Gene Transfer of Interleukin 10 to the Murine Cornea Using Electroporation

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Gene therapy is a promising approach to deliver anti-inflammatory genes to the eye to treat a number of corneal diseases. We have used electroporation to deliver plasmids expressing interleukin 10 (IL-10) to the corneas of mice and evaluated the duration of expression following gene transfer. Unlike expression of reporter genes driven from the cytomegalovirus immediate early promoter (CMV_{iep}), which remained high for 3 days, CMV_{iep}-driven IL-10 expression peaked at Day 1 and decreased 2-fold each day thereafter. In an attempt to increase the duration of expression, the long-acting ubiquitin C (UbC) promoter was used but, surprisingly, a similar half-life of gene expression was observed. This reduced duration was not due to promoter inhibition by expressed IL-10 or clearance of plasmids from the cornea. However, when DNA nuclear targeting sequences (DTSs) that promote DNA nuclear import were removed from the plasmids, contrary to findings in nondividing cells and tissues in which these sequences are needed for gene transfer, robust expression was observed, and the duration increased significantly. Although corneal cell turnover was detected, suggesting mitosis-dependent plasmid nuclear localization independent of a DTS, the patterns of expressing and dividing cells appeared different. These results suggest that DNA nuclear targeting sequences may act differently in the cornea than in other tissues. Exp Biol Med 232:362–369, 2007

Key words: electroporation; nuclear import; gene expression; transfection; IL-10; plasmid

Introduction

Ocular gene therapy is a promising strategy to correct genetic disorders, treat cancers, and control infectious diseases. The cornea has several experimental and therapeutic advantages that make it a desirable target for gene therapy (1). It has a simple histologic structure and excellent accessibility, and it is ideal for topical or direct gene delivery. In addition to providing treatment for diseases that have genetic components, such as glaucoma (2, 3); cataracts (2, 4, 5); and inherited epithelial, stromal, and endothelial dystrophies (6, 7), gene therapy also shows promise to treat infectious disease in the cornea.

The immunoregulatory cytokine interleukin 10 (IL-10) is a potent inhibitor of the inflammatory response. It can suppress proinflammatory cytokine and chemokine synthesis by resident corneal cells, and it can be used to modulate herpes simplex virus (HSV)–induced stromal keratitis (HSK), the leading cause of infectious blindness in the United States (8, 9). The pathology of the blinding illness in these ocular infections is not the result of viral replication, but rather of the ensuing host immunologic response to the virus. If recruitment of infiltrating immune cells into the corneal site of infection is prevented, no keratitis will develop. Intracorneal injection of recombinant IL-10 after HSV infection has been shown to prevent the development of necrotizing stromal keratitis and suppressed the severity of corneal disease (8, 9). The limitation of IL-10 therapy is that the injected protein has a very short half-life and requires repeated administration to elicit a therapeutic effect (9, 10).

Because of the rapid decay of injected IL-10 protein, gene therapy might be a better approach for IL-10 delivery, because the transferred vector should be able to produce a more steady level of IL-10. It has been reported that topical administration of plasmid DNA encoding IL-10 can resolve lesions of HSK (11). The expression of IL-10 lasted 8 to 11 days as measured by mRNA, but the time course of IL-10 protein is unknown. In addition, the amount of plasmid DNA delivered was 100 μg per mouse cornea, which is very high and may actually induce a CpG-mediated innate immune response itself. To decrease the amount of plasmid DNA, in this study we used electroporation following intracorneal injection of plasmid DNA (12).

In the current study we used our recently developed electroporation technique following intracorneal injection of

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plasmid DNA (12) to drive IL-10 gene expression in the cornea. We also analyzed the duration of gene expression in the cornea and attempted to extend the duration of plasmid-driven IL-10 expression. Finally, we further evaluated the efficacy of several different promoters and DNA sequences that have been demonstrated to promote plasmid nuclear entry in nondividing cells, including those isolated from the human cornea (13–15), and in certain tissues *in vivo* (16–18).

Materials and Methods

Plasmids. Plasmids were grown in *Escherichia coli* and purified using Qiagen Giga-prep kits, as described by the manufacturer (Qiagen, Chatsworth, CA). The concentration of plasmids was adjusted to 5 mg/ml in 10 m*M* Tris, pH 8.0; 1 m*M* EDTA; and 140 m*M* NaCl before injection.

