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The differential roles of D-Pax2 variants in regulating Drosophila eye and bristle development

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The differential roles of *D-Pax2* variants in regulating *Drosophila* eye and bristle development

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ABSTRACT

The ability to appropriately interact with the environment is crucial to an organism's survival. Establishing functional sensory systems, such as the bristles and eyes in *Drosophila*, is critical during the development of the organism. The transcription factor D-Pax2 is involved in the differentiation of the shaft and glial cells in the developing bristle (Kavaler, 1999) and of the cone and primary pigment cells in the developing eye (Fu and Noll, 1997). However, how D-Pax2 contributes to distinct pathways in different cell types is not known. Recent work by Anna Czechowski and Katharine Harmon (personal communication) identified a mutation in the *D-Pax2* gene that introduced a stop codon at the end of exon 9, effectively truncating the protein. This mutation affects bristle, but not eye, development. We thus suspected regions after exon 9 are required for D-Pax2 function only in the bristles and may also be associated with alternative splicing of the *D-Pax2* transcript. We plan to assess the role of the carboxy terminal region of the protein by establishing transgenic lines bearing rescue constructs of *D-Pax2* with either the complete coding sequence or with deletions of specific exons. To date, we have generated the first rescue construct bearing the complete coding region of the gene driven by a 3 kb upstream regulatory region of *D-Pax2* and are currently generating transgenic fly lines with this construct. Additionally, we have created a plasmid containing *D-Pax2* cDNA with exon 11 deleted to examine the phenotypic effect of a deletion of a carboxy terminus exon.

INTRODUCTION

The ability to appropriately interact with the environment is crucial to an organism's survival, and the establishment of functional sensory systems is a critical event during the development of the organism. The development of the *Drosophila* sensory systems is an ideal model to study such events because many of the key proteins and regulators involved in this process are homologs to those in humans. *D-Pax2*, a member of the paired box gene family, is involved primarily in regulating cell fate and neural development in many species. The mammalian *Pax2* gene is expressed in the developing kidney and in the central nervous system (CNS) and midbrain-hindbrain boundary region in mice. (Dressler, 1990, Dressler, 1992, Torres, 1996, Bouchard, 2005). Additionally, during *Drosophila* development, its temporal and spatial distribution is changed (Kavaler, 1999). In mammals, it is activated through phosphorylation by a kinase (Cai, 2002), which can be suppressed by Groucho, a transcription inhibiting factor (Cai, 2003). Groucho mutants show development of additional bristles, notably around the eyes. *Pax2* contains a well-conserved DNA-binding domain of 128 amino acids, known as the paired domain. Much of the research on the regulation of *Pax2* has been conducted in mice, rather than *Drosophila*.

The development of the *Drosophila* bristle sensory system stems from the division and differentiation of a sensory organ precursor (SOP) cell, which differentiates into four cells comprising the bristle (Figure 1 A, B). The SOP cell divides into two second-order precursor cells (pIIA and pIIB) (Hartenstein, 1989). Using the Notch inhibition pathway, these two cells differentiate into the shaft (trichogen) and socket (tormogen), and the sheath (thecogen) and neuron, respectively (Kavaler et al., 1999).

Alternatively, the *Drosophila* eye develops from the larval eye disc (Figure 1 C – E). Each eye is comprised of eight photoreceptors, four cone cells, and two primary pigment cells.

Initially, *shaven* (*sv*) mutants (Lees and Waddington, 1942), affecting bristle development, and *sparkling* (*spa*) mutants, affecting eye development, were considered the result of mutations in two distinct genes. *sv* mutants result in a lack of bristles and typical double-socket phenotype. *spa* mutants result in a disruption of the hexagonal structure of the eye. However, Fu *et al.* (1998) showed that mutations in two separate enhancers of *D-Pax2* caused the *spa* and *sv* phenotypes. These two phenotypes, which were once thought of as unrelated, are in fact a product of dysfunction of the same gene. *D-Pax2* is involved in the differentiation of the shaft cell in the developing bristle (Kavaler et al., 1999). D-Pax2 is expressed prior to the division of the SOP (14 hours after puparian formation (APF)), and continues to be present in its daughter cells. Thirty-two hours APF, D-Pax2 is only expressed in the shaft and sheath (Figure 1 B). *D-Pax2* is also expressed in the cone and primary pigment cells in the developing eye (Fu and Noll, 1997). With this knowledge, the focus of research of *D-Pax2* has shifted toward uncovering the mechanisms regulating these differential roles of Pax2. Pax2 is known to have alternative splice variants (Fu and Noll 1997), which could dramatically affect the function of the protein. Alternative splicing is known to have profound implications on retinal development (Tavassoli, 1997).

A new mutant sequenced by Anna Czechowski and Katharine Harmon (personal communication) showed that a stop codon at the end of exon 9 of *D-Pax2* affected bristle, but not eye, development. Regions after exon 9 are therefore suspected to be required for

D-Pax2 function only in the bristles and may also be associated with alternative splicing of the *D-Pax2* transcript. However, exons in the carboxy terminus of the Pax2 protein are not conserved across species, and there is no known literature on the effect of these exons on protein function. Exon 11, the largest of the remaining exons, may be of particular importance in determining bristle regulation, as exons 10, 12, and 13 are much smaller, each approximately 30-50 amino acids in length. In order to elucidate the regulatory effect of exon 11 on Pax2 determination, I decided to compare the abilities of a full length *D-Pax2* protein and one lacking exon 11 to rescue *D-Pax2* mutants. To this effect, I have created a rescue construct bearing the complete coding region of the gene driven by a 3 kb upstream regulatory region of *D-Pax2* and am currently generating transgenic fly lines with this construct. Additionally, I have provided the preparatory work for a rescue construct of *D-Pax2* with exon 11 deleted. Moreover, I have created labeled probes to determine, through *in situ* hybridization, the spatial differentiation of the localization of exon 11 in the developing larva.

MATERIALS AND METHODS

A summary of the oligonucleotides used as primers is presented in Table 1. A summary of the previously synthesized plasmids used in these experiments is presented in Table 2.

Protocols

Enzyme digests

For further ligations, 2μg of DNA was digested at 37°C for 2-3 hours in a 50μl reaction containing distilled water, the restriction enzyme(s), the DNA, and appropriate buffers. For diagnostic enzyme digests, 1μg of DNA was digested in a 20μl reaction.

KpnI – EagI sequential digest

2μg of DNA was digested in a 50μl reaction in Buffer 1 with KpnI for 2 hours at 37°C. 1μl 5M NaCl and 1μl EagI were then added to the tubes containing the digest mixture, and these tubes were incubated at 37°C for 2 hours.

PCR amplification tests

A 25μl reaction containing 0.5μl of each primer, 2.5μl of 10X Taq Buffer, 1μl of 2.5 mM dNTP, 0.2μl Taq Polymerase, 1μl DNA, and 19.8μl of dH_2O was added to each 200μl PCR tube (ISCBioExpress). These tubes were then placed in the thermocycler. The sequence of the PCR was 1 cycle of 95°C for 1 minute; 30 cycles of 95°C for 20 seconds, 55°C for 20 seconds, and then 72°C for 2 minutes; and finally 72°C for 10 minutes and holding the tubes at 10°C.

