One Gene Two Eyes?

Some Background and an Update on Data Obtained With Axolotl Embryos Homozygous for the Gene Eyeless

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"Eyeless" is a recessive gene. Embryos homozygous for the trait cannot produce eyes (Figure 1) and, as Van Deusen discovered, are also sterile (Van Deusen, 1973: Dev. Biol. 34:135-158).

induction capability of the mutant archenteron. Results following replacing the dorsal lip of the blastopores of wild-type early gastrulae with dorsal lips from eyeless mutant donors showed that the archenteron roof of the mutant was capable of inducing eyes in wild-type neurectoderm normally. However, replacing the prospective neurectoderm of wild-type early gastrulae with the analogous tissue from the eyeless mutant produced the eyeless mutant phenotype in wild-type hosts. Because of this result and also because there was evidence that the sterility problem of the mutant was caused by a deficient hypothalamus region, Van Deusen concluded that the anterior neurectoderm was the target of the gene eyeless.

In addition to the reciprocal grafting experiments between wild-type and homozygous eyeless early gastrulae, Van Deusen tested the

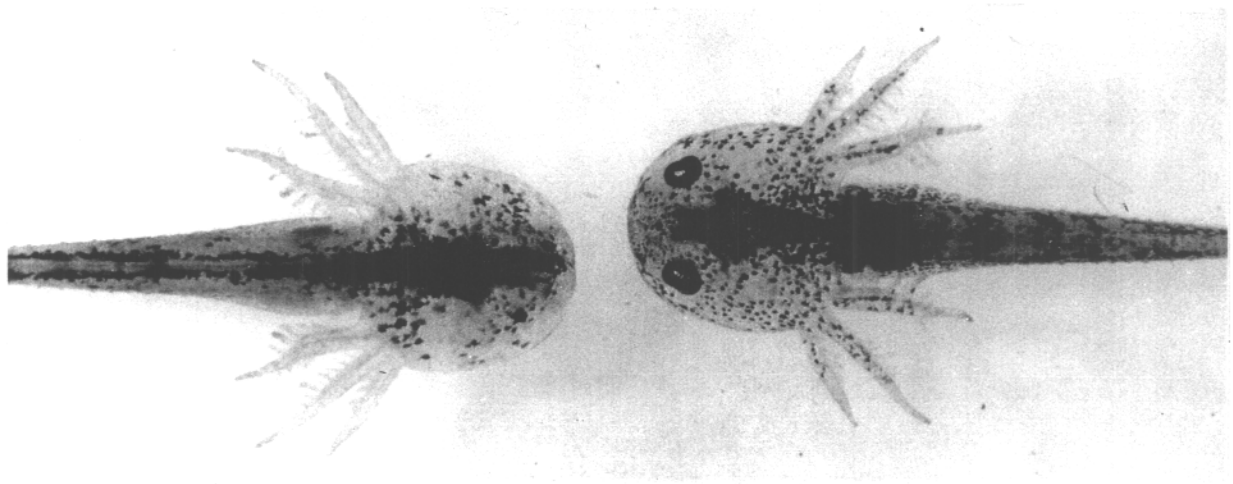


Figure 1

Fortunately, he also found a remarkable way to overcome the sterility problem, namely by replacing the head of the mutant with a head from a wild-type donor! Such head grafts produced fertile homozygous eyeless/wild-type chimeras. From these chimeric parents Van Deusen obtained embryos known to be homozygous for the trait. This allowed him to perform a variety of straightforward experiments using wild-type and homozygous mutant early embryos. First, he tested the eye prospective eye-region of the mutant by injection experiments. He dissociated the animal hemisphere of mutant late blastulae and injected the cells into the blastocoel of hosts that were wild-type for the gene eyeless, but carried the albino marker. This quite gorgeous experiment resulted in albino-type retinas that had smaller or larger black, wild-type spots. Clearly, the black cells in these mosaic albino-wild-type retinas originated from the eyeless mutant donor. Van Deusen speculated

that the sensory component of the retina, but not its pigmented layer, might be the target of the gene eyeless.

