Exogenous Gene Expression and Protein Targeting in Lens Fiber Cells

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PURPOSE. To test the ability of lens fiber cells at various stages of differentiation to transcribe and translate microinjected DNA templates.

METHODS. Expression plasmids encoding green fluorescent protein (GFP) or a GFP-tagged membrane protein (human CD46) were microinjected into organ-cultured embryonic chicken lenses. Protein expression was visualized by confocal microscopy.

RESULTS. GFP expression was detected within 12 hours of microinjection, evenly distributed throughout the cytoplasm of the injected cell. All nucleated fiber cells were competent to express GFP, whereas the anucleated central fiber cells were not. When GFP was fused to the C-terminal of CD46, the fusion protein was synthesized intact and properly inserted in the fiber cell plasma membrane. In contrast, N-terminal fusions were cleaved during synthesis, resulting in retention of the GFP tag in the endoplasmic reticulum.

CONCLUSIONS. Microinjection of expression plasmids is an effective technique for introducing exogenous genes into individual fiber cells. With this approach, the results show that fiber cells are transcriptionally and translationally competent until the time of organelle loss, and that fiber cells deep within the lens are capable of synthesizing new plasma membrane proteins. The techniques described here should have broad application in studies of fiber cell differentiation and provide a useful complement to conventional transgenic approaches. (Invest Ophthalmol Vis Sci. 1999;40:1435-1443)

In this article, we describe the use of a plasmid microinjection technique to test the transcriptional and translational competence of fiber cells at various stages of differentiation. Fiber differentiation involves a series of biochemical and morphologic changes that culminate in the rapid synchronous degradation of the cell nucleus¹ and other organelles.²⁻³ It is unlikely that fiber cells are capable of de novo synthesis of RNA and protein after denucleation. However, it is not known whether all nucleated fiber cells are capable of transcription and translation. It is possible, for example, that fiber cell nuclei are transcriptionally inactivated long before they are degraded. In fact, recent observations on the structure of the fiber cell nucleus late in differentiation are consistent with this view.⁴ Several investigators have addressed this question in the embryonic lens using the incorporation of ³H-uridine to measure RNA synthesis and tissue autoradiography to visualize the distribution of the tritiated label. Using this approach, Hanna⁵ found that RNA synthesis occurred in all nucleated fiber cells of the developing lens. In contrast, Reeder and Bell⁶ reported that ³H-uridine-labeled nuclei only extended partway into the body of the chicken lens at embryonic day (E)12, and Modak and Persons⁷ reported that nucleated central fibers are unlabeled as early as E8, 4 days before those cells lose their nuclei.² In view of these conflicting reports, we decided to examine the ability of differentiating fiber cells to synthesize green fluorescent protein (GFP) from a microinjected plasmid template as a direct measure of their transcriptional and translational competence.

During differentiation, fiber cells express lens-specific membrane proteins that are incorporated into an elaborately folded plasma membrane. Although mutations in fiber membrane proteins have been implicated in inherited cataract in mice and humans,⁸ little information is available on membrane protein synthesis or trafficking in the normal or diseased lens. In the present study, therefore, we explored the utility of GFP-tagged fusion proteins for visualizing the expression and trafficking of membrane proteins in intact living lenses.

MATERIALS AND METHODS

Chicken Lenses

All experimental procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Lenses from 15-day-old (E15) chicken embryos were dissected in warm (37°C) modified Ringer’s solution (90 mM NaCl, 4.5 mM KCl, 1 mM MgCl₂, 1.5 mM CaCl₂, 6 mM d-glucose, and 35 mM NaHCO₃ [pH 7.8]). After dissection, lenses were placed in 10% bovine serum albumin (Fraction V, Sigma, St. Louis, MO) in Ringer’s solution until use.
Three plasmids were constructed for use in this study (Fig. 1): plasmids, pEGFPN1 and pCCD46, were used as controls in Palo Alto, CA) containing the GFP coding sequence under the commercially available GFP expression plasmid (Clontech, GFP with CD46, a human membrane protein. Two additional and pFus26) containing transcriptional fusion constructs of chicken /3Bl-crystallin promoter and two plasmids (pFus115 (p/3Bgfpl4) containing GFP cDNA under the control of a DNA Constructs

