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Pax-6 is essential for lens-specific expression of ζ-crystallin

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ABSTRACT *Pax-6* **is essential for normal eye development and has been implicated as a "master gene" for lens formation in embryogenesis. Guinea pig ζ-crystallin, a taxon-specific enzyme crystallin, achieves high expression specifically in lens through use of an alternative promoter. Here we show that Pax-6 binds a site in this promoter, which is essential for lens-specific expression. Lens and lens-derived cells exhibit a tissue-specific pattern of alternative splicing of** ***Pax-6*** **transcripts and** ***Pax-6*** **is expressed in adult lenses and cells that support ζ-crystallin expression. These results suggest that ζ-crystallin is a natural target gene for Pax-6 and that this Pax family member has a direct role in the continuing expression of tissue-specific genes.**

Pax genes, which encode a family of proteins characterized by the paired domain (PD), a DNA-binding motif, have important roles in tissue differentiation during embryogenesis (1). A member of this gene family, *Pax-6*, is expressed in early developmental stages in eye and central nervous system in vertebrates (2–5) and is implicated in the determination of lens competence (4). Mutants in *Pax-6* have severe eye defects in mouse (Small eye) (6) and humans (aniridia) (7, 8). Remarkably, homologues of *Pax-6* play similar roles in eye development in invertebrates (9).

Crystallins are the structural proteins of the lens. During vertebrate evolution, the composition of the lens in particular lineages has been modified by the direct gene recruitment of existing enzymes as taxon-specific crystallins (10–12). Guinea pig ζ-crystallin is an NADPH:quinone oxidoreductase, which has been recruited to an additional, structural role in the eye lens (13–15). In this particular example, gene recruitment has occurred through the acquisition of an alternative lens-specific promoter in what would otherwise be the first intron of the enzyme gene (16, 17). The separation of lens and non-lens expression in alternative promoters makes ζ-crystallin an attractive model for examining tissue-specific gene expression and the mechanisms of gene recruitment.

The ζ-crystallin lens promoter exhibits differential DNase I protection in lens and non-lens cells. Lens cell extracts protect a single 50-bp region (−202 to −152), the ζ-protected element (ZPE) (16). Here we show that Pax-6 binds the ZPE and is essential for lens-specific promoter function. *Pax-6* may be a "master gene" for lens expression since parallel experiments describe Pax-6 binding in three other crystallin genes (18–20).

MATERIALS AND METHODS

Electrophoretic Mobility-Shift Assays (EMSAs). EMSA reaction mixtures contained 2 μg of poly(dI·dC), 1 μg of 1-kb DNA size ladder, 40 ng of competitor fragment, 0.5 ng of ZPE probe end-labeled with [γ^{32}P]ATP, and 30 μg of cell (21, 22) or nuclear extracts (22, 23) prepared by standard methods. Antibody treatment used 10-min preincubation on ice with 1 μl of antiserum. For Pax-6 binding, 100 ng of glutathione *S*-transferase (GST)–Pax-6 PD, derived from a cDNA for human Pax-6 PD fused to GST (24), was used.

Transient Transfection Promoter Analysis. N/N1003A cells (25) in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (BRL) were transfected with calcium phosphate (26). Host vector was pSV_0ATCAT (27); −756/+70.CAT contained the ζ-crystallin promoter (16); MUT−756/+70.CAT replaced the *Nsi* I/*Sac* I fragment (−183 to −7) with a PCR-generated fragment containing the mutant sequence of competitor N from Fig. 1*d*. After 24 hr, cells were harvested for chloramphenicol acetyltransferase (CAT) assays. All transfections included the β-galactosidase expression vector pCMVβ for normalization (16).

Transgenic Mice. The MUT−756/+70.CAT construction was tested in transgenic mice. A 2.4-kb *Bam*HI fragment was introduced into mice as described (16). Founder transgenic mice were screened by PCR and Southern blot analysis. Outbred F_1 progeny containing intact transgenes were assayed for CAT activity in various tissues (16).

