial β -galactosidase protein to the primary antibody solution was performed to confirm the specificity of the reactivity registered in the transgenic animals. Preadsorption of the primary antibody abolishes almost all reactivity in the tissue sections, suggesting that the response in the transgenic animals is due to a specific interaction with the expressed β -galactosidase transgene. Figure 7C shows the background reactivity in wild-type mice. Faint reactivity was registered in the lens, but was well below that observed in the lens of the transgenic animal. Light reactivity was observed within the cornea of both the transgenic and wild-type animals. Preadsorption of the primary antibody did not diminish the reactivity within the cornea, suggesting cross-

A



B

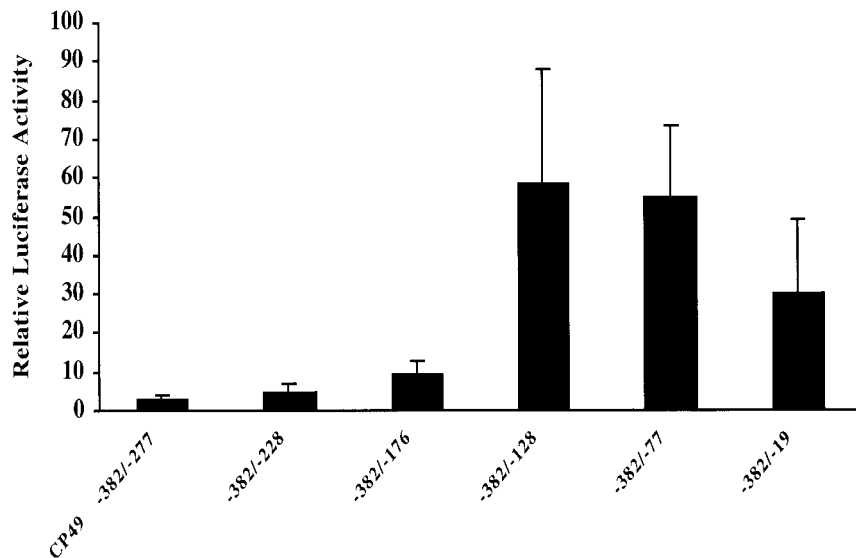


FIGURE 4. Promoter activity of the CP49 5'-flanking DNA sequences in the transfection of primary chicken lens cultures. (A) Luciferase activity of the human CP49 5'-deletion series in primary cultures of embryonic chicken lens cells. (B) Luciferase activity of the human CP49 3'-deletion series in primary cultures of embryonic chicken lenses. Errors bars represent standard deviation.

reactivity of the primary antibody. These results support the data from the luminescent assay and further establish the ability of the CP49 5'-flanking DNA sequence to drive lens-specific expression.

DISCUSSION

In an effort to characterize the transcriptional regulation of the human CP49 gene we have isolated the 5'-flanking DNA sequence and assayed for potential promoter activity. The 5'-deletion series exhibited lens-preferred activation of promoter gene expression in the transfection of the lens and nonlens cell

lines. Deletion of the distal end of the upstream sequence did not affect promoter activity, but truncation of the 3' end, in the -911/-277 construct, abolished the promoter activity observed with the other transfection constructs of this series. These results suggest that sequences within the proximal, or 3' end, are necessary to enhance expression of the reporter gene. Support for this was obtained in analogous transfection experiments with DNA sequences flanking the mouse CP49 gene. The 5'-deletion series also registered promoter activity when transfected into primary chicken lens cultures, a system that has been used in the characterization of lens fiber cell gene promoters, including CP115, the partner protein for CP49 in

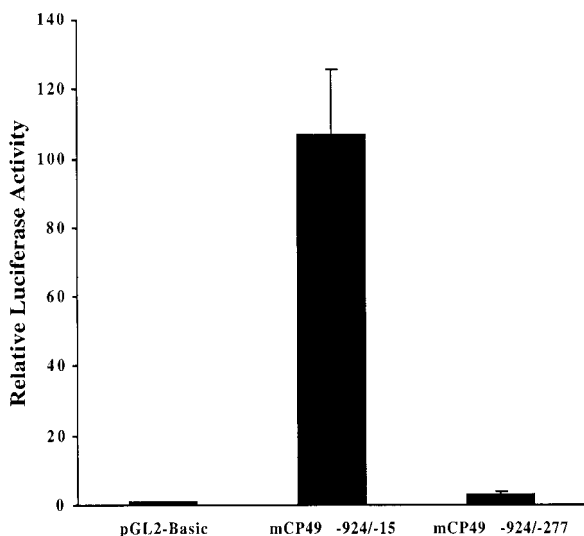


FIGURE 5. Transfection of mouse CP49 5'-flanking sequences in the N/N1003A cell line. The deletion of the proximal flanking sequences in the -924/-277 construct abolishes the promoter activity observed for the -924/-15 construct. Errors bars represent standard deviation.

formation of the beaded filament.²⁵ The 3'-deletion series was constructed to define further the location of regulatory elements within the proximal 5'-flanking sequence that contribute to promoter activity. The transfection of these constructs in the N/N1003A lens epithelial cell line and the primary chicken lens cultures indicates that sequences between -176 and -77 are necessary for maximal promoter activity.