FIGURE 1. Diagram of GFP and GFP/CD46 fusion protein expression plasmids. Each of the plasmids has a pEGFP vector backbone. In pBlBgfp14, the CMV promoter from the original vector is replaced with the chicken β1-crystallin gene promoter11 to ensure high-level GFP expression in lens fiber cells. To visualize the expression of an exogenous membrane protein in the lens, a GFP tag was fused to the N- or C-terminus (pFus26 and pFus115, respectively) of the human membrane protein CD46.

DNA Constructs

Three plasmids were constructed for use in this study (Fig. 1): a green fluorescent protein (GFP) expression plasmid (pBlBgfp14) containing GFP cDNA under the control of a chicken β1-crystallin promoter and two plasmids (pFus115 and pFus26) containing transcriptional fusion constructs of GFP with CD46, a human membrane protein. Two additional plasmids, pEGFPN1 and pCCD46, were used as controls in transient transfection experiments. The pEGFPN1 plasmid is a commercially available GFP expression plasmid (Clontech, Palo Alto, CA) containing the GFP coding sequence under the control of the cytomegalovirus (CMV) promoter. The pCCD46 plasmid was generated by inserting the CD46 coding sequence9 into the multiple cloning site of the pCAG expression vector described by Miyazaki et al.10 The lens GFP expression plasmid (pBlBgfp14) was generated from a pEGFPN1 plasmid vector backbone (Clontech). The AseI-Ecl136I1 fragment of pEGFPN1, containing the CMV promoter and enhancer, was replaced with a 464-bp (−434/+30) PuwII-cleaved polymerase chain reaction–generated fragment, containing the chicken β1-crystallin promoter.11 GFP/CD46 fusion constructs were obtained by ligation of a 1.1-kb KpnI-EcoRI fragment containing the CD46 cDNA sequence9 into pEGFPN1 and pEGFPc1 vectors (Clontech) double digested with KpnI and EcoRI. The resultant constructs (designated pFus115 and pFus26 respectively; Fig. 1) used the CMV promoter to drive GFP/CD46 fusion protein expression. In the pFus115 construct, the GFP sequence was transcriptionally fused to the C-terminus of CD46. In pFus26, the GFP sequence was fused to the N-terminus of CD46.

DNA Purification

Plasmid DNA (pDNA) was purified by CsCl gradient ultracentrifugation or with a kit (EndoFree Maxi Prep; Qiagen, Valencia, CA) before microinjection. In either case, the pDNA was subsequently purified with Triton X-114 (Sigma) as described12 to minimize cellular toxicity. pDNA was diluted to 20 ng/μl with TE buffer (pH 7.4), containing 0.2 mg/ml anionic tetramethylrhodamine dextran (molecular weight [Mwt], 3 kDa; Molecular Probes, Eugene, OR). All components were centrifuged for 10 minutes immediately before use.

Lens Microinjection

Lenses were immobilized in 1.5% low-melting-point agarose during injection. The agarose was dissolved in minimum essential medium (containing 50 mg/ml gentamicin), and 3 ml of molten solution was pipetted into a 35-mm petri dish. The gel was allowed to set and then overlaid with 1 ml of additional agarose solution. Lenses were positioned anterior face up in this molten upper layer, which was then allowed to set. Two small trenches were cut in the agarose immediately adjacent to the embedded lens. The trenches served to contain a collagenase solution (0.5 mg/ml in Ringer’s) for partially digesting a small region of the lens capsule, thereby facilitating the penetration of the microinjection pipette (Fig. 2A). The collagenase solution was added to the trenches for 2 minutes. The trenches were then rinsed with minimum essential medium to wash away any traces of collagenase. The embedded lenses were overlaid with 1 ml of minimum essential medium and maintained in a CO2 incubator before and after microinjection.

