Chapter 4: Experiments

> Our real teacher has been and still is the embryo who is, incidentally, the only teacher who is always right.
>
> - Viktor Hamburger, 1968

Introduction

When it comes to modeling biological systems, it is hardly ever the case that the modeler and the experimentalist are the same person. Instead, the work is usually done in collaboration. This leads to difficulties in that the modeler and the experimentalist don't always understand the intricacies and sticking points of the other discipline. Another problem is that the data used to build the model is not always the data ideally desired. For example, the binding coefficients listed in Table 3.1 were measured in cell cultures and not in chick or mouse. These facts lead the author to design and perform experiments relevant to the *Hox* system under investigation. Not only would the experiments be focused on testing and clarifying elements of the *Hox* model, they would also allow for better understanding of the problems and pitfalls in performing experiments in the biology lab.

In order to build the model it was necessary to make several assumptions. This chapter highlights one of those assumptions and describes an experiment that was performed to investigate and clarify an aspect of the model, namely the response of *Hoxa1* to retinoic acid (RA). This was accomplished by introducing a perturbation to the normal distribution of RA in the embryo.

The experiment described in this chapter was not the only model related experiment designed and pursued. In Appendix A, the reader will find the description of another experiment that was pursed. However, it turned out to be much more difficult than initially thought. This is not a rare occurrence in biology, and was one of the most important lessons about lab work that the author learned. While it is not possible to draw any definitive conclusions from the experiment in Appendix A, a great deal of work was done in paving the way for a continued investigation.

Before describing the retinoic acid perturbation experiment, there is a brief digression into the development of a method that made the experiments easier to perform.

Vital Stain

Any sort of work on early chick and quail embryos is complicated by the fact that they are nearly transparent and very difficult to see against the yellow yolk. By HH stage 9 (Figure 3.1 7) there are enough signs in the surrounding tissue (the position of the area opaca for instance) to enable harvesting, but in order to easily perform other work including electroporation (described in Appendix A) or bead implantation (described below), something needs to be done in order to see the embryo.

A typical solution is to use a mixture of 10% of India ink in a balanced salt buffer, and when this is injected beneath the embryo there is enough contrast to easily see the embryo. The problem with this mixture is that India ink is known to be toxic, especially to younger embryos. If it is used in situations where the eggs are placed back into the incubator for more than a few hours, there will always be a decrease in viability. This is especially true after manipulation that is hard on the embryo, like electroporation (in

which electricity is delivered to the embryo) or bead implantation (in which the egg is open for a long time and the neural tube is ripped). Despite these known problems, there were no readily identifiable solutions presented in the literature, but an inquiry of other laboratory members suggested a possible solution. It came in the form of an ancient stash of pale blue food coloring. Using this as a vital stain increased the survival dramatically, but the contrast was poor and it was still very difficult to see the embryo. Nonetheless, this suggested that food coloring might be a good solution. Two different sources of food coloring were acquired; powder from Spectra Colors Corp, and liquid from the local supermarket. Along with India ink, these were used in an experiment to compare the resulting contrast and subsequent embryo viability.

Fertile chicken eggs from a local supplier (AA Laboratories) were incubated at 38° C until stages 4-6, usually between 36 and 40 hours. After removal from the incubator, the eggs were rinsed with 75% alcohol and 3 ml of albumin was removed. The egg was windowed and a few drops of Hanks' Buffered Salt Solution (HBSS) were added to the embryo to keep it moist. Approximately 100 mL of vital stain was injected under the embryo, and the resulting contrast was noted. The egg was then resealed with packing tape and replaced into the incubator. The embryos were harvested after 24 hours and assayed for viability. The results of this experiment are summarized in Table 4.1 below. It should be mentioned that eggs are not always resealed successfully, and some of the morphology problems and deaths are certainly due to the embryo drying out. This was a problem that applied to all of the experiments equally, and so these numbers were not separated out.

