Characterization of the expression of \textit{DMPK} and \textit{SIX5} in the human eye and implications for pathogenesis in myotonic dystrophy

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The pathogenic mechanisms underlying myotonic dystrophy (DM), which results from a (CTG)$_n$ repeat expansion mutation in the 3′-untranslated region (3′-UTR) of the myotonic dystrophy protein kinase gene (\textit{DMPK}), remain obscure. The multisystemic nature and variable expressivity of the symptoms are unlikely to be explained by a defect in this gene alone. However, the location of the DM-associated (CTG)$_n$ repeat in the promoter region of \textit{SIX5}, immediately downstream of \textit{DMPK}, implicates it as a second candidate with a pathological role in DM. We hypothesize that dysfunction of \textit{SIX5}, which is homologous to the \textit{Drosophila} eye development gene \textit{sine oculis} (\textit{so}), is primarily responsible for the ophthalmic features of DM. We report an expression pattern for \textit{SIX5} in the normal adult eye that matches the sites of the ocular pathology in DM. \textit{SIX5} transcripts were detected in the adult corneal epithelium and endothelium, lens epithelium, ciliary body epithelia, cellular layers of the retina and the sclera. \textit{SIX5} expression was not detected in fetal eyes. We also report a restricted but partially overlapping expression pattern for \textit{DMPK} transcripts and \textit{DMPK} protein in normal fetal and adult eyes. \textit{DMPK} transcripts were detected in fetal eyes and in adult conjunctival and corneal epithelia, uvea, cellular layers of the retina, optic nerve and in the sclera. \textit{DMPK} protein was detected in the adult retina, conjunctival and ciliary body epithelia and in the smooth muscle of the ciliary body, pupillary sphincter and uveal blood vessels. We propose that the expression patterns of these two genes indicate their relative contribution to the ophthalmological dysfunction seen in DM. Furthermore, the expression of \textit{SIX5} and not \textit{DMPK} in the adult lens implicates a role for \textit{SIX5} dysfunction in the development of adult onset cataracts, the most frequently occurring eye phenotype in DM.

\textbf{INTRODUCTION}

Myotonic dystrophy (DM) is an autosomal dominantly inherited progressive neuromuscular disorder, with an incidence of 1 in 8000 in European and North American populations (1). Although characterized by myotonia and progressive muscle weakness and atrophy, DM is a heterogeneous disorder affecting a wide range of systems. In addition to skeletal muscle problems, DM is associated with a number of ophthalmic features as well as cardiac conduction defects, gastrointestinal problems, hyperinsulinism, hypersomnia, mental impairment, premature balding, reduced fertility, respiratory problems and testicular atrophy.

Ocular defects in DM can be numerous but the multicoloured iridescent cataract is the most prominent feature of the eye pathology. It is often the first and in some cases the only sign of the disease, occurring at a younger age than is expected for senile cataracts, in people who show no muscle symptoms or who carry a premutation size (CTG)$_n$ repeat allele. However, cataract development is not a common feature during childhood of DM patients or during infancy of congenitally affected patients, although it can occur later in life. Initially the DM-associated cataract is seen as a scattering of punctate white or multicoloured dust-like opacities in the posterior subcapsular region of the lens. As the cataract progresses and visual acuity diminishes, the lens surface becomes reticulated and the opacities radiate into the lens cortex. As general clouding increases, the iridescent opacities may be less conspicuous and the mature cataract is often difficult to differentiate from a senile cataract. This similarity and the occurrence of anticipation in DM initiated several investigations into whether senile cataract patients in the general population constituted a genetic reservoir of the DM mutation (2–5). However, in the majority of studies no premutation carriers were identified and therefore it was concluded that

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senile cataract patients were not at risk of carrying small (CTG)~n~ repeat expansions.

The retina, cornea, ciliary body, ocular muscles and the eyelids can also be affected in DM. Like the cataract, the retinal changes also lead to reduced visual acuity, usually as the result of a central macular lesion and less commonly because of pigmentary retinal degeneration. Abnormal electroretinograms are seen in most DM patients, including those with no ocular symptoms or fundus abnormality. Corneal lesions such as ulcers and keratoconjunctivitis sicca occur and the extraocular muscles display myotonia. Ptosis is generally symmetrical and forms an integral part of the characteristic facies of a DM patient. The low intraocular pressure may be attributable to abnormal ciliary body function.

The genetic defect associated with DM is the expansion of a (CTG)~n~ triplet repeat on human chromosome 19q13.3. The (CTG)~n~ repeat is polymorphic in the general population, with a range of 5–37 copies and is inherited in a stable manner. In DM patients the (CTG)~n~ repeat tract is expanded from 50 to thousands of copies and shows germline and somatic instability with a bias towards expansion. Despite the highly variable phenotypic expression of both genes (19,21–23). It has also been proposed that part of the DM phenotype may be mediated by a gain of function due to hypermethylation of a region (15–20). These changes in chromosomal structure may result in a field effect and, in particular, affect the transcription of DMPK and SIX5 in normal adult and fetal eyes at the RNA level by RT–PCR and DMPK protein by SDS–PAGE and western blotting and determined normal expression patterns. The specific sites of expression were further investigated by in situ hybridization and immunocytochemistry. We report that SIX5 is expressed in the normal adult eye in the cornea, lens, ciliary body, retina and sclera and that DMPK is expressed in normal fetal eyes and in normal adult cornea, conjunctiva, uvea, retina, sclera and optic nerve. DMPK protein was detected in normal adult uvea (pupillary sphincter, ciliary body and choroid), conjunctiva and retina. We propose that the expression patterns of these two genes indicate their relative contribution to the ophthalmological dysfunction seen in DM. In particular, the expression of SIX5 and not DMPK in the adult lens implicates a role for SIX5 dysfunction in the development of adult onset cataracts.