Microinjection pipettes were pulled from 1.0 mm (outer diameter) filamented borosilicate glass (WPI, Sarasota, FL) on a Flaming-Brown horizontal pipette puller (model P-97; Sutter Instruments, Novato, CA) to a tip diameter of 1 μm to 2 μm. The Shank of the pipette was filled with injection solution, and the pipette was connected to a manual air pressure microinjection system (Narishige, Tokyo, Japan). The microinjection system was mounted on an upright fluorescence microscope (Axioskop; Carl Zeiss, Thornwood, NY) equipped with water-immersion lenses. The microinjection pipette was inserted into the lens through the collagenase-treated region of the anterior lens capsule (Fig. 2A). Studies in other postmitotic cell types have indicated the importance of microinjecting pDNA directly into the cell nucleus or its immediate vicinity.13,14 Because of the extremely elongated morphology of lens fiber cells, we attempted to microinject the pDNA as near as possible to the fiber cell nuclei. To achieve this, the pipette tip was inserted into the lens bow region where the fiber cell nuclei are tightly clustered (Fig. 2B). The patency of the pipette tip was verified before, during, and after microinjection by observing the flow of fluorescent injection buffer through the pipette tip (Fig. 2C). Multiple injections (up to 50) were made in a single lens, with no obvious deleterious effects on lens structure or transparency. After microinjection, lenses were placed in organ culture overnight and examined for GFP expression the next day using confocal microscopy.

GFP Visualization in the Intact Lens

Lenses were removed from organ culture, placed in modified Ringer’s solution (containing 10% bovine serum albumin), and examined by confocal microscopy (model 410 LSM equipped with an argon-krypton laser; Zeiss). GFP fluorescence was visualized using the 488-nm laser line for excitation and a 530- to 565-nm band-pass emission filter. Extended focus software supplied with the instrument was used to visualize GFP expression throughout the full thickness of the lens.
FIGURE 2. Diagram of the procedure used for microinjecting pDNA constructs into embryonic chicken lenses. (A) Lenses were immobilized in low-melting-point agarose. Trenches cut in the solidified agarose served to contain collagenase solution during enzymatic digestion of the capsule. The injection pipette was inserted through the collagenase-treated region of the capsule. (B) In some lenses, microinjections were made at different depths into the lens from the superficial cortex to the nuclear fiber cells. In the superficial fibers, the pipette tip was always positioned as close as possible to the fiber cell nuclei. (C) In addition to the pDNA, the injection solution contained a fluorescent dextran. Fluorescence microscopy was used to visualize the outflow of dextran as a means of monitoring the patency of the pipette. In this case, the pipette had just been withdrawn from the lens after successfully microinjecting a superficial fiber cell.

Measuring GFP Expression at Different Depths in the Lens
To examine the expression pattern of GFP in lens fibers at different stages of differentiation, a series of injections was made at various depths in 10 E15 lenses. Usually, two parallel tracks of injections were used for each lens (see Fig. 5). In total, 50 injections were made per lens, evenly spaced from the center to the periphery. After overnight incubation, lenses were examined by confocal microscopy, and the position of each GFP-expressing fiber cell (or bundle of fibers) was recorded. Because the number of GFP-expressing cells at any one radial location was relatively small, the data were binned. To calculate the injection efficiency, the number of GFP-expressing cells in a given region of the lens was divided by the total number of injections made in that region.

GFP Visualization in Lens Slices
Lenses were fixed in 4% paraformaldehyde/phosphate-buffered saline and sectioned with a vibratome, as described. GFP fluorescence was visualized using the filter combination described earlier. Some lens slices were permeabilized with 0.1% Triton X-100 and stained with Texas Red-phalloidin. Phalloidin is a specific probe for F-actin. In lens fiber cells, F-actin is concentrated beneath the plasma membranes, and phalloidin thus serves as a convenient fluorescent counterstain for visualizing the tissue architecture. The Texas Red fluorochrome was excited using the 568-nm laser line, and fluorescence was detected through a 590-nm long-pass filter.