Western Blot Analysis. Cell or nuclear extracts (30 μg) were resolved by SDS/PAGE using 12% denaturing gels (Novex, San Diego). Western blots used a 1:4000 dilution of quail or zebrafish Pax-6 antiserum (28–30). Visualization used biotinylated secondary antibody (Vector Laboratories), streptavidin-linked horseradish peroxidase (Kirkegaard & Perry Laboratories), and diaminobenzidine tetrahydrochloride (DAB)/nickel/peroxidase solution (BRL). As a positive control, Western blot analysis was also performed on recombinant human Pax-6 generously provided by R. Maas (Harvard Medical School).

Reverse Transcription PCR (RT-PCR) of Pax-6. RT-PCR of mouse Pax-6 (2) used 1 μg of total RNA and 100 nM antisense primer 1 (TGGGCTATTTTGCTTACAACTT) (sequence positions 470–449) in 20 mM Tris·HCl, pH 8.4/50 mM KCl/3 mM $MgCl_2$/10 mM dithiothreitol/400 μM dNTP. SuperScript II reverse transcriptase (200 units) (BRL) was added at 42°C. Duplicate control reactions were set up without reverse transcriptase. After 30 min, reactions were terminated with RNase H. cDNA was amplified using 10 μM primer 1 and 10 μM sense primer 2 (CAGAAGACTTTAACCAAGGGC) (sequence positions 33–53) in 20 mM Tris·HCl, pH 8.4/50 mM KCl/1.5 mM $MgCl_2$/200 μM dNTP. After 5 min at 94°C, 5 units of *Taq* DNA polymerase (Promega) was added. Thirty cycles of PCR amplification were performed: 94°C for 1 min, 54°C for 30 s, 72°C for 2 min. Products were subcloned using the TA vector (Invitrogen) and sequenced with Sequenase 2.0 (United States Biochemical).

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Abbreviations: ZPE, ζ-protected element; PD, paired domain; RT, reverse transcription; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility-shift assay; DAB, diaminobenzidine tetrahydrochloride.

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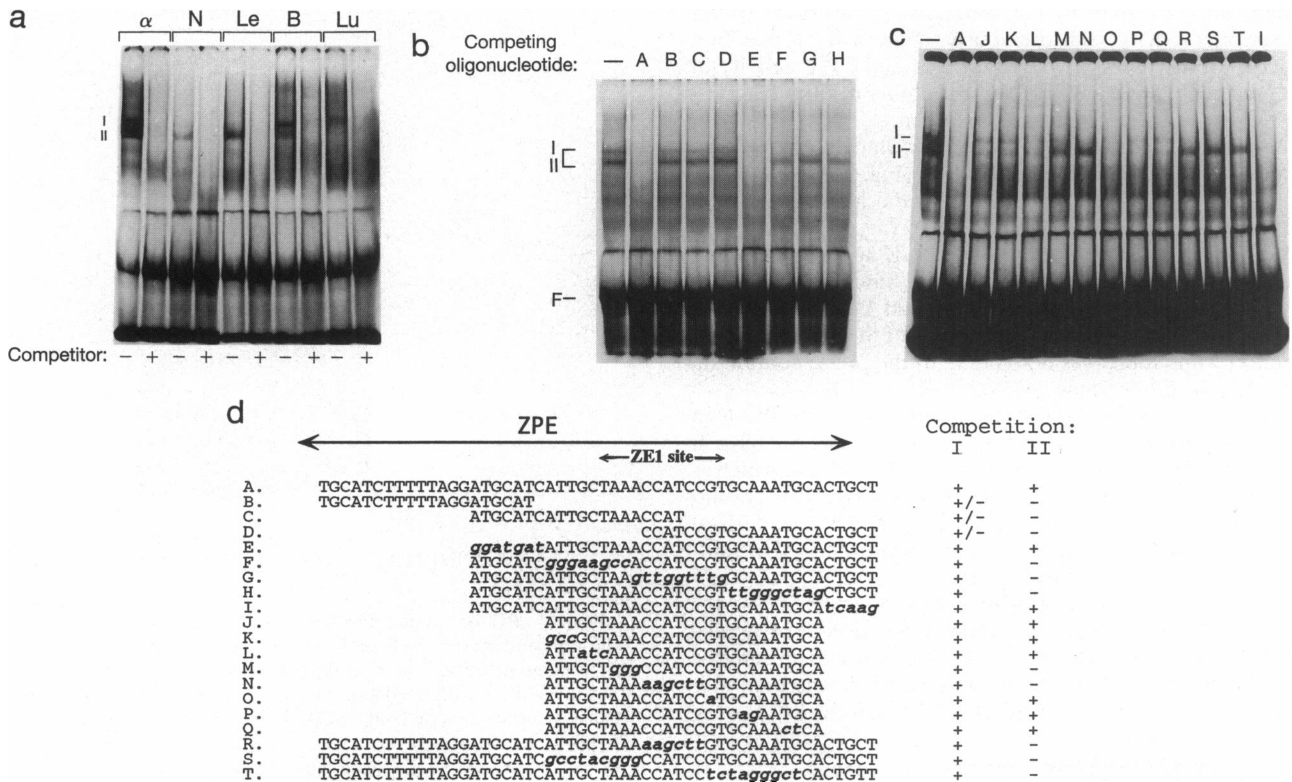

