Throughout development, the patterning of a field of cells is often achieved through the inductive interactions of signaling molecules produced by a discrete source. An example of such a signaling source is the apical ectodermal ridge (AER) of the vertebrate limb bud. The primary function of the AER is to control the outgrowth and patterning of the limb bud. This function appears to be mediated by members of the fibroblast growth factor family (notably Fgf-2, Fgf-4 and Fgf-8) which are produced by the AER. In animals from which the AER has been removed, limb bud outgrowth and patterning can be rescued and maintained by exogenous application of these signaling molecules (Tickle, 1995). Since the molecules produced by AER have such a strong inductive effect, the proper spatial and temporal control of the formation of this signaling source is critical for normal development.

The AER forms at the dorsal/ventral boundary of the limb bud and is morphologically identifiable in the chick around stage 18. AER formation, however, is thought to begin earlier as the earliest known marker of the AER, *fgf-8*, is expressed in ectodermal cells along the dorsal/ventral boundary at stage 16. Due in part to this expression pattern, *fgf-8* was proposed to play a role in the formation of the AER within the ectoderm. The function of Fgf-8, however, appears to be similar to that of the mature AER, as Fgf-8 has been proposed to stimulate the initial outgrowth of the limb field and later in development to control proliferation and patterning of the limb bud (Mahmood et al., 1995; Crossley et al., 1996; Vogel et al., 1996). Therefore, Fgf-8 may play a role in the formation of a morphologically recognizable AER, but its appearance marks the completed formation of the AER as a signaling source. It should be noted, however, that this ectodermal function of Fgf-8 is distinct from its proposed function in the mediation of signaling from the mesoderm to the ectoderm that allows AER formation to be initiated (Crossley et al., 1996; Vogel et al., 1996).

Recently the chick gene *Radical fringe* (*R-fng*) has been shown to be expressed prior to *fgf-8* and play a critical role in AER formation (Laufer et al., 1997; Rodriguez-Esteban et al., 1997). *R-fng* is first expressed in the dorsal ectoderm at stage 15 and is subsequently upregulated along the dorsal/ventral boundary at stage 16. The expression is then maintained in the morphologically recognizable AER, while it is gradually downregulated in the dorsal ectoderm. *Radical fringe* is homologous to the *Drosophila* gene *fringe* that has been shown to play a critical role in the formation of a signaling source analogous to the AER, the wing margin (Irvine and Wieschaus, 1994; Kim et al., 1995). In *Drosophila*, interactions between *fringe*-expressing cells and non-expressing cells, on either side of the dorsal/ventral boundary, result in the induction of the membrane bound ligand Serrate. Serrate, through interactions with its receptor, Notch, then triggers the formation of a functional wing margin through the induction of genes involved in wing growth and patterning. Similarly, experiments where new boundaries of *R-fng* expression are created through the ectopic expression of *R-fng* in the ventral compartment or the repression of *R-fng* in portions of the dorsal compartment, result in the formation of ectopic AERs (Laufer et al., 1997; Rodriguez-Esteban et al., 1997). This suggests that the boundary between *R-fng* expressing and non-expressing cells regulates the formation of the AER. Chick homologues of *Drosophila Serrate* and *Notch*, *Serrate-2* and *Notch-1*, have been cloned (Laufer et al., 1997; Rodriguez-Esteban et al., 1997). It is not clear, however, whether the inductive activities of *R-fng* are mediated through a pathway involving *Serrate-2* and *Notch-1*, nor is it clear whether a gene involved in limb bud outgrowth and patterning, *fgf-8*, is induced as a result of this pathway. If the mechanisms by which the *Drosophila* wing margin is formed are extendible to the formation of the vertebrate AER, then one would predict that the inductive functions of *R-fng* would be mediated by *Serrate-2* and *Notch-1* resulting in the induction of *fgf-8* and a functional AER.

Hypothesis: *fgf-8* expression is induced in the limb bud ectoderm during formation of the AER through the sequential actions of *R-fng*, and the *Serrate-2/Notch-1* signaling system.

Specific Aim 1: Determine whether the *Serrate-2/Notch-1* signaling system is required for the formation of the AER.

Serrate-2 is reported to be "expressed in the AER from the earliest stages of its formation" (Laufer et al., 1997) while *Notch-1* is simply reported as being expressed in the AER (Myat et al.,

1996). Because it is unclear from what stages these genes are initially expressed, I will first closely examine the expression patterns of these genes to confirm that they are expressed at the appropriate time and place to be required for the formation of the AER.