GFP/CD46 Fusion Protein Visualization
A double-labeling procedure was used to visualize independently the distribution of the GFP and CD46 components of GFP/CD46 fusion proteins. GFP was visualized by its intrinsic fluorescence using the filter combination described. CD46 was visualized using an immunofluorescence method. Fixed cells or lens slices were briefly permeabilized with 0.1% Triton X-100, blocked with 10% goat serum, and incubated overnight with a 1:500 dilution of CD46 monoclonal antibody (TRA2-10) in phosphate-buffered saline. Preliminary experiments determined that the TRA2-10 antibody did not cross-react with untransfected chicken cells. The preparation was then washed for 1 hour in phosphate-buffered saline and incubated with a 1:200 dilution of CY5-conjugated anti-mouse IgG (Molecular Probes). The CY5 fluorochrome was excited using the 670-nm laser line and detected through an 810-nm long-pass emission filter.

Fibroblast Transfection and Western Blot Analysis
GFP fusion protein expression was also examined by western blot analysis of lysates from transfected primary chicken embryo fibroblasts. Primary fibroblast cultures were prepared from E10 embryos. The limbs, head and gut were removed from the embryos and the trunks macerated and trypsinized. Trunk cells were suspended in 100% calf serum and gently centrifuged to pellet. Cells were resuspended in Dulbecco's modified Eagle's medium (Gibco, Gaithersburg, MD) containing 10% fetal bovine serum, 2% chicken serum, and 100 mg/ml penicillin-streptomycin and plated at high density. After two days in culture, the cells were trypsinized, counted, and replated at 5 to 7 × 10^5 cells per 100-mm petri dish. Transfections were performed the next day when cells had reached
50% to 80% confluency. Cells were transfected with pEGFPN1, pCCD46, pFus115, or pFus26 using Fugene-6 (Boehringer Mannheim, Germany), according to the manufacturer's instructions. After overnight incubation, cells were harvested and cellular lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under native or denaturing conditions (denaturing conditions were used for samples to be probed with anti-GFP and native conditions for samples to be probed with anti-CD46). Separated proteins were transferred to nitrocellulose and probed with a CD46 polyclonal antibody or anti-GFP (Molecular Probes). Bound antibody was detected using enhanced chemiluminescence (Amersham, Arlington Heights, IL).

RESULTS

GFP Expression in the Lens

To study the expression of an exogenous cytoplasmic protein in the lens, E15 chicken lenses were microinjected with the pBbgp14 expression plasmid. After overnight incubation, lenses maintained their transparency, and GFP fluorescence was readily detected in injected cells by confocal microscopy (Fig. 3). GFP expression was observed in cells of the annular pad and the lens cortex. Occasionally, epithelial cells were inadvertently microinjected; these cells also expressed GFP (data not shown). We did not observe GFP expression in microinjected nuclear fiber cells. In positive cells, GFP was evenly distributed in the fiber cell cytoplasm, from the posterior to the anterior tips. In annular pad cells, GFP expression (as estimated by fluorescence intensity) was maximal 8 to 10 hours after microinjection, whereas cortical fiber cell expression took 12 to 16 hours to reach maximal levels. Organ-cultured embryonic lenses remained viable for up to 3 days, and GFP fluorescence was visible throughout this period, decreasing slightly with time.