FIG. 1. The ZPE forms specific complexes in extracts of whole lens and lens-derived cells. (*a*) EMSA (22, 31) of the ζ-crystallin ZPE with whole cell extract of rabbit lens N/N1003A cells (N) (25), mouse lens αTN4-1 cells (α) (32), and nuclear extracts of mature mouse lens (Le), brain (B), and lung (Lu). Complexes I and II are indicated. (*b* and *c*) Complex-binding regions of the ZPE in N/N1003A extract. Unlabeled competitors contained sequence changes as shown in *d*. (*d*) Sequences of double-strand DNA competitors. Mutated bases are indicated in lowercase, italicized letters. Critical region for complex II formation, ZE1, is indicated. The ability of each fragment to compete is summarized on the right.

Immunohistochemistry. Eyes from 3-month-old guinea pigs were fixed in formalin. Paraffin sections (5–7 μm) were prepared by standard methods and incubated overnight with primary antibody to the PD of quail Pax-6 (28) or guinea pig ζ-crystallin (13). Sections were washed and treated with biotinylated secondary antibody (Vectastain ABC kit, Vector Laboratories) according to the manufacturer's instructions. Staining used Vectastain ABC reagent and DAB and 0.001% hydrogen peroxide. For Pax-6, staining was enhanced with 0.045% nickel chloride.

RESULTS AND DISCUSSION

The ζ-crystallin lens promoter ZPE forms two complexes (Fig. 1*a*) in EMSA with cell or nuclear extracts. While most non-lens tissues, such as lung, form only complex I, lens nuclear extract forms only complex II. Extracts of rabbit N/N1003A (25) and mouse αTN4-1 (32) lens-derived cells and mouse brain produce both complexes I and II. The presence of complex II in brain suggests that factors important for expression in lens are shared by brain. This is consistent with the observation that a truncated ζ-crystallin promoter containing the ZPE has low expression in brain in addition to high expression in lens in transgenic mice (16). It also appears that populations of lens-derived cell lines have both lens and non-lens characteristics.

Sites required for complex II formation were defined by competition of unlabeled mutated competitors with the full-length labeled ZPE probe (Fig. 1 *b* and *c*). While mutated competitors throughout the ZPE were still able to compete with complex I, the ability to compete with complex II required a defined core sequence, designated ZE1 (33).