Some history. For about ten years my friend H.R. Kobel and I tried to produce adult "frogs" (*Xenopus*) following replacing the egg pronucleus with the genome of various fully differentiated cells. All we obtained were sick larvae that did not make it to the feeding stage. This was also true for the results I had obtained with nuclei from erythroblasts in 1978 (Dev. Biol 65:271-284). I brought these results with me from Geneva, Switzerland to Bloomington Indiana. The paper was written in Bloomington while on the look out for some new, perhaps less frustrating work.

While familiarizing myself with the various mutants available in the collection of the Axolotl Colony, I was quite intrigued by the eyeless mutant. One gene one enzyme—may be. But one gene two eyes?? Since Van Deusen had found that the mutant was not capable of forming (sensory) retina, could its epidermis at least form a lens? In order to stimulate the epidermis of the mutant to form lenses, I decided to graft prospective optic vesicle from wild-type donors under flank epidermis of mutant recipients. This approach was clearly based on the experiments published by Lewis in 1904 (J. Anat. 3:505-536). His results were commonly interpreted to provide evidence that ectopic lenses could be induced following grafting of prospective optic vesicles under flank epidermis, for example.

Since there were no chimeric eyeless/wild-type animals left in the Axolotl Colony, the Lewis experiment had to be performed with spawnings from parents heterozygous for the mutant gene. Obviously, only 25% of the embryos in such spawnings were homozygous for the trait. I decided to perform the grafts at the stage when the neural folds just close. At this stage of development, however, I was unable to identify the homozygous eyeless neurulae and separate them from their wild-type (or heterozygous) sibs. To overcome the difficulty of not knowing the genotype of the embryos, two neurulae were randomly selected from spawnings obtained with heterozygous parents to form a pair. One partner was used as the donor and the other as the recipient. The excision of the prospective optic region was performed unilaterally. The rationale was to use the unoperated side for identification of the genotype, once the eyes had developed in wild-type larvae.

The answer to the question whether homozygous eyeless embryos could produce a lens was a somewhat ambiguous yes. Lenses did indeed form in mutant recipients (5%), but not as frequently as in wild-type/wild-type controls (34%). There was, however, an answer for a question that wasn't asked. Since the genotype of the donor and the recipient neurulae was unknown at the time of grafting, prospective eye regions of embryos that later were revealed to be homozygous for the gene eyeless were also grafted under the flank epidermis of wild-type hosts. Surprisingly, the mutant material produced eye-cups in wild-type hosts almost as frequently (86%) as did the wild-type controls (94%). However, the eye region of the wild-type donors seemed to have more difficulties differentiating under the flank epidermis of the mutant (74%). Therefore, I argued that the epidermis of the mutant could be a possible target of the mutant gene. (In an effort to reconcile this interpretation with Van Deusen's opinion that the neurectoderm was the target of the eyeless gene, I think I found evidence that his neurectoderm grafts could have been contaminated with epidermis. For details see Fig. 4 in Brun, 1978, Amer. Zool. 18:273-279).

To generate further evidence that the epidermis of the mutant actually was involved in causing the eyeless mutant phenotype at least partially, the epidermis covering the prospective eye-region of the mutant was replaced with flank epidermis from wild-type donors. In 3 out of 12 such grafts, complete eyes formed in the mutant *in situ* (25%). I concluded that the epidermis might indeed be a target for the mutant gene (Brun, 1978, reference above).

A year later, a TEM/SEM study of the eyeless mutant was published that pointed towards several differences in the developing eye region of wild-type and eyeless mutant embryos (Ulshaver and Hibbard, 1979, Anat. Embryol. 156:29-35). For example, mesenchyme cells filled the space between the forebrain and the overlying epidermis in mutant but not in wild-type embryos. This opened up the possibility that these cells might inhibit physical and/or biochemical inductive interactions between the neural tube and the overlying epidermis, necessary for optic vesicle formation. Since these head mesenchyme cells are neural-crest-cell derivatives, the observations provided evidence that the neural crest might be a target of the eyeless mutant gene. This led to the question whether or not wild-type neural crest, grafted into the eye region

of the mutant, could rescue eyelessness.

