Transcription Factors of the Anterior Neural Plate Alter Cell Movements of Epidermal Progenitors to Specify a Retinal Fate

Kristy L. Kenyon,‡ Norann Zaghloul,† and Sally A. Moody*,†‡†1

*Department of Anatomy and Cell Biology, Institute for Biomedical Sciences, †Genetics Program, and ‡Neuroscience Program, The George Washington University, Washington, D.C. 20037

The embryonic progenitors that give rise to the vertebrate retina acquire their cell fate identity through a series of transitions that ultimately determine their final, differentiated retinal cell fates. In Xenopus, these transitions have been broadly defined as competence, specification, and determination. The expression of several transcription factors within the anterior neural plate at the time when the presumptive eye field separates from other neural derivatives suggests that these genes function to specify competent embryonic progenitors toward a retinal fate. In support of this, we demonstrate that some transcription factors expressed in the anterior neural ectoderm and/or presumptive eye field (otx2, pax6, and rx1) change the fate of competent, ventral progenitors, which normally do not contribute to the retina, from an epidermal to a retinal fate. Furthermore, the expression of these factors changes the morphogenetic movements of progenitors during gastrulation, causing ventral cells to populate the native anterior neural plate. In addition, we experimentally demonstrate that the efficacy of pax6 to specify retinal cells depends on the position of the affected cell relative to the field of neural induction. Thereby, otx2, pax6, and rx1 mediate early steps of retinal specification, including the regulation of morphogenetic cell movements, that are dependent on the level of neural-inductive signaling. © 2001 Academic Press

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INTRODUCTION

The elucidation of how fate decisions are directed throughout the span of development is of central importance to understanding the mechanisms by which specific phenotypes are produced. Attainment of a differentiated phenotype is regulated at multiple levels and influenced by maternal and zygotic signaling factors and transcriptional regulators. The visual system has been utilized extensively for studying the molecular mechanisms that orchestrate the cellular transitions from a progenitor (uncommitted) state to a final differentiated phenotype (Perron and Harris, 1999). Classical experiments involving ablation and/or transplantation of tissues at different times of eye development defined when progenitor cells first acquired competence for a retinal fate (e.g., epidermal vs. neural), when competent cells became specified (e.g., neural vs. retinal), and when cells undergo differentiation into a specific, differentiated phenotype (e.g., ganglion vs. amacrine vs. bipolar). Understanding these broadly defined transitions, i.e., competence, specification, and differentiation, at the molecular level has been aided greatly by the identification of a number of genes that are expressed at these transitions. However, how these molecules function to direct progenitor cells from a state of competence to differentiated phenotypes is not known.

From an experimental standpoint, the developing Xenopus retina provides an excellent model for studying the cellular mechanisms involved in the process of cellular determination. Amphibian retinal progenitors are especially accessible for in vivo experimental manipulation throughout all stages of development. Each retina in Xenopus descends from a consistent subset of nine animal blastomeres at the 32-cell stage, each of which contributes unique percentages of cells and amacrine cell subtypes (Huang and Moody, 1993, 1995, 1997). These nine animal blastomeres, however, are not committed to a retina fate at

1 To whom correspondence should be addressed. Fax: 202-994-8885. E-mail: anasam@gwumc.edu.
cleavage stages. If ventral animal and equatorial blastomeres, which normally do not contribute any cells to the retina (Moody, 1987), are transplanted to a dorsal site, they contribute significant numbers of progeny to the retina (Huang and Moody, 1993). If the most retinogenic blastomere is transplanted to a ventral vegetal site that normally gives rise to gut and tail, it will form neural structures but not retina (Gallagher et al., 1991). These data indicate that position within the animal hemisphere plays an important role in determining which blastomere progenitors become specified to contribute to the retina.

Growing evidence indicates that the signaling environment within the animal hemisphere is responsible for the positional determination of retinal progenitor competence. Ectopic expression of several growth factor (Activin, FGF, BMP) pathway components demonstrates that blastomeres acquire the competence to express a retinal fate by repressing BMP signaling (Moore and Moody, 1999). Specifically, expression of BMP4 in a blastomere that normally is a major contributor to the retina blocks this lineage, whereas disrupting BMP signaling by expression of a dominant-negative BMP receptor or a BMP4 antagonist in a blastomere that gives rise to epidermis induces a retinal lineage. Consistent with the current model for neural induction (Harland, 2000), these data indicate that the inhibition of BMP in the animal hemisphere defines the domain of presumptive neural plate and thereby is the next step in retinal determination. Next, the anterior neural plate becomes subdivided into fields that give rise to different CNS domains. The retina descends from a subfield of cells called the eye field (Adelmann, 1929, 1937; Eagleson and Harris, 1990; Eagleson et al., 1995), which is defined by a number of molecular markers (Dirksen and Jamrich, 1995; Casarosa et al., 1997; Hirsch and Harris, 1997; Li et al., 1997; Mathers et al., 1997). Transplantation experiments demonstrate that the entire presumptive neural plate of the midgastrula embryo is capable of forming retinas, further indicating that the initial step in retinal specification is to acquire neural competence. But, eye field fate is not specified until neural plate stages (Saha and Grainger, 1992; Perron and Harris, 1999), concomitant with the restricted expression of a number of transcription factors. It is not clear how cells distinguish whether they will proceed in the retinal developmental pathway versus other anterior neural fates nor the role these transcription factors play in this process.

Herein, we demonstrate that a subset of genes expressed in the anterior neural plate, including the presumptive eye field, likely function in the specification of competent blastomere progeny to contribute to the retina. Pax6 (Hirsch and Harris, 1997; Li et al., 1997) and rx1 (Casarosa et al., 1997; Mathers et al., 1997) are initially expressed during late gastrulation in the presumptive anterior neural ectoderm, become restricted to the eye field, and continue to be expressed during retinal differentiation. Otx2 is expressed during gastrulation, then in the anterior neural ectoderm, and later in the developing retina (Acampora et al., 1995; Blitz and Cho, 1995; Matsuo et al., 1995; Kablar et al., 1996; Perron et al., 1998; Andreazzoli et al., 1999). Ectopic expression of each of these genes in ventral epidermal progenitors causes progeny to express a retinal fate. Previous studies showed that pax6 and rx1 can cause ectopic retinal tissue to form when misexpressed in dorsal retinal progenitors (Mathers et al., 1997; Andreazzoli et al., 1999; Chow et al., 1999). Herein, we further demonstrate that pax6, otx2, and rx1 also shift ventral blastomeres from an epidermal/placodal fate to a retinal fate. Rather than creating an ectopic or expanded neural field, these transcription factors cause small numbers of cells to disperse early and maintain an anterior position during gastrulation movements, thereby populating the native anterior neural plate. In addition, we experimentally prove the recent suggestion (Chow et al., 1999) that the ability of pax6 to cause these ectopic retinal tissues depends on a cell’s position within the field of neural induction. These studies demonstrate that otx2, rx1, and pax6 mediate early steps in retinal specification, including the regulation of morphogenetic cell movements, that are dependent on the level of neural-inductive signaling.

