Developmental Expression Patterns of "Eyes Absent" Genes I, II, and III in *Xenopus laevis*

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Abstract
The original eyes absent gene was first located in *Drosophila* (fruit fly) and its crucial role in embryological sensorial development has stimulated research which has been conducted on this family of genes in mouse, frog, chick and human. Dr. Martin Offield isolated Eya1, 2 and 3 in *Xenopus laevis* (African clawed frog) in Rob Grainger's lab at the University of Virginia (Personal communication). The present study was a follow up study using the process of *in situ* hybridization to record the expression patterns of these genes using embryos at various stages of development. After a staining procedure, embryos were photographed as whole mounts and histology was used to view the gene expression in different tissue layers. Further study was conducted to locate an Eya4 gene in *Xenopus* by searching large DNA databases using mouse and human Eya4 protein sequences. Eya1 in *Xenopus tropicalis* was also located using the same search techniques.
Developmental Expression Patterns of “Eyes Absent”

Genes I, II, and III in *Xenopus laevis*

The “eyes absent” or Eya genes were first isolated in *Drosophila* (fruit fly) during a winery population screening in Australia completed by Sved (1986). Sved (1986) used “chromosome homozygosis via the Cy/Pm technique (repeated backcrossing of generations)” and found the gene to “be associated with an inversion with breakpoints at 22D and 34B (specific points along the second chromosome) (p. 169).” It was later identified as a transcriptional regulator by Bonini, Leiserson, and Benzer (1993) who also generated 35 additional alleles by screening for mutations produced by chemical, X-ray and P-element insertion that failed to complement the original Eya allele. Further research identified the gene’s DNA sequence to encode for certain proteins necessary to normal embryological development. These mutations caused a disruption in the cell specification and differentiation process leading to mutants with no eyes or death revealing the importance of the task that the gene performed (Bonini, Leiserson, and Benzer, 1998).

Using the *Drosophila* sequences, other researchers discovered mouse DNA sequences that were similar using homology screening and identified three homologs (Pin-Xian, Woo, Her, Beier, and Maas, 1997). After these gene patterns were published, Offield and Grainger at the University of Virginia identified three of the four mouse homologs present in *Xenopus laevis* gene sequences (Personal communication).

In the hierarchy of genomic control, master controller genes are activated and induce the activation of sub-genes according to a specific pattern of the developing
embryo at the proper stage. The Eya gene family has been shown to be controlled by Pax6 in mouse (Pin-Xian et al., 1997; Tomarev, 1997). Without Pax6, the Eya proteins are unable to return to the nucleus to act as transcriptional cofactors, blocking their normal function. Null mutants of Pax6 result in significant defects in the development of vertebrate eyes (Hanson, 2001).

The mouse genetic data revealed the areas of expression that could be expected in the frog, and ideas of what the genes accomplish in the anterior development of embryos (Pin-Xian et al., 1997). Focusing on eye development of the mouse, these genes have been found to be expressed in the lens, iris, corneal ectoderm, retinal tissue, optic nerves and the optic sheath (Pin-Xian et al., 1997). Following these expression patterns, close attention was given to the anterior portions of the Xenopus embryos being studied. The anterior anatomy of developing Xenopus embryos, including placode position, was acquired from other in situ studies of Xenopus laevis (David, Ahrens, Weldich, and Schlosser, 2001).

The Eya genes specifically code for proteins that play essential roles in normal Drosophila eye development (Bui and Zimmerman, 2000). Further work has indicated Eya gene products function as transcriptional co-factors (Bonini et al., 1998), which work together with other proteins at a specific site on the DNA to activate or repress the transcription of downstream genes. These downstream genes have been shown to code for proteins that play key roles in the development of the eye (Hsiao, Williams, Davies, and Rebay, 2001; Zygar, Cook, and Grainger, 1998). Mutations of the Eya gene family in mouse and humans have been documented to cause problems with the developing ear,
eye and kidney, even causing late onset deafness (Abdelhak, Kalatzis, Heilig, Compain, Samson, Vincent, Weil, Cruaud, Sahly, Leibovici, Bitner-Glindzicz, Francis, Lacombe, Vigneron, Charachon, Boven, Bedbeder, Van Regemorter, Weissenbach, and Petit, 1997). The family of four Eya homologs present in human and mouse are so closely related to one another that if one of the genes is damaged, the remaining genes are able to cover for the deficient protein production (Abdelhak et al., 1997). Through biochemical analysis, the specific proteins that Eya encoded proteins cooperate with are DNA binding proteins called “sine oculus” and “dachshund.” These interactions involve a specific activation domain (the Eya domain) of Eya proteins which activate transcription, resulting in expression of mRNA (Pignoni, Hu, Zavitz, Xiao, Garrity, and Zipursky, 1997; Chen, Amoui, Zhang, and Mardon, 1997).