To perform these neural-crest grafts, Spemann's well-known fate map of the prospective eye tissues was used (Figure 2 from Spemann, 1938, *Embryonic Development and Induction*, Yale Univ.P.).

Figure 2
From Spemann, 1912/38

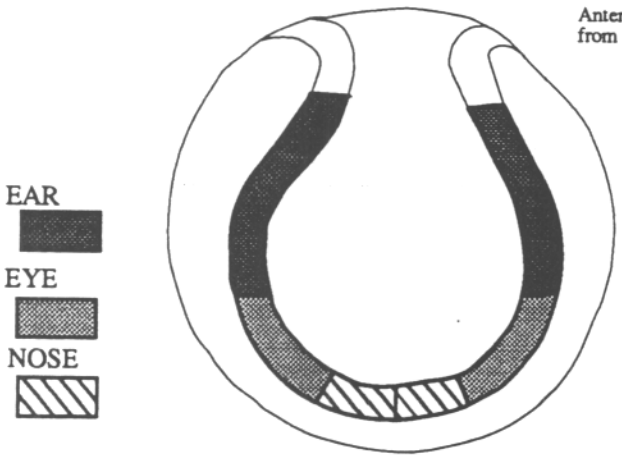
The result was that unilateral homotopic grafting of antero-lateral neural fold from wild-type donors into mutant hosts produced eye rudiments quite frequently. I could, however, not exclude the possibility that the eye structures that formed in the mutant larvae originated from the wild-type donor material, not from the tissues of the mutant. By using the albino/wild-type marker system, I found that the homotopic neural crest grafts were indeed contaminated with prospective retina. Was Spemann's fate map wrong? The map suggested to me that the prospective retina was still in the anterior neural plate at the time the antero-lateral neural folds had formed. In the paper in which the fate-map was first published, Spemann warned about precisely this difficulty in locating the prospective optic tissues. He knew of course that the prospective retina was in the anterior neural plate only before the neural folds form (Spemann,1912, Zool. Jahrbuch, allg. Zool., 32:1-98). As the folds appear, the eye primordia move from the neural plate into the rising neural folds (Brun, 1981, Dev. Biol. 88:192-199). In a nutshell, the artist who drew Spemann's fate-map over emphasized the neural folds.

To avoid grafting neural crest material contaminated with wild-type eye tissues into eyeless mutant recipients, I therefore decided to perform heterotopic grafts. The rationale was to avoid neural crest grafts that were contaminated with prospective retina. For this reason I excised neural crest material from the posterior prospective ear regions, not from antero-lateral positions where the eye tissues were located.

To perform these neural crest transfers from wild-type donors into eyeless mutant hosts, embryos from heterozygous parents were again randomly selected to form pairs. Part of the prospective ear region was excised from a donor embryo at a stage when the posterior neural folds had just formed. This neural fold tissue was then implanted into the antero-lateral area of a recipient at a time when the folds had not yet formed in these regions (Stage 14+, Schreckenberg and Jacobson, Dev. Biol. 42:391-400). As the folds would form in this area, the eye primordia of the eyeless mutant would move from the anterior neural plate into neural fold now containing wild-type neural crest cells because of the wild-type graft. The operation was again performed unilaterally to identify the genotype of the partners in each pair after the eyes had formed in wild-type larvae.

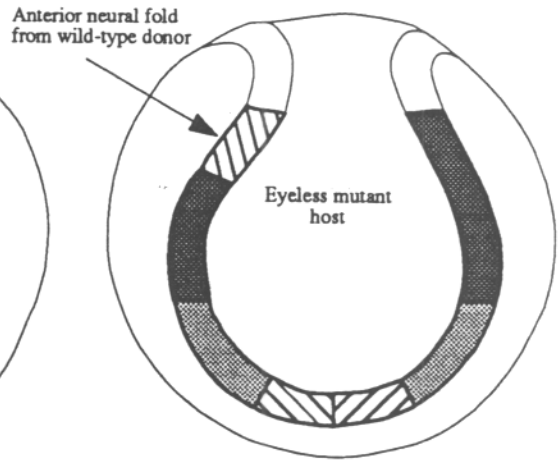
In contrast to the previous homotopic neural crest grafts, two such heterotopic grafting experiments produced no eye structures in eyeless mutant recipients!