Solution used	# Injected	Viable	% Viable
10% India ink in HBSS	9	7	78 %
Dec-a-Cake	7	1	14%
10% Dec-a-Cake in HBSS	7	5	71%
Stock pale blue	8	8	100%
10% Spectra Red #40 In HBSS	6	5	83%
10% Spectra Blue #1 in HBSS	5	5	100%
10% Spectra Blue and Red in HBSS	8	7	88%

Table 4.1 Vital stain results. Viable is defined as embryos that are alive and look to have normal morphology. All of the solutions were diluted or mixed with HBSS. India ink actually faired better than expected. This was probably helped by the use of a freshly opened bottle: there is anecdotal evidence that using old ink decreases viability. The India ink solution also affected the surrounding tissue of an embryo, and there were often clumps of ink globules visible beneath the embryo. The Dec-a-Cake solution was the worst of the bunch, almost certainly due to the preservatives included, and while the diluted mix was much better than straight, it is still on the same level as India Ink. The stock pale blue provided excellent viability, but the contrast was very poor. The different mixes of the Spectra F.D&C. food coloring all resulted in good viability, and the contrast from the Blue and Red combination was very strong.

Since this experiment, the author has used food coloring exclusively for all experiments and the viability has been much better. In addition, the use of food coloring as a vital stain has collected a steady following in the Fraser and Bronner-Fraser laboratories and a half dozen people use it regularly. It has also been used at the Stowers Institute for Medical Research, and a member of the House Ear Institute used it to

successfully perform an experiment that was otherwise unsuccessful using India ink (A. Collazo, personal communication).

Having to solve the problem with the vital stain was just one of the many examples of the issues that need to be resolved before the experiment of interest can be performed.

Retinoic Acid Bead

As mentioned in Chapter 3, the act of building the model caused a shift in thinking about how the system might become initiated. It became clear that a constant source of RA is not needed, and in fact a constant source leads to simulation results that are in disagreement with laboratory results. To better understand the connection between RA and *Hoxa1*, an experiment was undertaken to introduce RA into the system and determine the effects on *Hoxa1* expression. *Hoxa1* was picked as the assay because it is the first *Hox* gene to appear and unpublished work has shown that culturing embryos in the presence of RA causes a broad pattern of expression (R. Krumlauf, personal communication). In addition, RA appears to be the sole input to *Hoxa1*, as opposed to *Hoxb1* which also has a retinoic acid response element, but is also cross regulated by *Hoxa1* and auto-regulated.

There are a variety of methods for introducing RA into a biological system. These include oral administration (Pasqualetti et al., 2001), bathing an embryo in a culture medium containing RA (Godsave et al., 1998), or using a bead soaked in RA (Eichele et al., 1984). Using a bead is particularly attractive as it provides an effective way to deliver a local release. But the most important aspect of the bead is the local

delivery helps create an artificial gradient that can be used to test the connection between *Hoxa1* and RA, and, in particular, whether the implementation chosen for the transcription of *Hoxa1* is supported.

Embryos

Instead of using eggs from the local supplier, fertile pathogen free chicken eggs were acquired from Charles River Laboratories. The change in egg supplier occurred because eggs from the local supplier were unreliable: many were unfertilized, and the development was inconsistent. Before the change, a great deal of time was spent dealing with eggs that were substandard. On a typical day only 2 dozen of 5 dozen eggs pulled from the incubator would be usable. The River Laboratories eggs were significantly more expensive (~$20 a dozen vs. $3.50 a dozen for AA Laboratory eggs), but they were consistently reliable, both in fertility and development time. This was yet another object lesson on the difficulty of laboratory work.

The eggs were incubated at 38°C until the proper stage of development, usually between 30 and 40 hours. The eggs were rinsed with 75% alcohol and 3 ml of albumin was removed. The eggs were windowed and a solution of .1% food coloring (equal amounts of FD&C Red #40 and FD&C Blue #1 from Spectra Colors Corp.) in HBSS was injected beneath the blastoderm to provide contrast.