Quick ligation

50ng of the vector DNA was combined with a 3-fold molar excess of the insert DNA. The volume of this solution was adjusted to 10μl with dH_2O. 10μl of 2X Quick ligation buffer, and 1 μl of Quick T4 DNA Ligase were added and the solution was

mixed thoroughly. After brief centrifugation, the ligation mixture was incubated at room temperature for 5 minutes and then chilled on ice. The mixture was then either stored at -20°C or was transformed into competent cells.

Transformation

Competent cells were thawed on ice for 10 min, after which 2µl of the ligation mixture was added to 50µl of cells in a microcentrifuge tube. The cells were then mixed by gently tapping the microcentrifuge tube. The cells were heat shocked for 30 seconds at 42°C and then were chilled on ice for 5 minutes. For New England Biolabs (NEB) turbocompetent cells, 950µl of room temperature SOC medium was added to the tubes. For Invitrogen 1 shot Top10 cells, 250µl of pre-warmed SOC medium was added to the tubes. The cells were incubated while shaking at 225-250 rpm for 1 hour. These cells were spread onto Luria-Bertani (LB, Genesee) plates containing ampicillin in dilutions of either 100µl and 900µl (NEB turbocompetent cells) or 20µl and 200µl (1 shot Top10 cells). These plates were then incubated overnight at 37°C.

PCR amplification testing of plate colonies

A pre-warmed LB-amp plate served as the master plate. The master plate was divided into sections for each colony to be streaked with permanent marker on the back of the plate. Each separate colony from the plates from a transformation to be tested was picked with a toothpick and gently streaked onto the master plate in a separate section. The toothpick was then agitated in 10µl of TE in a 200µl PCR tube, dislodging the remaining cells on the toothpick. The tubes were then boiled in the thermocycler for 5

minutes, and used for further PCR amplification. For each reaction, 1µl of each primer, 1µl of the boiled cell mixture, 2.5µl of 10X Taq Buffer, 0.2µl of TAQ polymerase, and dH_2O to 25µl were added to a 200µl PCR tube. The sequence for the thermocycler was 1 cycle of 95°C for 2 minutes; 30 cycles of 95°C for 20 seconds, 55°C for 20 seconds, and then 72°C for 1 minute; and finally 72°C for 10 minutes and holding the tubes at 10°C.

Plasmid DNA purification (QIAQuick Spin Miniprep Kit)

Cells picked from the appropriate colonies on the master plate were grown in 5ml LB-amp overnight. 3ml of these cells were pelleted in 1.5ml microcentrifuge tubes. The LB-amp was removed, and the cells were resuspended in 250µl of Buffer P1. 250µl of Buffer P2, the lysis buffer, was added, and the tubes were mixed thoroughly. 350µl of Buffer N3 was added to the tubes, and the tubes were mixed thoroughly. Buffer N3 caused the contaminating proteins to precipitate from the solution. These tubes were centrifuged at 13,000 rpm for 10 minutes. The supernatant was then pipeted from these tubes to separate QIAprep spin columns. These columns were centrifuged for 60 seconds at 13,000 rpm, and the flow-through was discarded. 750µl of Buffer PE was added to the columns, and the columns were centrifuged for 60 seconds at 13,000 rpm. The flow-through was discarded, and the columns were centrifuged once again for 60 seconds to remove any residual ethanol, which would inhibit subsequent enzymatic reactions. The columns were then placed in separate, clean 1.5ml microcentrifuge tubes. 50µl of Buffer EB, the elution buffer, was added to the column. The column was let stand for 60 seconds, then centrifuged for 60 seconds, and then discarded. The DNA in the elution buffer remained in the clean microcentrifuge tube.

Site-directed mutagenesis

A PCR amplification mixture was created containing 10µl 5X Phusion HF Buffer, 1µl 10mM dNTPs, 1.25µl of each primer, 0.5µl of Phusion Hot Start DNA Polymerase, 1 µg of template DNA, and dH_2O to 50µl. The sequence for the Touchdown PCR reaction was 1 cycle at 96°C for 30 seconds; 20 cycles at 96°C for 10 seconds, 70 – 60°C (decreasing 0.5°C every cycle) for 30 seconds, then 72°C for 5 minutes; 10 cycles at 96°C for 10 seconds, 60°C for 30 seconds, then 72°C for 5 minutes; finally, the amplified DNA was placed at 72°C for 10 mintues and then held at 4°C. The resulting linear DNA was then electrophoresed on an agarose gel to ensure success of the PCR amplification. The amplified DNA was then ligated, following the quick ligation procedure, and then transformed into NEB turbocompetent cells following the transformation procedure. The resulting clone was tested both with PCR amplification and an enzymatic digest.

Creation of the pCE1 construct

The previously developed Pax2 cDNA pBluescript SK plasmid (pJK12) was used as the basis for the pCE1 rescue plasmid. pBluescript is an advantageous plasmid to use because it is smaller, and thus more manageable for future insertions. The 3 kb upstream regulatory region of Pax2 from pSJ6 (courtesy of Seth Johnson) was cloned into the pJK12 plasmid using BamHI. The 3 kb insert and the pJK12 vector were ligated using Quick Ligase and a standard quick ligation procedure. This plasmid was transformed into *E. coli* (New England Biolabs Turbo competent cells), following the New England Biolabs transformation protocol, and purified thereafter following the QIAquick Plasmid

Purification protocol. The success of the ligation and transformation was tested via PCR amplification of plate colonies using primers OL3 and OL210. These primers bind to sites around the junction of *D-Pax2* and the 3 kb regulatory region. If the 3 kb region was inserted and ligated successfully, a DNA strand about 930 base pairs in length should be amplified. Because we only used one enzyme to open the ligation site, there was a possibility that the 3 kb region would be ligated in the opposite orientation, in which case a DNA strand about 3 kb in length would be synthesized. We named the resulting plasmid pCE1 (Figure 2 A).

Creation of pCE2

pCE1 contained the appropriate *D-Pax2* cDNA and regulatory region; however, it was in a vector which is not designed for injections of embryos to create transgenic flies. As such, these regions needed to be inserted into another vector designed for injections, pCaSpeR4. pCE1 and pCaSpeR4 were digested with KpnI and EagI, using a sequential digest protocol. These digests were electrophoresed on a low-melt agarose gel. The bands of vector and insert DNA were cut from the gel, taking care to limit the amount of agarose in the slice, because the EDTA in the gel is known to inhibit further reactions. In place of using a standard DNA gel-extraction procedure, the gel was melted and appropriate amounts of the gel/DNA mixture were added to the quick ligation protocol. This ligated the *Pax2* cDNA and the 3 kb upstream regulatory region with pCaSpeR4, our injection vector. This plasmid was transformed into *E. coli* (New England Biolabs turbo competent cells), following the New England Biolabs transformation protocol, and purified following the QIAquick Plasmid Purification protocol. The success of the

ligation and transformation was tested following a PCR testing of plate colonies procedure using primers B75 and OL23. These primers bind to sites around the junction of the 3 kb region and the pCaSpeR vector. If the insertion and ligation were successful, a DNA strand about 750 base pairs in length should be amplified. Additionally, the restriction enzyme ClaI digests the plasmid twice – once inside the 3 kb regulatory region and once inside the pCaSpeR4 vector, which should result in bands of DNA 9.5 kb and 5.2 kb in length with successful insertion and ligation. Therefore, a diagnostic digest with ClaI was used in order to secure our findings from the PCR amplification. We named the resulting plasmid pCE2 (Figure 2 B).