MATERIALS AND METHODS

Generation and Collection of Embryos

Fertilized Xenopus laevis embryos were obtained by the natural mating of gonadotropin-induced adult frogs. After chemical removal of the jelly coat, embryos were selected if the first cleavage furrow bisected the gray crescent to accurately determine the dorsal midline (Moody, 1999). Only those embryos demonstrating stereotypic cleavage patterns (Jacobson and Hirose, 1981; Moody, 1987) were microinjected to consistently target the appropriate 32-cell progenitors (blastomeres V1.2.1, V1.1.1, V1.1.2, and V2.1.1; Fig. 1).

Targeted Microinjection of Synthetic mRNAs

Plasmids for pax6 (Hirsch and Harris, 1997), otx2 (Blitz and Cho, 1995; Panness et al., 1995), Xfh4 (Dirksen and Jamrich, 1995), rx1 (Mathers et al., 1997), noggin (Smith and Harland, 1992), and bmp4 (Dale et al., 1992) were used as templates to synthesize in vitro capped mRNAs (Ambion, Inc.). GFP and pgal mRNAs were synthesized for use as both lineage tracers and controls for RNA injection. Each experimental mRNA was mixed with tracer mRNA (200 pg/100 nl) for the following working solutions: pax6 (30–50 pg/100 nl), rx1 (50 pg/100 nl), otx2 (50 pg/100 nl), Xfh4 (25 pg/100 nl), noggin (10 pg/100 nl), pax6/noggin (30–50 pg/250 pg/100 nl), pax6/BMP4 (30–50 pg/50 pg/100 nl). Blastomeres were injected with approximately 1 nl of each RNA solution. The amount of test mRNA for injection was determined by whether: (1) the dose was sufficient to reproduce previously published phenotypes for each test mRNA; and (2) the dose did not produce signs of cell toxicity (see Moody, 1999 for description of these effects).

Cell Fate Analysis and Immunofluorescence

To determine whether mRNA ectopic expression caused progeny to populate the retina, embryos were injected with a mixture of
the test mRNA and GFP mRNA. They were fixed in 4% paraformaldehyde, 3% sucrose, 0.1 M phosphate buffer and serially sectioned at 14 μm with a cryostat. To monitor whether a blastomere contributed to the retina, every tissue section was scored for GFP-expressing cells between the dorsal-ventral and anterior-posterior boundaries of the retina at 200× magnification. The frequencies at which injected blastomeres contained clones in the retina were compared by the χ² test. To measure the extent of contribution, single GFP-labeled cells were counted when the clones were smaller than 100 cells (~10% of retina; Huang and Moody, 1993), or the area of retina occupied by GFP-labeled cells was measured from tissue sections when the clones were >100 cells. Both measurements were converted to a percentage of the total number of retinal cells in order to compare mean clone sizes (by the χ² test). To ascertain whether other cell fates were altered, fate maps of the entire head were constructed from serial sections as previously described (Moody, 1987; Moore and Moody, 1999). The proportions of embryos expressing each fate in control and experimental embryos were compared by the z-test of proportions (Sigma Stat, Jandel). To ascertain whether ectopic retinal tissue was produced as a result of RNA misexpression, tissue sections were immunostained as previously described (Huang and Moody, 1995), using antibodies raised against photoreceptor cells (4D2; Hicks and Molday, 1985) and retinal pigmented epithelium (XAR-1; Sakaguchi et al., 1997). Primary antibodies were diluted (1:300, 4D2; 1:20, XAR-1) and combined for an overnight incubation at 4°C. After washing, a Texas Red-conjugated goat anti-mouse IgG antibody (diluted 1:100; Jackson Laboratories) was applied for 2 hours at 4°C. Since primary antibodies were applied simultaneously and visualized with the same secondary antibody (to allow identification of

FIG. 1. Blastomeres of the 32-cell Xenopus embryo, as seen from an animal pole-lateral view of the right side. Arrows indicate the midsagittal cleavage furrow. Dorsal is to the top. Blastomeres containing percentages are those that normally contribute to the retina. Percentages represent the number of retina cells that descend from individual blastomeres (data from Huang and Moody, 1993). The four ventral blastomeres that were targeted in the experiments described herein are indicated by the nomenclature of Jacobson and Hirose (1981). These correspond to A3 (V1.2.1), A4 (V1.1.1), B4 (V1.1.2), and D4 (V2.1.1), according to the nomenclature of Nakamura and Kishiyama (1971).

Percentage of Embryos with V1.1.1 Progeny in the Retina

FIG. 2. Percentage of embryos with GFP-positive cells in the retina after mRNA injection into blastomere V1.1.1. As a control, GFP mRNA was injected alone to confirm previous fate maps (Moody, 1987; Huang and Moody, 1993) that V1.1.1 does not contribute to the retina. These results demonstrate that otx2, rx1, and pax6 translocate epidermal precursors to the retinal lineage. However, Xfh4, a gene also expressed in the early eye field, did not translocate V1.1.1 progeny.
mRNA-expressing cells by GFP fluorescence), cellular morphology using DIC optics determined whether ectopic staining corresponded to photoreceptor cells or RPE. To ascertain whether eye field genes alter the gastrulation movements of expressing clones, blastomeres V1.1.1 or V1.1.2 were injected with a mixture of the test mRNA and βgal mRNA. Embryos were fixed at several gastrula stages, and processed for βgal histochemistry (Sive et al., 2000).

In Situ Hybridization

To ascertain whether ectopic expression of the injected mRNAs locally upregulated neural induction, embryos were injected with a mixture of the test and βgal mRNAs. They were fixed at neural plate stages, processed for βgal histochemistry, and then by in situ hybridization according to standard protocols (Sive et al., 2000). Full-length, digoxigenin-labeled antisense RNA probes for sox2, sox3 (Penzel et al., 1997; Zygar et al., 1998), and bmp4 (Schmidt et al., 1995) were synthesized according to manufacturer's instruction (Boehringer-Mannheim). To determine whether the neural plate was expanded, the widths of both the injected (βgal-positive) and uninjected sides were measured with an eyepiece micrometer, and the mean percent difference between sides was compared to similar measurements from control embryos (βgal-injected) by the t test (Sigma Stat, Jandel).