The heterotopic grafting experiments just described left a wound in the posterior neural fold of the donor. There was also a piece of antero-lateral prospective neural fold left over in the operation dishes that had to be excised from the recipient to make room for the graft. In a third experiment I decided (don't ask me why!) to graft the anterior tissue from the "recipient" back into the wound left in the "donor." Both partners became "donors" and "recipients" in this way. After the eyes had formed, I recorded the genotype of each larva, using presence or absence of eyes. The overall result was that fewer than 25% of all the operated larvae showed the eyeless mutant phenotype (although larvae with small eyes and eye rudiments were present). I could



Fate-map of the prospective head sense-organs.

Figure 3



Antero-posterior (a-p) graft.

Figure 4

hardly find the combination: anterior neural fold from wild-type implanted into posterior neural fold of mutants. [Please see Figure 3, which is a fate map of the head sense-organs based on Carpenter, 1937; Zwilling, 1941; and Brun, 1981. Figure 4 illustrates the antero-posterior (a-p grafts).]

In further experiments I found that out of a total of 75 operated pairs, the just mentioned combination was actually observed only three times. If one assumed that the operations had no effect on the phenotypes, 14 such pairs (3/16 x 75) should have been present. I concluded that anterior prospective neural fold tissues from wild-type grafted into posterior neural fold of mutants was capable of rescuing the eyeless mutant phenotype at a rate of 79%. In controls, the situation was the following: posterior neural fold from wild-type grafted homotopically into mutant hosts: 49% rescue; anterior neural fold from wild-type into anterior neural fold of mutants: 8% rescue; whereas posterior neural fold from wild-type into anterior neural fold of mutants had no effect. I concluded that the eyeless mutant phenotype might be generated by an antero-posterior morphogenetic system out of balance.

These new results provided additional evidence that the eye region of the mutant was capable of eye-formation but inhibited to differentiate. That the brain area of the mutant was capable of forming eyes had already been the main result of the analysis published in 1978. However, a new, quite intriguing result of the antero-posterior grafting experiment was that following unilateral operations on the neural folds of the mutant, eyes were observed not only on the operated side of the larvae but also on the unoperated, contralateral side. Unilateral grafting had a bilateral effect (Brun,1987, J. Neurogenetics 4:29-46)!

Shortly after this paper became available, Cuny and Malacinski's publication entitled "Axolotl retina and lens development: mutual tissue stimulation and autonomous failure in the eyeless mutant retina" (1986, JEEM 96:151-170) came out. They analyzed the behavior of wild-type and eyeless mutant tissues *in vitro*. Cuny and Malacinski (1986) found that almost all prospective retina tissues from wild-type neurulae cultured together with wild-type epidermis differentiated retina (95%), whereas no retina developed *in vitro* from the analogous tissues of the mutant. If neural plates from eyeless donors were co-cultured with epidermis from wild-type donors, two out of six cultures formed neural retina (33%), one of which also continued to

differentiate pigmented retina. They concluded from these results that the neural plate of the eyeless mutant was intrinsically defective to form retina.

This interpretation was quite surprising to me because one third of their cultures actually did produce retina if cultured with epidermis from wild-type (their Figure 9, line 2). In addition, already Van Deusen (1973) had shown that injection of cells from the blastocoel roof of eyeless mutant blastulae into the cavity of wild-type host blastulae could form pigmented retina in larvae carrying the albino marker. Data that I published in 1978 also showed that the prospective eye region of the mutant could differentiate normally. In my opinion, the striking difference between retina formation in wild-type cultures (95%) and retina differentiation in mutant tissues culture *in vitro* (0%) probably does not provide evidence for an autonomous failure of the mutant neural plate to form retina, but might be the result of a deficient eye promoting process in the mutant. Because of abnormal microenvironmental factors, the process leading to the determination of the prospective eye tissues might be suppressed. Therefore, the tissues of the mutant might not be capable of differentiating autonomously *in vitro*. In contrast, in the tissues of wild-type embryos, the determination process probably started already at the gastrula stage. Therefore at the time the cultures are prepared at the beginning of neurulation, the wild-type tissues are fully determined and therefore capable of differentiating *in vitro*.