The nomenclature of all plasmids is promoter–gene product–DNA nuclear targeting sequence (DTS). pCMV-Lux-DTS and pCMV-Lux express firefly luciferase from the CMV immediate early promoter (CMV_{iep}) and either contain or lack the SV40 DTS downstream of the luciferase gene (19). pCMV-GFP-DTS and pCMV-GFP express green fluorescent protein (GFP) from the CMV_{iep} with or without the SV40 DTS downstream of the GFP gene, respectively (named pGFP-DTS and pGFP, respectively, in Ref. 18). pCMV-IL10-DTS and pCMV-IL10 similarly express human IL-10. Human IL-10 cDNA amplified by polymerase chain reaction (PCR) with appropriate restriction sites flanking the coding sequence open reading frame was cloned into the *Not*I sites of pCMV-βGal (Clontech, Palo Alto, CA), replacing the *lacZ* gene.

The 1.2-kb human ubiquitin C (UbC) promoter was amplified from pUB6/V5-His/*lac*Z (Invitrogen, Carlsbad, CA) using PCR with primers containing *Eco*RI and *Xho*I restriction sites flanking the promoter fragment. This *Eco*RI–*Xho*I UbC promoter fragment was cloned into pCMV-Lux, pCMV-Lux-DTS, pCMV-IL10, and pCMV-IL10-DTS plasmids, replacing the CMV promoter to create plasmids pUbC-Lux, pUbC-Lux-DTS, pUbC-IL10 and pUbC-IL10-DTS, respectively. All plasmids were verified by DNA sequencing.

Mice. Female Balb/c mice weighing 16 to 24 g were purchased from Charles River (Wilmington, MA). Three or four mice (i.e., 6–8 eyes) were used at each data collection point. Animal protocols were approved by Northwestern University Institutional Animal Care and Use Committee, and all experiments complied with the recommendations of the "Guide for Care and Use of Laboratory Animals."

Plasmid DNA Injection and Electroporation. Plasmid solutions were injected into the corneas of Nembutal (pentobarbital sodium; Abbott Laboratories, Chicago, IL)–anesthetized Balb/c mice as previously described (12, 20). Briefly, a 30-gauge needle was used to make a nick in the epithelium and stroma in the midperiphery area of mouse cornea, and 1 μl plasmid solution containing 5 μg plasmid was injected into the stromal layer using a 50-μl Hamilton syringe and a WPI syringe pump (WPI, Sarasota, FL). Immediately following plasmid injection, gold-plated Genetrode electrodes (Genetronics, San Diego, CA) were placed on the cornea on either side of the area injected with plasmid DNA (3-mm gap). An ECM 830 square wave electroporator (BTX Instruments, Holliston, MA) was used to deliver eight pulses of 10-msec duration at a field strength of 200 V/cm. Following injection and electroporation, animals were allowed to recover and were returned to the vivarium.

Measurement of Luciferase Activity. Following euthanasia by pentobarbital sodium overdose and cervical dislocation, eyes were excised, the lenses were removed, and the eyes were snap frozen in liquid nitrogen. The eyes were ground into powder in a 2-ml glass tissue grinder bathed in liquid nitrogen. The ground tissue was suspended in 400 μl Promega reporter lysis buffer (Promega, Madison, WI) supplemented with 1 m*M* dithiothreitol (DTT). Homogenized samples were subjected to three freeze–thaw cycles before centrifugation (5 mins at 14,000 rpm at 4°C) to remove debris. Luciferase activity in the supernatant was assayed in duplicate using a Promega luciferase assay kit in a Turner luminometer (Turner Biosystems, Sunnyvale, CA). Purified recombinant luciferase (Promega) was used as standard.

IL-10 Quantification. Eye extracts were prepared as described above. IL-10 levels in the supernatant were measured in duplicate using a Human IL-10 ELISA Kit II (BD Biosciences Pharmingen, San Diego, CA) and quantified using recombinant human IL-10 as a standard.

Quantification of Plasmid DNA in the Cornea. After homogenization of excised eyes as described above, the pellets from the centrifugation step were stored at −80°C until DNA isolation was performed using TRIZOL reagent, as described by the manufacturer (Invitrogen Life Technologies, Carlsbad, CA). TRIZOL reagent (200 μl per pellet; i.e., per eye) was used. Total extracted DNA was quantified by spectrophotometry, and 1 μg total DNA was used for each PCR reaction.

Immunohistochemical Analysis. GFP expression was detected using a rabbit polyclonal antibody against GFP (Clontech) and an alkaline phosphatase–labeled secondary antibody (Vector Laboratories, Foster City, CA). Diaminobenzidine (DAB) was used as a substrate for the enzyme-linked secondary antibody.