RESULTS

**otx2, pax6, and rx1 Each Cause Epidermal Precursors to Enter the Retinal Lineage**

In Xenopus, each retina descends from nine blastomeres, all but one of which lie in the dorsal animal quadrant (Huang and Moody, 1993). Although the majority of ventral animal blastomeres do not contribute to the retina, they are competent to do so if transplanted to a dorsal position or if they move into that position after a dorsal blastomere is deleted (Huang and Moody, 1993). Moreover, inhibiting BMP signaling redirects ventral animal blastomeres to contribute progeny to the retina (Moore and Moody, 1999). These results indicate that the mechanisms that specify cells to the retinal lineage are repressed on the ventral side by a high level of BMP signaling. Genes that are expressed in the presumptive anterior neural plate are candidates for mediating the next step in retinal determination, which involves the eye field acquiring an identity distinct from other neural plate domains. We tested whether four transcription factors that are expressed early in the anterior neural plate and/or the presumptive eye field (Xfh4, otx2, pax6, rx1) cause epidermal precursors to enter the retinal lineage. The ventral midline animal blastomere (V1.1.1; Fig. 1) was used because it normally does not contribute to the retina but is competent to do so (Moody, 1987; Huang and Moody, 1993).

Ot2, pax6, and rx1 each cause some V1.1.1 descendants to express a retinal fate, pax6 having the greatest activity (Figs. 2 and 3). The sizes of the retinal clones were highly variable, ranging from less than 10 cells to large, coherent cell clusters (Fig. 3) in which all retinal cell layers were represented. In contrast, expression of Xfh4 in V1.1.1 did not have this effect, demonstrating specificity for the phenotype. Likewise, GFP mRNA did not have this effect, consistent with previous fate maps (Moody, 1987; Huang and Moody, 1993).

The test blastomere (V1.1.1) gives rise to descendants populating tissues derived from all three germ layers, not just epidermis (Moody, 1987). To distinguish whether these genes altered other fates as well, the tissue distribution of all progeny in the head was examined and compared to the normal distribution of V1.1.1 progeny, as determined from GFP-labeled controls and previous fate maps (Moody, 1987). Control V1.1.1 gives rise to cement gland (76.5% of embryos, n = 34), olfactory placode (55.9%), head epidermis (100%), lens (61.8%), cranial ganglia (100%), otocyst (94.1%), forebrain (29.4%), hindbrain (8.8%), branchial and dorsal head mesoderm (70.6%), and foregut (61.8%). The distribution of Xfh4-expressing cells was not significantly
FIG. 4. Ectopic retinal structures form after expression of pax6 in ventral blastomeres. (A–D) Transverse sections through stage-44 to -45 embryos in which pax6 was injected into the V1.2.1 blastomere. (A) DIC image of an inverted eye-like structure near the otic vesicle. Le, lens-like tissue; arrow, RPE-like tissue. (B) Extension of the retinal pigmented epithelium (left arrow) toward the brain and displaced photoreceptor (PH) cells within a pax6/GFP-expressing clone within the retina. (C) Ectopic photoreceptor cells (arrows) in an ectopic eye-like structure. (D) Retina appears duplicated, as indicated by the double-cup arrangement of photoreceptors (red) associated with an enlarged lens (le) that is expressing pax6 (green). (E, F) Transverse sections through stage-44 to -45 embryos in which pax6 and noggin mRNA were coinjected into the V1.1.1 blastomere. (E) Ectopic photoreceptor (PH) located in a circular, GFP-positive structure near the heart (ht). (F) Ectopic site of RPE (arrow) near a normal retina. Injection of noggin alone into V1.1.1 does not cause the formation of ectopic retinal tissue (Table 1).
different from controls (n = 17; P > 0.05). As predicted from previous misexpression studies (Blitz and Cho, 1995; Gammill and Sive, 1997), otx2 expression in V1.1.1 caused ectopic cement glands to form (data not shown). In addition, it caused a significant decrease in the frequency of V1.1.1's contributions to the otocyst (66.7% of embryos; n = 27) and foregut (14.8%) (P < 0.05), but all other contributions besides retina were not significantly different from controls. As described previously (Chow et al., 1999), pax6 caused ectopic lens-like structures (as well as ectopic retina cell types; see below). In addition, it caused a significant decrease in the frequency of V1.1.1's contributions to cement gland (12.5% of embryos, n = 24) and otocyst (58.3%), and an increase to hindbrain (33.3%) (P < 0.05). rx1 did not induce any ectopic tissues when expressed ventrally. However, it caused a significant decrease in the frequency of V1.1.1's contributions to cement gland (28.6% of embryos, n = 31), olfactory placode (23.3%), lens (3.2%), cranial ganglia (58.1%), and otocyst (36.7%) (P < 0.05). These results indicate that the action of otx2, pax6, and rx1 to direct clones toward a retinal fate is accompanied by varying decreases in head placodal fates. There was a trend for all three factors to cause concomitant increased contributions to brain, but these changes reached a significant level only for pax6 in the hindbrain.

One of these transcription factors overcame the vegetal inhibition of retinal fate. Previous studies demonstrated that the vegetal blastomere (V2.1.1, Fig. 1), which normally gives rise to endoderm and tail, is maternally blocked from contributing to the retina. Neither transplantation to a retinogenic site (Huang and Moody, 1993) nor expression of components of mesoderm or neural-inducing pathways (Moore and Moody, 1999) overrides this block. Likewise, the expression of otx2 (n = 9), pax6 (n = 22), or rx1 (n = 17) in the V2.1.1 lineage did not cause any of its progeny to express a retinal fate in any embryo. Fate maps made from these clones revealed that: (1) pax6 caused no significant changes in the V2.1.1 lineage; (2) the only significant change caused by otx2 was an increased frequency of contribution to head mesoderm (0% of embryos for controls, n = 32; 25% for otx2, n = 8); and (3) the only significant change caused by rx1 was a decreased frequency of contribution to nephric mesoderm (47% of embryos for controls, n = 32; 0% for rx1; n = 13) and trunk neural crest (63% for controls; 23% for rx1). These data indicate that the retinogenic transcription factors can only affect blastomeres that are maternally competent to express a retinal fate; they are not sufficient to override the maternally derived vegetal inhibition of retinal fate. Thus, their ability to cause expression of a retinal fate is context-dependent.