Creation of pCE3 and pCE4, plasmids with exon11 deletion

Two mutagenized plasmids were created – one with and one without the upstream 3 kb regulatory region. This was done in case the length of the plasmid proved a confounding factor in plasmid mutagenesis. Exon 11 was deleted from pCE1 and pJK12 following the NEB Phusion Site-Directed Mutagenesis protocol using the primers A39 and A40 (Figure 3). The mutagenized plasmid was ligated following a quick ligation procedure and transformed into NEB turbo competent cells, following the New England Biolabs transformation protocol. The success of the mutagenesis was tested by PCR testing of plate colonies procedure using primers OL207 and OL68. These two primers flank exon 11, so that the amplified DNA includes the deleted region. This allowed us to determine whether the mutagenesis was successful or not. The amplified strand would be either about 1300 base pairs without successful deletion or 800 base pairs with successful exon 11 deletion. Additionally, the restriction enzyme HindIII digests these plasmids

once on each side of exon 11, which should result in bands of DNA 8.7 kb in length and either 800 base pairs without successful deletion or 300 base pairs in length with successful deletion of exon 11. Therefore, a HindIII digest was used in order to corroborate our findings from the PCR amplification. We named the resulting plasmids pCE3 (Figure 2 C), which contained the 3 kb region, and pCE4 (Figure 2 D), which did not contain the 3 kb region.

Injection of embryos with plasmid

pCE2 was purified using an ethanol precipitation protocol and was mixed with an injection dye. The pCaSpeR4 vector contains P-element transposons, to transpose the plasmid DNA into the fly genome, as well as the *white* gene, coding for red eyes, to serve as a marker in adult flies. w^{1118} embryos were laid on grape juice plates, which were then washed with water. These embryos were then aligned on microscope slide covers in approximately five columns of ten embryos each, and were dried using ethanol. The embryos were then covered with halocarbon oil, and enough of the injection solution to create a spot in the embryo was microinjected posteriorly.

Collection of injected larvae and selection of offspring

After injection, embryos remained on the slide cover and were placed on a fresh grape juice plate. After 24 hours, larvae that survived the injection were transferred to a vial containing a fly food medium consisting of a standard yeast-cornmeal-molassas agar mix. After 8 days, the emerged flies were transferred to a fresh vial and crossed with 5

w^{1118} flies. Offspring were collected starting 10 days later and were selected for colored (red) eyes.

RNA probe preparation

Two μg of the target plasmid was digested in a 50μl reaction. For antisense probes, the plasmid was digested with a restriction enzyme at the 5' end of the gene. For sense probes, the plasmid was digested with a restriction enzyme at the 3' end of the gene. The digested plasmid was then extracted via phenol/chloroform extraction. For each enzymatic reaction, the volume of the DNA solution was measured and an equal volume of Tris-saturated phenol was added to the tube and was mixed well. The samples were placed on ice for a few minutes, to allow the aqueous and organic phases to separate, and the tubes were centrifuged at 13,000 rpm for 2 minutes at 4°C. The organic phase was removed and was placed into a fresh 1.5ml microcentrifuge tube. This procedure was then replicated using chloroform. The extracted DNA was then precipitated with an ethanol precipitation. The volume of the DNA solution was measured and 2.5μl of 4M sodium acetate, pH 5.0, was added for every 100μl of DNA. Additionally, 2μg of carrier tRNA was added. Two volumes of 100% ethanol was added to the tube, and the tube was placed at -20°C overnight. The microcentrifuge tubes were spun at 13,000 rpm for 10 minutes, and the supernatant was removed. An additional 500μl of 100% ethanol was added to the tubes. The tubes were again spun at 13,000 rpm for 10 minutes and the supernatant was removed. The pellet was dried in a vacuum microfuge and the DNA was redissolved in 8μl dH_2O.

In order to visualize the riboprobe, the RNA probe was labeled with uracil-bound digoxygenin, a steroid. The RNA probe was then transcribed from the purified DNA in a 20µl reaction containing 2µl 10X transcription buffer, 1µl RNAsin (or RNase block), 2µl 10 mM rA, rC, rG mix, 2µl rU/dig-rU mix (6.5/3.5 mM), 4µl of the plasmid DNA, 1 µl T3 or T7 RNA polymerase, and 8µl dH_2O. This mixture was incubated at 37°C for two hours, at which point 1µl of 0.5M EDTA was added to stop the reaction and the volume was adjusted to 90µl with dH_2O. For probes of pJK12, 10µl of 0.1M sodium carbonate, pH 10.2 was added and the mixture was heated to 65°C for 30 minutes to break the RNA into smaller pieces so the probes could enter the nucleus. 5µl of glacial acetic acid was then added to adjust the pH of the solution. For both pJK12 and pAC2 probes, 7.5µl of 4M sodium acetate, 1µl of 20mg/ml tRNA, and 250µl 100% ethanol were added to the tubes. The tubes were placed at -20°C for 1 hour and were spun at 13,000 rpm for 10 minutes. An additional 500µl of 100% ethanol was added to the tubes, which were again spun at 13,000 rpm for 10 minutes. The supernatant was removed. The RNA was redissolved in 250µl dH_2O and stored frozen.

The plasmid containing *D-Pax2* exon11, pAC2, was either digested with BamHI-HF and transcribed using T3 polymerase or digested using HindIII and transcribed using T7 polymerase to create the antisense and sense RNA probes, respectively. pJK12 was either digested with BamHI-HF and transcribed with T7 polymerase or digested using KpnI and transcribed with T3 polymerase to create the antisense and sense RNA probes, respectively (Figure 4 A – D).

Dissection of fly larvae and in situ hybridization

Late third-instar larvae were dissected by Prof. Joshua Kavaler in a glass depression dish (3 well spot plate) in KS+ phosphate buffer solution (PBS). Larvae were cut in half and inverted so that the discs were outside. Larvae were then placed in 300µl of 4% paraformaldehyde in PBS at room temperature for 30 minutes in a separate 3 well spot plate. An additional 300µl of 1X PBS with 0.1% Tween-20 (PBT) was added to the wells, and the larvae were incubated at room temperature for 30 minutes. The larvae were washed 6 times with PBT by removing the previous liqud and adding 400-500µl of PBT and placing the plate on a nutator for 5 minutes. The larvae were then equilibrated in washing grade In Situ Hybridization Buffer (ISHB), which contains 50% formamide, 5X saline-sodium citrate (SSC) buffer, 50µg/ml heparin, and 0.1% Tween-20. The complete ISHB also contains 100µg/ml ssDNA and 100µg/ml tRNA. Equilibration was achieved by removing the PBT and incubating the larvae in 25% ISHB:75% PBT at room temperature for 10 minutes on the nutator. This solution was then replaced with 50% ISHB:50% PBT, then 75% ISHB:50% PBT, and finally 100% ISHB, allowing the cells to adjust for the different osmotic pressure. The larvae were then moved to 1.5ml microcentrifuge tubes with 500µl complete ISHB and prehybridized by incubating at 55°C for 1 hour. During this time, 4µl of the thawed probe was added to 400µl of complete ISHB in a 200µl PCR tube. The probe was denatured by heating to 80°C for 5 minutes. This solution was then chilled on ice. The probes were then added to the samples, and the larvae were hybridized at 55°C for over 24 hours. The larvae were moved to a new 3 well spot plate and were washed 3 times for 5 minutes with pre-warmed washing grade ISHB. Larvae were then equilibrated into PBT, using the reverse

procedure as previously outlined, and washed 6 additional times for 10 mintues in PBT. 1µl of anti-digoxygenin-AP antibody (Roche Diagnostics) was added to 1ml PBX with 2% bovine serum antibody (BSA). Wells containing the larvae were drained and 500µl of this anti-dig antibody was added. Larvae were incubated at 4°C overnight. Unbound antibody was removed by washing the samples 6 times with PBT for 10 minutes each time. After the final wash, the larvae were washed in alkaline phosphatase staining buffer (APSB). APSB contains 100mM NaCl, 50mM MgCl2, 100mM Tris (ph 9.5), and 0.1% Tween-20. Next, 4.5µl of Nitro-Blue Tetrazolium Chloride (NBT) and 3.5µl of 5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt (BCIP) (prepared solutions from Promega) were added to 1ml of APSB, which was divided between two wells. The larvae were then placed on the nutator and the staining was monitored visually.