Bead Preparation and Implantation

AG1-X2 ion-exchange resin beads (mesh size 200-400, for an effective size between 50 and 150 μm) were purchased in chloride form from BioRad. They were

rederivatized to formate form by inserting them into a column and rinsing with three bed volumes of1M formic acid. They were then rinsed with water until the wash was approximately pH 5. All-trans RA was purchased from Sigma corporation and a 10^{-2} M solution of RA in DMSO was made fresh each day. This was subsequently diluted to the working concentration of 10^{-3} M. It was learned through the course of these experiments that RA degrades very quickly, even when stored under argon in a -20°C freezer. The formate beads were soaked in a 10 μ L drop of RA solution for 20-40 minutes, then rinsed in a 10 μ L drop of tissue culture media 3 times for 5 minutes each. This final step helps remove the DMSO from the beads, and the red dye in the tissue culture media stains the beads, which in turn helps make placement easier. The beads were then implanted into the hindbrain or midbrain of an embryo using the technique described in the caption of Figure 4.1 below.

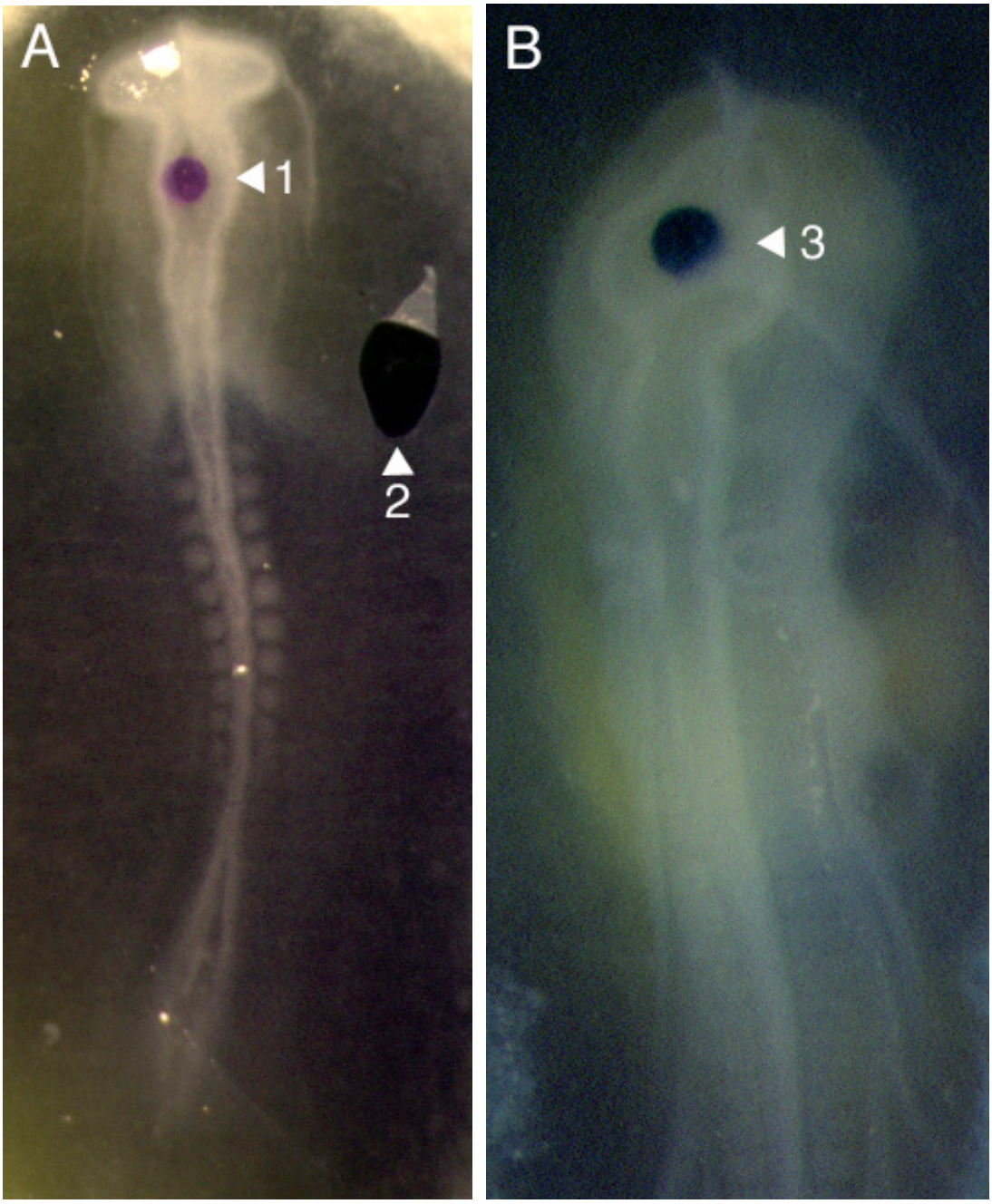

Figure 4.1 Bead implantation. (A) A 4x view of a stage 9 embryo with a bead (marked by 1) implanted into the midbrain of the embryo. To place the bead, a hole was torn in the vitelline membrane (marked by 2) using an electrolytically sharpened tungsten needle. The needle is then used to incise a small section of

the (potentially) closed neural tube at the mid and hindbrain level (see Figures 3.1.7 through 3.1.9). The bead is plucked from a dish with a pair of #5 forceps and placed into the hole then pushed under the vitelline membrane. After that, the bead is pushed from above the vitelline membrane into the neural tube and maneuvered into the desired position. Due to the surface tensions of the fluid, it is not possible to actually place the bead into the right position and expect it to stay there, especially if the vitelline membrane is completely removed. The white speck just anterior to the bead is a piece of eggshell that fell into the work area. **(B)** This 5x picture of a different embryo was taken 8 hours after bead implantation. The embryo is now at HH stage 12 and is starting to turn, but the bead (marked by 3) is still clearly visible in the midbrain.