**Misexpression of pax6 in Ventral Blastomeres Induces Ectopic Retinal Tissue**

The small numbers of cells that populate the retina after each transcription factor (otx2, pax6, rx1) are expressed in V1.1.1 and their lack of effect in ventral vegetal blastomeres suggests that these three factors are effective only in descendants that are exposed to a particular environment. Since each factor is normally expressed predominantly in neural tissue, their ability to specify cells to a retinal fate may require some level of neural induction. If so, then expression of these genes in a blastomere lineage that normally receives moderate levels of neural-inductive signals should produce a greater effect. To test this, pax6 was expressed in a ventral lateral blastomere (V1.2.1; Fig. 1), whose progeny contribute significantly to the CNS (Moody, 1987, 1989) but only very small numbers to the retina (Huang and Moody, 1993). The percentage of embryos in which V1.2.1 contributed to the retina was significantly increased after pax6 expression (Table 1; cf. controls, 44%, n = 45). In addition, the size of the retinal clones derived from V1.2.1 was larger; in 41% of the embryos with labeled cells in the retina (n = 29), the clone comprised more than 15% of the retina compared to <1% in controls (Huang and Moody, 1993). Furthermore, the eye tissue was expanded in a large percentage of these embryos (Table 1). The retina and lens were enlarged and/or duplicated, the RPE layer was extended around the entire retina or toward the neural tube, and photoreceptor cells were displaced (Fig. 4). Ectopic

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**TABLE 1**

Frequency of Retinal Phenotypes after pax6 Misexpression

<table>
<thead>
<tr>
<th>Blastomeres: mRNAs:</th>
<th>V1.2.1 pax6</th>
<th>V1.1.1 pax6</th>
<th>V1.1.1 noggin</th>
<th>V1.1.1 pax6 + noggin</th>
<th>V1.1.2 pax6</th>
<th>V1.2.1 pax6 + bmp4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contribution to retina</td>
<td>66* (44)</td>
<td>43 (42)</td>
<td>42 (19)</td>
<td>46 (22)</td>
<td>17 (17)</td>
<td>0 (29)</td>
</tr>
<tr>
<td>Enlarged retina/lens</td>
<td>43 (44)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0 (17)</td>
<td>0 (29)</td>
</tr>
<tr>
<td>Extension of RPE</td>
<td>66 (44)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0 (17)</td>
<td>0 (29)</td>
</tr>
<tr>
<td>Ectopic RPE</td>
<td>43 (44)</td>
<td>21 (28)</td>
<td>0 (19)</td>
<td>45§ (22)</td>
<td>12 (17)</td>
<td>0 (29)</td>
</tr>
<tr>
<td>Ectopic PH</td>
<td>33 (42)</td>
<td>—</td>
<td>0 (19)</td>
<td>18 (22)</td>
<td>0 (17)</td>
<td>0 (29)</td>
</tr>
</tbody>
</table>

Note. RPE, retinal pigmented epithelium; PH, photoreceptor. _, Phenotype was not scored in these embryos. *, Significant difference from controls (44%, n = 45) by χ² analysis, P < 0.05. §, Significant difference from V1.1.1-pax6 and from V1.1.1-noggin groups by χ² analysis, P < 0.05.
eye-like structures also were identified by DIC optics in 39% of the embryos. These masses were composed of multiple, organized cell layers including an RPE-like layer on the outer surface (Fig. 4C). Some specimens were stained with antibodies against RPE and photoreceptor cells, which confirmed that these ectopic structures contained retinal cells (Fig. 4; Table 1). Ectopic retinal structures often were located either near the otic vesicle, the neural tube, or the heart, consistent with the normal tissue distribution of the V1.2.1 lineage (Moody, 1987). Thus, there is an increased contribution to retina and ectopic retinal structures when pax6 is expressed in a ventral animal blastomere that normally experiences neural-inductive signaling.

Levels of Neural-Inductive Signaling Influence the Efficacy of pax6 Effects

Chow et al. (1999) demonstrated that pax6 overexpression in dorsal blastomeres produced ectopic retinal phenotypes similar to those described herein for the ventral lateral blastomere (V1.2.1), including the formation of ectopic “eyes.” They report that these phenotypes occur only when pax6 is misexpressed in dorsal blastomeres that give rise to the CNS and not in ventral blastomeres, and thereby suggest that a high level of neural induction is necessary for pax6 to specify cells to a retinal fate. Our observations show that, in contrast, two ventral animal blastomeres also respond to pax6 by expressing a retinal fate. But, in accord with the prediction of Chow et al. (1999), the level of responsiveness of the ventral blastomeres correlates with the level of native neural-inductive signals that its progeny receive, as estimated from their contributions to neural structures (Moody, 1987). The ventral midline blastomere (V1.1.1) produced a consistent, albeit small, fate change, whereas the ventral lateral blastomere (V1.2.1) produced a larger fate change. To directly test that responsiveness to ectopic pax6 expression is amplified in accord with levels of neural-inductive signaling, pax6 was expressed in different neural-inductive environments.

First, noggin and pax6 mRNAs were coexpressed in V1.1.1 to test whether the frequency of retinal cell formation within the lineage increased. Noggin alone caused V1.1.1 progeny to contribute to the retina at the same frequency as pax6 alone (Table 1). Coexpression of pax6 and noggin mRNAs did not significantly increase this frequency. A comparison of the mean size of the retinal clones showed that the coexpression of pax6 and noggin caused a significant increase (22.5% of retina) over both pax6 alone (1% of retina) and noggin alone (12% of retina). Furthermore, noggin did not cause the ectopic development of retinal structures (RPE or photoreceptors; Table 1), whereas the coexpression of pax6 and noggin significantly increased the frequency of these phenotypes above that observed for pax6 alone (Table 1). These data demonstrate that increasing the level of neural induction enhanced pax6-mediated expression of a retinal fate.

Conversely, increasing BMP4 levels should reduce the ability of pax6 to elicit the expression of a retinal fate. First, pax6 was expressed in a competent blastomere that lies in the ventral equatorial zone (V1.1.2; Fig. 1), a position that places its progeny more posterior, further from native neural-inductive signals (Moody, 1987; Feledy et al., 1999) and well within the BMP4-expressing epidermal domain (Schmidt et al., 1995; Gamill and Sive, 2000). By whole-mount analysis, injected embryos exhibited a normal distribution of GFP-expressing cells (Figs. 5D–5F). Yet, examination of serial sections revealed that 12% of these embryos had small ectopic sites of RPE tissue and 17% contained GFP-expressing cells in the retina (Table 1). Likewise, GFP-positive cells populated the retina at similar frequencies after the V1.1.2 blastomere was injected with either rx1 (14.3%; n = 7) or otx2 (13.3%; n = 15) mRNAs, indicating that the effects of these two genes also are responsive to the neural-inductive environment. Second, bmp4 mRNA, at a concentration that does not cause head malformations, was coinjected with pax6 mRNA into the V1.2.1 lineage. None of these embryos contained V1.2.1 progeny in the retina nor did ectopic eye-like structures form (Fig. 5, Table 1). Both experiments demonstrate that increasing the level of BMP signaling inhibits pax6-mediated changes in fate from epidermal to retinal.