Four larvae were hybridized with each antisense probe. The larval eye disc and developing inner ear were the areas of focus for Pax2 expression. The inner ear, because it contains hair mechanoreceptors, was used as a bristle-equivalent.

Sequencing of plasmids

For each primer used, a 10µl reaction was prepared containing 2µl of sequence buffer, 0.5µl of the 20µM primer, 1µl BigDye 3.1, 100-500ng plasmid DNA, and dH_2O to 10µl. These reactions were then run with the thermocycler to amplify sequenced DNA. The thermal cycles were: 1 cycle of 95°C for 5 minutes; 40 cycles of 95°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes; and the samples were held at 4°C until ready to purify. These sequencing samples were purified with 0.06g/ml 50-G Sephadex. 800µl of the sephadex solution was added to a centricep column in an

appropriate base. Water was pushed through the column with a dropper bulb, and then the columns were centrifuged at 3,000 rpm for 2 minutes to remove remaining water. The centricep columns were then placed inside a 1.5ml microcentrifuge tube. The sequence reaction was added to the dry sephadex and the columns were spun at 3,000 rpm for 2 minutes. The sephadex was discarded and the microcentrifuge tubes were spun in the Speedvac until dry. Samples were then stored at -20°C until analysis. All sequence reactions were analyzed by Patricia Easton in the Sequencing Laboratory at Colby College (Figure 5). pCE1 will be sequenced using primers OL4, 9, 10, 123, 124, 193, and 205-214. pCE2 was sequenced using primers B75, OL4, 123, and 205-214. pCE3 will be sequenced using primers A4-10, 13-20, OL9, 10, and 205-214. pCE4 will be sequenced using primers OL9,10, and 205-214. pCE5 will be sequenced using primers B75, OL4, 123, and 205-214.

RESULTS

Creation of constructs

In order to create a rescue construct containing full length *D-Pax2* cDNA, we cloned *D-Pax2* cDNA and the 3kb upstream regulatory region into pBluescript SK. Thermocycling of the ligation of pJK12 and the 3 kb upstream regulatory region using primers OL3 and 210 resulted in amplification of a DNA strand approximately 1 kb in length in two colonies (Figure 6). This indicated that the 3 kb region was inserted in the correct orientation into pBluescript SK containing *Pax2* cDNA, creating pCE1.

This insert was then transformed into pCaSpeR4, creating pCE2. PCR of this plasmid using primers B75 and OL123 resulted in amplification of a DNA strand 750

base pairs in length in 9 colonies (Figure 7), indicating that the insert had been ligated correctly into pCaSpeR4. Additionally, digesting the plasmid with ClaI resulted in two bands. One was 9.5 kb in length and one was 5.2 kb in length (Figure 8). These lengths are identical to the lengths hypothesized from our proposed plasmid sequence.

In order to determine the effect of exon 11 on protein function and ability to rescue bristle development, exon 11 was deleted from *D-Pax2*. Using two primers flanking exon 11 and facing away from the exon, we amplified the DNA of the entire plasmid except for exon 11, creating the exon 11 deletion. Using primers A39 and A40, exon 11 was successfully deleted from pCE1 and pJK12, creating pCE3 and pCE4. PCR of these plasmids using the primers OL207 and OL168 resulted in the amplification of a DNA strand 800 base pairs in length in 10 colonies for pCE3 and 12 colonies for pCE4 (Figure 9 A, B). These amplified strands were 500 base pairs shorter than the amplified pieces from pCE1 and pJK12, indicating that these new plasmids had been successfully mutagenized, as exon 11 is 500 base pairs in length. Additionally, the digest with HindIII created two strands of DNA, one 8.7 kb and one 300 base pairs in length (Figure 10 A, B). These lengths are identical to the lengths hypothesized from our proposed plasmid sequence. The mutagenized insert has yet to be successfully ligated into pCaSpeR4.

Creation of transgenic flies

In order to examine the phenotypic rescue of bristle development in *shaven* mutants, we injected 700 embryos with pCE2 over four days. From the 75 surviving larvae, 39 virgins were crossed to w^{1118} flies. None of the resulting offspring showed

expression of the injected gene through the red eye phenotype. Therefore, we were unable to successfully microinject our plasmid into w^{1118} embryos.

Creation of RNA probes and in situ *hybridization*

In order to visualize the spatial localization of exon 11 in *D-Pax2* mRNA, synthesis of RNA probes for exon 11 was attempted. However, these probes were not successfully synthesized (Figure 11). Probes directed toward *D-Pax2* appeared to be correctly synthesized when electrophoresed on the RNA Flash gel. These probes were used for *in situ* hybridization; however, the staining seen was extraneous and not specific to the eye and inner ear tissue. Probe transcription was attempted two additional times, with no greater success.

DISCUSSION

Four of the five constructs have been successfully ligated and transformed. We have successfully created a *D-Pax2* rescue construct, including the 3 kb upstream regulatory region, both in pBluescript and the injection vector pCaSpeR, pCE1 and pCE2. Additionally, we have successfully deleted exon 11 from pCE1 and pJK12, creating pCE3 and 4, which are both in pBluescript. The sequencing constructs have been created for all of these plasmids, and one plasmid, pCE2, has been successfully sequenced. This is a multi-year project, and much of the setup has been done.

The data from *in situ* hybridization are inconclusive; the pattern of staining seen is indicative of non-specific probes. In order to determine the cause of the difficulty in synthesizing these probes, we digested pAC2, to examine whether or not the DNA was

degraded. The plasmid was determined to still be structurally complete. Therefore, these results may be due to either the primers or the polymerase, which potentially resulted in inexact transcription of the RNA probes.

One significant obstacle during the course of this work was successfully creating clones. This was not specific to my work; it was encountered by many members of the lab. In order to determine which step created the obstacle for the clones, we examined the results of each process. All PCR amplifications and digests of plasmids were electrophoresed on agarose gels and were determined to be correct. We therefore determined the interference to be in either the ligation or transformation stage. On our first attempt to create pCE2 from ligation of the insert from pCE1 and the pCaSpeR vector after gel extracting the digests, we noticed that many of the cells transformed with the vector only were growing into colonies when plated, and that there were few plated colonies resulting from cells transformed with the vector and insert ligated. We attempted a digest of the vector and the insert followed by gel extraction and then phenol and chloroform extraction. This again showed low rates of vector and insert ligation and the possibility that the vector was ligating with itself. We additionally attempted another gel extraction of the digests, and electrophoresed the results of the gel extraction on an agarose gel to determine whether the extraction was degrading the DNA. We determined that the DNA was still intact, but hypothesized that reagents used during the gel extraction protocol might inhibit the ligation or transformation reactions. To test this, we electrophoresed the results of the digest on a low-melt agarose gel, and added this gel/DNA mixture directly to the ligation reaction. This proved successful, indicating that the gel extraction was inhibiting future processes. We have attempted the same

procedure in order to create pCE5, the mutagenized *D-Pax2* in pCaSpeR; however, we have not yet been successful, indicating that there may be another confounding factor inhibiting the ligation and transformation reactions.