RESULTS

Analysis of DMPK and SIX5 expression by RT–PCR

The expression of DMPK and SIX5 in normal human fetal and adult eyes was analysed initially by RT–PCR on RNA extracted from whole fetal eyes and pooled samples of cornea, lens, uvea, retina, optic nerve and sclera dissected from adult eyes. Genomic DNA was also used as a template and confirmed that the reaction conditions were appropriate for amplifying the larger genomic fragment and that the primers were gene-specific, as only one gene product was amplified (data not shown). The use of primers within different exons of the genes enabled the cDNA PCR products and genomic DNA PCR products to be differentiated. DMPK transcripts were amplified using a forward primer in exon 2 of DMPK (CLW2F) and a reverse primer in exon 4 of DMPK (CLW4R) (Fig. 1). Transcripts were detected as 233 bp PCR products in the fetal eye and in adult uvea, retina, optic nerve and sclera but not in the adult lens or cornea (Fig. 2A). A genomic DNA PCR product of 546 bp was amplified only in the corneal sample, due to genomic DNA contamination in the corneal total RNA extract.

SIX5 transcripts were amplified using a forward primer in the homeobox of exon A of SIX5 (KJDMF) and a reverse primer in exon B of SIX5 (SIX5-R-SEH) (Fig. 1). Transcripts were detected as 307 bp PCR products in the adult lens, cornea, uvea, retina and sclera but not in the optic nerve or the fetal eyes, where a faint ladder of bands was observed (Fig. 2B). A genomic DNA PCR product of 1195 bp was detected only in the corneal sample, due to genomic DNA contamination in the corneal total RNA extract.

The identity of the DMPK and SIX5 PCR products as spliced cDNAs was confirmed by DNA sequence analysis (data not shown).

Analysis of DMPK and SIX5 expression by in situ hybridization

To identify the specific sites of DMPK and SIX5 expression in the eye, transcripts were analysed by in situ hybridization of gene-specific digoxigenin-labelled riboprobes to sections of whole normal eyes from six human fetuses and eight adults. Sense and antisense riboprobes were generated from the 3’-UTR of DMPK (plasmids pDMPK15a and pDMPK15b) and from the 5’- and 3’-UTRs of SIX5 (pSIX51 and pSIX52) (Fig. 1). The UTRs...
Figure 1. Diagram of the DM locus on human chromosome 19q13.3. The genes are drawn telomere to centromere, the direction of transcription. Exonic regions are represented by boxes and intronic and intergenic regions by linking lines. Only exons 2–4 and 15 of the DMPK gene are drawn, linked by a dashed line that represents the genomic region of 7.6 kb. All three exons of SIX5 are shown (A, B and C). The promoter region of SIX5 is situated within the 3.5 kb CpG island in the 3′-end of the DMPK gene (double-headed arrow). The (CTG)n repeat in the 3′-UTR of DMPK and the promoter of SIX5 are marked. The locations of the gene-specific PCR primers are represented by short lines above the relevant exons: CLW2F, CLW4R, KJDMF, SIX5-R.SEH. The regions of the genes used for the in situ hybridization riboprobes, pDMPK15, pSIX51 and pSIX52, are shown by thick black lines.

Figure 2. (A) RT–PCR analysis of DMPK expression in human fetal and adult eye tissues. RT–PCR products were analysed by electrophoresis in 2.5% (w/v) agarose. The expected cDNA PCR product of 233 bp and the genomic PCR product of 546 bp are indicated. Lane 1, 1 kb DNA ladder (Gibco BRL); lane 2, adult lens; lane 3, adult retina; lane 4, adult cornea; lane 5, adult sclera; lane 6, adult uvea; lane 7, optic nerve; lane 8, fetal eyes; lane 9, first water control; lane 10, second water control. (B) RT–PCR analysis of SIX5 expression in fetal and adult eye tissues. RT–PCR products were analysed by electrophoresis in 2.5% (w/v) agarose. The expected cDNA PCR product of 307 bp and the genomic PCR product of 1195 bp are indicated. Lane 1, 1 kb DNA ladder (Gibco BRL); lane 2, adult lens; lane 3, adult retina; lane 4, adult cornea; lane 5, adult sclera; lane 6, adult uvea; lane 7, adult optic nerve; lane 8, fetal eyes; lane 9, first water control; lane 10, second water control.

**DMPK Gene**

<table>
<thead>
<tr>
<th>CLW2F</th>
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**SIX5 Gene**

- pDMPK15
- pSIX51
- pSIX52

were used to minimize cross-reactivity between the riboprobes and related genes that have homology within the coding regions.