otx2, pax6, and rx1 Alter the Gastrulation Movements of Ventral Animal Clones

Because the ability of otx2, pax6, and rx1 to elicit a retinal fate depends on a neural-inductive environment, these genes might cause cells to change from an epidermal to retinal fate by increasing levels of neural-inductive signaling when expressed in ectopic sites. For example, high levels of otx2 can induce the expression of the neural marker NCAM, indicating that it has some neural-inductive activity (Gammill and Sive, 2000). This potential mechanism would be evidenced either by expansion of the neural plate on the side of the mRNA injection or by ectopic sites of neural-specific gene expression. To test this, injected embryos were processed for expression of sox2 or sox3, both of which are transcribed in an early response to neural-inductive signaling (Penzel et al., 1997; Mizusaki et al., 1998). Comparison of the width of the neural plate on control versus injected (otx2-labeled) sides demonstrated no expansion of the neural plate (Fig. 6), which was confirmed by comparing the mean differences between sides in control (otx2, 9.5 ± 1.4, n = 29; pax6, 6.1 ± 1.1, n = 38; rx1, 10.0 ± 1.1, n = 36) animals (P > 0.05, t test). Furthermore, no ectopic patches of sox2/3 expression were observed in the epidermis occupied by otx2-, pax6-, or rx1-expressing cells (Fig. 6). Although some members of the transcription factor-expressing clone overlapped sox2/3-expressing placodes in the lateral ectoderm (Fig. 6), the sizes of the placodes were not larger than in controls (data not shown). These data further indicate that the genes do not expand neurally...
The formation of retinal phenotypes by ventral \textit{pax6} expression is dependent on a blastomere's position in the field of neural induction. (A) Lateral view of tadpole in which the V1.2.1 blastomere was injected with GFP alone, demonstrating the normal distribution of this clone in the head epidermis. ey, eye; gt, gut. (B, C) Lateral views of two tadpoles in which V1.2.1 was coinjected with \textit{pax6} and GFP mRNAs. (B) A large ventral mass (arrow) is associated with RPE-like tissue (arrowheads) that extends towards the brain. Tissue section analyses showed that the ventral mass contained ectopic eye structures and the native retina contained GFP-expressing cells (Table 1). (C) Ectopic RPE-like tissue (arrowheads) extends from the retina towards the brain (arrowheads). In addition, the retina is enlarged, irregular in shape, and \textit{pax6}-expressing cells (green) stream posteriorly from the lens (arrow). (D–F) The same tadpole in which the V1.1.2 blastomere was coinjected with \textit{pax6} and GFP mRNAs. Injected cells (green) populate the trunk epidermis (D), consistent with the normal fate map (Moody, 1987). There is no evidence of ectopic, \textit{pax6}-expressing retinal structures in the head (E), as observed when V1.2.1 was injected. Furthermore, the eye on the injected side (right side in E and F) looks normal and has no extensions of RPE-like tissue (F, arrowhead). Tissue section analyses demonstrated that there were no \textit{pax6}-labeled cells in the native retina, nor were there ectopic retinal structures (Table 1). (G–I) The same tadpole in which blastomere V1.2.1 was coinjected with \textit{pax6}, \textit{bmp4}, and GFP mRNAs. Injected cells (green) are more posterior (G) than controls (A), and are not in the retina (G). The retina on the injected side is normal in size (H, I) and there are no ectopic extensions of RPE-like tissue (I, arrowhead). Tissue section analyses confirmed that there were no GFP-labeled cells in the native retina, nor were there ectopic retinal structures (Table 1).

Anterior views of neural plate (stage 15–16) embryos. Sox3 expression (blue) delineates the neural plate and cranial placodes (pl). Red cells are \textit{β-gal}-positive descendants of blastomere V1.1.1 that also are expressing the indicated transcription factor. In \textit{β-gal}-injected controls (ctrl), the most anterior cells of the clone are distant from the neural plate, whereas in transcription factor-injected embryos these cells overlap the anterior boundary of the neural plate. There are no ectopic patches of \textit{sox3} expression. The same results were obtained with a \textit{sox2} probe.
FIG. 7. otx2, pax6, and rx1 alter the positions of ventral blastomere clones during gastrulation. (A) Animal cap view of stage-11.5 control V1.1.1 clone. Cells, centered on the animal pole (ap), are coherent with interdigitation along the border. (B) Animal cap view of a stage-11.5 otx2-injected V1.1.1 clone. Some cells (arrows) are dispersed distant from the coherent members of the clone, which are centered on the animal pole (ap). (C) Ventral view of stage-11.5 pax6-injected V1.1.1 clone, showing dispersed cells that have invaded the marginal zone. Arrow depicts the equator. Animal pole (ap) is to the left. (D) Stage-11.5 rx1-injected clone. Same as in (C). (E) Animal cap view of a stage-12 control V1.1.1 clone, which is retreating from the animal pole (ap). (F) Animal cap view of a stage-12 otx2-injected V1.1.1 clone that is more dispersed and remains predominantly at the animal pole (ap). (G) Animal cap view of a stage-12 pax6-injected V1.1.1 clone is similar to (F). (H) Animal cap view of a stage-12 rx1-injected V1.1.1 clone is similar to (F). (I) Anterior view of a stage-13 control V1.1.1 clone. The anterior-most cells are distant from the anterior border (indicated by red line) of the presumptive neural plate (np). Dorsal is to the top. (J) The stage-13 otx2-injected V1.1.1 clone overlaps the anterior border (red line) of the presumptive neural plate (np). Same orientation as in (I). (K) Stage-13 pax6-injected V1.1.1 clone, as in (J). (L) Stage-13 rx1-injected V1.1.1 clone, as in (J). (M) Ventral view of a stage-12 control V1.1.2 clone that has retreated from the animal pole (ap) and is mostly in the vegetal half of the embryo. yp, location of yolk plug. (N) Ventral view of a stage-12 otx2-injected V1.1.2 clone. Cells are more dispersed and many remain at the animal pole (ap). Cells do not reach the vegetal yolk plug (yp). (O) Ventral view of a stage-12 pax6-injected V1.1.2 clone, as in (N). (P) Anterior view of a stage-13 rx1-injected V1.1.2 clone, which overlaps the anterior rim (red line) of the presumptive neural plate (np). Dorsal is to the top.
indistinguishable from b mRNA. These embryos, examined at stage 12 (neural plate genes, embryos were injected with whether these effects were specific to these three anterior and retarded in their vegetal spread (Table 3). To discern clones were dispersed across the animal cap (Figs. 7F–7H) and many cells to be located more anterior than in controls transcription factors caused clones to be more dispersed, and most typically were in the marginal zone several cell diameters coherent (Table 3). The anterior limits of these clones plate are first distinguished, many control clones remained changes. At early stage 13, when the outlines of the neural in controls (Table 2). Thus, expression of these genes inductive fields. Thus, the retinogenic effects of these factors do not result from ectopic neural induction.