The ZE1 site was mutated in the context of the lens-specific −756/+70.CAT promoter construct (16). In transient transfection in N/N1003A cells, this mutated promoter was almost completely inactive (Fig. 2). This result was confirmed in transgenic mice. Mice from two independent lines were ex-

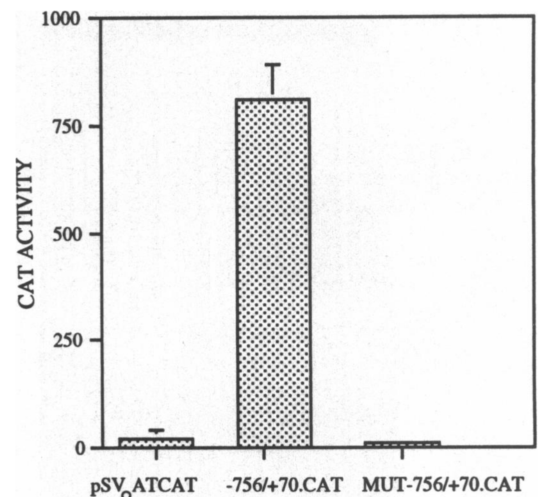

FIG. 2. Mutation in ZE1 inactivates the lens promoter. Wild-type and mutant constructs of the ζ-crystallin lens promoter were assayed by transient transfection in N/N1003A cells. pSV$_0$ ATCAT is the host vector with no promoter insert (27), −756/+70.CAT contains a wild-type ζ-crystallin promoter (16), while MUT−756/+70.CAT contains the noncompetitive sequence N (see Fig. 1*d*). All experiments were performed in triplicate. CAT activity is normalized to cotransfected β-galactosidase activity as described (16). Error bars indicate SD for triplicate measurements. Error bar for the mutant construction is too small to visualize at this scale. Mutant promoter was also nonfunctional in transgenic mice.

amined for CAT activity by TLC assay. In contrast to the strong lens-specific activity seen previously in the wild-type −756/+70.CAT transgenics (16), neither line had CAT activity in any tissue (data not shown). Thus, the ability to form complex II correlates with lens promoter function.

The sequence containing ZE1 closely matches the *in vitro* consensus site for the PD of Pax-6 (24) (Fig. 3*a*). Antisera to both the PD of quail Pax-6 (28) and to the highly conserved C-terminal region of the zebrafish Pax-6 (29, 30) were tested in EMSA and both abolished complex II without affecting complex I (Fig. 3*b*). The ability of Pax-6 to bind the ZPE region was then confirmed by using recombinant human Pax-6 PD (24) (Fig. 4). The PD gave a specific shift of the wild-type ZPE sequence, while there was no binding to the probe containing the mutated ZE1 sequence.

While these results suggest an essential role for Pax-6 in lens-specific expression of the ζ-crystallin gene, to have a significant role in crystallin gene expression, Pax-6, which is best known for its essential role in embryogenesis, would require continuing expression in adult lens and in lens-derived cells. Accordingly, the presence of Pax-6 was examined in adult mouse tissues and in guinea pig lens.

Western blots detected a protein of the expected size for Pax-6, 46 kDa (2), in adult brain and lens and lens-derived cells (Fig. 5*a*). No cross-reactive protein was detected in lung or adipose tissue (data not shown). Pax-6 cDNA was then amplified from the same tissues by RT-PCR of total RNA using primers from the known mouse sequence (2). cDNA of the expected size was amplified from mouse lens and brain and from αTN4-1 cells, while no transcripts were detected in lung (Fig. 5*b*) or adipose tissue (data not shown). The region

a
ZE1
E-box
C/EBP Oct1
A T T G C T A A A C C A T C C G T G C A A A T G
G N T A A N T C A T G C G T G A A a a T
Pax6

b ANTISERUM
Pax6-2
Pax6-1
PI
I –
II –
Lens N1003A

FIG. 3. Role for Pax-6 in the formation of complex II. (*a*) Essential ZE1 region compared with the reverse complement consensus for Pax-6 binding (24). Two positions where a base is favored at a lower frequency are shown in lowercase letters. (*b*) Antisera to both zebrafish (Pax6-1) (30) and quail (28) (Pax6-2) Pax-6 abolish complex II formation in EMSA with both mouse lens and N/N1003A extracts. PI, preimmune control.