DMPK expression was detected consistently in a series of adult eyes in the corneal epithelium (Fig. 3A), conjunctival epithelium (Fig. 3B) and the ganglion cells, the cells of the inner nuclear layer and the photoreceptor cells of the retina (Fig. 3D). DMPK transcripts were not detected in the lens (Fig. 3E) or in any other structures of the adult eye (data not shown). The expression pattern obtained by in situ hybridization was not exactly the same as determined by RT–PCR, as transcripts were not detected by in situ hybridization in the uvea, sclera or optic nerve but were detected in the cornea. However, both methods consistently detected DMPK transcripts in the adult retina and not in the lens (Figs 2 and 3). The localization of DMPK transcripts in fetal eyes is currently being analysed by in situ hybridization.

SIX5 expression was detected consistently in the adult corneal epithelium and endothelium (Fig. 4A and C), inner and outer epithelia of the ciliary body in both the pars plicata (Fig. 4D) and pars plana (Fig. 4E), lens epithelium (Fig. 4F) and the ganglion cells, cells of the inner nuclear layer and photoreceptor cells of the retina (Fig. 4G) but not in any other structures of the adult eye or in fetal eyes (data not shown). The expression pattern was consistent with the RT–PCR expression data, except for the detection of SIX5 transcripts in the sclera by RT–PCR but not by in situ hybridization. Expression was not detected in the adult optic nerve or in the fetal eyes by either method.

The specificity of the antisense riboprobes and the expression data were confirmed by hybridizing the eye sections with sense
Figure 3. *In situ* hybridization (A–E) and immunocytochemical analyses (F–I) of DMPK expression in normal human adult eyes. An antisense riboprobe generated from pDMPK15a (A, B, D and E) and a sense riboprobe generated from pDMPK15b (C) were used for the *in situ* hybridization experiments. DMPK transcripts were detected with a blue/black stain and photographed at an original magnification of ×400, with interference contrast optics. (A) Anterior surface of the cornea. DMPK was detected in the epithelium (E) but not in the cells of the stroma (S). (B) Conjunctiva. DMPK was detected in the epithelium (E) but not in stromal cells (S). (C) Conjunctiva. There was no staining in the negative control eye using the DMPK sense riboprobe. (D) Retina. DMPK was detected in the ganglion cells (GC), cells of the inner nuclear layer (INL) and in the photoreceptor cells (PR), predominantly in the inner segments of the photoreceptors. (E) Lens. Both the epithelium (E) and the lens fibres (S) were negative. A 1/50 dilution of 6G8 anti-DMPK monoclonal antibody was used for the immunocytochemistry. DMPK protein was detected with a red stain and photographed at an original magnification of ×400. Haematoxylin was used to counterstain the cell nuclei blue. Intrinsic melanin pigment appears yellow/brown in (G) and (I). (F) Lens. Both the epithelium (E) and the lens fibres (S) were negative. (G) Ciliary body. DMPK was detected strongly in the smooth muscle cells within the ciliary body and faintly in the ciliary body epithelium, predominantly in the inner non-pigmented layer. (H) Retina. DMPK was detected strongly in the smooth muscle cells of choroidal blood vessels (V).
Figure 4. *In situ* hybridization analysis of normal adult eyes with a *SIX5* antisense riboprobe (A and C–G) and a *SIX5* sense riboprobe (B and H), generated from pSIX51. *SIX5* transcripts were detected with a blue/black stain and photographed with interference contrast optics at an original magnification of ×400. Intrinsic melanin pigment appears yellow/brown in (D) and (E). (A) Anterior surface of the cornea. *SIX5* was detected in the epithelium (E) but not in the cells of the stroma (S). (B) Anterior surface of the cornea. There was no staining in the negative control eye using the *SIX5* sense riboprobe. (C) Inner (posterior) surface of the cornea. *SIX5* was detected in the endothelium (En) but not in stromal cells (S). (D) Pars plicata and (E) pars plana of the ciliary body. *SIX5* was detected in both the inner and outer pigmented epithelia (E) but not in the stromal cells (S). (F) Anterior axial lens. *SIX5* was restricted to the anterior lens epithelium (E) and was not detected in the lens capsule (C) or the substance of the lens cortex (fibres) (S). (G) Retina. *SIX5* was detected in the ganglion cells (GC), cells of the inner nuclear layer (INL) and the photoreceptor cells (PR), both around their nuclei in the outer nuclear layer (ONL) and in the inner segments. (H) Retina. There was no staining in the negative control eye using the *SIX5* sense riboprobe.
The expression of DMPK mRNA was only detected in the adult sclera and optic nerve by RT–PCR and only in the corneal epithelium by in situ hybridization. There are several potential explanations for these incongruities. For example, RT–PCR is a more sensitive method of detection and therefore it is possible that transcripts expressed from eight adults (Fig. 3). Localization of DMPK protein expression in normal fetal and adult eyes was analysed using four different methods, which resulted in some discrepancy in the expression patterns (summarized in Fig. 6 and Table 1). The differences might be attributed to the methods of detection and tissue preparation (RT–PCR, in situ hybridization, western blot or immunocytochemistry) or the molecules being detected (mRNA or protein).

**DISCUSSION**

The expression of DMPK in normal fetal and adult eyes was analysed using four different methods, which resulted in some discrepancy in the expression patterns (summarized in Fig. 6 and Table 1). The differences might be attributed to the methods of detection and tissue preparation (RT–PCR, in situ hybridization, western blot or immunocytochemistry) or the molecules being detected (mRNA or protein).