The creation of the mutagenized plasmids likewise proved difficult. Initially, we attempted mutagenesis using two primers, B36 and 37, which are similar to A40 and 39. A40 has an additional 5' TTTG, compared to B36; A39 and B37 are identical. We continually noticed improper binding of the primers resulting in incorrect mutagenesis results, despite lowering the annealing temperature during PCR and altering the thermocycling procedure. We additionally sequenced the results of the mutagenesis around exon 11 in order to determine where the primers were binding. We found that B36 was binding non-specifically, resulting in improper mutants. We then attempted the mutagenesis using A40 and 39, and successfully created mutagenized *D-Pax2* plasmids, pCE3 and pCE4. We thus hypothesize that the previous primers were the cause of the incorrect mutagenesis results.

Because much of the work done is preparatory in nature, many of the next steps in this research are to finalize the results. One primary goal is to successfully inject embryos with the mutagenized and full-length plasmids. Once these transgenic flies are created and maintained, they can be crossed with known *shaven* mutants. The full-length *D-Pax2* should completely rescue bristle development in the *sv* mutant. We hypothesize that the exon 11-deleted *D-Pax2* will result in a lack of rescue of bristle development. This experiment would truly determine the phenotypic effect of exon 11 on bristle development. Additional studies would include attempting to perform *in situ*

hybridizations again and examining the structural and functional differences in the mutated protein.

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LITERATURE CITED

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Appendix 1: Figures and Tables

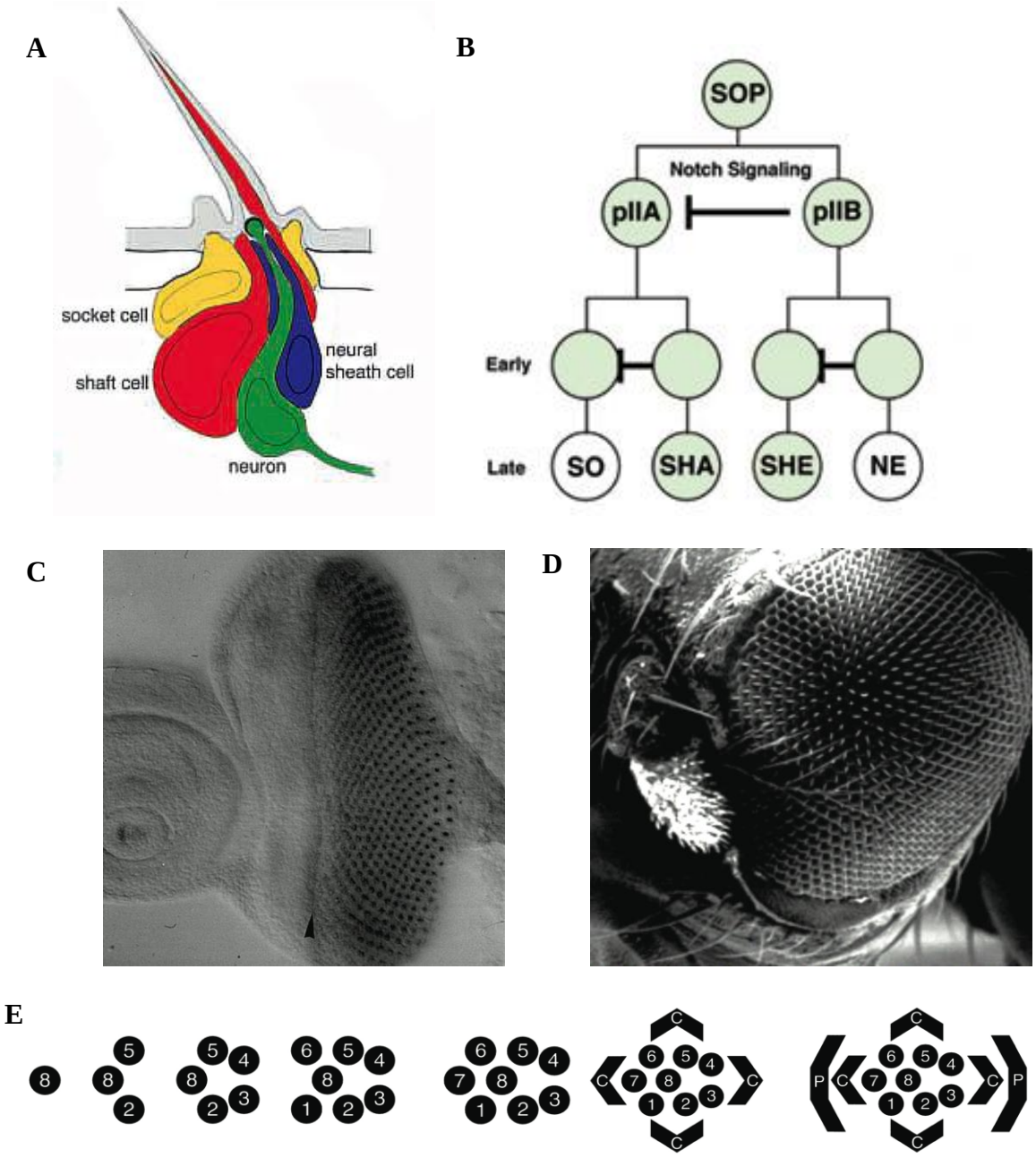

Figure 1. Depictions of *Drosophila* bristle and eye sensory system development. (A) Schematic illustration of the four cells comprising the bristle structure (from Adam, 1998). (B) Illustration of the division of the sensory organ precursor (SOP) into the four cells comprising the bristle and the cell fate determination using Notch signaling. Cells in green express *D-Pax2*. (C) Larval eye disc stained for *D-Pax2*. The organization of the compound eye can be seen. (D) Adult compound eye. (E) Development of the eight photoreceptor cells, four cone cells, and two primary pigment cells in the adult eye.

Table 1. Primer names and sequences. These primers were previously synthesized, and the sequences are listed in 3' – 5' fashion.

Primer	Sequence (3' – 5')
A4	ACGTAACTTCACCGGAATACC
A5	GTGCTATCGACTTGTTAAAGCCC
A6	AGTTACCCACCGTTTAGCATGCG
A7	GATGACGGTAAATGAACACCTG
A8	CGATAGTAACACCAGTCAGCTC
A9	GTAACAATACGCGATCCAGACG
A10	CTCATCCCTTCTCATTCAGTGAC
A13	CGTTTCCAACCATATAAAGTGTGATC
A14	TTGAAGTTATCCGTCCAGATTCTAG
A15	GCTAGGTACTGGAAATGCTACTG
A16	TCCTTACGTGGTTAATTCGTGC
A17	TGCCGTGCGTCTTTGTCTCTG
A18	AATTGGCATCCCTGAACGAC
A19	GTCCTGACTGCTTAAAGTTTCC
A20	CAAGGCGGCTTTACGGTTGG
A39	GCTGCAGCTCAGTTGTACCCG
A40	CTGTGTAAAAGGGAGGGACTGTTTG
B75	TTAACCCTTAGCATGTCCGTGG
OL4	GAAGCGGGGATCCTATTTCAAAC
OL9	AATTAACCCTCACTAAAGGG
OL10	GTAATACGACTCACTATAGGGC
OL123	CTTGTCCGTGGATCCATCTTTA
OL124	ACACACGGATCCACGGACATAC
OL125	CAACCATATAAAGCTTGATCAGG
OL193	CTGAGTCAAGGATCCTAAGCC
OL205	GCCAATACGACGCCATCTGC
OL206	GAGATACGAGATCGACTTTTG
OL207	CGGAATCAAGTTGTAAACGC
OL208	ACACAGCTCTAGCATTTGAC
OL209	TCGATCAGATGTTGAGCTCA
OL210	CAGATGGCGTCGTATTGGC
OL211	ACGCTGGGAACATTATCTTG
OL212	CTCATCATGTGCTTCAATTC
OL213	GTCAAATGCTAGAGCTGTGTA
OL214	GTGTTGAATAAAGGCCTACG