The interest in studying developmental genes in mice, frogs and fruit flies is rooted in the long-term goal of understanding the genetics of human embryological development, which eventually will have important medical applications. Once the homologs of a gene family are located and sequenced, a map can be made of when and where the genes are active. Due to the moral and ethical issues surrounding direct research on human embryos, studying animals gives the insight needed to eventually be able to better understand human development.

Seeing where and when genes are active during development gives clues to what the gene does during embryogenesis. To record this information, a technique called in situ hybridization is used. Through a series of washes over a period of five days, embryos are prepared to receive a specific probe that binds to RNA produced by the
activation of the target gene. Using a combination of antibodies and staining, the areas of tissue with the activated gene are stained. These genes have an impact on the sensorial development of embryos. This led to a specific interest in observing the anterior portion of the embryos to indicate which “placodes” the expression was located. Placodes are the tissues that will develop into future structures, such as the otic (ear), nasal, and lens (eye) placodes.

Methods

All processes of harvesting, fertilizing, preserving, and staining of embryos were taken from Sive, Grainger, and Harland (1996). The following text is a brief summary of the processes used for this study.

Harvesting, Fertilization and Storage

Fertilization of albino *Xenopus laevis* embryos began by injecting a female with 700-800 units of human chorionic gonadotropin into dorsal lymph sacs to induce egg laying. The high variation depends on the size of the female in question. The testis of a male were also obtained through dissection and stored in L-15 cultured media. After nine to ten hours, eggs were harvested into a Petri dish by gently holding the frog’s legs open and massaging the belly with lateral and vertical stimulation. The female pushed the eggs from a storage sac near the cloaca while remaining relaxed if the technique was properly administered. To fertilize the eggs, a small amount of well diced testis was stirred into the eggs in a solution of high salt concentration in 1x MBS (Modified Barth’s Saline). After approximately 15 minutes the eggs were de-jellied for easier handling through a 2% cysteine wash of two to four minutes. After being washed thoroughly with
1/10x MBS multiple times over 10 minutes, the eggs were placed into 1/10x MBS medium and allowed to mature for one and a half hours.

The eggs showing signs of cleavage were removed and placed in 1/10x MBS medium for continued development. The stages of growth that were collected were from the neurula stages, 12 (13 HAF22 [hours after fertilization at 22°C]), 14/15 (17 HAF22), and 18/19 (20.5 HAF22), to the early tadpole stages of 23 (24.75 HAF22), 28 (32.5 HAF22), and 34 (44.5 HAF22) (Niewkoop and Faber, 1967). Each stage was visually confirmed by external morphological features before the embryos were preserved. Then the protective vitelline envelope was removed manually from the embryos and they were immediately fixed for preservation in a 4% formaldehyde solution. The last step before storage of the embryos was washing them with a dehydration gradient from water based solutions to ethanol. The embryos were then placed at -20°C, where they were stored until needed for in situ hybridization.

**RNA Probe Production**

The plasmids containing the Eya gene sequences were obtained and characterized through a screening of a neurula stage *Xenopus* cDNA library using the mouse Eya gene sequences. The plasmids were placed in bacteria and set up to have T3 and/or T7 viral promoter sequences which allow for the corresponding RNA polymerases to transcribe either sense or antisense RNA sequences. Cultures of bacteria containing the plasmid DNA were allowed to grow overnight at 37°C. The DNA was isolated from the resulting solution by alkaline lysis followed by ion exchange chromatography (Qiagen, DNA Large Prep Kit).
This plasmid DNA was then placed in solution with either T3 or T7 viral RNA polymerases to generate antisense RNA sequences. The solution contains the raw materials needed by the RNA polymerases to copy the sequences, along with a specific uracil RNA nucleotide labeled with digoxygenin. Digoxygenin (dig) is the epitope recognized by the immunostaining procedure. To ensure that only one of the two strands of DNA was copied, the DNA was linearized to provide a termination point for the RNA by a restriction enzyme. Once transcription had run for 10 minutes, the unused nucleotides were removed using a Sephadex spin column. The remaining solution was exposed to ethanol, which precipitated the RNA. The supernatant was removed and the remaining pellet was re-suspended in sodium carbonate and bicarbonate.

The resulting RNA, containing the sequence of the specific gene, were anywhere from 1500-3000 nucleotides long. A sequence this large could have affected the efficiency of the sequence from penetrating the cells of the embryo during hybridization, which would give high levels of background staining and weak primary staining. To avoid this problem, the probes were hydrolyzed through exposure to sodium hydroxide. This cut the probes at random into fragments of approximately 300 nucleotides to ensure better penetration. The RNA was reprecipitated, the hydrolytic solution removed, and then, the RNA diluted to 10 or 20 ug/ml using hybridization buffer. These 10x and 20x stock solutions were kept at -20°C until they were diluted in small amounts as needed for each *in situ* procedure.