**Analysis of DMPK protein expression by western blot**

To complement the analyses of DMPK mRNA, the protein was also analysed. Proteins were extracted from the same adult and fetal eye tissues from which the RNA had been obtained and analysed by western blotting using 6G8, an anti-DMPK monoclonal antibody (Fig. 5). No proteins from the adult optic nerve were available for this analysis. A band of the predicted size of DMPK, 71 kDa, was immunodetected in the protein extracted from the adult retina and uvea and a band of 67 kDa was also immunodetected in the adult uvea. No proteins were immunodetected in the fetal eye extract or in the adult cornea, lens and sclera extracts. Prior to the analysis of the proteins extracted from the human fetal and adult eyes, the specificity of the anti-DMPK monoclonal antibody was checked against human and mouse proteins extracted from fetal and adult skeletal muscle and heart and a number of recombinant GST–DMPK fusion proteins that contain the region used as the antigen. A 71 kDa protein was immunodetected in the tissue extracts and the GST–DMPK recombinant proteins were also immunodetected (C.L. Winchester, unpublished data).

**Analysis of DMPK protein expression by immunocytochemistry**

To identify the specific sites of DMPK protein expression, 6G8 monoclonal antibody was used to analyse sections of whole eyes from eight adults (Fig. 3). Localization of DMPK protein in normal fetal eyes is currently being investigated. DMPK protein was immunodetected in normal adult eyes in the smooth muscle of the ciliary body (Fig. 3G), pupillary sphincter (data not shown) and blood vessels of the choroid (Fig. 3I) and in the ganglion cells, cells of the inner nuclear layer and photoreceptors of the retina (Fig. 3H). Relatively weak and inconsistent staining was also found in the conjunctival epithelium and the epithelia of the pars plicata of the ciliary body (data not shown). DMPK protein was not immunodetected in the lens (Fig. 3F). These data concur with the western analysis.

**Figure 5.** Western blot analysis of proteins extracted from normal human fetal and adult eyes with a 1/100 dilution of 6G8, an anti-DMPK monoclonal antibody. The immunodetected DMPK proteins of 71 and 67 kDa are indicated. Lane 1, fetal eye extracts; lane 2, adult sclera extract; lane 3, adult retina extract; lane 4, adult lens extract; lane 5, adult cornea extract; lane 6, adult uvea extract.

**Figure 6.** Diagram summarizing the expression data of DMPK and SIX5 in normal adult eyes. CEp, corneal epithelium; CEn, corneal endothelium; CjEp, conjunctival epithelium; I (PS), iris (pupillary sphincter); LEp, lens epithelium; CBEp (PPli), ciliary body epithelia (pars plicata); CBEp (PPla), ciliary body epithelia (pars plana); S, sclera; Ch, choroid; R, retina; ON, optic nerve. (A) RT–PCR analysis of DMPK expression. Transcripts were detected in the uvea (iris, ciliary body and choroid), retina, optic nerve and the sclera. (B) In situ hybridization analysis of DMPK expression. Transcripts were detected in the corneal epithelium, conjunctival epithelium and the ganglion cells, cells of the inner nuclear layer and the photoreceptors of the retina. (C) Western blot analysis of DMPK expression. Protein was detected in the uvea (iris, ciliary body and choroid) and the retina. (D) Immunocytochemical analysis of DMPK expression. Protein was detected in the conjunctival epithelium, epithelia of the pars plicata of the ciliary body, smooth muscle of the ciliary body, pupillary sphincter (iris) and choroidal blood vessels and in the ganglion cells, cells of the inner nuclear layer and photoreceptors of the retina. (E) RT–PCR analysis of SIX5 expression. Transcripts were detected in the cornea, lens, uvea (iris, ciliary body and choroid), retina and sclera. (F) In situ hybridization analysis of SIX5 expression. Transcripts were detected in the corneal epithelium and endothelium, lens epithelium, ciliary body epithelia of the pars plicata and pars plana and in the ganglion cells, cells of the inner nuclear layer and the photoreceptor cells of the retina.
at low levels (i.e. adult sclera and optic nerve) might not be detected by in situ hybridization. It is also possible, due to the difficulties of dissecting fresh eyes, that the scleral sample could have been contaminated with both conjunctival or corneal epithelium or retina or that the optic nerve sample could also have been contaminated with retina. These tissues were shown by in situ hybridization to contain DMPK transcripts and therefore might account for the RT–PCR product in the sclera and optic nerve samples. An alternative method to circumvent this problem would be to microdissect individual cell types from fixed paraffin sections using direct microscopic control (38).

The western blot and immunocytochemical analyses of the DMPK protein gave more consistent results. Protein was detected by both methods in the adult uvea (which includes the ciliary body and pupillary sphincter) and in the retina. Dissection of the adult eyes might have resulted in the loss of the conjunctival epithelium and therefore protein extracts from this tissue would not have been present on the western blot. Two proteins were immunodetected in the adult uvea and it is possible that the anti-DMPK monoclonal antibody interacted with the full-length DMPK protein (71 kDa) and one of the truncated DMPK isoforms (67 kDa) encoded by an alternatively spliced transcript. A number of splice isoforms which could result in a lower molecular weight DMPK protein of 66–68 kDa have been described. These include cDNAs with 150 bp deleted from exon 7, exon 13 deleted, exon 14 deleted or introns 13 and 14 retained (39–43).