Although cell culture systems have been valuable in the study of lens fiber cell gene expression the production of transgenic mice allows for the assessment of promoter specificity *in vivo*. Transgenic lines carrying 1.8 kb of the CP49 5'-flanking DNA sequence upstream of the bacterial β -galactosidase gene were produced and characterized for reporter gene expression. The analysis of several tissues showed that β -galactosidase activity was increased solely within the lens for three of the seven lines, whereas the remaining four lines did not display enhanced reporter gene expression within any tissue evaluated. The amount of reporter gene activation within the lens may be underrated, in that the protein concentration within the lens is extremely high compared with other tissues, and this effectively lowers the relative amount of tissue

assayed. Immunohistochemical analysis of transgene expression within ocular tissues confirmed the lens specificity produced by the proximal promoter sequence. β -Galactosidase expression was limited to the lens fiber cell and was absent from the anterior lens epithelium. The specificity of the 5'-flanking DNA sequence of the CP49 gene in both the transfection and transgenic experiments confirms that this region of DNA can drive lens-specific expression. Although the transgene construct contained a significant portion of the upstream sequence, the transfection data suggest that it is the proximal promoter region, within a few hundred base pairs of the coding region, that is responsible for the promoter activity.

The analysis of the proximal flanking DNA for potential transcription factor-binding sites reveals the presence of consensus sequences for a number of regulators. The proximal promoter region harbors binding sites for AP-1, AP-2, OCT-1, PAX6, GATA-1, NF- κ B, and USF. There were no TATA or CAAT boxes detected in this region of the promoter, as was the case in the characterization of the mouse Cp115 promoter.²⁵ Pax6 has been shown to act as a positive regulator of crystallin genes expressed within the lens epithelium and as a negative regulator of fiber cell-specific crystallins.²⁶ Two of the putative binding sites for AP-2 are located within the -77 to -176 region of the flanking DNA sequence that appears to be responsible for promoter activity in our transfection experiments. AP-2 is involved in the regulation of CP115 and MIP, two other lens fiber cell-specific genes, through interaction with proximal promoter elements.^{25,27} The CP49 and CP115 gene products are coordinately expressed, and it seems likely that both genes use similar regulatory mechanism(s) to establish a common expression pattern. This type of sharing of promoter elements is also seen in the coordinated expression of keratin protein pairs of the intermediate filament family.^{28,29} Ubiquitous factors, such as AP-1 and AP-2, have been identified as regulators of differentiation-specific cytokeratin expression within the epidermis, the group of intermediate filament genes with which CP49 shows highest homology.³⁰⁻³²

The collection of crystallin genes has been evolutionarily recruited for high-level expression within the lens through the use of common specific transcription factor-binding sites. Recent studies have revealed that the most widely conserved binding site, located within the proximal promoter region, interacts with a lens-specific member of the Maf transcription factors.¹² c-Maf, as it is referred to in mice, is critical in enacting lens fiber cell differentiation, and in the absence of this factor, lens development does not proceed beyond the formation of the lens vesicle.¹⁴⁻¹⁶ No primary or secondary lens fiber

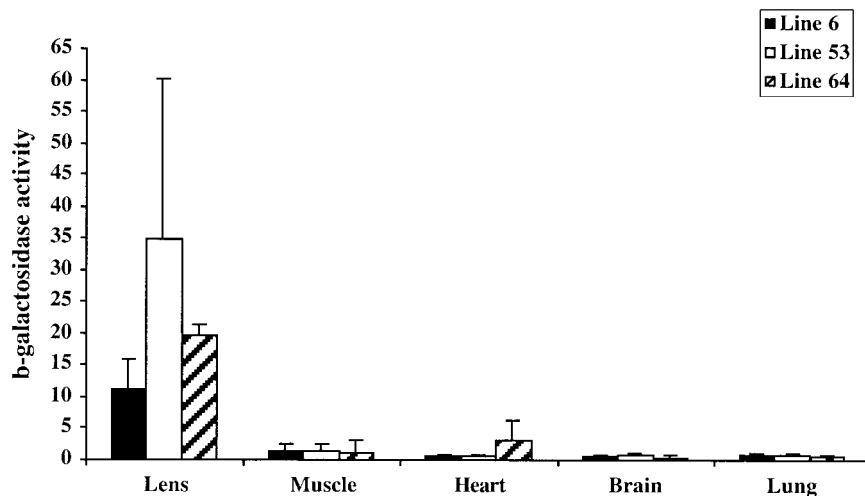


FIGURE 6. Analysis of transgene expression driven by 5'-flanking DNA sequence of the human CP49 gene. Founder mice were bred and the offspring analyzed for transgene expression through the use of a luminescent β -galactosidase assay. Tissue samples were taken from various tissues, and cell lysates were analyzed for β -galactosidase activity. The activities are displayed as multiples of induction over the observed activity within the wild-type animals. Errors bars represent standard deviation.