Table 2. Description of previously synthesized plasmids. pAC2 and pJK12 were previously synthesized, while all CE plasmids were not. pBluescript is a general vector, pCaSpeR4 is our injection vector, containing transposons and the *white* gene.

Plasmid Name	Description
pBluescript	General vector, containing ampicillin resistance and a multiple cloning site (MCS) region. SK/KS denotes orientation of the MCS.
pCaSpeR4	Injection vector, containing transposons and the *white* gene
pAC2	Previously created plasmid containing *D-Pax2* cDNA in pBluescript KS
pJK12	Previously created plasmid containing *D-Pax2* cDNA in pBluescript SK

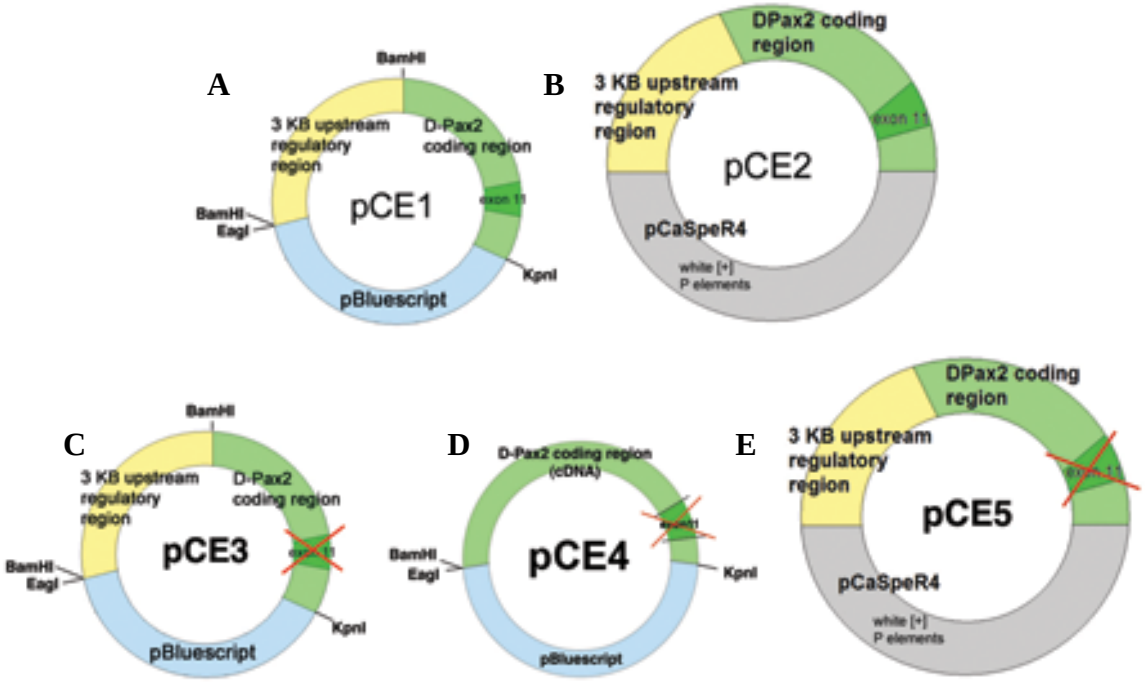

Figure 2. Depictions of synthesized and proposed plasmids. (A) pCE1, *D-Pax2* cDNA and 3 kb regulatory region in pBluescript SK, 9.5 kb in length. (B) pCE2, *D-Pax2* cDNA and 3 kb regulatory region in pCaSpeR4, 14.7 kb in length. (C) pCE3, mutagenized (exon 11 deletion) *D-Pax2* cDNA and 3 kb regulatory region in pBluescript SK, 9 kb in length. (D) pCE4, mutagenized (exon 11 deletion) *D-Pax2* cDNA in pBluescript SK, 6 kb in length. (E) pCE5, mutagenized (exon 11 deletion) *D-Pax2* cDNA and 3 kb regulatory region in pCaSpeR4, 14.2 kb in length.

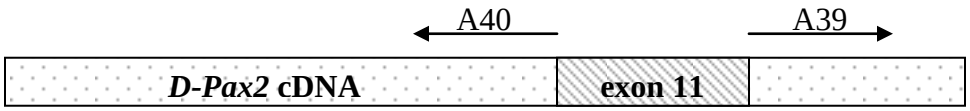

Figure 3. Depiction of exon 11 deletion synthesis. Primers flanking exon 11 (A39 and 40) amplified the DNA of pCE1 and pJK12 except for exon 11.

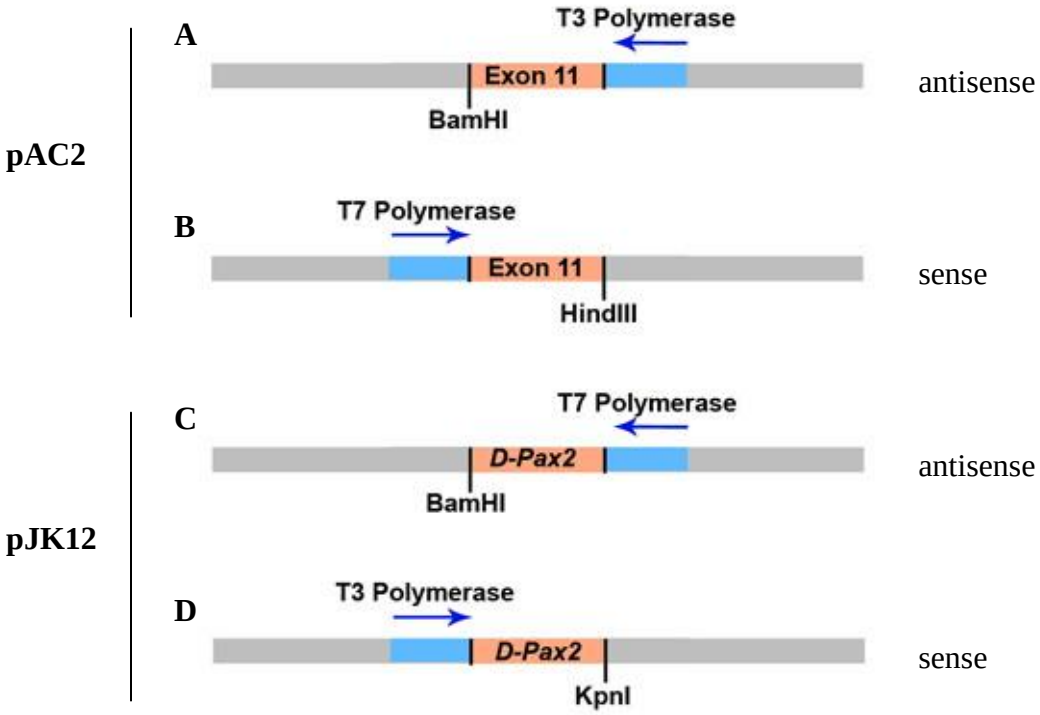

Figure 4. Depiction of RNA probe synthesis. This general procedure was used to synthesize RNA probes containing only the gene or exon of interest. (A) Synthesis of the antisense probe of pAC2 by digesting the plasmid with BamHI-HF and transcribing using T3 RNA polymerase. (B) Synthesis of the sense probe of pAC2 by digesting the plasmid with HindIII and transcribing using T7 RNA polymerase. (C) Synthesis of the antisense probe of pJK12 by digesting the plasmid with BamHI-HF and transcribing using T7 RNA polymerase. (D) Synthesis of the sense probe of pJK12 by digesting the plasmid with KpnI and transcribing using T3 RNA polymerase.