*In Situ Hybridization*

This multiple day process placed *Xenopus* embryos through a series of washes
and incubations whereby the dig labeled RNA probe is used to label cells of the embryo that contain RNA’s with complementary sequences. Antibodies to the dig are then used to stain the cells using immunohistochemical techniques.

Embryos of each harvested stage were taken from -20°C ethanol and equilibrated to phosphate buffered saline solution through a series of washes. Vials were set up to contain various stages of embryos according to the desired gene to be mapped. Once organized, each vial went through a series of washes in order to prepare the embryos to be receptive to the probe. The probe was applied at high temperatures in special hybridization buffer overnight. Because of the high sensitivity of the probe to RNAases, which are present on the surface of skin, protective gloves were worn during the first day of washes.

The embryos were put through a series of washes at high temperatures to remove any unbound probe. Treatment with RNAase caused all remaining single stranded RNA to be destroyed. Embryos were incubated in lamb serum and BM Blocking Reagent (obtained from Roche Biomedical Inc.) to prevent non-specific binding of the antibody. This assured that the antibody labeling would only mark the RNA probe. The last step of the second day was to add antibodies to the solutions which recognized the dig on the RNA probe. This was incubated overnight at 4°C.

The third day consisted of multiple extended detergent washes to remove the extra unbound antibody. This was crucial to achieving proper contrast levels during the staining of the embryos. The last wash was extended overnight at 4°C.

The embryos were put through a series of buffer washes designed to change and
stabilize the pH of the solution to 9.6 before being exposed to the color reagent. The dye reagent Alkaline phosphatase substrate BM purple-AP (obtained from Roche Biomedical Inc.) was added to each vial of embryos, placed on an aggitator, and covered with foil. This reagent was catalyzed to a blue precipitate by the Alkaline Phosphatase conjugated antibody which was bound to the labeled RNA probe. This reaction marks where the gene in question was active in the embryo. This staining reaction lasted anywhere from two to three hours up to multiple days depending on the success of the probe hybridization. The process was carefully monitored to prevent over-staining which would result in a high background levels making visual contrast and comparison impossible. The properly stained embryos were stored short term in a detergent solution for microscopic examination and photography, and then placed into a glycerol solution for long-term storage. This was useful for later data verification as well as selecting which embryos to section using the microtome.

Embryo Histology

Stained embryos were processed for histological analysis using standard techniques. Stained embryos were taken from -20°C glycerol and placed in a solution of formaldehyde detergent to post-fix the tissues prior to the process of embedding. Detergent washes were used to remove formaldehyde and the embryos were dehydrated into methanol through a series of gradient washes resulting in solutions comparable to the storage conditions of unstained embryos. After achieving these conditions, the methanol was replaced with two washes of isopropyl alcohol, which has better mixing properties with the Paraplast™ wax in which the embryos were eventually embedded. Two washes
of isopropanol / Paraplast™ at a 50:50 ratio were completed and the embryos were
washed twice with 100% Paraplast™, all at 60°C. To ensure the best conditions, fresh
un-melted Paraplast™ was heated and used for a final wash for 30 minutes. Unless the
Paraplast™ completely infiltrates the entire embryo, sectioning would not be successful
in obtaining proper samples.

After embryos were completely infiltrated with Paraplast™, individual embryos
were embedded and positioned in one of three ways. Anterior orientation of the embryo
gave sectioning with the embryo facing the microtome, lateral sectioning allowed the
embryo to lie on its side, and dorsal sectioning sliced the embryo from the stomach
through to the back. The embryos were placed in each position quickly to avoid
movement during the cooling of the wax, one embryo in each plastic mold. The blocks
of wax were allowed to cool at room temperature for several hours, and placed in
refrigeration overnight to ensure uniform temperature and complete solidification.

Each block was taken from refrigeration, removed from the plastic mold and
trimmed so that there was only a small amount of wax around the position of the embryo
in a trapezoidal configuration, with the large side down, parallel to the intended cut of the
microtome blade. The microtome was set to cut in 10 μm increments, and the wax began
to come off the cells in parallel ribbons. Microscope slides were labeled and heated to
60°C to receive the wax ribbons. Water containing gelatin and BSA buffer was
vigorously boiled to remove all gas and then placed by pipet onto the receptive slides.
The wax ribbons containing slices of embryo were floated onto this bubble of water held
in place by surface tension. As the water evaporated, the wax ribbons dried to the slides.
The final steps before viewing the microtome embryos was the removal of the wax and placing a cover slip on the slides to ensure preservation. This was done by three 30 second washes in xylene. A line of Permount™ was placed along the side of a dried slide free of wax and a thin glass cover-slip was applied in such a manner as to allow capillary action to guide the glue to create an air free thin layer between the slide and the cover slip. The completed slides were allowed to dry and were then viewed and photographed with the use of a Zeiss Axioscope™ equipped with a digital camera.