Probe: ZPE MUT-ZPE
Competitor: − + ZPE + MUT ZPE − + ZPE + MUT ZPE

FIG. 4. PD of human Pax-6 binds the ZPE region. EMSA of wild-type and mutant ZPE probes with recombinant human Pax-6 PD (24). Lanes ZPE used the wild-type probe with sequence A from Fig. 1*d*. Lanes MUT-ZPE used a probe identical to the ZPE region except that it incorporates the complex II-abolishing mutations of sequence N shown in Fig. 1*d*.

a 1 2 3 4 5
46 kDa→
30 kDa→
b αTN4-1 Lens Brain Lung
bp M + − + − + − + −
1353
1078
872
603
310
281

FIG. 5. Pax-6 mRNA and protein are present in adult lens. (*a*) Western blot with Pax-6 antisera. Extracts were as follows: lane 1, N/N1003A cells; lane 2, αTN4-1 cells; lane 3, mouse lens; lane 4, mouse brain. Lane 5 shows human Pax-6 provided by R. Maas. (*b*) Agarose gel showing RT-PCR products using mouse Pax-6-specific primers with RNA from αTN4-1 cells, mouse lens, brain, and lung. Upper band in brain corresponds to a product of 437 bp, which includes the 42 bp of exon 5a. Lower band in brain, αTN4-1 cells, and lens corresponds to a product of 395 bp, which lacks exon 5a. Bands were subcloned and sequenced for verification.