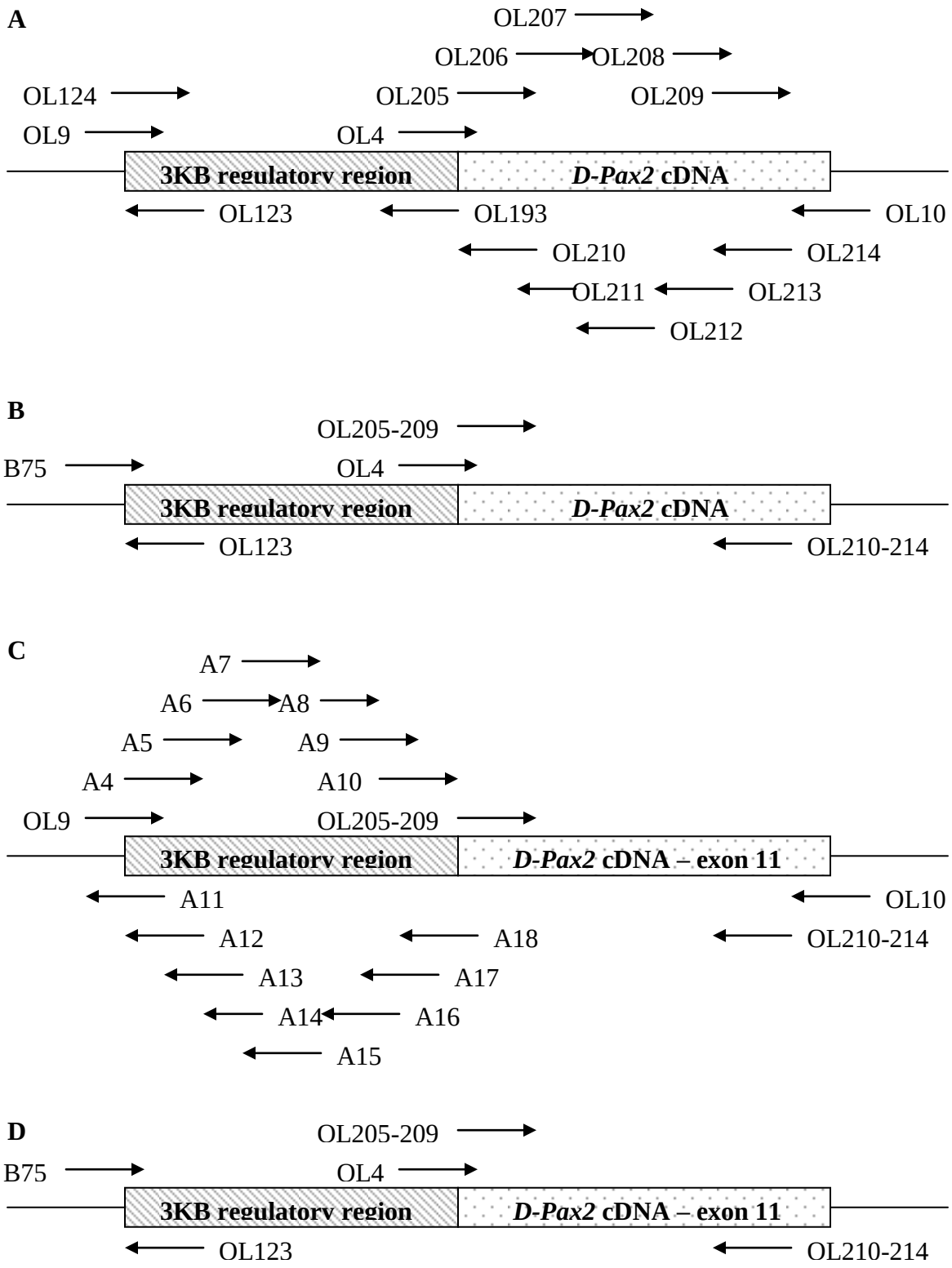

Figure 5. Sequencing constructs. (A) Sequencing construct of pCE1, in pBluescript SK. (B) Sequencing construct of pCE2, in pCaSpeR4. (C) Sequencing construct of pCE3, in pBluescript SK. (D) Sequencing construct of pCE5, in pCaSpeR4. The sequencing

construct of pCE4 is identical to the sequencing construct of pCE3, without the oligonucleotides associated with the 3 kb regulatory region, and was left out for simplicity.

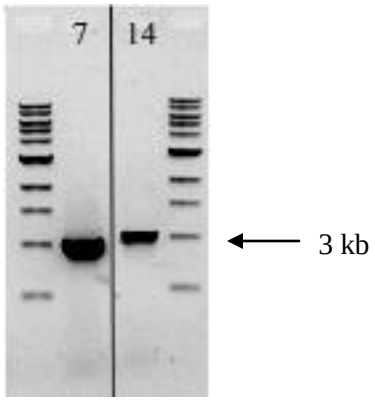

Figure 6. PCR test of pCE1 using OL3 and OL210. According to the proposed sequence of the plasmid, the DNA synthesized should be approximately 1 kb in length, which was what was seen in two of the colonies: 7 and 14.

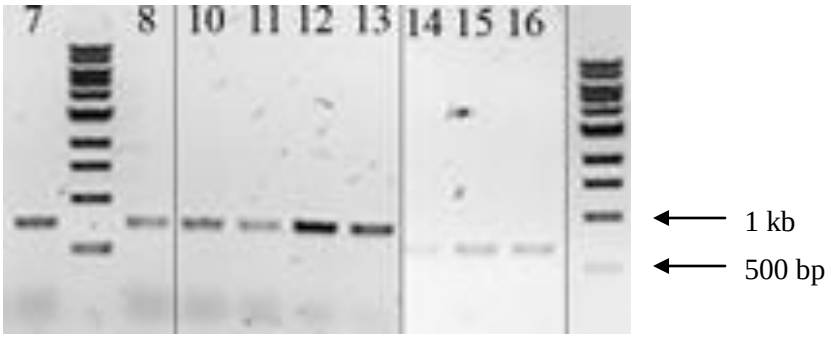

Figure 7. PCR test of pCE2 using primers B75 and OL123. According to the proposed sequence of the plasmid, the DNA synthesized should be approximately 750 base pairs in length, which was seen in colonies 7, 8, and 10-16