Transcripts or protein were detected consistently in the adult uvea and retina but not in the lens, by each method. These data differ from a report by Dunne et al. (44), in which DMPK mRNA was detected in the adult lens by RT–PCR and a 67 kDa DMPK isoform was detected in the adult lens by western and immunocytochemical analyses with a polyclonal antisera. One possible explanation for these differences is that DMPK is expressed at such low levels that the methods described in this report were not sensitive enough to detect the low abundance of DMPK transcripts or protein in the adult lenses. However, this is unlikely, as four independent methods were used on a total of 11 adult eyes (three adult eyes for the RT–PCR and western blot analyses and eight adult eyes for the in situ hybridization and immunocytochemical analyses). The amount of RNA and the RT–PCR protocol used by Dunne et al. (44) are not described in detail in their publication. As a result, it is not possible to resolve whether different technical approaches could generate such anomalies in sensitivity between the two studies. The discrepancy at the protein level might be explained by differences in the epitopes detected by the respective antibodies used. Dunne et al. (44) immunodetected a 67 kDa DMPK isoform with a polyclonal antisera. It is possible that this particular isoform is not recognized by 6G8 (anti-DMPK monoclonal antibody) and that the full-length DMPK protein (71 kDa) recognized by 6G8 is not present in adult lenses.

The expression of SIX5 was analysed only at the RNA level, by two different methods, and a consistent expression pattern was determined. To date there are no antibodies available for the analysis of SIX5 protein. RT–PCR and in situ hybridization analyses detected SIX5 transcripts in the adult cornea, lens, ciliary body and retina, all sites of pathological change in DM. Transcripts were detected in the sclera by RT–PCR, which could be attributed to the lower sensitivity of in situ hybridization or be the result of contamination by other tissues, as discussed above. SIX5 transcripts were not detected in normal fetal eyes (aged between 6 and 14 weeks) by either method. However, it is possible that SIX5 is expressed at other times during development and therefore we are continuing to collect and analyse fetal eyes over a range of gestational ages.

Histological examination of the eyes of DM patients have shown that a number of pathological changes can occur. The cornea is often thinner and the cellular organization disrupted and in some cases the corneal endothelium is lost. Decreased expression of SIX5 in the corneal epithelium and endothelium or decreased expression of DMPK in the corneal epithelium could contribute to these pathological changes. The lack of DMPK expression in the lens (Figs 2, 3E and F and 5) and the detection of SIX5 transcripts by RT–PCR (Fig. 2) and in situ hybridization (Fig. 4F) implicate SIX5 dysfunction in the development of cataracts. Changes to the ciliary body of DM patients include vacuolation and degeneration and hyperplasia of both epithelial layers. Both SIX5 and DMPK are expressed within these layers. One can speculate that decreased levels could result in the histological changes and might lead to the reduced intraocular pressure of DM patients. Likewise changes in the levels of DMPK expression in the smooth muscle of the ciliary body could be responsible for the pressure changes. Cystic atrophy with pigment epithelial proliferation produces the pigmented retinopathy in DM. This could be attributable to DMPK dysfunction as overexpression of human DMPK in a transgenic mouse resulted in a phenotype similar to retinitis pigmentosa (45). A complete absence of retinal photoreceptors and foci of retinal pigment epithelial cell migration around retinal blood vessels was observed. Another pathological change in the retina of DM patients is a reduction in the number of ganglion cells. Expression of both DMPK and SIX5 was observed in the ganglion cells.

The significance of DMPK and SIX5 in the ocular phenotype of DM can only be hypothesized at this stage, but the expression data in the normal adult eye and the homology between SIX5 and the Drosophila eye development gene sine oculis promote SIX5 as a strong candidate. Furthermore, the sites of SIX5 expression in the adult eye more closely match the areas affected in adult onset DM than the sites of DMPK expression. It seems that SIX5 is predominately expressed in the adult as it was not detected in fetal eyes and there is no known ocular fetal abnormality in DM. SIX5 is a member of the Six family of transcription factor encoding genes, which includes sine oculis. To date six mammalian Six genes have been identified (Six1, Six2, Six3, Six4/AREC3, Six5 and Optx2) (36,37,46,47) and homologues have also been isolated in Drosophila, Caenorhabditis elegans, Xenopus laevis, chick, newt, killifish medaka and zebrafish (47–54). The Six family proteins are characterized by a distinctive and diverged homeodomain that shares ≤30% homology with previously described homeodomains (55,56). Gel retardation assays and DNase I footprinting analyses have shown that the Six proteins bind DNA in a specific manner, confirming their roles as transcription factors (36,37,57; S.E. Harris and K.J. Johnson, personal communication). The region immediately flanking the 5′-end of the homeobox, encoding 110 amino acids, is also conserved in the Six genes and is called the Six domain. It is thought to be involved in modulating DNA binding specificity and to be important for protein–protein interactions. The Six domain of the Drosophila sine oculis protein has been shown, in yeast and in vitro, to physically interact with the conserved domain of another Drosophila eye development transcription factor, the eyes absent (eya) protein (58).
The expression of the *sine oculis* in the developing eye and in the adult compound eye is well documented (55,59). However, the mammalian *Six* genes have only been analysed in the retina, where expression of mouse *Six2*, *Six3*, *Six4* and *Six5* was detected in the ganglion cells, the inner and outer nuclear layers and the pigment epithelium (36). The expression of human *Six5* in the adult retina closely matches the expression pattern observed for its mouse orthologue, *Six5*, and that of *Six2*, *Six3* and *Six4*. *Six5* was not expressed in fetal eyes and, to date, *Six3* and chicken *optx2* are the only *Six* genes shown to be expressed in the vertebrate developing eye (47,49,53,54,60). There have been no reports of *Six* gene expression in the lens but the ectopic expression of mouse *Six3* in killifish medaka embryos resulted in ectopic lens formation in the otic vesicles (61).