**BLAST Searching for Gene Homologs**

The search for new genes in *Xenopus* began with the amino acid sequences already decoded from mouse and human. The primary goal of searching for homologues was to find an EST (Expressed Sequence Tag) clone. This EST clone DNA is DNA that has been taken from an embryo during a certain developmental stage, cut into specific lengths according to enzyme activity, sequenced and categorized for computer searching. This data is made available online at research websites such as the Sanger Center, the DOE Joint Genome Institute (JGI), and the National Center for Biotechnology Information (NCBI) for the purpose of electronically submitting amino acid or base pair sequences for comparison. The servers at these institutions take the sequence submitted and attempt to align it across an entire archive of EST clones obtained from a genome of the researcher’s choice, and formulate a report that displays the best matches obtained.

When matches are found, a process called back-blasting occurs, which confirms the identity of the clone before it is ordered from the institution in physical form for use in further study. For example, a researcher submits an amino acid sequence for gene “x”
from mouse to the Sanger center BLAST server for the *Xenopus tropicalis* EST clone library. This gives a list EST clones from *Xenopus tropicalis* that have high levels of matching sequences to the amino acid sequence of gene x from mouse. The researcher takes the sequence of the EST clone obtained from the server, and BLASTS it back to NCBI, which contains more than just EST clones allowing for matching of gene identities. If the sequence from *Xenopus* “backblasts” to NCBI and results in high levels of matching to either the original sequence blasted to the Sanger center or homologs of the same gene x in humans and other animals, then the probability is very high that the EST is the match of gene x in *Xenopus*.

The purpose of finding homologs in EST clones and ordering them is to begin the process of identifying what the gene accomplishes during development. This is done first through *in situ* procedures which tell where the gene is expressed during different stages of development, and then other tests are run to both knock out the expression or overexpress the gene and record its impact upon normal development.

**Results and Discussion**

*Expression Patterns in Xenopus laevis*

Each gene was studied at stages 12, 14/15, 18/19, 23/24, 28, and 34, which represent major developmental stages from the neurula to the tadpole. Both whole mount and slide photographs of sectioned embryos were used to deduce the physical location and the tissue layer where expression occurred. The lack of a standard anatomical reference to the development of *Xenopus laevis* has necessitated the use of descriptive language along with extensive diagrams to effectively record the locations of expression...
and how they were modified during development through the researched stages. Most of the microtome slides presented use the anterior orientation.

*Xenopus laevis - Expression of Eya 1*

Two main symmetrical areas of expression are present at stage 12 in Figure 1. The foremost is located in presumptive placodal tissue lateral to the developing neural plate denoted by A in Figure 1. The second set of stain is located dorsally, lateral to the midline near the top of the neural plate marked by B in Figure 1. This is close to the placodes denoted for the beginning of the spinal chord. The sections shown in D and E indicates stain A as an ectoderm stain while B is located in both ectodermal and mesodermal derivatives.

Figure 1. Eya 1 staining stage 12 - (A) anterior view showing two main areas of staining - A is located in
presumptive placodal tissue next to the developing neural plate, and B is a deep midline dorsal stain near the tissue that will become the front of the spinal chord, (B) dorsal view, (C) lateral view, (D) anterior sectioning of the embryo showing A in the ectoderm while B is expressed in multiple layers, (E) B expression in the posterior section of the embryo.

At stage 14/15 (Figure 2), the same two main areas of staining are present, but have matured into broader and darker areas. The first expression A has now outlined the entire anterior portion of the neural crest including the developing sensory placodes. Expression B has elongated with the embryo extending further toward the posterior formation of the neural plate/crest. Sections of this region shows expression in the neural plate to exist in both ectodermal and mesodermal derivatives. The dorsal expression is superficially or in the outer ectodermal layer, but weak expression is seen deeper in the tissue that will become the somites.
Figure 2. Eyal staining at stage 14/15: Anterior view – (A) Expression A has extended to outline the entire anterior neural plate, while B has migrated posterior and remained deep in expression. (B) Dorsal view – B has elongated along with the length of the embryo, (C) lateral view - A has widened to encompass a larger number of developing sensory placodes, (D) anterior microtome sectioning showing the depth of the B expression into developing somites.

At stage 18/19 (Figure 3), the two areas of expression traced from stage 12 are still present in the same patterns, and a third symmetrical expression has developed. Based in the most anterior portion of the neural folds, expression C is based in the presumptive olfactory region. Expression A’s pattern has matured into outlining the tissue lateral to the neural fold with an addition of a hotspot in the otic (ear) placode. Expression B has maintained its posterior migration, now stretching in parallel lines
down 75% of the dorsal side of the embryo.