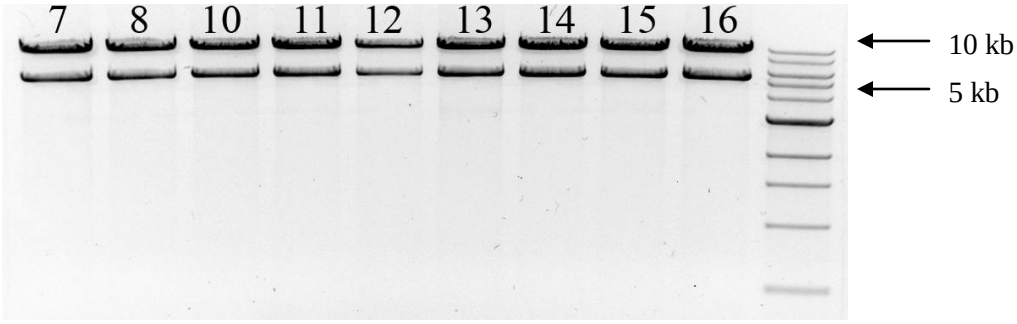

Figure 8. ClaI digest of pCE2. According to the proposed sequence of the plasmid, the restriction enzyme should cut twice, creating two bands of DNA approximately 9.5 kb

and 5.2 kb in length. These lengths were confirmed in plasmids in all colonies selected from the previous PCR test.

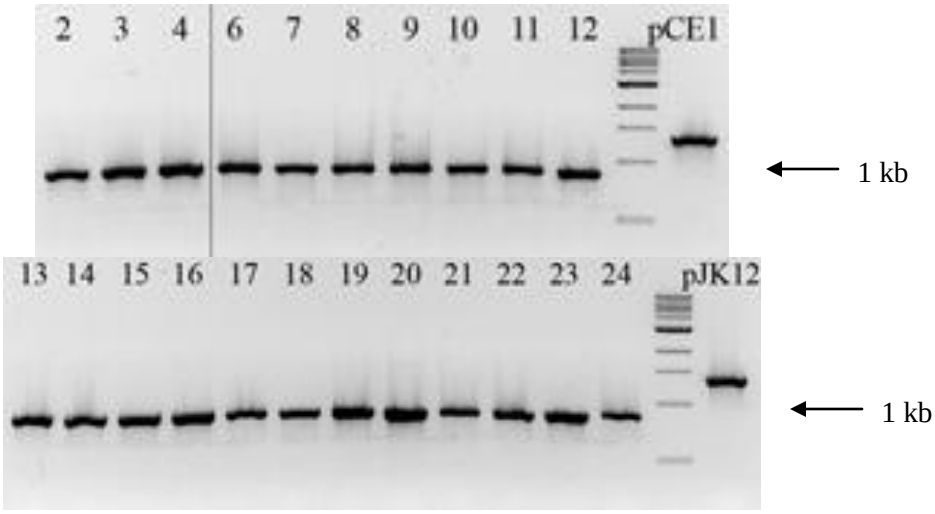

Figure 9. PCR test of (A) pCE3 and (B) pCE4 using primers OL207 and OL168. According to the proposed sequence of the plasmid, the DNA synthesized should be approximately 800 base pairs in length with successful exon 11 deletion, or 1300 base pairs in length without the deletion. Plasmid from colonies 2-4 and 6-12 showed successful deletion of exon 11 from pCE1. Plasmid from colonies 13-24 showed successful deletion of exon 11 from pJK12. The full length plasmids are shown to compare with the deleted plasmids.

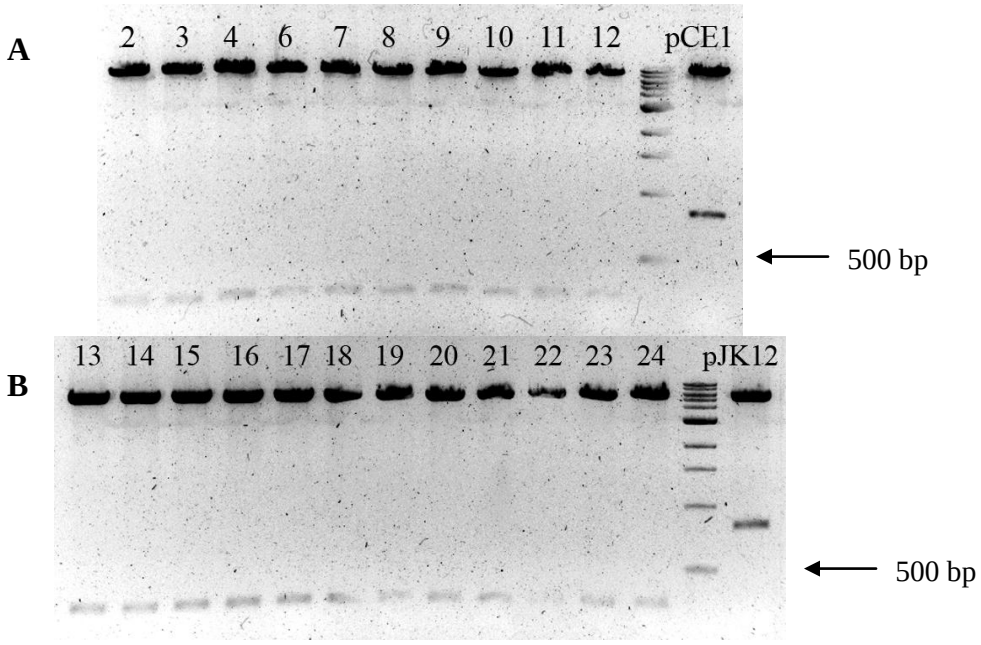

Figure 10. HindIII digest of (A) pCE3 and (B) pCE4. According to the proposed sequence of the plasmid, the restriction enzyme should digest the plasmid twice. One band should be 8.7 kb in length, regardless of whether the plasmid was mutagenized. The other band should be either 300 base pairs in length with successful exon 11 deletion or 800 base pairs without the deletion. Plasmid from colonies 2-4 and 6-12 showed successful deletion of exon 11 from pCE1. Plasmid from colonies 13-24 showed successful deletion of exon 11 from pJK12. The full length plasmids are shown to compare with the deleted plasmids

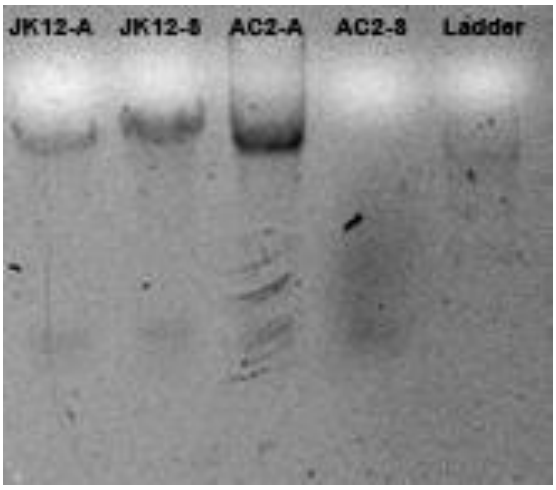

Figure 11. Visualization of RNA probes on a RNA Flash gel. A and S denote antisense and sense RNA strands, respectively. Unfortunately, the ladder proved difficult to visualize with detail. The probes for pJK1 seemed to be of the correct length – around 3.5 kb. However, the probes for pAC2 either were not synthesized correctly or could not

be visualized accurately. The band in the lane corresponding to the pAC2 antisense probe was hypothesized to be residual DNA.