Bromodeoxyuridine (BrdU) Incorporation. Cell proliferation was measured by BrdU labeling. Twenty-four hours after plasmid injection and electroporation, 500 μl BrdU solution (2 mg/ml in phosphate-buffered saline [PBS]) was injected intraperitoneally into a 20-g mouse. The animal was sacrificed as above 60 mins after BrdU injection, and the eyes were removed along with the surrounding skin tissue. Eyes were fixed overnight in 70% ethanol with 50 m*M* glycine, embedded in paraffin, and 6-μm thin sections were prepared. BrdU was detected using the BrdU In-Situ

Detection Kit (BD Pharmingen), following the manufacturer's instructions.

Statistics. The nonparametric Mann-Whitney *U* test was performed to determine statistical significance.

Results

Expression of IL-10 in the Mouse Cornea. Previous studies have shown that expression of luciferase from plasmids (pCMV-Luc-DTS) delivered to the cornea by electroporation can last for 3 days (12). Using the technique optimized in our lab, 5 μg plasmid DNA encoding IL-10 (pCMV-IL10-DTS) was delivered to the mouse cornea by electroporation. Unlike expression of luciferase in the cornea, IL-10 expression started to decline 2 days after plasmid delivery (Fig. 1). One day after gene transfer, the level of IL-10 in the cornea was about 200 pg/eye. The IL-10 level decreased by 60%, to about 80 pg/eye, by Day 2. This decline continued on Day 3 to 20% of the levels of IL-10 seen on Day 1, and by Day 7 only 8 pg/eye IL-10 was detected, approximately 4% of that on Day 1. This level of gene expression is similar to that of luciferase on Day 7, which is about 5% of that on Day 1 (12). The expressed IL-10 and its decrease over time was not due to the injection itself as opposed to expression from the plasmid, since injection and electroporation of a luciferase-expressing plasmid resulted in no significant increases or decreases in corneal IL-10 expression.

To improve the duration of gene expression, the UbC promoter was used to replace the CMV promoter based on reports that this promoter continues to express for up to 6 months *in vivo* (21). Four plasmids were constructed expressing luciferase or IL-10 with or without a downstream SV40 DTS, a *cis*-acting DNA sequence that has been shown to be required for plasmid nuclear uptake in nondividing cultured cells (13, 15). All four constructs expressed in the mouse cornea (Fig. 2). The duration of gene expression of pUbC-Lux and pUbC-IL10 was greatly increased compared with that driven by the CMV promoter. Luciferase expression from pUbC-Lux lasted for at least 4 weeks, with a maximum of 817 ± 100 pg/eye on Day 2 (100% ± 12%; Fig. 2A). Expression decreased moderately, to 35% ± 6% of Day 2 levels on Day 7 ($P \leq 0.05$ compared with Day 1), and after that luciferase levels continued to decrease, to 8.7% ± 1.3% of Day 2 levels by 4 weeks ($P \leq 0.05$ compared with Day 1). IL-10 expression from pUbC-IL10 was maintained at a fairly constant level (170–230 pg/eye) for 4 days (Fig. 2B). By contrast, plasmids containing a SV40 DTS, which was used to improve nuclear import of plasmid DNA, exhibited a shorter duration of gene expression (Fig. 2).

To determine whether loss of plasmid from the tissue over time could account for the decreased expression, pUbC-IL10-DTS DNA was extracted from the electroporated corneas and detected by PCR. Plasmid DNA was still present in the mouse cornea 7 days after gene transfer, when gene expression was less than 5% of that seen on Day 1 (Fig. 3). Similar retention of DNA over 7 days was seen with other plasmids as well (data not shown).

Another possible explanation for the limited expression of IL-10 from the UbC promoter is that the expressed IL-10 protein itself may inhibit expression from the UbC promoter in an autocrine manner. To test this hypothesis, a UbC promoter–driven luciferase-expressing plasmid, pUbC-Lux-DTS, was co-injected into the mouse cornea with pUbC-IL10-DTS (2.5 μg of each plasmid DNA). For comparison, pUbC-Lux-DTS also was co-injected with another plasmid expressing β-galactosidase (pUbC-βgal-DTS) but not IL-10. If IL-10 inhibits UbC promoter–driven gene expression, luciferase expression in corneas co-injected with pUbC-IL10-DTS should be much lower than that in corneas co-injected with pUbC-βgal-DTS, because β-galactosidase is not expected to inhibit gene expression. As seen in Figure 4,