Figure 3. Eya1 staining at stage 18/19 – (A) Anterior view – maturation and migration of stains A, B, and C, (B) dorsal view, (C) lateral view, (D) anterior microtome section showing invagination of the otic placode hotspots contained in stain A along with other stain A contained in sensorial placodes, (E) anterior microtome sectioning showing the further development of stain B into deep as well as surface germ layers,
(F) anterior microtome sectioning showing heavy staining of A near the anterior portion of the embryo.

Stage 23/24 embryos show higher differentiation of the three traced areas of expression. As can be seen in Figure 4, expression A has begun to outline the developing eye anlages while highlighting the otic placode and beginning to trace the epibranchial regions. As expression B extended along the dorsal midline of the embryo, the expression closest to the posterior remained solid, while the tissue which expressed the gene earlier has differentiated into lines denoting somites, the muscular structure surrounding the developing spinal chord and backbone. Expression C has condensed into a more defined region marking the olfactory placodes.
Figure 4. Eyal staining at stage 23/24 – (A) Anterior view showing the developing eye anlages surrounded by expressions A and C while B demarcates the elongation of the embryo on the sides of the developing spinal chord, (B) dorsal view showing the division of the B stain among the developing somites of the muscular structure as well as the dark portion of the stain near the back of the spinal chord, (C) lateral view showing the beginnings of the epibranchial regions by stain A, (D) anterior microtome sectioning containing the eye anlages along with small portions of the nasal placodes containing expression C and the developing epibranchial / otic regions containing stain A, (E) anterior microtome
sectioning showing the location of expressions A vs B along with the developing neural tube and spinal chord, (F) anterior microtome sectioning showing the somatic expression B in the posterior portion of the embryo.

Stage 28 shows the same patterning in Figure 5 with maturation as the stage 23/24 embryo. Expressions continue in the epibranchial region, the otic placodes, the somites, and the olfactory placodes.
Figure 5. Eya1 staining at stage 28 – (A) anterior view showing the positioning of the expressions along with the development of the cement gland below the nasal placodes which contain expression C, (B) dorsal view which reveals the same continuing patterns of expression positions as earlier stages, (C) lateral view which shows the maturation of expression A’s patterning in and around the epibranchial region and a well developed hotspot in the otic placode, (D) anterior microtome section showing the relationship of
expression A to expression C, both being deep in the endoderm at this stage. (E) anterior microtome section showing great detail of expression A in and around the otic placodes, (F) anterior microtome section showing the weak expression of A around the eye anlanges and the relationship of the epibranchial regions to the otic placode, (G) anterior microtome sectioning showing the increased intensity of expression B near the posterior of the developing somites next to the neural tube and spinal chord.

Stage 34 continues the same patterning with slight modifications (Figure 6). The anterior somite expression developed hotspots at their ventral tips. The anus region, possibly remnants of the blastopore shows the only completely new expression. All other expressions continue in previously denoted regions.
Figure 6. Eya 1 staining at stage 34 – (A) anterior view showing a more developed cement gland, the expression C in the nasal placodes and expression A continuing in the otic and epibranchial regions, (B) dorsal view confirming 6-A while showing the hotspot of expression B at the most posterior somites, (C) lateral view showing highly developed expression patterning of A in the epibranchial region and otic placode; expression C with A completely outlines the eye anlage; the expression of B includes the ventral tips of the developing somites and at the hotspot near the posterior end, (D) anterior microtome sectioning
relating the continued development upon the positions of expression A in the epibranchial region and the otic placode, (E) anterior microtome sectioning revealing deep intense expression C in the nasal placodes just above the cement gland, (F) anterior microtome sectioning showing the highly matured otic placode and the surrounding tissue which contains expression A, (G) anterior microtome sectioning showing the posterior hotspot of expression B.

_Xenopus laevis - Expression of Eya 2_

At stage 12 indicates (Figure 7), Eya2 has major expressions in two locations, a deep expression antero-dorsal just lateral to the midline marked as A, and a hotspot located in the region of the future adenohypophyseal placode marked as B. Histology reveals that both A and B are expressed in inner germ layers.
At stage 14/15 (Figure 8), expression B has moved to outline the entire neural crest including staining of the otic placode. Expression A has remained at the dorsal portion of the neural crest. A new expression has developed deep in the center of the neural plate, which combined with expression B has outlined the future eye anlages. All expression is in multiple germ layers and not in the surface tissue.