Interestingly, the epidermis seems to be somehow involved in the process: mutant neural plates cultured with mutant epidermis cannot form retina, whereas neural plates from mutant donors cultured with wild-type epidermis form retina in about one third of the cases. This quite clear-cut result obtained *in vitro* by Cuny and Malacinski (1986) fits together with the results *in vivo*. Following grafting of epidermis from wild-type donors over the eye region of mutant hosts, eyes did form in the mutant (Brun, 1978).

It had been quite apparent for some time that the precision of the experimental designs could only be improved if embryos of known mutant genotype became available again. Not knowing the genotype of the embryos at the time of experimentation forced a statistical approach comparing expected frequencies of the mutant phenotype with actual observed ratios. The sterility problem of homozygous animals had to be solved by using Van Deusen's method to generate fertile mutant/wild-type chimeras.

In a rapid communication I reported the results obtained with just one such spawning from mutant/wild-type chimeras. The only chimeric male that I had at this time died shortly after the experiment was performed. Two main results were obtained in this experiment. Various antero-posterior neural fold rearrangements produced retina in the eyeless mutant *in vivo*, and unilateral operations had a bilateral effect. Eyes did not form only on the side of neural fold rearrangements but also on the non-operated, contralateral side (Brun, 1990, J. exptl. Zool. 254:107-113).

Since the above mentioned publication, the chimeric animals in my lab have produced over a dozen homozygous eyeless mutant spawnings. Experiments performed with these embryos have made it quite clear that excision of posterior neural fold (prospective ear region) produces eyes more frequently than excision of anterior neural fold (prospective nose area). This difference is statistically significant. Furthermore, the experiments with these homozygous eyeless mutant embryos confirm that unilateral neural fold operations frequently result in bilateral eye formation. Interestingly, the eye that forms on the side from which part of the posterior neural fold (part of the prospective ear region) was excised is bigger than the one on the contralateral, unoperated side (Figure 5). This also statistically significant difference in eye size disappears, however, if the posterior neural folds are excised bilaterally. In this case, the emerging eyes are of similar size. A manuscript reporting on these recent data is in preparation. An abstract has recently been published in the American Zool. 30, 1990, #471.

Is it possible to explain all the data obtained by the various investigators with the eyeless mutant axolotl from just one point of view? Thanks to data originally obtained with *Drosophila* embryos, but more recently also with embryos from vertebrates especially in the mouse and *Xenopus* systems, a hypothesis explaining the eyeless mutant phenotype might be attempted. There is good evidence that the antero-posterior organization of a vertebrate embryo, including its developing brain, depends on the proper activation of homeobox genes (Hox), paired-box (pax) and POU genes (see Kessel and Gruss, 1990, Science 249:374-379). Boundaries between regions of the brain very likely develop because