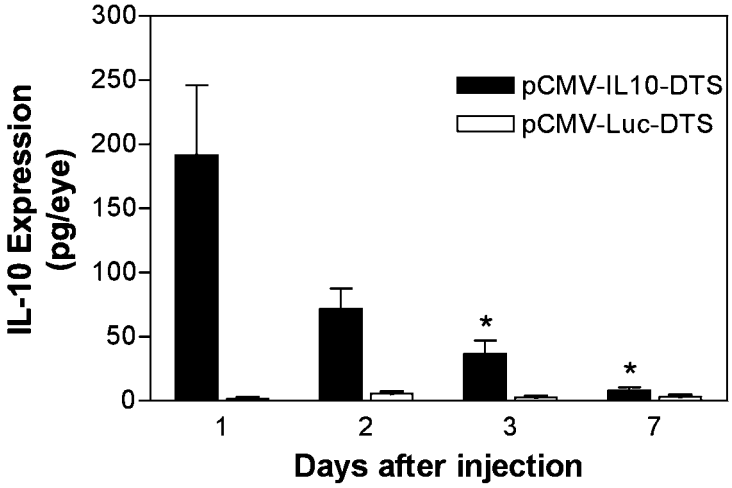

Figure 1. Transient expression of pCMV-IL10-DTS. Eyes were intracorneally injected with 5 μg of either pCMV-IL10-DTS ($n = 6$) or pCMV-Luc-DTS ($n = 6$), followed by electroporation at 200 V/cm using eight pulses of 10 msec in duration. At the indicated time points mice were euthanized, and IL-10 protein levels were measured in corneal extract, shown as mean ± SEM. *$P \leq 0.04$ compared with that at Day 1.

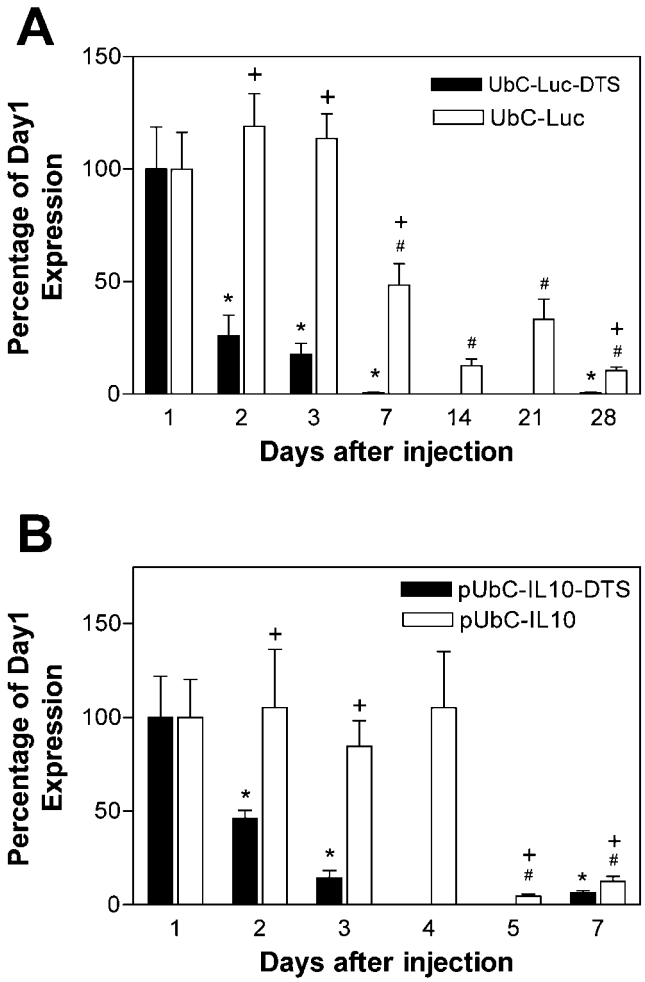

Figure 2. Duration of gene expression driven by UbC promoter. Eyes ($n = 6$ to 8) were injected with 5 μg plasmid containing (filled bar) or lacking (open bar) the SV40 DTS, followed by electroporation at 200 V/cm. At the indicated times mice were euthanized, and gene products were measured in corneal extract (mean ± SEM). (A) Expression of luciferase. *$P \leq 0.03$ compared with pUbC-Luc-DTS at Day 1. #$P \leq 0.05$ compared with pUbC-Luc at Day 1. +$P \leq 0.004$ compared with pUbC-Luc at each given day. No time points at 14 and 21 days were assayed for the DTS-containing plasmid. (B) Expression of IL-10. *$P \leq 0.02$ compared with pUbC-IL10-DTS at Day 1. #$P \leq 0.01$ compared with pUbC-IL10 at Day 1. +$P \leq 0.02$ compared with pUbC-IL10 at each given day.