Figure 7. Eya2 staining at stage 12 – (A) anterior view showing expression A to be located in deep tissue in the dorsal region of the developing neural plate and expression B in the future adenohypohyseal placode, (B) dorsal view showing the placement of expression A, (C) lateral view showing the spatial relationship of expression A vs B, (D) anterior microtome sectioning showing deep levels of expression A about halfway through the neural plate.
Figure 8. Eya2 staining at stage 14/15 – (A) anterior view expression B has outlined the neural crest, expression A has remained in place at the dorsal section of the neural crest, and new expression has developed in the center of the neural plate denoted as expression C; A faint outline of the future eye anlanges is visible, (B) dorsal view, (C) lateral view, (D) anterior microtome sectioning showing deep expression of B around the neural plate, (E) anterior microtome sectioning showing the deep expression of A in the dorsal neural plate.

As can be seen in Figure 9, stage 18/19 staining expression B has matured into a heavy hotspot in the nasal placodal region. Expression C has concentrated into the closing neural groove, and expression A has remained stationary in the dorsal anterior position. Histological analysis in panels D & E reveals expression B has deep expression in the nasal tissue and hotspots developing in the otic placodes.
Figure 9. Eya2 staining at stage 18/19 – (A) anterior view. Expression B has matured into the nasal placode, expression A has remained stationary and expression C has concentrated in the closing neural fold, (B) dorsal view, (C) lateral view, (D) anterior sections showing the depth of expression B in the nasal placode, (E and F) anterior microtome section showing the depth of expression B around the neural plate and developing hotspots in the otic placodes.

In the stage 24 embryos with Eya2 stain show an increased specificity in their expression regions (Figure 10). Expression B has now patterned itself in the highly specific nasal placodes, has outlined the developing eye anlagen and continued up into the otic placodes. Expression A has remained strongest in the anterior region of the developing dorsal nerve ridge and has extended in a gradient fashion, diminishing into the posterior dorsal region. Microtome sectioning confirms highly specific nasal, otic,
and somatic expression. Expression C diminished with the closing of the neural tube.

Figure 10. Eya2 staining at stage 24 – (A) anterior view showing the specificity of expression B in the nasal placodes as well as the otic hotspots; expression A has elongated to the posterior of the dorsal ridge, (B) dorsal view showing the gradient of expression A from the anterior to the posterior of the embryo, (C)
the lateral view showing the breadth of expression A, the otic placodes and the expression of B within the nasal placodes, (D) anterior microtome section showing the depth of the tissue expression of B in the nasal placodes, (E) anterior microtome section the otic placode expression and its relation to the expression of B behind the developing eye anlage, (F) anterior microtome sectioning showing expression A along the sides of the dorsal ridge.

Figure 11 shows the changes occurring in the expression patterns of Eya2 from stage 23/24 to stage 28. Expression B has continued to follow the developing nasal placodes, has present weak expression in the eye anlages and the pharyngeal pouches, and has continued to develop within the otic placode. Expression A has diversified showing some patterns resembling expression B of Eya1 by elongating along the entire region of developing somites, with hotspots at their ventral tips. Microtome sectioning shows an increased intensity in the deep tissue expression of the nasal placodes, continued expression in the otic placodes and the expression in the somites.
Figure 11. Eya2 staining at stage 28 – (A) anterior view showing the continued presence of expression B in the nasal placode, (B) dorsal view showing expression B in the nasal and otic vesicles; also showing the gradient of expression A from the anterior to the posterior of the embryo, (C) lateral view showing the same as 11-B, (D) anterior section showing the continued interior intensity of expression B in the nasal placodes, (E) anterior section showing expression B weakly in the pharyngeal pouches behind the eye anlagenes and the strong expression in the otic placodes, (F) anterior microtome sectioning showing the diminishing expression of A into the posterior of the embryo.
Eya2 expression from stage 34 is examined (Figure 12). All of the expressions from stage 28 remain in the same areas, with no notable differences. Histological analysis reveals no changes in the expression of A or B in any of the germ layers.
Figure 12. Eya2 staining at stage 34 – (A) anterior view revealing the continued presence of expression B in the nasal placodes, (B) dorsal view which shows the spatial relationship of A and B along with their presence in the dorsal somites and the nasal and otic placodes, (C) lateral view revealing the positioning
and gradient of expression A and the various locations of expression B, (D) anterior section containing nasal expression, (E) Otic placode expression along with expression B in the pharyngeal pouches, (F) expression A in the posterior of the embryo along the somites.

*Xenopus laevis – Expression of Eya3*

Stage 12 Eya3 stained embryos (Figure 13) show expression in the tissues around and within the midline of the developing neural plate labeled as expression A. The outer tissue layers have been partially removed due to protease K treatment designed to increase the penetration of the *in situ* hybridization probe. Potential hotspots exist near the otic and lens placodes labeled as B.

*Figure 13. Eya3 staining at stage 12 –* (A) an anterior view which shows expression A following the outline of the developing neural plate, while a potential hotspot marked B is located in the future otic and lens placodes (B) a lateral view showing the same expressions, and (C) anterior sectioning approximately half way through the developing neural plate showing the strength and depth of expression A.