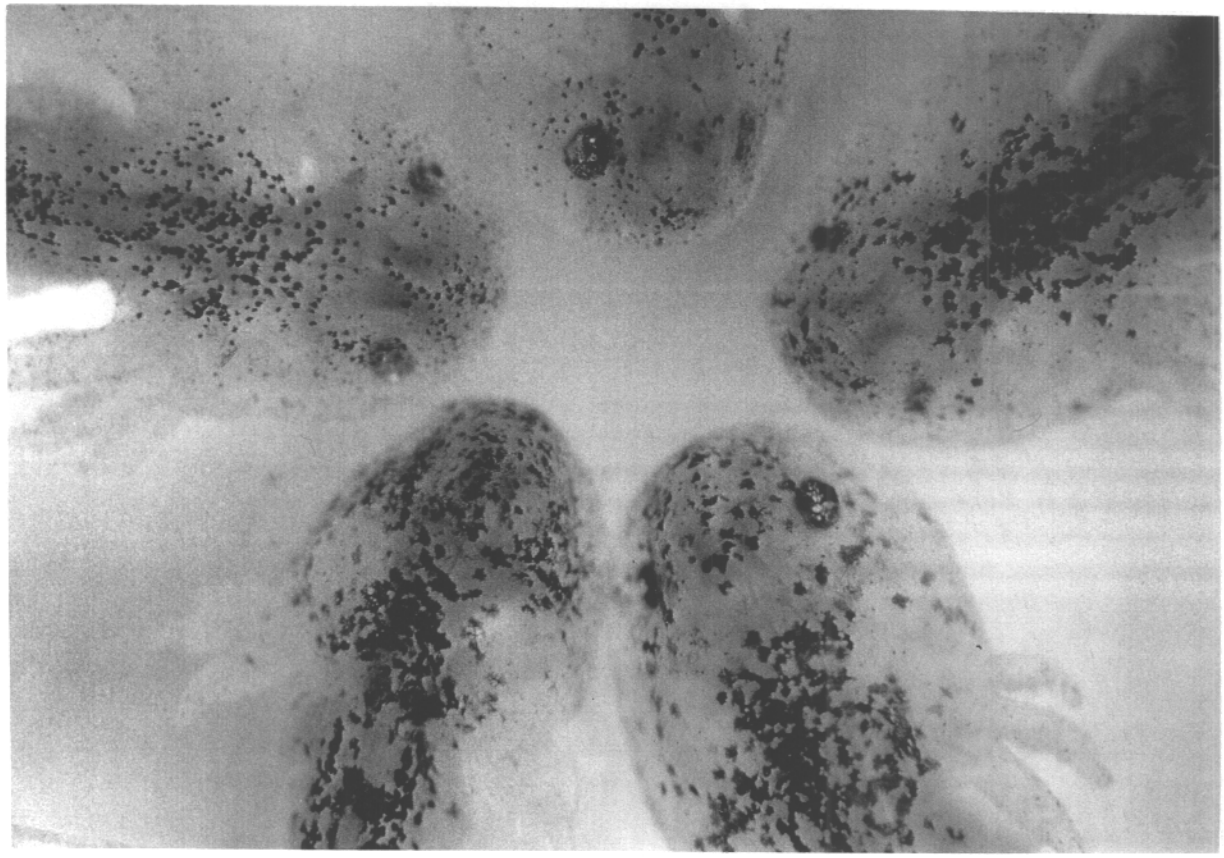


Figure 5. Larvae homozygous for the gene eyeless from which part of the prospective ear region on the right side was excised at the beginning of neural fold formation (stage 14+).

of differential activation of such genes that specify regions. These gene products, probably their ratios, are involved in the specification of brain segments.

These position specifying genes might be turned on selectively by various concentrations of a diffusible morphogen, such as retinoic acid (see: Dolle et al., 1989, Nature 342:767-772). For example, a particular Hox gene might be turned on by a low concentration of retinoic acid, whereas other position specifying genes might turn on at higher concentrations of the morphogen. In short, there might be a gradient of a diffusing substance in the developing neuroepithelium. This gradient might be formed by a morphogen source in posterior areas and a sink in anterior regions. The proper antero-posterior activation of brain segments specifying genes might depend on the correct antero-posterior concentration of the gradient. In the eyeless mutant, this gradient might be abnormal, too steep for example. Perhaps posterior brain regions overproduce the morphogen. This might result in the full or partial failure of the eye-region specifying control gene(s) to be turned on. As a consequence, the diencephalon of the brain might be too small. This fits together with the finding that the hypothalamo-hypophyseal system in the eyeless mutant is underdeveloped (Eagleson and Malacinski, 1986, Anat. Rec. 215:317- 327). This in turn might cause abnormal hormone production, thereby preventing maturation of the germ-cells in the mutant (Van Deusen, 1973).

It will be interesting to find out whether the genetically homozygous eyeless mutant larvae that developed eyes following operations on their neural fold at the neurula stage will develop into fertile adults. At the time of this writing there are no definitive indications of whether or not this might indeed be the case.

I would like to conclude this update on the analysis of the eyeless mutant by extending an invitation to anyone interested in homozygous eyeless mutant embryos. At this time I have enough spawnings from mutant/wild-type chimeras. I look forward to sharing the material!