After the bead implantation, the eggs were returned to the incubator for 6-8 hours. The embryos were then harvested and fixed in 4% paraformaldehyde solution either overnight at 4°C or for 1 hour at room temperature. After the paraformaldehyde treatment and a rinse in phosphate buffer saline (PBS), the embryos were dehydrated through a series of methanol/PBS washes, and were placed in a –20° C freezer for storage. Embryos stored in this manner can be kept in a freezer for upwards of a year, but in this case they were not in the freezer for more than a couple weeks. After storage, the embryos were re-hydrated with through a series methanol/PBS washes and subjected to *in situ* hybridization.

In situ hybridization is a molecular biology technique that allows the identification and localization of a particular nucleic acid sequence, in this case a specific

strand of messenger RNA. Recall that the Central Dogma of Molecular Biology states that mRNA is the ribonucleic acid transcribed from DNA and is the template from which a protein is translated. One method of detecting the mRNA for a particular protein in the organism is to create a probe: a complementary mRNA strand with specially modified nucleic acids. If the mRNA of interest is present in an organism, the probe will stick to it. The excess probe is then washed away, and an antibody to the modified nucleic acids is added to the mix. Finally, a dye that reacts to the antibody is added and the result is a visual readout on the location of the mRNA of interest.

Despite the brevity of the description, this process takes 4 days to complete, and so only one experiment can be performed a week. The complete protocol used can be found in Appendix C, and is a modified version of one described in the literature (Wilkinson, 1992).

The probe used for the assay was *Hoxa1*, and typical results are shown in Figure 4.2. The most striking feature of the expression pattern in Figure 4.2B is that there appears to be a gradient of expression in section of the neural folds marked by the arrows. This is, in fact, a real measurable gradient as seen in Figure 4.3.