co-injection of the IL-10–expressing plasmid did not affect luciferase expression from the same promoter. Rather, it appeared that co-injection of the β-galactosidase plasmid caused a slight but statistically significant decrease in expression of luciferase at Day 2 compared with Day 1 ($P \leq 0.02$). Although the level of luciferase obtained with β-galactosidase co-injection was higher on Day 1 than that seen with the IL-10 co-injection, the difference was not statistically significant.

Effect of Multiple Doses of DNA and Electroporation on Gene Expression. As an alternative approach to further increase the duration of IL-10 gene expression, we tested whether multiple doses of plasmid and electroporation could result in prolonged expression. pUbC-IL10 was injected and electroporated into the cornea, and 4 days later, when gene expression was shown to decrease (Fig. 2B), a second dose of DNA was administered using the same parameters as the first dose (Fig. 5). Following the first injection, expression declined to about 50% of Day 1 values by Day 4 ($P \leq 0.04$). One day after the second injection, IL-10 expression was restored to the same level seen after one day following the first injection ($P \leq 0.015$ compared with that at Day 4). The decline in expression after the second dose was roughly the same as that seen following the first injection. These results suggest that the duration of exogenous IL-10 expression, or other genes, can be further improved with multiple dosing of plasmid DNA.

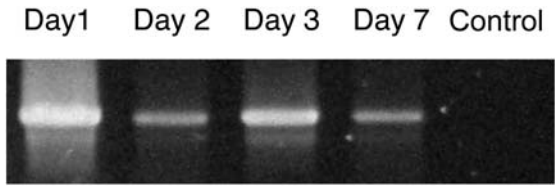

Figure 3. Plasmid DNA retained in mouse cornea. Following intracorneal injection of TE (control) or 5 μg pUbC-IL10-DTS and electroporation, eyes ($n = 4$) were excised and homogenized at the indicated times. Plasmid DNA was extracted and subjected to PCR to amplify a plasmid specific sequence. Equal amounts of total DNA were used for PCR reactions and separated by agarose gel electrophoresis. A representative gel is shown.

Effect of DTS on Gene Expression. In previous studies of gene expression following naked DNA transfer with or without electroporation, it was found that an SV40 DNA nuclear targeting sequence (SV40 DTS) is critical for efficient gene expression in tissues composed of non-dividing or slowly dividing cells (16–18). Thus, we investigated the effect of this sequence on exogenous gene expression in the cornea in hopes of increasing the level and extending the duration of IL-10 gene expression.

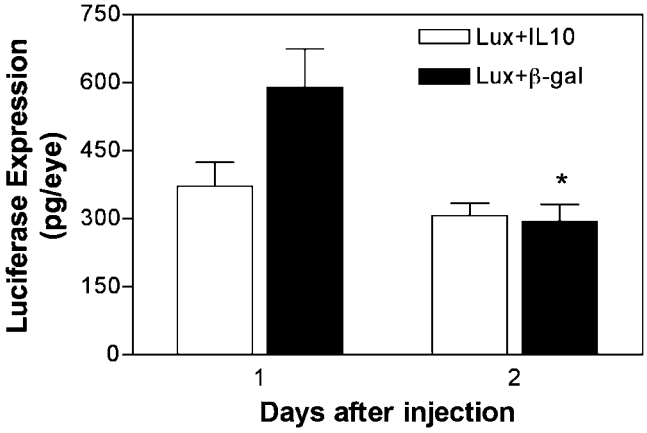

Figure 4. Comparison of luciferase expression from pUbC-Luc-DTS co-injected with pUbC-IL10-DTS or pUbC-βgal-DTS. Eyes ($n = 6$) were injected and electroporated with 2.5 μg pUbC-Luc-DTS and either 2.5 μg pUbC-IL10-DTS or 2.5 μg pUbC-βgal-DTS. At the indicated times mice were euthanized, and luciferase activity was measured in corneal extract, shown as mean ± SEM. *$P \leq 0.02$ compared with pUbC-Luc-DTS + pUbC-βgal-DTS on Day 1.