Stage 14/15 Eya3 expression shows a continued trend of strong expression outlining the neural crest labeled A. There is continued expression near the anterior-dorsal portion of the neural crest, which is no longer considered to be in the otic placode,
but as precursors of the somite development marked as B. A developing expression in the neural plate is marked as C. The expression in the neural crest has two weak spots corresponding to the future eye anlages. Histology shows the expression of A and B to be deep in the developing neural crest.

Figure 14. Eya3 at Stage 14/15 – (A) anterior view showing expression A outlining the neural crest with weaker expression near the future eye anlages, expression B in the future somitic tissue, and deep expression C in the neural plate, (B) dorsal view showing the future somitic expression, (C) lateral view, (D) microtome selection showing neural crest expression, and (E) showing deep somitic expression.

Eya3 at stage 18/19 (Figure 15) shows a wider expression of A as the neural fold is closing which encompasses the entire anterior face of the embryo. Expression B has remained constant at the top of the neural crest, and has also widened.
Figure 15. Eya3 at stage 18/19 – (A) anterior view showing the widening of expression A as the neural fold closes while B remains at the anterior-dorsal region of the neural crest, (B) dorsal view, (C) lateral view, (D) microtome showing deep expression of A near the neural folds, and (E) microtome showing the somitic expression of B in deep tissue.

At stage 23, Eya3 expression (Figure 16) encompasses the entire anterior portion of the embryo except for the developing ventral anterior cement gland. Expression A includes the nasal placode, the eye anlanges and the otic placodes. Expression B is distinctly now in the developing pharyngeal pouches.
At stage 28, Eya3 expression has similar patterns as previous stages, with addition of minor somitic expression. Expression A in Figure 17 still encompasses the nasal placode, the eye anlagenes and the otic placodes. Expression B continues to be present in the pharyngeal pouches. A new expression D has developed among the maturing somites. Microtome sections show consistent depth of Eya3 expression in the anterior expression A region as well as expression B in the pouch regions.
Figure 17. Eya3 expression at stage 28 – (A) anterior view of the continued expression of A in the nasal placodes, (B) lateral view showing expression A presence in the eye anlages and the remainder of the anterior embryo while expression B remains in the pharyngeal pouches. There is faint markings of Eya3 in the maturing somites denoted as expression D. (C) microtome section of the anterior portion of the embryo through the eye anlages showing a consistent depth of Eya3 expression, (D) microtome sectioning through the pharyngeal pouches confirm the depth of expression B.

During stage 34 of Eya3 the only notable difference that has occurred from stage 28 is the continued development of expression D in the maturing somites. All the anterior embryo staining has remained the same.
Figure 18. Eya3 at stage 34 – (A) anterior view of expression A continuing in the nasal placode, (B) lateral view showing identical expression patterns of A and B with a maturation of expression D into a constant line among maturing somites, (C) microtome section of the nasal placodal area, showing deep consistent expression A without including the cement gland, (D) microtome sectioning through the eye anlages showing a consistent depth of expression A, (E) microtome sectioning through the otic placode and the pharyngeal pouches showing expression B and the beginnings of expression C.

Identifying the Xenopus tropicalis Eya4

The first step was to take protein sequences from mouse and human Eya 4 and BLAST them to the EST clones databases of Xenopus laevis and tropicalis at the Sanger Center. High level matches were found, but the process of back-blasting revealed that all of the notable matches were conserved protein sequences with other members of the Eya family (1-3) (which had already been located and documented), but mostly Eya 3. Using
alignment programming, the mouse and human Eya 4 protein sequences were matched with their counterparts of Eya 3. This alignment found two separate sequences that were unique only to Eya 4 in mouse and human (Figure 19).

Figure 19. Alignment of Human and Mouse Eya3 and Eya4 protein sequences showing the unique sequences available from the Mouse Eya4 position 190 to 229 and from the Human Eya4 from position 214 to 229. The colors were added with the following key: yellow is a completely unique sequence, blue is 2 of the four sequences matching, red is the augmentation of a 2nd set of 2 of the four sequences matching, and green in when 3 of the 4 sequences match.

Searching for these sequences in a database for EST clones would eliminate the possibility of the highest found competitor, Eya 3. Unfortunately, the BLAST results for both *Xenopus tropicalis* and *laevis* showed no matches with high enough relevancy to
pursue further.

It is frequently observed that simple, less complex organisms have fewer genes and more complex organisms have several versions of a particular gene with diversified functions (Hogan, 1996; Krumlauf, 1994). This tentatively explains why *Drosophila* only contains one eya gene while mouse and human contain four homologs. Therefore, the search was guided to look for Eya 4 in organisms, less complex that *Xenopus*, which would support the notion that Eya 4 should exist in Xenopus. Two databases of EST clones were used in this style of search: Zebrafish and Fugu (a small fish indigenous to Japan). The unique sequences of Eya 4 were taken to the Zebrafish database first and no matches of significance were located. Accomplishing the same method in the fugu database located a gene that had not been identified as a working Eya 4, but had been predicted by a sequencing comparison program to be the location of Eya 4 in the genome. Backblasting this result had high frequencies of Eya 1 and 3', but the main matches were Eya 4. These results suggest that Eya 4 should be present in *Xenopus*, an amphibian considered to be more complex than fish.