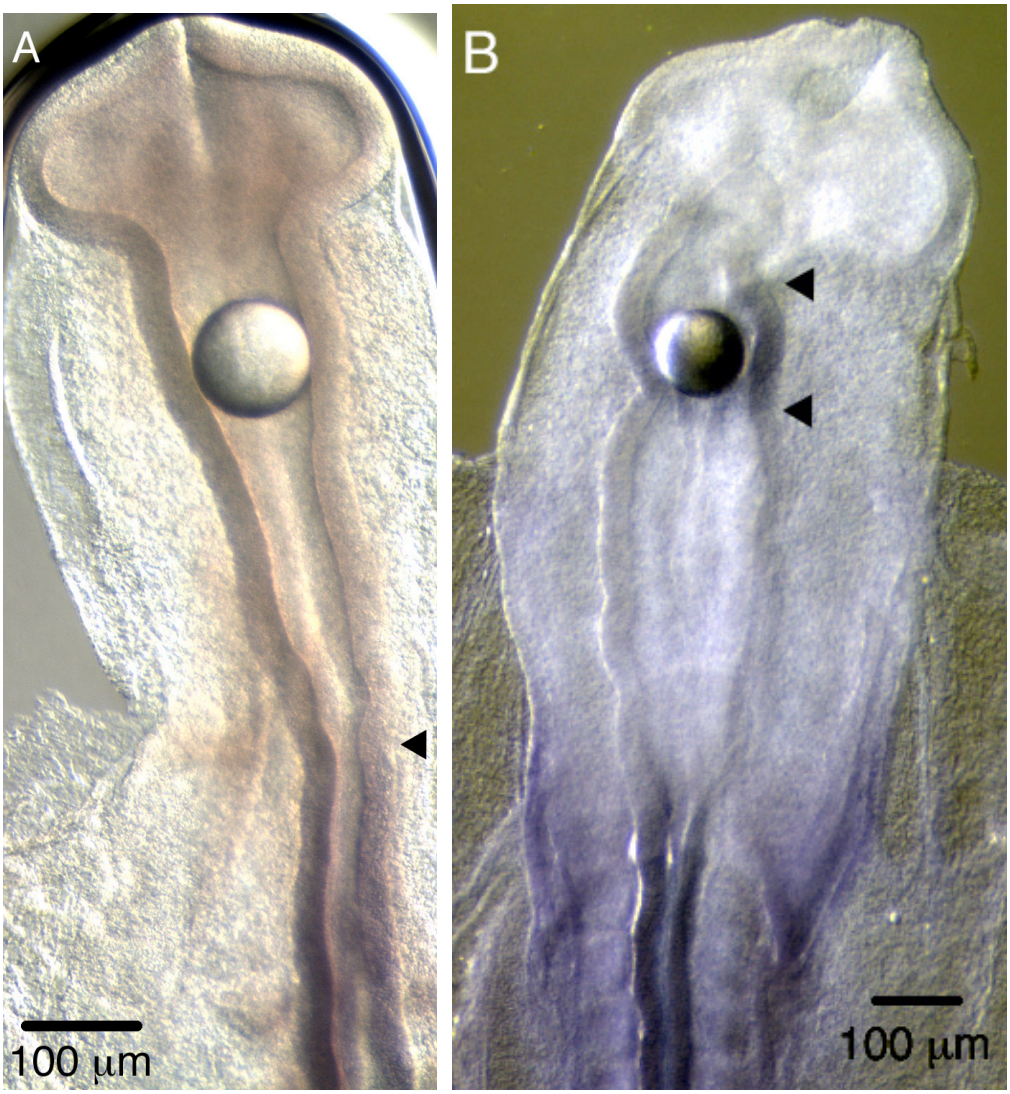

Figure 4.2 Hoxa1 expression patterns. (A) 6.3x picture of a stage 11 embryo stained for *Hoxa1*. The purple/blue stain marks the localization of the gene, and the deeper the color, the stronger the expression. A control bead soaked in only DMSO was implanted into the midbrain of a stage 9 embryo and collected at stage 11. This picture is a bit unique in that the bead remained in place through the entire *in situ* protocol. This is not often the case, as the bead usually becomes dislodged during one of the many washes. *Hoxa1* is clearly expressed (as