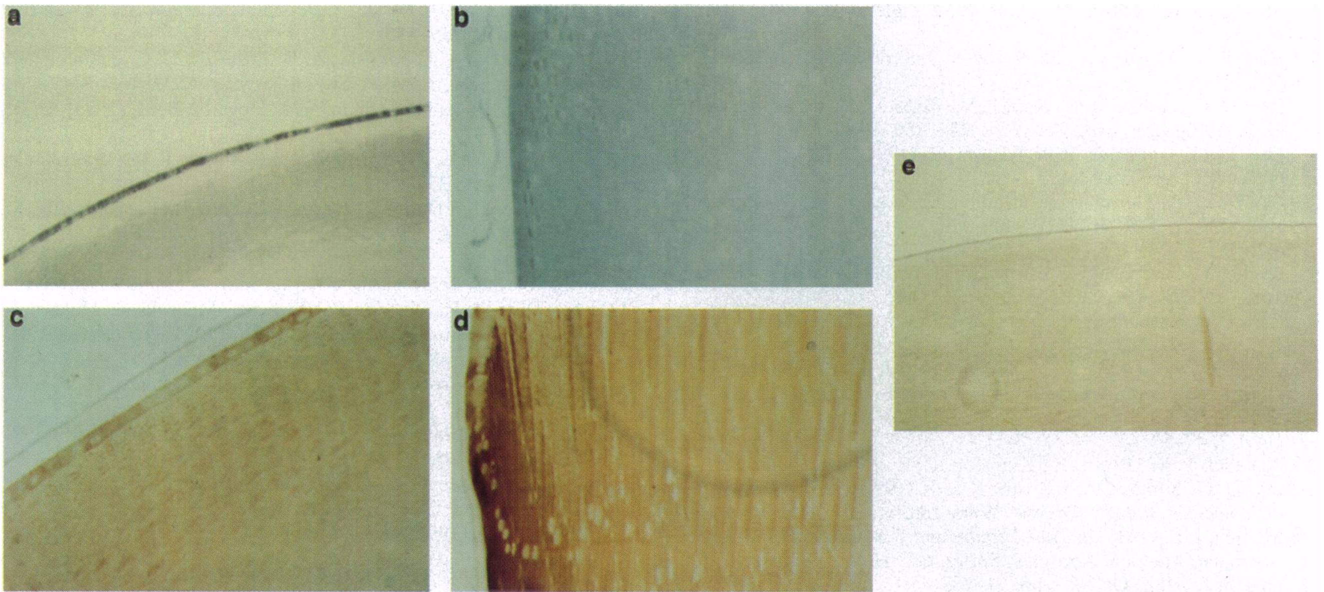

FIG. 6. Pax-6 and ζ-crystallin in adult guinea pig lens. Immunohistochemistry of guinea pig eye lens. (*a* and *b*) Stained with antisera to Pax-6. (*c* and *d*) Stained with antisera to ζ-crystallin. (*e*) Results for preimmune serum. *a, b,* and *e* used nickel enhancement of DAB color reagent, while *c* and *d* show only DAB color reagent. *a, c,* and *e* show regions of lens epithelium with cortical fibers underlying. *b* and *d* show equatorial region of lens where epithelial cells undergo massive elongation into cortical fibers. Thin dark line in *e* is associated with the surrounding lens capsule and not the epithelial cells. (*a* and *e*, ×10; *b–d*, ×20.)

amplified covers an alternatively spliced exon in the PD (2), thereby yielding products of two sizes. Both products were subcloned and confirmed by sequencing. In mouse brain, PCR products for both splicing forms of Pax-6 mRNA were of similar abundance. In contrast, the shorter form, lacking the alternative exon, greatly predominated in lens and in αTN4-1 cells. The alternative exon introduces an additional peptide into the DNA-binding PD of Pax-6 and only the product of the shorter transcript can bind to the *in vitro* consensus (24, 34). This raises the possibility of tissue discrimination in Pax-6 DNA binding, with a major form in lens capable of recognizing one sequence found in the ZPE and higher abundance of a different form in brain.

Pax-6 and ζ-crystallin were localized in adult guinea pig lens by immunohistochemistry (Fig. 6). Staining for Pax-6 was intense in cell nuclei of lens epithelium (Fig. 6*a*). In the equatorial region (Fig. 6*b*), where cells are differentiating into elongated lens cortical fibers, Pax-6 staining was still detectable in cell nuclei. Staining was weak or undetectable in the nuclei of fiber cells in deeper (older) layers. In contrast, staining for ζ-crystallin was entirely cytoplasmic. There was clear staining in epithelial cells (Fig. 6*c*), but maximal staining for ζ-crystallin was in the equatorial cortical region in cells that had most recently undergone differentiation (Fig. 6*d*). These results show that Pax-6 is expressed in adult guinea pig lens cells that synthesize ζ-crystallin and that, as judged by staining intensity, maximum levels of Pax-6 are achieved prior to maximum levels of ζ-crystallin during lens cell differentiation.

Thus, Pax-6 is present in tissues and cultured cells that support expression of the ζ-crystallin lens promoter and is absent from cells that do not. Its continuing expression in adult lens could have important implications for the progression of cataract in aniridia in humans. Indeed, Pax-6 may be a key factor in the expression of many lens-specific genes. Parallel experiments have detected Pax-6 in complex binding to functional promoter elements of chicken and mouse αA-crystallin (18, 19) and to the enhancer region of chicken δ1-crystallin (20). Pax-6 binding sites have also been identified in the promoter of the mouse gene for the neural cell adhesion molecule L1 (35). Of these sites, that in ζ-crystallin conforms most closely to the *in vitro* consensus (24).

In the case of ζ-crystallin, the initial event in gene recruitment may have been the acquisition of a binding site for Pax-6 in an intron of the enzyme gene. Although Pax-6 expression is not limited to lens, tissue preference might be conferred through differential splicing of *Pax-6* transcripts. Lens specificity of the ζ-crystallin promoter could subsequently have been fine-tuned by the addition of other cis elements to the recruited promoter, suppressing expression in brain and other tissues that contain Pax-6 (ref. 16; unpublished data).

Changes in the expression of pattern-forming genes can produce significantly altered developmental programs. In the same way, acquisition of binding sites for factors like Pax proteins could radically alter the protein composition of a tissue, such as the lens, in one evolutionary step.

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