Instead of the neural plate domain expanding to incorporate members of the transcription factor-expressing clones, all three transcription factors caused cells to populate the neural plate (Fig. 6) at frequencies significantly higher than in controls (Table 2). Thus, expression of these genes appears to cause epidermal cells to move ectopically into the correct position for expression of a retinal fate. To determine when this aberrant cell movement occurred, injected clones were monitored through gastrulation. Consistent with a previous study (Bauer et al., 1994), clones derived from control, βgal-injected V1.1.1 were coherent and spread across the animal cap through stage 11.5 (Fig. 7A; Table 3). However, otx2, pax6, and rx1 each caused the cells of the clone to be more dispersed (Figs. 7B–7D; Table 3). In addition, these clones had prematurely spread into the animal marginal zone (Figs. 7C and 7D; Table 3). At stages 12–12.5, control clones remained mostly coherent with interdigitation along the borders of the clone (Fig. 7E). The posterior cells of the clones had spread variable distances toward the vegetal pole (Table 3), and the anterior cells began to withdraw from the dorsal side of the animal cap (Fig. 7E; 69.2% of cases). In contrast, otx2, pax6, and rx1 clones were dispersed across the animal cap (Figs. 7F–7H) and retarded in their vegetal spread (Table 3). To discern whether these effects were specific to these three anterior neural plate genes, embryos were injected with Xfh4 mRNA. These embryos, examined at stage 12 (n = 18), were indistinguishable from βgal-injected controls, demonstrating that cell movement effects correlated with retinal fate changes. At early stage 13, when the outlines of the neural plate are first distinguished, many control clones remained coherent (Table 3). The anterior limits of these clones typically were in the marginal zone several cell diameters ventral to the anterior neural plate (Fig. 7I), and most extended to the yolk plug (Table 3). In contrast, all three transcription factors caused clones to be more dispersed, and many cells to be located more anterior than in controls (Table 3), often contributing to the anterior neural ridge (Figs. 7J–7L; otx2, 56.3% of cases; pax6, 50%; rx1, 75%).

To test whether this effect on cell movement was limited to the more inducible V1.1.1 blastomere, these experiments were repeated in the animal equatorial blastomere (V1.1.2; Fig. 1). The results were very similar (Table 3). At stage 11.5, βgal-injected control clones were coherent and confined to the ventral side of the animal cap, whereas experimental clones contained dispersed cells and had spread into the vegetal marginal zone. At stages 12–12.5, control clones were mostly coherent, had extended into the ventral marginal and vegetal zones, and the majority had left the animal pole (Fig. 7M; Table 3). In contrast, the experimental clones were more dispersed, and in all cases still maintained significant numbers of cells in the animal cap (Figs. 7N and 7O). In fact, one-third of the pax6-expressing clones were confined entirely to the animal hemisphere (Table 3). At stage 13, control clones were mostly coherent, and no longer occupied the animal cap (Table 3). All three transcription factors caused V1.1.2-derived cells to be dispersed more frequently and located more anterior (Fig. 7P; Table 3). Thus, each transcription factor that alters ventral fates from epidermal to retinal also alters the gastrulation movements of ventral clones. Cells disperse earlier and a higher proportion of the clone remains anterior in the animal cap. This change in cell movements appears to allow cells to gain access to the anterior neural plate and eye field.

**DISCUSSION**

The potential of embryonic cells to give rise to the retina is gradually restricted through a series of interactions, culminating in the expression of several different retinal cell phenotypes. In Xenopus, vegetally localized maternal determinants define which blastomeres are competent to give rise to the retina (Huang and Moody, 1993; Moore and Moody, 1999). During gastrulation, further restrictions occur within the broad ectodermal domain as a result of neural-inductive signaling. Inhibition of BMP signaling is a critical step that allows the induction of the presumptive neural ectoderm and specifies which progenitors will participate in forming the eye (Moore and Moody, 1999). The presumptive neural ectoderm is divided into primitive anterior and posterior domains during gastrulation as a result of anterior-repressing signals from the posterior end of the embryo (Gamse and Sive, 2000). At neural plate stages, the anterior neural domain is divided into presumptive brain regions (Eagleson and Harris, 1990; Eagleson et al., 1995), and eye field fate is determined (Saha and Grainger, 1992; Perron and Harris, 1999). The molecular mechanisms that control this transition from competent to specified progenitor cells are not fully understood, but the expression of key transcriptional regulators in regionally specific domains likely plays a critical role.

The paired homeodomain gene pax6, the paired-like homeodomain gene rx1, the homeobox gene otx2, and the

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**TABLE 2**

Transcription Factors Cause Epidermal Cells to Populate the Neural Plate

<table>
<thead>
<tr>
<th>Blastomere</th>
<th>mRNA</th>
<th>N</th>
<th>% with cells in neural plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>V1.1.1</td>
<td>βgal</td>
<td>26</td>
<td>7.7</td>
</tr>
<tr>
<td></td>
<td>otx2</td>
<td>33</td>
<td>81.8</td>
</tr>
<tr>
<td></td>
<td>pax6</td>
<td>38</td>
<td>84.2</td>
</tr>
<tr>
<td></td>
<td>rx1</td>
<td>37</td>
<td>89.2</td>
</tr>
<tr>
<td>V1.1.2</td>
<td>βgal</td>
<td>26</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>otx2</td>
<td>26</td>
<td>80.8</td>
</tr>
<tr>
<td></td>
<td>pax6</td>
<td>37</td>
<td>91.9</td>
</tr>
<tr>
<td></td>
<td>rx1</td>
<td>37</td>
<td>97.3</td>
</tr>
</tbody>
</table>
winged helix gene Xfh4 have been implicated as important regulators of retinal development because of their restricted expression in the anterior neural ectoderm and eye field. Loss-of-function studies revealed that several of these factors play critical roles in the formation of the vertebrate eye (Hogan et al., 1988; Hill et al., 1991; Gridley et al., 1995; Matsuo et al., 1995; Mathers et al., 1997; Huh et al., 1999). Furthermore, rx1 and pax6 can cause ectopic retinal tissue to form in Xenopus when overexpressed in dorsal CNS progenitors (Mathers et al., 1997; Andreazzoli et al., 1999; Chow et al., 1999). In this study, we demonstrate that otx2, pax6, and rx1 may modulate the earliest steps of retinal specification prior to or during the formation of the eye fields. All three genes cause progeny of ventral, competent epidermal precursors (V1.1.1, V1.2.1, and V1.1.2) to express a retinal fate. Each affects the movements of cells so they occupy a domain conducive to a retinal fate. Xfh4 has none of these effects, demonstrating specificity to the observed phenotypes. We further demonstrate that the level of neural-inductive signaling to which cells are exposed significantly impacts the ability of otx2, pax6, and rx1 to cause cells to express a retinal fate.