evidenced by the purple color) in the neural tube posterior to the point marked by the arrow. As expected, there is no *Hoxa1* expression near the bead **(B)** 5x picture of a stage 11 embryo stained for *Hoxa1*. An RA coated bead was implanted into the midbrain at stage 9. Notice the strong purple expression of *Hoxa1* in the area between the black arrows. This picture is typical of the results, but is particularly nice in that the bead stayed in place and the expression of *Hoxa1* near the bead is so prominent. If the bead is implanted at ages older than stage 10, there is a reduced chance that there will be any change in the expression of *Hoxa1*. This is compared to earlier stages when the hind and midbrain are still able to respond to the RA, and is consistent with other reports of RA perturbation experiments (Dupe and Lumsden, 2001; Gale et al., 1996).

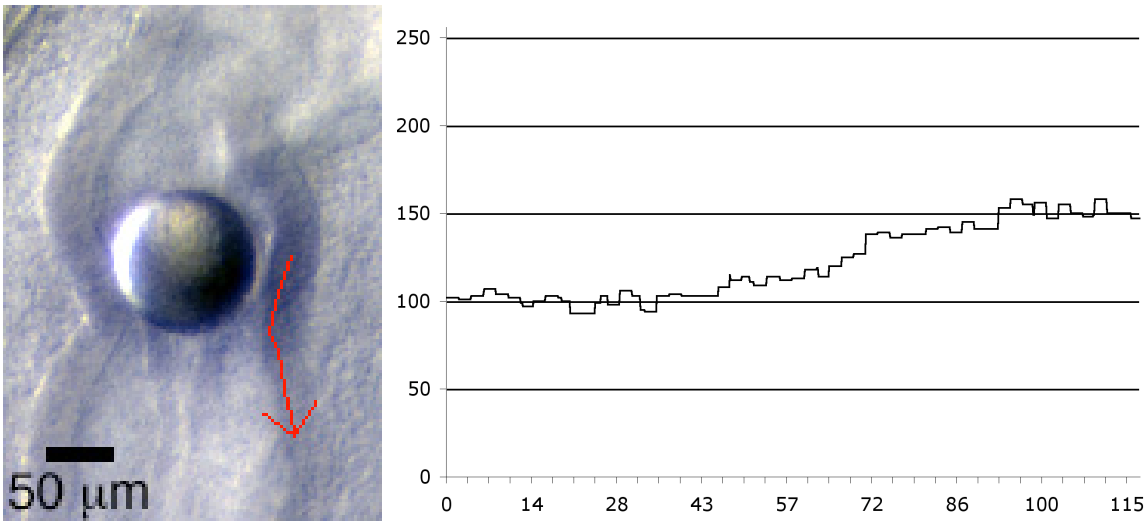

Figure 4.3 *Hoxa1* expression near the RA bead. This closeup of Figure 4.2 focuses on the expression of *Hoxa1* near the RA coated bead. There is an area of

expression just posterior to the bead that extends nearly 50 microns from the bead, and the expression in the neural tube is evident, especially on the right side. Using the 510LSM image analysis software from Zeiss, the change in intensity was measured along the red trajectory, and the results are show in the chart above. The ordinate is pixel intensity, and the abscissa is the microns along the path. Recall that lower intensities correspond to darker colors. Along the120 micron path the intensity pixels changes about 20%, with the first 40 microns holding relatively steady, followed by a gradual change starting before the bend in the red arrow. After a gradual change along the next 40 microns of the path, the intensity values level off to background intensity.

Determining the number of RA molecules on the bead can only be done in an indirect way. A study showed using radioactive RA that after about 30 minutes, approximately 25% of the radioactivity in the solution had been depleted (Eichele et al., 1984). Therefore, assuming a concentration of 10^{-3} M for the solution a theoretical maximum uptake by the bead is approximately 2.4×10^{12} molecules. As for the depletion, approximately 40% of the RA is released by the bead in the next 8 hours (Eichele et al., 1984; Langer and Peppas, 1981). This means that the bead is, in effect, a saturating source with over 9.6×10^{11} molecules released from the bead into the surrounding tissue during the 8 hours it is in the embryo.