The expression patterns and functional studies, including the analysis of *Drosophila* *sine oculis* mutants (59,62) and DNA-binding studies, have confirmed the role of the *Six* genes as transcription factors regulating the development and formation of the eye. The mouse *Six5* protein has been shown to bind to the same regulatory elements as *Six4/AREC3*, in the Na*,K*+-ATPase α1 subunit gene (36). In this report we show that *SIX5* is expressed in the lens epithelium in the normal adult eye, whereas *DMPK* is not. One can speculate that dysfunction of *SIX5* binding to the Na*,K*+-ATPase α1 subunit gene could affect ion balance in the lens leading to osmotic problems and cataract development. Failure of Na*,K*+-ATPase activity in controlling the water content of the lens is thought to be the cause of cataracts in the Nakano mouse (63,64). It has been estimated that there are at least 30 loci responsible for autosomal dominant cataracts in man, some of which are homeobox genes (65). A novel homeobox gene, *PITX3*, was recently isolated and shown to be mutated in families with autosomal dominant cataracts and anterior segment mesenchymal dysgenesis (66). Many other homeobox genes, including *PAX6*, have been implicated in eye development and have been found to be mutated in eye disorders (67,68). Furthermore, the Na*,K*+-ATPase α1 subunit gene is important for cellular ion homeostasis (36) and a decrease in Na*,K*+-ATPase activity and incorrect regulation of ion homeostasis have been implicated in myotonic dystrophy (69–77).

**Table 1. Summary of the expression of DMPK and SIX5 in normal adult and fetal eyes**

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<th>Eye structure</th>
<th>DMPK expression (method of analysis)</th>
<th>SIX5 expression (method of analysis)</th>
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<tr>
<td>Adult cornea</td>
<td>– (RT–PCR, western, ICC) + (ISH) corneal epithelium (ISH)</td>
<td>+ (RT–PCR, ISH) corneal epithelium and endothelium (ISH)</td>
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<tr>
<td>Adult lens</td>
<td>– (RT–PCR, ISH, western, ICC)</td>
<td>+ (RT–PCR, ISH) lens epithelium (ISH)</td>
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<tr>
<td>Adult uvea</td>
<td>+ (RT–PCR, ISH, western, ICC) pupillary sphincter muscle (ICC), ciliary body epithelia (ICC), ciliary smooth muscle (ICC), smooth muscle cells of choroidal blood vessels(ICC)</td>
<td>+ (RT–PCR, ISH) epithelia of the pars plicata and pars plana of the ciliary body (ISH)</td>
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<tr>
<td>Adult retina</td>
<td>+ (RT–PCR, ISH, western, ICC) ganglion cells, cells of the inner nuclear layer and photoreceptor cells (ISH, ICC)</td>
<td>+ (RT–PCR, ISH) ganglion cells, cells of the inner nuclear layer and photoreceptor cells (ISH)</td>
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<tr>
<td>Adult optic nerve</td>
<td>– (ISH, ICC) + (RT–PCR) (not analysed by western)</td>
<td>– (RT–PCR, ISH)</td>
</tr>
<tr>
<td>Fetal eyes</td>
<td>+ (RT–PCR) (not analysed by ISH, western, ICC)</td>
<td>– (RT–PCR, ISH)</td>
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The expression of both genes was analysed by RT–PCR on structures dissected from human adult eyes (cornea, lens, uvea, retina, sclera and optic nerve) and from whole human fetal eyes and *in situ* hybridization (ISH) on whole human adult eyes. DMPK protein was analysed by western blot analysis (western) of proteins extracted from eye structures dissected from human adult eyes and from whole human fetal eyes and immunocytochemistry (ICC) on whole human adult eyes. Detection of gene expression in a specific eye structure is denoted + and no expression is denoted –.
MATERIALS AND METHODS

RNA and protein preparation

Three adult human eyes were collected at post mortem and two human fetal eyes (aged 12 weeks) were obtained from the MRC Tissue Bank (Imperial College School of Medicine, University of London, Hammersmith Hospital, London, UK) and snap frozen in liquid nitrogen. The three adult human eyes were thawed and the cornea, lens, optic nerve, retina, sclera and uvea (iris, ciliary body and choroid) dissected from each eye and pooled. Total RNA and protein were extracted from the pooled adult eye tissues and the fetal eyes using TRI Reagent (Sigma), according to the manufacturer’s instructions. The total RNA pellets were resuspended in DEPC-treated water and the concentrations estimated by measuring the UV absorbance of the solution at wavelengths ranging from 200 to 300 nm. The integrity of the RNA samples was confirmed by electrophoresis in a 1.35% (w/v) agarose. The protein pellets were resuspended in 8 M urea and then dialysed against 1× phosphate-buffered saline. The concentration of the protein samples was estimated using the Bio-Rad protein assay according to the manufacturer’s standard protocol. The integrity of the protein samples was confirmed by electrophoresis in a 10% (w/v) SDS–polyacrylamide gel.