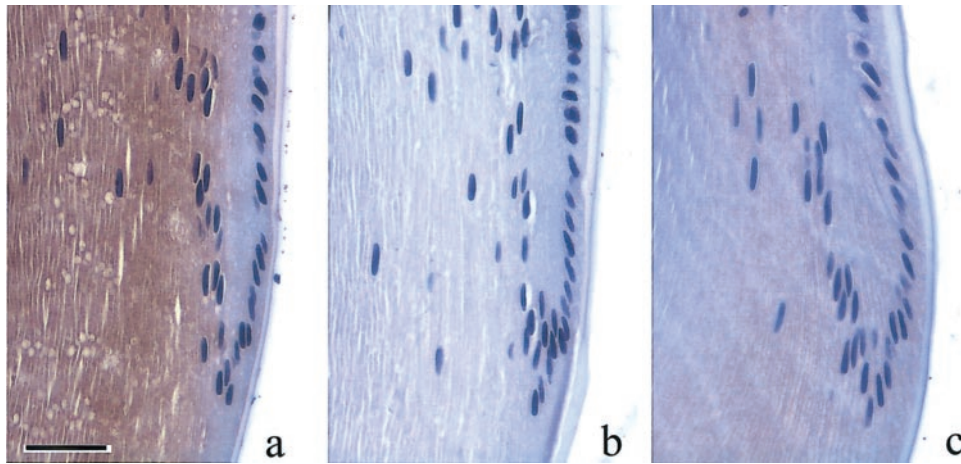


FIGURE 7. Immunohistochemical localization of transgene expression within ocular tissues. Eyes from transgenic and wild-type mice were isolated and analyzed for the expression of the β -galactosidase transgene. Reactivity corresponds to *brown* coloration. The bow region of the (A) transgenic lens, (B) transgenic lens with preadsorbed primary antibody, and (C) wild-type lens shows the anterior lens epithelium at the right side of each section, as well as the elongating and fully elongated lens fiber cells. Scale bar, 30 μ m.

cell formation is observed in these mice. Exogenous expression of L-Maf, a lens-specific Maf in chicken, in cultures of neural retinal cells initiates transdifferentiation of these cells into lens fiber cells. Analysis of gene expression within these transfected cells by RT-PCR shows expression of several different crystallin genes, as well as CP115.¹² These results suggest that c-Maf is a driving force in fiber cell formation and the characteristic gene expression of lens fiber cells.

Analysis of the 5'-flanking region of the human CP49 failed to identify any potential binding sites for Maf transcription factors. The δ 1-crystallin gene found within the lens of chicken uses an enhancer element within an intron that contains a binding site for Maf transcription factors.³³ To test for additional regulatory elements, five of the six introns located within the coding region of the human CP49 gene were also assayed for enhancer activity in transfection experiments. None of the intron sequences modulated reporter gene expression in either a positive or negative manner (data not shown). The remaining intron, which is in excess of 20 kb, was screened for potential regulatory sites by sequence analysis. It does not appear that a Maf factor plays a role in the activity of the proximal promoter of the human CP49 gene or is used in the intronic sequences as part of an enhancer element.

The expression of the noncrystallin genes of the lens fiber cell have generally been shown to be under the influence of a different set of transcription factors than the crystallin genes. Whereas crystallin expression is directly dependent on the involvement of factor such as Pax6, Sox-1, and c-Maf, the characterization of the CP49 and MIP proximal promoter regions, at this point, does not indicate a similar role in the regulation of these genes. In the case of the CP115 gene, a putative c-Maf binding site has been identified within the proximal promoter sequence that appears to be used in CP115 gene expression, as observed in the transfection of chicken retinal cell cultures with a c-Maf expression vector.^{12,25} The differences in promoter elements may be due in part to the significant disparity in the level of gene expression between crystallin and noncrystallin lens fiber cell genes. The MIP and CP115 promoters contain consensus sequence elements for transcription factors such as AP-1, AP-2, and Sp-1. Potential binding sites for some of these factors are also found within the proximal promoter sequence of the human CP49 gene, which has been shown to contribute to the lens-specific expression of this gene. The presence of shared regulatory elements within the promoter regions of the noncrystallin genes suggests that a common regulatory mechanism governs the expression of these genes.

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