To test the requirement for the *Serrate-2/Notch-1* signaling system during AER formation I will carry out ectopic expression studies in the chick limb bud using a replication-competent retroviral vector (Laufer et al., 1997; Rodriguez-Esteban et al., 1997). Since Serrate-2 is the proposed ligand for the Notch-1 receptor, the ectopic expression of either gene alone should not be sufficient to affect AER formation unless the ectopically expressing cells come in close proximity to cells expressing the other gene. However, a truncated form of the *Notch-1* homologues in *Drosophila* and *C. elegans*, consisting of only the intracellular domains, have been shown to be constitutively active *in vivo* (Struhl et al., 1993). The high degree of structural similarity between Notch family members suggests that a similarly truncated form of *Notch-1* will also be constitutively active. The ectopic expression of constitutively active Notch-1 and analysis of the resulting phenotypes will allow the role of the *Serrate-2/Notch-1* signaling system during AER formation to be determined.

To further test the requirement for the *Serrate-2/Notch-1* signaling system, the mouse homologues of both of these genes will be cloned by screening a cDNA library with probes complementary to the chick genes. Once cloned, mice lacking either *Serrate-2* or *Notch-1* function will be generated using standard homologous recombination techniques. Phenotypic analysis of the mutant mice will then be carried out with a focus on the developing limb bud.

Note: It is possible that when the expression patterns of *Serrate-2* and *Notch-1* are examined more closely they will not be expressed until after *fgf-8* is first expressed in the limb bud ectoderm, i.e. after formation of the AER is complete. If this is the case, then either (1) *fgf-8* is induced by *R-fng* signaling, but through a *Serrate-2/Notch-1* independent pathway or (2) *fgf-8* is induced by a mechanism other than *R-fng* signaling, and *R-fng* signaling through *Serrate-2/Notch-1* acts simply to maintain *fgf-8* expression in the AER. If the *Serrate-2/Notch-1* signaling system is also found to be required for the proper function of the AER, then the most likely scenario is (2). These results would disprove the hypothesis I have proposed and (2) would become my working hypothesis.

Specific Aim 2: Determine whether the *Serrate-2/Notch-1* signaling system functions downstream of *R-fng* in the formation of the AER.

It has been shown that ectopic AERs are formed when new boundaries of *R-fng* expressing and non-expressing cells are created. If *Serrate-2* is induced as a result of the juxtaposition of these cells, as *Serrate* is in *Drosophila*, then it should be expressed during the formation of such ectopic AERs. To test this ectopic AERs will be induced through the ectopic expression of *R-fng* in the ventral compartment or the repression of *R-fng* in portions of the dorsal compartment (Laufer et al., 1997; Rodriguez-Esteban et al., 1997) and the expression of *Serrate-2* examined.

To further test whether the *Serrate-2/Notch-1* signaling system functions downstream of *R-fng* boundary interactions, the recessive mutant chick strain *limbless* will be used. *limbless* homozygous limb buds have previously been shown to be bidorsal in character and lack an AER. Consistent with these observations, *R-fng* is expressed throughout the early limb bud ectoderm before it regresses due to failure of AER formation (Laufer et al., 1997; Rodriguez-Esteban et al., 1997). I will examine *limbless* mutant limb buds for *Serrate-2* expression. If *Serrate-2* expression requires *R-fng* boundary interactions, then no *Serrate-2* expression should be observed. Additionally, if *R-fng* boundary interactions act through the *Serrate-2/Notch-1* signaling system to induce AER formation as proposed, then ectopic expression of the constitutively active form of Notch-1 (see above) should rescue a *limbless* limb bud.

Specific Aim 3: Determine whether *fgf-8* is downstream of the *Serrate-2/Notch-1* signaling system.

To test this the expression of *fgf-8* will be examined in chick limb buds where the *Serrate-2/Notch-1* signaling system has been ectopically activated or inactivated. Activation of the signaling system will be achieved through the ectopic expression of the constitutively active form of Notch-1,

while inactivation will be mimicked through the ectopic expression of either *Serrate-2* or *Notch-1* alone. In the later case it will be important to visualize the areas of retroviral infection using an antibody to a retroviral protein epitope (Laufer et al., 1997).

Specific Aim 4: Identify, clone and examine the expression pattern of the R-fng receptor or partner.

R-fng encodes a putative secreted signaling protein and therefore must have a corresponding receptor or partner protein with which it interacts in order to mediate its effect on target cells. To identify the R-fng receptor or partner protein I will perform a yeast two hybrid screen using the R-fng protein to screen a chick limb bud expression library (Fields and Song, 1989; Fields and Sternglanz, 1994). The identification and eventual cloning of genes that directly interact with R-fng will enable the elucidation of the mechanism by which a boundary between R-fng expressing and non-expressing cells results in the formation of an AER.

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