RT–PCR

First strand cDNAs were synthesized from 2.5 µg of total RNA with an Oligo(dT) 12–18 primer (Gibco BRL) using the Superscript Preamplification System for first strand cDNA synthesis (Gibco BRL). The manufacturer’s protocol was used with the following modifications: 1 mM each dNTP and 20 U RNasin RNase inhibitor (Promega) were included in the reaction mix and 400 U Superscript II (Gibco BRL) were used. Negative control (–RT) samples were also generated by omitting the Superscript II reverse transcriptase enzyme. First strand cDNA aliquots and –RT control samples, corresponding to 250 ng RNA equivalents, or 200 ng of genomic DNA were used as templates for the gene-specific PCR reactions. Aliquots of the same samples were used for all the PCR analyses, so that the expression patterns could be compared. The first strand cDNAs and –RT negative controls were checked by PCR with human GAPDH primers. Products were obtained from the first strand cDNAs but not the –RT controls (data not shown). This confirmed that the first strand cDNA samples had been synthesized from the mRNA in the total RNA extracted from the adult eye tissues and whole fetal eyes.

*DMPK* was amplified using a forward primer from exon 2, CLW2F (5′-GAA TTC AGG CTT AAG GAG GTC CGA CT-3′), and a reverse primer from mouse Dmpk exon 4, CLW4R (5′-GAA TTC GCA AAG TGC AGC TGT GTG ATC-3′). These primers were chosen because this region of the gene is present in all the previously described alternatively spliced isoforms of *DMPK* (39–43). The reaction mix contained 0.4 µM each primer, 0.2 mM each dNTP, 1× PCR buffer containing 1.5 mM MgCl2 (Perkin Elmer) and 1.25 U AmpliTaq Gold Taq polymerase (Perkin Elmer). The samples were denatured at 95°C for 10 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 61°C for 1 min and extension at 72°C for 1 min. The amplification was finished with an extension at 72°C for 7 min.

The PCR products were electrophoresed in 2.5% (w/v) agarose and the separated DNA samples stained with 500 ng/ml ethidium bromide and visualized using a UV transilluminator (wavelength 254 nm). The PCR products (generated by amplification of the first strand cDNAs) were excised from the agarose gel and the DNA extracted using the QIAquick Gel Extraction kit (Qiagen) according to the manufacturer’s instructions. Aliquots of 250 ng of PCR product were sequenced with the gene-specific forward primers. The –RT controls were also analysed by PCR with *DMPK* primers (CLW2F and CLW4R) and SIX5 primers (KJDMF and SIX5-R.SEH) and no cDNA products were obtained (data not shown). These analyses also confirmed that the first strand cDNA samples had been synthesized from the mRNA in the total RNA preparations. A genomic DNA product was amplified in the corneal –RT sample, due to genomic DNA contamination in the cornea total RNA extract. Genomic DNA PCR products were not amplified in any other samples due to the lack of genomic DNA contamination in the total RNA extracts.

Subcloning and generation of riboprobes

Digoxigenin (Boehringer Mannheim)-labelled riboprobes were generated by *in vitro* transcription of fragments of *DMPK* or *SIX5* subcloned into the phagemid pBluescript SK(+) (Strategene). The riboprobes were designed to specifically detect only *DMPK* mRNA or *SIX5* mRNA, respectively. The *DMPK* riboprobe is mainly from the 5′-UTR of the gene and contains only five (CTG) repeats. The *SIX5* riboprobe generated from pSIX51 is mainly from the 5′-UTR and does not include any regions that are similar to the other human SIX genes. One microgram of each template was linearized and the DNA transcribed with 40 U T7 or T3 RNA polymerase (Promega) at 37°C for 4 h. The reaction mix contained 10 mM DTT, 20 U RNasin RNase inhibitor (Promega), 1× transcription buffer (Promega), 1 µM ATP, 1 µM CTP, 1 µM GTP, 0.65 mM UTP and 0.35 mM digoxigenin-11-UTP (Boehringer Mannheim). The riboprobe was precipitated and resuspended in 40 µl of DEPC-treated water.

The *DMPK* subclones, pDMPK15a and pDMPK15b, were generated by ligating a 660 bp *HincII* fragment (positions 12 832–13 492 of Genbank accession no. L08835) in both orientations into *HincII* cut pBluescript SK(+) (Life Technologies). The *HincII* fragment was cut from plasmid 8C (a gift from C.L. Moncrieff, University of Glasgow, UK), which was generated by amplifying genomic DNA using a forward primer in intron 14 of *DMPK*, AO15F (5′-CCA CAC ATG GCC GTA GCC-3′), and a reverse primer in exon 15 of *DMPK*, AO15R (5′-TTT GGC AAA AGC AAA TTT CC-3′). The PCR product was subcloned into the T vector pMOBlue (Amersham) to produce plasmid 8C. The sense riboprobe was generated by linearizing pDMPK15b with EcoRI and transcribing with T7 RNA polymerase (Promega) and the antisense riboprobe was generated by linearizing pDMPK15a
with EcoRI and transcribing with T7 RNA polymerase (Promega).