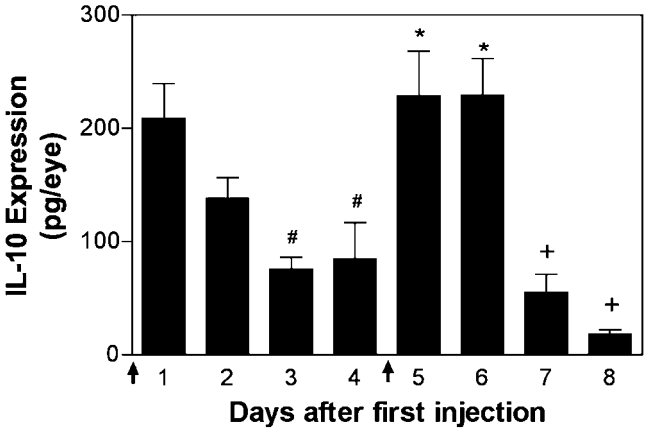

Figure 5. Repeated doses and electroporation of pUbC-IL10. Eyes ($n = 6$ to 8) were injected with 5 μg pUbC-IL10 and electroporated. Four days after the first injection, another 5 μg pUbC-IL10 was injected into each cornea, followed by electroporation using the same parameters. At the indicated time points mice were euthanized, and IL-10 was measured in corneal extract (mean ± SEM). #$P \leq 0.04$ compared with that at Day 1. *$P = 0.015$ compared with that on Day 4. +$P \leq 0.01$ compared with that at Day 5.

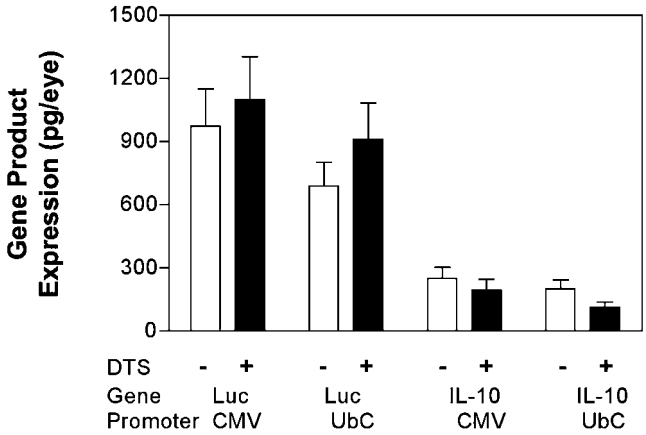

Figure 6. Effect of the SV40 DTS on gene expression. Eyes ($n = 6$ to 8) were intracorneally injected with 5 μg plasmid either containing (filled bar) or lacking (open bar) the SV40 DTS. Eyes were electroporated at 200 V/cm immediately after injection. The levels of gene expression were measured 1 day after gene transfer (mean ± SEM). No statistical differences were detected between any matched sets of vectors (±DTS).

Plasmids expressing luciferase or IL-10 from either the CMV or UbC promoters with or without the SV40 DTS downstream of the reporter gene were injected into the corneas at equal concentrations and electroporated. One day later gene expression was determined. In contrast to previous reports in the vasculature (18) and skeletal muscle (16, 17), the SV40 DTS appeared not to be required for gene expression in the mouse cornea (Fig. 6). Each pair of matched plasmids (with or without the SV40 DTS) gave similar levels of gene expression with no statistical differences.

One possible explanation for the difference may be in the proliferation state of the tissues, since the assumption for seeing maximal DTS activity is that the target tissues are nondividing. It is possible that injection of the cornea, with or without electroporation, may be inducing a wound-healing response that could result in cell proliferation. To evaluate this, 1 day following corneal injection and electroporation of either pCMV-GFP-DTS (Fig. 7, A–C) or pCMV-GFP (Fig. 7, D–F), BrdU was injected 60 mins prior to sacrifice, and BrdU incorporation was evaluated by immunohistochemistry. Indeed, a significant number of cells showed incorporation of BrdU, indicating that they were undergoing DNA synthesis and cell division (Fig. 7, A and D). However, there was no apparent correlation between the areas of proliferating cells and those expressing reporter gene. BrdU-positive cells were located mostly in the epithelial layer of the cornea, with a few in the endothelial layer (Fig. 7, B and E). By contrast, gene expression was located mostly in stromal layer, which showed little DNA synthesis at 24 hrs after injection (Fig. 7, C and F). It should also be noted that with either construct there was little sign of inflammation or infiltrating cells.