At higher magnification, individual fiber cells were visible (Fig. 4A). Because fiber cells are around the lens, it was difficult to obtain images in which entire cells lay in a single focal plane. This problem was overcome using extended depth-of-focus confocal microscopy. Imaged in this fashion, GFP-expressing cells could be visualized in the intact lens, optically isolated.
Microinjections were made at regular intervals from the surface to the center of the lens (Table 1). The number and position of GFP-expressing fibers were recorded (see FIG. 5). Examined by confocal microscopy for the presence of GFP-expressing fiber cells, were readily resolved. Several GFP-expressing fibers were usually observed in the center of the lens. Lenses were injected multiple times along two parallel injection tracks (*). After overnight incubation, lenses were examined by confocal microscopy for the presence of GFP-expressing cells. The slices were cut perpendicular to the optical axis and incubated with Texas Red-phalloidin to stain F-actin within the lens. Immunofluorescence staining and GFP expression were covisualized by confocal microscopy (Fig. 4B). In cross-section, fiber cells have a flattened hexagonal appearance that was highlighted by the F-actin staining pattern. The expression pattern of GFP resulting from several injections of the pβBgfpl4 was shown in Fig. 4B. Approximately 50% of successful injections resulted in GFP expression in single fiber cells. The remaining cases, small bundles of fiber cells expressed GFP. In single GFP-expressing cells, the GFP did not diffuse into neighboring fiber cells, suggesting that the extensive network of gap junctions that interlink cortical lens fiber cells is not permeable to the 27-kDa GFP protein. Presumably, the clusters of GFP-expressing cells resulted from the inadvertent injection of the GFP expression plasmid into more than one fiber cell.

The loss of cell nuclei (and other organelles) is a striking feature of lens fiber cell differentiation. With the disappearance of the fiber cell nuclei, the opportunity for transcriptional regulation is lost. However, it is possible that the changes in nuclear morphology that precede fiber cell denudation are indicative of the transcriptional inactivation of the nuclei. We used GFP expression as a direct measure of a fiber cell's ability to transcribe a DNA template and translate the corresponding mRNA. In 10 E15 lenses, a series of microinjections of pβBgfpl4 were made at regular intervals from the surface of the lens to the center (Fig. 5). After overnight incubation, intact lenses were imaged in the equatorial plane, and the number and position of GFP-expressing cells were determined. These data are presented in Table 1. The maximum success rate for the injections (30.6%) was observed in the midcortical region at a depth of 15% to 30% of the lens radius (r). The success rate in any region of the lens varied considerably, evidenced by the size of the SD associated with each of the measurements. However, a general decline in injection success rate was evident in the deeper layers falling to 2% at a depth of 60% to 75% r. The deepest GFP-expressing cell was observed at a depth of 70% r. No expression was observed in the central region of the lens (75%-100% r).

**GFP/CD46 Fusion Protein Expression in the Lens**

To visualize the fate of an exogenous membrane protein in the lens and to test the GFP-tagging technique, we examined the expression of CD46/GFP fusion proteins. CD46 is a well-characterized integral membrane protein that is widely expressed in human tissues, where it plays an important role in inhibiting complement activation on host cells. In the present study, the expression of CD46 was used to probe membrane protein synthesis and trafficking in lens fiber cells.

To test the stability of newly synthesized GFP/CD46 fusion proteins, both domains of the fusion protein were visualized independently in transfected fibroblasts or microinjected lens fiber cells using intrinsic fluorescence to localize GFP and immunofluorescence to localize CD46. Figure 6 shows the expression pattern of GFP or CD46/GFP fusion protein constructs in transfected fibroblasts or microinjected lens fiber cells. In transient transfection experiments with the pEGFPN1 plasmid, GFP fluorescence was present throughout the cytoplasmic volume of the fibroblasts, including the nucleus (Fig. 6D). Similarly, microinjection of pβBgfpl4 into lens fiber cells resulted in strong homogeneous cytoplasmic fluorescence (Fig. 6D). In contrast to the diffuse cytoplasmic fluorescence observed with the GFP expression plasmids, both the N- and C-terminal fusion constructs resulted in membranous expression patterns. Interestingly, when GFP was fused to the N-terminus of CD46, the two components of the chimeric molecule did not colocalize (Fig. 6C,D). Immunofluorescence for CD46 revealed that in fibroblasts (Fig. 6C) or lens fibers (Fig. 6D) the CD46 protein was properly trafficked to the plasma membrane. In fibroblasts, the CD46 immunofluorescence was continuous in the plasma membrane (Fig. 6C), but in lens fibers, the CD46 immunofluorescence was restricted to the plasma membrane and the JTC.