### Anterior Neural Plate Transcription Factors Cause Epidermal Progenitors to Express a Retinal Fate

Loss-of-function and gain-of-function analyses clearly show that otx2, pax6, and rx1 have fundamental roles in the formation of the eye. The effects of these genes on the end point of retinal development (i.e., either lack of or ectopic presence of differentiated retinal cell types) have been established by these studies. Yet, how these regulators function during early stages of eye development (competence, specification, and determination) still requires investigation. Importantly, the results reported herein are the first demonstration that each of these factors can direct a

#### TABLE 3

Transcription Factors Alter Ventral Clone Movements during Gastrulation

<table>
<thead>
<tr>
<th>mRNA (n)</th>
<th>Coherent</th>
<th>Across animal cap only</th>
<th>Some in animal cap</th>
<th>Extended to animal marginal</th>
<th>Extended to vegetal hemisphere</th>
</tr>
</thead>
<tbody>
<tr>
<td>V1.1.1:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage 11.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ßgal (20)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>41.2</td>
<td>0</td>
</tr>
<tr>
<td>otx2 (17)</td>
<td>11.8</td>
<td>58.8</td>
<td>100</td>
<td>38.9</td>
<td>0</td>
</tr>
<tr>
<td>pax6 (18)</td>
<td>55.6</td>
<td>61.1</td>
<td>100</td>
<td>62.5</td>
<td>0</td>
</tr>
<tr>
<td>rx1 (8)</td>
<td>12.5</td>
<td>37.5</td>
<td>100</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Stage 12-12.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ßgal (13)</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>46.2</td>
<td>53.8</td>
</tr>
<tr>
<td>otx2 (13)</td>
<td>23.1</td>
<td>23.1</td>
<td>100</td>
<td>76.9</td>
<td>0</td>
</tr>
<tr>
<td>pax6 (17)</td>
<td>5.9</td>
<td>70.6</td>
<td>100</td>
<td>29.4</td>
<td>0</td>
</tr>
<tr>
<td>rx1 (19)</td>
<td>21.1</td>
<td>21.1</td>
<td>100</td>
<td>42.1</td>
<td>36.8</td>
</tr>
<tr>
<td>Stage 13:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ßgal (20)</td>
<td>40.0</td>
<td>0</td>
<td>20.0</td>
<td>40.0</td>
<td>60.0</td>
</tr>
<tr>
<td>otx2 (16)</td>
<td>18.8</td>
<td>0</td>
<td>100</td>
<td>43.7</td>
<td>56.3</td>
</tr>
<tr>
<td>pax6 (16)</td>
<td>25.0</td>
<td>0</td>
<td>50.0</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>rx1 (8)</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>62.5</td>
<td>37.5</td>
</tr>
</tbody>
</table>

| V1.1.2:  |
| Stage 11.5 |  
| ßgal (10) | 100 | 100 | 100 | 63.6 | 0 |
| otx2 (11) | 0 | 36.4 | 100 | 23.5 | 27.3 |
| pax6 (17) | 17.6 | 17.6 | 100 | 58.9 | 0 |
| rx1 (11) | 27.3 | 27.3 | 100 | 45.4 | 0 |
| Stage 12-12.5 |  
| ßgal (27) | 59.3 | 0 | 25.9 | 51.9 | 48.1 |
| otx2 (10) | 10 | 0 | 100 | 20.0 | 80.0 |
| pax6 (9) | 0 | 33.3 | 100 | 11.1 | 55.6 |
| rx1 (10) | 0 | 0 | 100 | 40.0 | 60.0 |
| Stage 13:  |
| ßgal (17) | 100 | 0 | 5.9 | 94.1 | 58.8 |
| otx2 (21) | 47.6 | 0 | 52.4 | 38.1 | 61.9 |
| pax6 (10) | 0 | 0 | 50 | 50.0 | 50.0 |
| rx1 (6) | 16.7 | 0 | 83.3 | 33.3 | 66.7 |
subset of ventral epidermal progeny to a retinal cell fate and suggest that these factors modify the response of progenitor cells at the time of retinal competence and/or specification. The ventral animal blastomeres that were targeted in these experiments are under the influence of high levels of BMP4 signaling (Fainsod et al., 1994; Hemmati-Brivanlou and Thomsen, 1995), that normally inhibit the competence of these blastomeres to form retina (Moore and Moody, 1999). Since the studied transcription factors normally are expressed in response to neural-inductive signaling, how does this change in cell fate (epidermal to retinal) occur in a nonconducive environment?

One possibility is that these factors directly neuralize cells; by initiating a neural fate in cells located in a BMP4-expressing domain, the cells then may be able to respond to the retina-forming activity of the genes. If this were the mechanism, one expected result would be the downregulation of BMP4 transcription. However, a second predicted result would be that genes that are directly transcribed in response to neural-inductive signaling (e.g., sox2, sox3; Penzel et al., 1997; Mizuseki et al., 1998) would be ectopically expressed. We found that otx2, pax6, and rx1 neither expanded the neural plate domain nor induced ectopic patches of neural tissues, indicating that these genes are not likely to neuralize ventral epidermal cells at the doses used. It remains a formal possibility that these genes maintain the expression of molecules that promote the competence of ectodermal cells to respond to retinogenic signaling.

A second possibility is that otx2, pax6, or rx1 activate a “retina-forming” program when cells are located in a sufficiently conducive environment. Other studies have shown that each of these genes can induce the ectopic expression of other anterior neural and eye field genes. Otx2 induces the expression of anterior neural markers in tissue explants in a dose-dependent manner (Blitz and Cho, 1995; Gammill and Sive, 2000). Likewise, pax6 and rx1 ectopically induce the expression of otx2, six3, and each other (Andreazzoli et al., 1999; Chow et al., 1999). Overexpression of other eye field genes, six3 and six6 (Opx2x), expands the expression of pax6 and rx1 (Bernier et al., 2000). Thus, the ability of otx2, pax6, and rx1 to cause epidermal cells to express a retinal fate, and of pax6 and rx1 to cause ectopic retinal tissue (data herein, Mathers et al., 1997; Andreazzoli et al., 1999; Chow et al., 1999) likely results from the initiation of this “retina-forming” program. But otx2, pax6, and rx1 are not acting simply as “master” switches of cell fate; they elicit a retinal fate only in the appropriate environment. First, not all cells of the injected ventral animal lineages became retinal, suggesting that subtle differences in the cellular environment impacted whether these genes could effect a retinal fate. We identified one element in that environment to be the level of neural-inductive signaling (see below). Second, no cells of injected vegetal lineages became retinal. Expression of these genes in the ventral posterior quadrant was not sufficient to override either a vegetal maternal block to retinal fate (Moore and Moody, 1999) or posteriorizing neural signals (Gammill and Sive, 2000).