Discussion

Although viral vectors have been reported to express IL-10 for over 3 weeks in corneas *ex vivo* (22, 23), the duration of exogenous IL-10 gene expression following local delivery of naked plasmid DNA *in vivo* is not clear. Here we report the successful gene transfer of an IL-10–encoding plasmid into the mouse cornea using electroporation. With a constitutively active UbC promoter, a single dose of 5 μg plasmid DNA was able to give sustained expression for 4 days, and with a second dose expression was detected for 8 days. This method is efficient, simple, safe, and inexpensive (12). In addition, plasmid DNA encoding IL-10 can be delivered repeatedly without compromising its ability to modulate inflammation (24). These results suggest that IL-10 gene delivery to the eye by electroporation may be a viable option as an anti-inflammatory treatment.

The duration of gene expression is especially important to achieve IL-10 immune modulation, because IL-10 has very short half-life (10). There are several possibilities that can cause loss of persistent gene expression of plasmid DNA transferred to tissue *in vivo*, including loss of plasmid by cell turnover or intracellular degradation, transcriptional silencing, and immune response to the gene product. As loss of gene expression after several days with the CMV promoter is observed commonly (18, 25), we replaced the CMV promoter with the UbC promoter to overcome this problem caused by probable promoter inactivation. In the absence of the SV40 DTS, the UbC promoter was able to support longer gene expression in the cornea.

We also explored other possibilities that could cause a loss of gene expression of plasmid DNA, such as loss of plasmid or inhibition of the UbC promoter by IL-10. Both had insignificant effects on the duration of IL-10 expression. While evidence shows that there is cell turnover in the

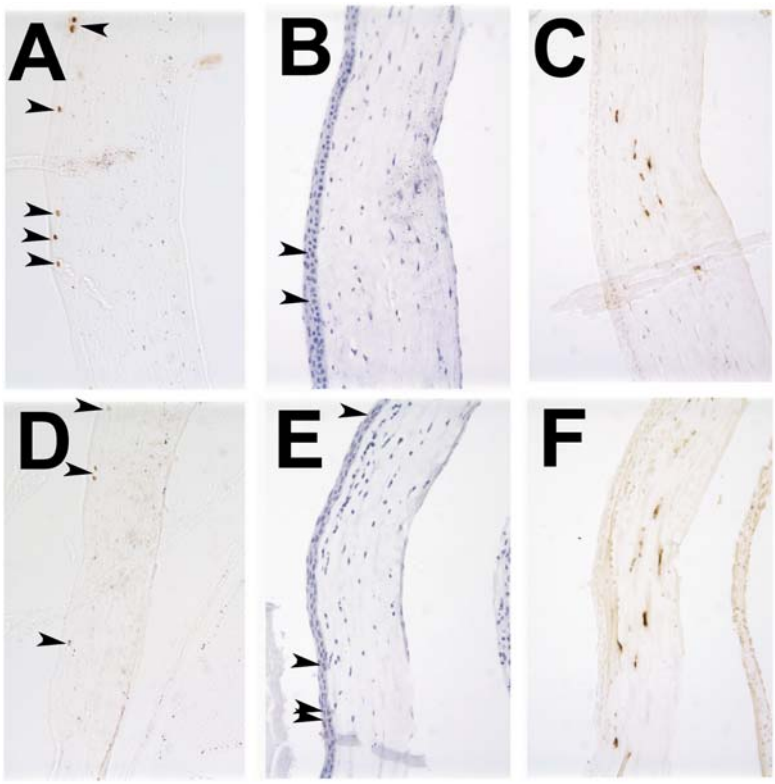

Figure 7. Cell proliferation and GFP gene expression in injected and electroporated mouse eyes. Eyes ($n = 4$) were injected with 5 μg of either pCMV-GFP-DTS (A–C) or pCMV-GFP (D–F), followed by electroporation at 200 V/cm. Twenty-four hours after gene transfer, BrdU was injected intraperitoneally, and mice were euthanized 60 mins later. Eyes were removed, embedded in paraffin, and thin sectioned. Immunohistochemistry was used to detect BrdU (A, B, D, and E) or GFP (C and F) using DAB to visualize antibody staining. Sections in panels B and E were counterstained with hematoxylin.

wounded cornea (26), which could cause a loss of plasmid being retained in those cells, there was still a significant amount of plasmid retained in mouse cornea 7 days after gene transfer. Similarly, using quantitative PCR Gill and colleagues demonstrated that transient gene expression of luciferase in the lung is not mediated by loss of plasmid DNA: 2 weeks after gene transfer, the amount of plasmid DNA remaining in the lung was the same as that on Day 2, but luciferase expression was less than 1% of that on Day 2 (21).