**Table 1. Microinjection Efficiency in Different Regions of the Lens**

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<tr>
<th>Injection depth (% Lens radius)</th>
<th>0-15</th>
<th>15-30</th>
<th>30-45</th>
<th>45-60</th>
<th>60-75</th>
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<tr>
<td>Percent injections resulting in GFP expression*</td>
<td>15.9 ± 19.8</td>
<td>30.6 ± 24.6</td>
<td>17.4 ± 14.2</td>
<td>12.0 ± 11.8</td>
<td>2.6 ± 5.5</td>
<td>0 ± 0</td>
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<td>(n = 10)</td>
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*Data are expressed as mean percentages ± SD.
Figure 6. Confocal images of primary fibroblast cells (A, C, E) and lens fiber cells (B, D, F) expressing GFP (A, B) or GFP/CD46 fusion proteins (C, D, E, F). GFP (green) was localized by its intrinsic fluorescence, CD46 (red) was localized by immunofluorescence using TRA2-10 antibody (see the Results section for details). (A) Primary fibroblasts transfected with pEGFPN1. GFP fluorescence was diffuse and cytoplasmic (arrow). (B) Lens fiber cell microinjected with pEGFPN1. GFP fluorescence was diffuse and cytoplasmic (arrow). (C) Primary fibroblast transfected with pFus26. The two components of the CD46/GFP fusion protein were apparently cleaved from each other during processing. The CD46 was properly targeted to the plasma membrane. In contrast, GFP was located in internal membranes, including the nuclear membrane (arrow). (D) Microinjection of pFus26 into fiber cells resulted in the separation of the GFP tag from the CD46 protein. The GFP signal was retained within the fiber cell, whereas CD46 was localized to the plasma membrane. Note the unstained patches of membrane (arrows) reminiscent in size and distribution to fiber cell gap junction plaques. (E) Fibroblast transfected with pFus115. The C-terminal construct CD46 and the GFP tag colocalized to the plasma membrane (the yellow color indicated the superimposition of the red and green signals). (F) Superficial fiber cell microinjected with pFus115. The GFP and CD46 largely colocalized to the plasma membrane.
fibers, the lateral plasma membrane staining was fenestrated by regions of unstained membrane (Fig. 6D). These unstained regions correspond in size and distribution to the gap junction plaques that are a prominent feature of the plasma membranes of lens fiber cells.\(^{18}\) In contrast to the distribution pattern of CD46, the GFP tag was located in the internal membrane plaques that are a prominent feature of the plasma membranes of lens fiber cells.\(^{18}\) In well-spread fibroblasts, the internal GFP staining localized to a reticular intracellular membrane system—probably the endoplasmic reticulum. The fact that the CD46 immunofluorescence did not colocalize with the GFP fluorescence suggested that the fusion protein was cleaved during synthesis or trafficking. When GFP was fused to the C-terminus of CD46, both components of the fusion protein colocalized to the plasma membrane in transfected fibroblasts (Fig. 6E) or microinjected lens fibers (Fig. 6F). Because GFP alone did not localize to the plasma membrane (Fig. 6A, 6B), these data suggest that the C-terminal fusion was intact and properly trafficked to the plasma membrane along with its attached GFP tag.