Bead Model

Modeling the effects of the RA soaked bead proceeded concurrently with the laboratory work. The simulation was modified to provide a constant saturating source of RA diffusing laterally into the tissue. At each time step anywhere from 20 to 2000 molecules of RA were introduced into each cell. This effectively provides a saturating source, because each of the cells contains approximately 2000 free receptors for the RA. The source is not symmetric, as it appears from the position of the bead that it is able to provide more RA to the anterior cells as compared to the posterior cells. Recall that the *Hoxa1* mRNA transcription was implemented used a combination of a Hill function and first order reaction. The accumulation of transcription factors (in this case the bound RAR/RXR dimers), would lead to the activation of the *Hoxa1* gene, and once this occurred the gene was activated and mRNA could be transcribed. But with the large accumulation of bound dimers provided by the constant source of RA, there was little chance that the gene would become unactivated. If a bound dimer dissociated from the gene, another was present to take its place. This implementation does not allow for a differential in *Hoxa1* expression due to varying amounts of RA. This results in an indiscriminate up-regulation of *Hoxa1* as shown in Figure 4.4A below.

Because the results did not accurately capture the new data, the model required a change to incorporate the data gathered from the embryo. This is in accordance with the quote from Hamburger at the beginning of the chapter. Therefore, the model was changed so that the *Hoxa1* mRNA was transcribed using a proportionality function (*i.e.*, the probability of transcription of *Hoxa1* mRNA proportional to the number of bound RAR/RXR dimers present) instead of the sequential Hill equation to activate the gene,

and a first-order reaction transcription from the activated gene. The other change to the model was the deletion of the parameters for the activation/unactivation of the *Hoxa1* gene. After making these changes, the model was able to capture the results that were gathered in the laboratory, as seen in Figure 4.4B.