A third possibility is that early expression of otx2, pax6, and rx1 initiates cellular programs that allow cells to move into a retinogenic environment. The misexpression of these factors in the V1.1.1 and V1.1.2 lineages caused epidermally derived cells to be incorporated within the endogenous retina, indicating that cell movements were affected. We demonstrate that indeed these transcription factors altered gastrulation movements such that cells became appropriately positioned to enter the eye field. In general, some cells dispersed earlier than in controls and maintained more anterior positions. As a result of these changes in morphogenetic movements, a subset of epidermal cells moved into an environment that promoted neural and retinal fates.

The ability of these transcription factors to mediate changes in cell movement is supported by a few recent studies. Zebrafish retinal progenitors express rx genes during their lateral migration to form the optic primordia, and ectopic expression in forebrain precursors appears to impair normal cell movements (Chuang and Raymond, 2001). Several studies implicate pax6 in the regulation of cellular migrations in the developing cerebellum (Engelkamp et al., 1999), cerebral cortex (Warren et al., 1999), and basal ganglia (Chapouton et al., 1999). How cell movements might be regulated is not yet known, but it has been reported that cells lacking Pax6 have reduced adhesive properties (Collinson et al., 2000).

These studies suggest that otx2, pax6, and rx1 may normally play a role in regulating cell dispersal. More specifically, these factors may regulate the movement of cells during the early formation of the presumptive anterior neural plate. Lineage-tracing studies reported that cells fated to form the anterior neural ectoderm and presumptive eye field disperse during gastrulation movements (Jacobson and Hirose, 1978; Wetts and Fraser, 1989; Huang and Moody, 1993; Bauer et al., 1994). This dispersal occurs prominently in the presumptive eye field, and is thought to account for the bilateral origin of each retina (Huang and Moody, 1993). In addition, cellular dispersal may lead to reorganization of cells into functional domains. For ex-
ample, treatment of animal cap ectoderm with low levels of Noggin causes increased cell dispersal and reorganization into forebrain marker-expressing domains (Knecht and Harland, 1997). Considering that such movements likely impact the formation of the eye field, it may be that these transcription factors (otx2, pax6, rx1) normally regulate the process of cell mixing in the retinogenic domain of the anterior neural plate. The onset of expression of otx2 occurs during early gastrulation and pax6 and rx1 are initially expressed at late gastrulation. Thus, expression of transcriptional regulators and signaling factors during the establishment of anterior neural/retinal competence and specification may play an important role in the cellular reorganizations that establish the various forebrain domains. By expressing these factors in ventral epidermal lineages that normally remain coherent, our assays have revealed a role for these factors in cellular movements and patterning.

The Ability of pax6 to Elicit a Retinal Fate Depends on Position within the BMP4/noggin Signaling Field

When pax6 was expressed in ventral blastomeres, there was both an increase in the number of cells contributed to the native retina and the frequency of ectopic formation of retinal cell types. The latter phenotype is similar to those shown by Chow et al. (1999) in response to pax6 overexpression in dorsal blastomeres, albeit at a lower frequency. Those authors argued that pax6 misexpression can only specify cells to retinal cell fates in dorsal animal progenitors. The demonstration herein of similar effects in ventral blastomeres clearly indicates that the responsiveness is not simply a dorsal–ventral difference. Both studies suggest that the responsiveness of cells to pax6 activity is dependent on the level of neural-inductive signaling. This idea is supported by two sets of experiments. First, the position of a blastomere within the normal field of neural induction correlates with the ability of pax6 to elicit a retinal fate. When pax6 was expressed in CNS/retina progenitors, large ectopic retinas formed (Chow et al., 1999). When pax6 was expressed in ventral lateral blastomere (V1.2.1), whose progeny lie on the edge of the neural-inductive field (based on its contributions to neural structures; Moody, 1987), there was a significant increase in the frequency that embryos contributed to retina, the number of cells contributed to the retina, and the frequency of ectopic retinal cell formation. When pax6 was expressed in a ventral animal blastomere (V1.1.1), which does not contribute to the retina but does produce small numbers of primary spinal neurons (Moody, 1987, 1989), consistent but small contributions to the retina and small patches of ectopic retinal tissues were observed. When pax6 was expressed in a ventral equatorial blastomere (V1.1.2), whose progeny do not contribute to the nervous system (Moody, 1987) and thus are not exposed to anti-BMP factors, retinal contributions and ectopic retinal cell types were observed at an even lower frequency. Second, changing the level of BMP signaling in these blastomeres altered the effectiveness of pax6 misexpression. Increasing the level of Noggin concomitant with pax6 expression in V1.1.1 significantly increased the frequency of ectopic retinal cell types. Conversely, increasing the level of BMP4 concomitant with pax6 expression in V1.2.1 inhibited cells from a retinal fate. Thus, the effectiveness of pax6 to produce retinal phenotypes is in inverse proportion to a cell’s position within the BMP4 gradient.

The different results obtained in our experiments and those of Chow et al. (1999) regarding ventral misexpression of pax6 may be explained by differences in experimental approaches. They targeted pax6 mRNA to ventral blastomeres at the 16-cell stage. If the mRNA became localized to the equatorial daughter (V1.1.2) rather than the animal daughter (V1.1.1) of this cell, no ectopic retinal phenotypes would have been observed by whole-mount analysis because they are very small (see Figs. 5D–5F). We only detected retinal contributions from these altered lineages by screening serially sectioned tissue. Nonetheless, although our study predicts a different boundary of pax6 effectiveness in retinal specification, both studies show that the ability of pax6 to specify cells to a retinal fate depends on the level of neural induction.

In summary, the experiments described herein demonstrate that three anterior neural plate transcription factors, otx2, pax6, and rx1, cause epidermal cells to express a retinal fate in proportion to levels of neural-inductive signaling. This phenotype results not from ectopic neural-inductive signaling but by altered morphogenetic cell movements that allow cells to access the retinogenic environment. These experiments indicate that otx2, pax6, and rx1 have multiple effects on cells, which are context-dependent, to cause the acquisition of a retinal fate. Several studies define multiple roles for pax6 in tissue-specific fate decisions (Altmann et al., 1997; Chow et al., 1999), identity of brain regions (Bishop et al., 2000; Yun et al., 2000; Schwartz et al., 2000), and cell-type identity (Ericson et al., 1997; Perron et al., 1998; Mastick and Andrews, 2001). As transcription factors, otx2, pax6, and rx1 are likely to have multiple roles in the establishment of retinal competence and specification. Since these genes also are expressed in tissues other than the developing eye, it will be important to identify the downstream targets that direct their tissue-specific functions at the different stages of eye development. The identification of these target genes will provide important insights as to how progenitor cells transition through the stages of retinal competence, specification, and determination to ultimately acquire their differentiated retinal cell fates.

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