**Fusion Protein Analysis**

To analyze fusion protein stability further, primary fibroblasts were transfected with pCCD46, pEGFPN1, pFus26, and pFus115. The cells were incubated overnight and analyzed by western blot using antibodies to GFP or CD46 (Fig. 7). Transfection with plasmids encoding GFP or CD46 (pEGFPN1 and pCCD46, respectively) led to the expression of proteins of the expected size. On western blot, the CD46 band was relatively broad, as noted previously.\(^{19}\) Transfection with pFus26 did not result in the expected single band corresponding to the molecular weight of the fusion protein; instead, two bands were detected. The smaller band was recognized by the GFP antibody. Although the protein was slightly larger than the wild-type GFP. The second, larger, band was recognized by the CD46 antibody. These data support the notion that the N-terminal fusion protein was cleaved during processing. In contrast, transfection with the pFus115 plasmid (encoding a C-terminal CD46/GFP fusion protein) generated a single high-molecular-weight band recognized by both anti-GFP and anti-CD46 suggesting that the C-terminal fusion protein was intact.

**DISCUSSION**

In this study, we used pDNA microinjection to introduce exogenous genes into individual lens fiber cells. Because of the unique cellular architecture of the lens, several modifications to a standard microinjection protocol were necessary to obtain a reasonable success rate with the microinjections. For example, a brief collagenase treatment was used to facilitate the penetration of the injection pipette through the lens capsule. The positioning of the pipette within the lens was also critical. We observed GFP expression only when pDNA was injected directly into the bow region of the lens, close to the fiber cell nuclei. Interestingly, in other postmitotic cells, high levels of gene expression are only observed when pDNA is introduced directly into cell nuclei. Cytoplasmic injection usually fails, presumably because of sequestering and degradation of pDNA in the cytoplasm.\(^{12,14,20}\) Given these data, the relatively high success rate of cytoplasmic pDNA injections in the superficial lens fiber cells is intriguing. Recently, a 240-bp motif of the SV40 virus genome (containing the origin of replication, portions of two promoters, and the SV40 enhancer) has been shown to facilitate the active transport of pDNA to the nucleus.\(^{14}\) The family of expression vectors used in the present work contains this SV40 motif, perhaps explaining the relatively high success rate of cytoplasmic injections of pDNA reported here for lens fiber cells. It is still likely, however, that the distance between the site of cytoplasmic injection and the cell nucleus is critical because attempts to achieve GFP expression by injection into distal regions of the fiber cells were unsuccessful.

**βB1-Crystallin Promoter**

We incorporated the βB1-crystallin promoter into the pβBglp14 lens expression plasmid to achieve strong fiber-specific GFP expression. Although this study was not designed to investigate the properties of the βB1-crystallin promoter, it is informative to compare the expression pattern observed here with those obtained previously with this promoter. As expected, the βB1-crystallin promoter drove high-level GFP expression in the fibers. In addition, we obtained reasonable

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**Figure 7.** Western blot analysis of fusion protein stability in primary embryonic chicken fibroblasts. Fibroblasts were transfected with one of four plasmid constructs: pEGFPN1, pCCD46, pFus26, or pFusN115. Blotted proteins were probed with anti-CD46 (lanes 2, 4 and 6) or anti-GFP (lanes 1, 3 and 5). Transfection with pEGFPN1 resulted in the synthesis of an approximately 27 kDa band recognized by anti-GFP (lane 1). Transfection with pCCD46 resulted in a broad band with an apparent molecular weight of approximately 55 kDa recognized by anti-CD46 (lane 2). The CD46/GFP N-terminal fusion protein plasmid (pFus26) produced two bands: one at approximately 34 kDa (lane 3; recognized by anti-GFP) and the other at approximately 60 kDa (lane 4; recognized by anti-CD46). Transfection with pFus115 (the C-terminal fusion protein construct) resulted in a single band of approximately 75 kDa recognized by both anti-GFP (lane 5) and anti-CD46 (lane 6).
expression levels in annular pad cells and epithelial cells. The GFP expression in annular pad and epithelial cells was surprising in view of the fact that endogenous βB1-crystallin protein is not expressed in this region in vivo.21 The (-434/+30) βB1-crystallin promoter has been widely used for in vitro transfection experiments and in transgenic mice. In transfection experiments, the promoter drove high level expression of a CAT reporter gene in cultured epithelial cells.22 In contrast, in transgenic mice, the expression of the transgene was restricted to the lens fiber cells.23 Thus, the tissue specificity of the βB1-crystallin promoter varies depending on the context in which it is used. A plausible explanation for the difference between the results obtained in vivo (i.e., with transgenic mice) and in vitro (cell transfection or microinjection) could lie in the role of Pac-6, an important lens transcription factor. In vivo, Pax-6 is believed to repress the βB1-crystallin promoter.22 In transfection and microinjection techniques a large number of copies of the exogenous gene are introduced into the cell. In epithelial cells, it is possible that these templates deplete the pool of endogenous repressors, such as Pax-6, thereby derepressing the βB1-crystallin promoter.