If the loss of gene expression is not caused by the loss of plasmid DNA, it could be caused by promoter inactivation. We showed in this study that downregulation of the UbC promoter, if any, is not caused by the expressed IL-10. Co-injection of an IL-10–expressing plasmid did not affect UbC-driven luciferase expression compared with the control group. In addition, the second dose of plasmid DNA was able to booster the gene expression, indicating that immune response did not suppress gene expression. Thus, other mechanisms must be involved in modulating gene expression.

One rather surprising finding was that the presence of the SV40 DTS adversely affected the duration of UbC promoter–driven gene expression in the cornea. Expression of luciferase by pUbC-Lux lasted for more than 4 weeks, but the luciferase level dropped to nearly background at Day 7 with pUbC-Lux-DTS. However, this negative effect of SV40 DTS was not seen in plasmids with a CMV promoter. The duration of gene expression was the same with or without the SV40 DTS (data not shown). It is not clear how the SV40 DTS affected the duration of UbC-driven gene expression. In mouse skeletal muscle, CMV_{iep}-driven luciferase expression was more persistent, with a tandem repeat of three 72-bp elements from SV40 enhancer, which is part of SV40 DTS in the plasmids used in this study (16). Although the authors did not discuss the mechanism of the increased duration of gene expression in the presence of SV40 enhancer elements, it has been shown in cells and other tissues that the SV40 enhancer promotes nuclear import of plasmid DNA (14, 18), and that those plasmids retained in cell nucleus are protected from degradation (18).

Previous studies from our lab and other labs have shown that the SV40 DTS is essential to transfect nondividing cells, including corneal epithelial cells and keratocytes in cell culture, due to its DNA nuclear import activity (15, 27). Plasmids lacking the SV40 DTS that were microinjected into the cytoplasm of cultured cells remained in the cytoplasm, and hence no gene expression was

observed, suggesting that in cultured nondividing cells a nuclear import sequence is needed for plasmid nuclear import and gene expression (13–15). The ability of the SV40 DTS to increase nuclear import and gene expression also has been detected in several tissues *in vivo*. Blomberg *et al*. and Li *et al*. independently demonstrated that inclusion of the SV40 DTS into an expression plasmid increased gene transfer and expression in mouse skeletal muscle *in vivo* by 20-fold (16, 17). Similarly, Young and colleagues showed that the SV40 DTS increased gene expression 20- to 100-fold in the intact vasculature of rats due to its ability to promote plasmid nuclear import (18).

However, the SV40 DTS did not show a similar effect on plasmid expression following gene transfer into the cornea *in vivo*. Four plasmids, pCMV-Lux, pCMV-IL10, pUbC-Lux, and pUbC-IL10, all were able to express in the mouse cornea after intracorneal injection followed by electroporation, suggesting that the SV40 DTS is not required for gene transfer in this tissue. Similarly, gene expression has been detected in a number of tissues *in vivo* from plasmids lacking a DTS, especially skeletal muscle. Using BrdU labeling, cell proliferation was detected in the treated corneas, suggesting that plasmids without a SV40 DTS could readily enter the cell nucleus during mitosis, when the nuclear envelope is disassembled. However, it should be pointed out that cells showing BrdU incorporation did so 24 hrs after DNA injection and electroporation. Thus, luciferase-expressing cells must already have gone through mitosis before BrdU administration, and this could account for the fact that the two classes of cells (luciferase-expressing and BrdU-positive) appeared distinct. Cell division alone may not explain the gene expression obtained with plasmids lacking the SV40 DTS in cornea, since as seen in Figure 7, most of the BrdU-positive cells were in the epithelial layer, whereas gene expression was seen mostly in the stroma. How plasmids without a DTS entered non-dividing keratocytes in the stroma is not clear. It is possible that with a large amount of plasmid DNA being injected in mouse cornea, a large number of plasmids were delivered into cell cytoplasm. Even if a very small percentage of cytoplasmic plasmid entered the nucleus *via* a non–sequence-specific pathway, this could be enough to detect gene expression. Thus, the mechanisms of DTS activity, including its apparent modulatory effects on the UbC promoter, in the cornea must be investigated further.

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