The SIX5 subclone, pSIX51, was generated by first amplifying genomic DNA using a forward primer in the 3′-UTR of DMPK, FAS (5′-TCC TCA CTG CGC TGC TCT C-3′), and a reverse primer in exon A of SIX5, RAS (5′-TGC GCA GTC GAT ACT TGT CCA C-3′). The PCR product was subcloned into the T vector pMOSBlue (Amersham) and then cut with EcoRI and BamHI to release a SIX5 fragment of 652 bp (positions 662–1314 of GenBank accession no. X84813). The fragment was ligated unidirectionally into EcoRI + BamHI cut pBluescript SK(+), to create pSIX51. The sense riboprobe was generated by linearizing pSIX51 with BamHI and transcribing with T7 RNA polymerase (Promega) and the antisense riboprobe was generated by linearizing pSIX51 with EcoRI and transcribing with T3 RNA polymerase (Promega).

In situ hybridization

Normal adult eyes from eight individuals (total eight) and eyes from six fetuses (from 6 to 14 weeks) were analysed in situ hybridization. These were obtained from the archive of the Ophthalmic Pathology Laboratory, Western Infirmary, Glasgow. Sections were cut at 4 µM from paraffin blocks of whole adult eyes and whole fetal eyes that had been fixed in glutaraldehyde or formalin and mounted onto slides coated with 3-aminopropyl-triethoxysilane (APES). After rehydration the sections were treated with 0.2 M HCl, followed by 0.3% (v/v) Triton-X100 to extract lipid membrane components. Formalin-fixed sections were treated with 100 µg/ml proteinase K and glutaraldehyde-fixed sections were treated with 300 µg/ml proteinase K at 37°C for 30 min. The sections were fixed in 2% (w/v) paraformaldehyde before a prehybridization treatment in 50% (v/v) formamide for 30 min. The sections were fixed in 2% (w/v) Triton-X100 to extract lipid membrane components. Formalin-fixed sections were treated with 100 µg/ml proteinase K and glutaraldehyde-fixed sections were treated with 300 µg/ml proteinase K at 37°C for 30 min. The sections were fixed in 2% (v/v) paraformaldehyde before a prehybridization treatment in 50% (v/v) formamide at 37°C for 2 h. In control experiments sections were treated with 100 µg/ml RNase A at 37°C for 30 min before the prehybridization step. The riboprobes were diluted to the previously determined optimum concentration in hybridization buffer (10 mM Tris–HCl, pH 7.5, 12.5× Denhardt’s solution, 2× SSC, 0.5% w/v SDS, 50% v/v formamide, 10% w/v dextran sulphate and 0.25 mg/ml salmon sperm DNA), boiled for 3 min and quenched on ice. The riboprobes were hybridized with the sections at 70°C for 2 min, then at 55°C overnight. In control experiments sections were incubated with hybridization buffer without riboprobe. The sections were washed in 2× SSC at room temperature for 30 min, in 0.1× SSC at room temperature for 10 min and in 0.1× SSC at 50°C for 30 min. In control experiments sections were treated with 100 µg/ml RNase A at 37°C for 30 min before the immunodetection steps. After rinsing, the sections were incubated with a 1/2000 dilution of an alkaline phosphatase-conjugated digoxigenin antibody (Boehringer Mannheim) in 10% (v/v) swine serum, at room temperature for 2 h. The sections were washed in water and mounted in Glycerol (Sigma). The sections were analysed under a microscope and the eye structures that stained blue/black, indicating hybridization and therefore gene expression, were photographed.

Western blot analysis

Fifty micrograms of each protein sample, extracted from the human adult eye tissues and human fetal eyes, were separated by electrophoresis in a 10% (w/v) SDS–polyacrylamide gel with 6 µg of low molecular weight marker proteins (Pharmacia Biotech) and 16 µg of kaleidoscope prestained protein standards (Bio-Rad). After electrophoresis the proteins were transferred by wet blotting onto Trans-Blot transfer medium PVDF membrane (Bio-Rad) and analysed with a 1/100 dilution of 6G8, an anti-DMPK monoclonal antibody (a gift from G.E. Morris, North East Wales Institute, UK). The antibody was raised against a recombinant human DMPK protein spanning the kinase and coiled coil domains (83). The interactions were detected using anti-mouse HRP-conjugated IgG (Scottish Antibody Production Unit, Law Hospital, Scotland, UK) with ECL western blotting detection reagents (Amersham). The molecular weight of the immunodetected proteins was estimated with reference to standard curves compiled using the logarithms of the known molecular weight markers (listed above) plotted against the distance migrated by these protein markers.

Immunocytochemistry

Sections were cut at 4 µM from paraffin blocks of whole adult eyes and whole fetal eyes that had been fixed in glutaraldehyde or formalin and mounted on APES-coated slides. After rehydration the sections were treated with 0.5% (v/v) H2O2 to block endogenous peroxidase activity and then microwaved in 0.01 M citrate buffer, pH 6. The sections were blocked in 2% (w/v) bovine serum albumin and then incubated with 1/50, 1/100 or 1/1000 dilutions of 6G8 anti-DMPK monoclonal antibody at 4°C overnight. The antibody–protein interactions were detected colorimetrically (red) using a HRP duet kit (Dako) and VIP kit (Vector Laboratories) according to the manufacturers’ instructions. The sections were counterstained with haematoxylin, which stained the cell nuclei blue. The sections were dehydrated and mounted with Histomount (Hughes and Hughes). The specificity of the immunodetection was checked by performing the analysis without the 6G8 antibody. These sections did not stain red (data not shown). The sections were analysed under a microscope and the eye structures that stained red, indicating immunodetection of DMPK, were photographed.

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