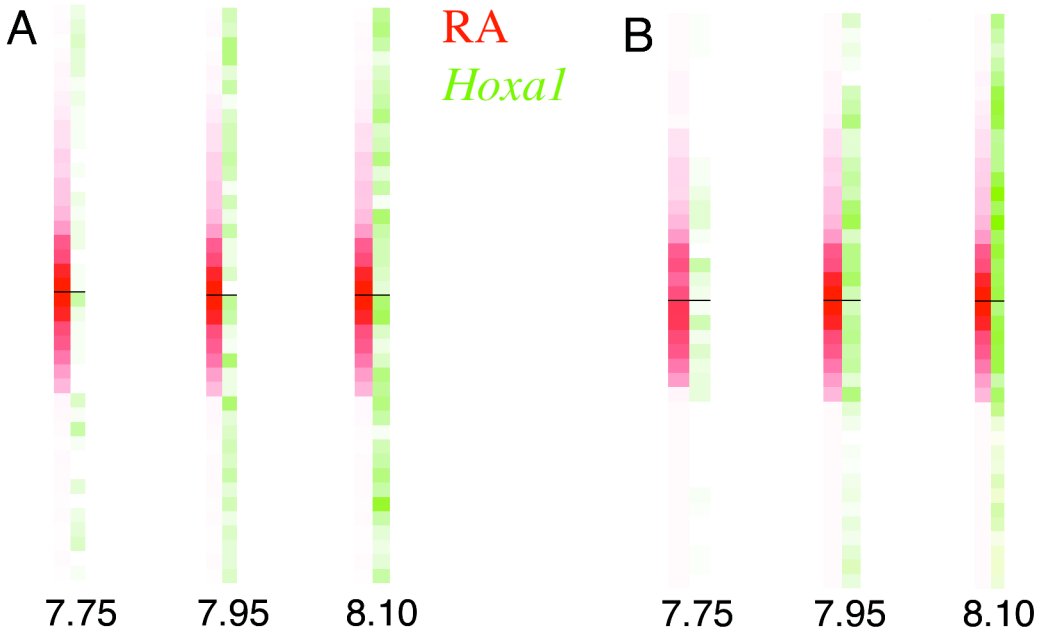

Figure 4.4 *Hoxa1* expression from a constant RA source. (A) Notice that there is no visible change in the levels of mRNA for *Hoxa1* due to the differing levels of RA. Increasing the number of free receptors by an order of magnitude does not affect the qualitative results. Because the bead was kept in the embryo for only 6-8 hours, the model was stopped after 8.1 dpc. **(B)** After making a change that ties the transcription of *Hoxa1* to the number of transcription factors present, the model now captures the type of behavior seen in the lab, namely more RA leads, in general, to a stronger expression of *Hoxa1* mRNA. There is still moderate

expression in the anterior (top) end of the figure however, maybe more than one would hope. This is not terribly surprising however, given that 200 molecules of RA are introduced at each time step and they have a cumulative effect. But in the posterior (bottom) section of the figure, in which there are 10 times fewer RA molecules introduced at each time step than in the anterior end, the expression is lower in general. Most importantly, the strongest expression of *Hoxa1* mRNA in Figure 4.4B is nearest the largest collection of RA, *i.e.,* the center of the figure. This is not true in Figure 4.4A: More RA does not in general lead to a stronger expression of *Hoxa1* mRNA.

The changes to the function for the transcription of *Hoxa1* were made to the baseline model, and the wild-type scenario was run again. The results of the simulation are shown in Figure 4.5 below.

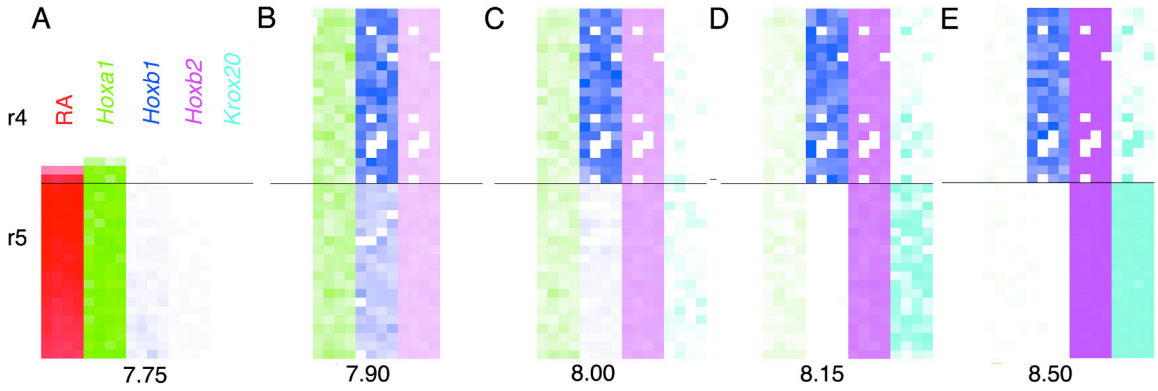

Figure 4.5 Wild type. This time slices in this picture are exactly the same as in Figure 3.7. Notice that the results are qualitatively the same. The only changes to the parameters were the deletion of the cell division, and a change in the transcription rate of *Hoxa1*. It might be tempting to make a comment about the number of blank cells in the second column of the *Hoxb1* and *Hoxb2*, but any conclusions would be erroneous; the only difference between that column and the first column is the random number seed used. This results shows that the model was insensitive to this change in the implementation of *Hoxa1* transcription.

An experiment that was relevant to the model under investigation provided a test of one of the key interactions of the model. The resulting data led to a change in the implementation of the RA and *Hoxa1* connection. The gradient of expression resulting from an RA coated bead has not been reported in the literature, and this novel result continues to support the view that RA concentration plays a role in the patterning of the hindbrain.

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