When the search of EST sequences failed, the next step was to move to the genomic databases, in this case JGI, which contains most if not all of the DNA sequences, including the sections of DNA that are not transcribed as genes. Using the entire mouse Eya 4 sequence, the JGI database dedicated to *Xenopus laevis* and *tropicalis* were BLASTed. High matches from that blast were taken and manually compared to locate matches that contained the unique Eya 4 sequences. Four candidates were found, but two probable matches resulted from this data due to *Xenopus laevis* being
pseudotetraploid which gives two pairs of the same sequence located at different locations within the genome.

These matches from the genomic database were in DNA format, while it is best to BLAST EST clone databases with a protein sequence. Transcription machinery for DNA reads in triplets, which allows for 64 different combinations of three base pairs of DNA to “code” for one of 20 amino acids. A strand of DNA can be read three different ways, each called a reading frame. So the DNA sequences were analyzed manually to locate the correct reading frame by translating all three reading frames into protein sequence and matching them to the original search sequence of the mouse Eya 4. These two protein sequences were BLASTed into the Sanger Xenopus EST clone databases, and a match statistically significant for using genomic DNA as the submitted sequence was located in the *Xenopus tropicalis* EST catalog of $P(N) = 3.2 \times 10^{-6}$. The presence of the Eya 4 in the genomic DNA and the lack of it in the EST clone database results in one of three options. One, the EST clone that contains Eya 4 has not been cataloged into the database. Two, Eya 4 in *Xenopus (laevis or tropicalis)* is not a large factor in the stages of embryological development and activates later in the life cycle as maintenance or as part of the maturation metamorphosis from a tadpole in to an adult frog. Finally three, Eya 4 possibly doesn’t exist in *Xenopus (laevis or tropicalis)* due to this disagreement between databases.

*Locating Eya 1 in Xenopus tropicalis*

This project involved only one series of testing, using a closely related sequence from another member of the genus *Xenopus*. Eya 1 alpha protein sequence from *Xenopus*
*laevis* was taken and blasted into the Sanger Center EST clone database for *Xenopus tropicalis*. The first three matches that had highest priority were backblasted to NCBI which revealed Eya 3 conserved sequences. The fourth match however backblasted to match *Xenopus laevis*, mouse and human Eya 1 sequences. Further testing of alignment shows high conservation of Eya 1 *laevis* to *tropicalis* at positions 1-160 of the protein sequence. Therefore the EST clone AL658918.1 of *Xenopus tropicalis* is most likely the homolog of Eya 1.

**Future Applications of the In Situ and Gene Location Results**

Eya1 is expressed in the earliest stages of the olfactory epithelium development. This could mean that it is involved in the activation or maintenance of the normal developmental cascade which guides the cells in that placode to differentiate normally. This train of thought could be applied to every region where expression of Eya1, 2, or 3 was present in the embryos.

For a specific interest in sensorial development, the presence of Eya1, 2, and 3 in the eye, nasal, otic, somitic and pharyngeal placodes continue to present opportunities for further investigation. Methods of causing a loss of function of the gene within a placode or entire embryo are available. Recording the affects of this loss of function can give insight into the specific nature of the gene’s purpose. Also overexpressing the gene, which causes too much of the encoded protein to be produced, provides another possible way to analyze these genes function.

Comparing the patterns of Eya expression to other genes expressed in the same placode at the same stage could begin to map out the interactions that the produced
protein has with the other constituents available in the developing cell. Of particular interest would be the expression patterns of the genes that are known to interact with Eya such as pax genes (homologs of the Drosophila eyeless gene) and dachshund and the six genes (vertebrate homologs of the Drosophila sine occulis). These studies have begun to answer the questions regarding Eya’s areas of action, but it also provides many more questions that still need to be addressed.

Conclusion

This study represents a gateway into continued highly specific genetic research dealing with sensory developmental embryology. Using the expression patterns presented for Eya1, 2 and 3, specific analyzing of placodal expression analyization will lead to a better understanding of the role these genes play in during the development of Xenopus embryos. Using the possible Eya4 Xenopus tropicalis genomic DNA, in situ hybridization should eventually be possible in Xenopus laevis and Xenopus tropicalis. This is a small step in the larger project of the Grainger lab at UVa seeking to understand the sensorial development of Xenopus African clawed frogs and ultimately the development of human embryos as well.
References