Transcriptional Activity during Fiber Cell Differentiation

Fiber cell maturation in the lens is accompanied by the sudden loss of nuclei and other organelles resulting in the formation of a central organelle-free zone.2 In the chicken lens, programmed organelle loss begins in the central cells on about E12, and by E15, the central region of the lens is devoid of organelles.2 Loss of the fiber cell nuclei is preceded by alterations in the nuclear lamina and redistribution of fibrillarin and colin in the coiled body and nucleolar compartments.4 These morphologic changes are suggestive of transcriptional shutdown. By injecting the pβBgfpl4 plasmid into fibers situated at different depths in the lens, we were able to assess directly the ability of a fiber cell to transcribe and translate a synthetic template. A general observation was that the injection efficiency (number of GFP expressing cells in a region/number of injections made in that region) was decreased in the deeper fiber cell layers. This may reflect inherent physical and/or biochemical differences between the superficial fibers and the inner fiber cells. It is possible, for example, that fibers located near the center of the lens are more susceptible to damage by the microinjection pipette. Alternatively, the activity of the βB1-crystallin promoter may be greater in the superficial layers, consistent with the expression pattern of the endogenous gene.21 The pβBgfpl4 expression plasmid may be inherently less stable in the cytoplasm of fiber cells near the border of the organelle-free zone. In this region, cells are beginning to degrade their nuclear DNA, and cytoplasmic nucleases may also attack exogenous plasmid DNA. Whatever the explanation for the decrease in injection efficiency in the deeper cell layers, we observed GFP expression in the region of 60 to 75% r with the most deeply located GFP-expressing fiber cell located at a depth of 0.7 r. Previous data suggest that, at E15, the border of the organelle-free zone is located at a depth of 65 to 70% r.2 Because of the correspondence between these values, we conclude that fiber cells are transcriptionally and translationally competent until the point of organelle loss.

CD46/GFP Fusion Protein Expression

To monitor the expression and trafficking of exogenous membrane proteins in lens fiber cells, we constructed C- and N-terminal variants of GFP-tagged CD46. CD46 is a human glycoprotein that is abundantly expressed in the plasma membranes of many cells. By observing the fate of the GFP and CD46 portions of the fusion proteins independently, we showed that the fusion protein was stable and properly trafficked to the plasma membrane only when the GFP tag was fused to the C-terminus of CD46. N-terminal fusions resulted in cleavage of the fusion protein and retention of the GFP tag in the ER. The CD46 protein contains a signal peptide at its N-terminus, and it is likely that in N-terminal fusions the GFP tag was removed along with the signal peptide by specific signal peptidases in the ER.

This hypothesis is supported by the observation that the cleaved GFP tag was slightly larger than wild-type GFP (Fig. 7). The increase in size may be caused by the presence of the signal peptide sequence.

A basolateral targeting sequence is present at the C-terminus of CD46.24 This sequence was sufficient to direct the CD46/GFP C-terminal fusion protein to the lateral membrane of the fiber cells. One interesting feature of CD46 incorporation in lens fiber cells was the exclusion of the protein from plaque-like regions of the lateral membrane. We hypothesize that these sequestered areas correspond to fiber cell gap junctions which, in this species, account for approximately 50% of the membrane surface.18

Acknowledgments

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References


