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Exogenous retinoic acid induces a stage-specific, transient and progressive extension of *Sonic hedgehog* expression across the pectoral fin bud of Zebrafish

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ABSTRACT We have performed a time course analysis of the expression of *Sonic hedgehog (shh)* and *patched1 (ptc1)* in response to exogenous retinoic acid (RA) application to get some insight into the mechanism(s) underlying the formation of a mirror-image duplication of *shh* and *ptc1* domains of expression in the pectoral fin buds of zebrafish. We have shown that RA exposure during the early stages of pectoral fin development first results in a rapid decrease or complete loss of *shh*/*ptc1* expression. This is followed by reappearance of transcripts in the normal posterior domain, then by a stage-dependent and progressive expansion of the *shh* domain from the ZPA towards the anterior margin of the bud. *Shh* transcripts are induced in mesenchymal cells underlying the ventral ectoderm at the base of the bud. Once *shh* expression is activated in the most anterior cells, the number of *shh*-expressing cells increases in this region, possibly through an amplification mechanism involving signals from the apical ectodermal ridge. At this time, *shh* expression disappears from cells centrally located in the bud, resulting in the formation of the two distinct domains. An anterior extension of *shh* expression is also obtained in *syu* mutants with impaired shh function, suggesting that *shh* induction across the fin bud is independent of shh signaling. This study suggests the existence of complex mechanisms controlling the spatial and temporal expression of *shh* in the developing fin bud.

KEY WORDS: *zebrafish, fin development, sonic hedgehog, patched1, retinoic acid*

Introduction

Development of the tetrapod limb depends on diffusible signals emanating from specific centers, the apical ectodermal ridge (AER), the zone of polarizing activity (ZPA), and the dorsal ectoderm (Cohn and Tickle 1996; Johnson and Tabin 1997; Tickle and Munsterberg 2001). Proper growth and patterning of the limb also relies on interactions between the different signaling centers. The ZPA located in the posterior mesenchyme of the limb bud is involved in the patterning of the antero-posterior axis of the limb as demonstrated through grafting experiments. When placed at the anterior margin of the limb bud, the ZPA induces the formation of a mirror-image duplication of digits (Saunders and Gasseling 1968). Sonic hedgehog (shh), a signaling molecule expressed in the ZPA, and retinoic acid (RA) have been shown to play critical role in mediating the polarizing activity. Like the ZPA, ectopic shh or RA at the anterior margin of the limb can induce a mirror-image duplication of the digits (Tickle *et al.*, 1982; Riddle *et al.*, 1993; Chang *et al.*, 1994). In these experiments, RA can induce *shh* expression suggesting that it is acting upstream of shh (Helms *et al.*, 1994). However, it has been shown that shh by itself has low polarizing activity and in order to produce the same activity as the ZPA, it would need the synergistic cooperation of a factor inducible by RA (Ogura *et al.*, 1996). The mechanisms by which RA regulates *shh* expression are currently unclear. However, the induction of *shh* expression following RA-bead implantation at the anterior margin of the chick wing does not happen before 24 hours, clearly indicating an indirect mechanism of action (Riddle *et al.*, 1993).

The paired fins of fish, composed of the pectoral and pelvic fins, are phylogenetically related to the tetrapod limbs. In the teleost zebrafish, *Danio rerio*, the early pectoral fin buds are morphologically similar to the tetrapod limb buds (Grandel and Schulte-Merker 1998).

Abbreviations used in this paper: ptc1, patched 1 gene; RA, retinoic acid; shh, sonic hedgehog; ZPA, zone of polarizing activity.

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Development initiates at 28 hours post fertilization (hpf) with the proliferation of mesodermal cells on the dorsal side of the yolk sac at the level of the second and third somites. The pectoral fin buds are rimmed along their antero-posterior axis by a thickened epidermis which resembles the tetrapod AER; however, the functional homology of the apical ectodermal fold and the AER has not yet been confirmed. By 36 hpf, the apical ridge folds on itself and beginning around 48 hpf, this apical fold distally elongates to form the fin fold that is later invaded by mesenchyme. The continued elongation of the fin fold will give rise to the part of the fin containing the dermal ray skeleton.

Although limb and fin development rapidly diverge, gene expression analyses have shown that the molecular mechanisms underlying the early steps of their development are similar (Ekker *et al.,* 1992; Krauss *et al.,* 1993; Akimenko and Ekker 1995; Akimenko *et al.,* 1995; Sordino *et al.,* 1995; Neumann *et al.,* 1999). For example, the early phase of expression of the 5'members of the Hox clusters are conserved in tetrapod and zebrafish (Sordino *et al.,* 1995). In tetrapods and zebrafish, *engrailed1*, a member of the *engrailed* homeobox gene family, is expressed in the ventral ectoderm of the limb/fin bud (Hatta *et al.,* 1991; Ekker *et al.,* 1992). Transcripts of the zebrafish ortholog of *shh* are found in the posterior mesenchymal region of the pectoral fin buds (Krauss *et al.,* 1993). Its receptor *patched1* (*ptc1*), which is a target of shh signaling, is expressed in the posterior part of the fin bud in a domain encompassing the *shh* domain (Concordet *et al.,* 1996). There is considerable evidence indicating that shh plays a similar role in antero-posterior patterning of the fin and limb buds. Analysis of zebrafish embryos carrying the *sonic you* (*syu*) mutation that disrupts the *shh* gene suggests that shh is necessary for the establishment of some aspects of antero-posterior polarity but not all (Schauerte *et al.,* 1998; Neumann *et al.,* 1999). For example, in the absence of shh signaling, there is no posterior activation of *hoxd-13* and *hoxa-13.* In contrast, *hoxd-11, hoxd-12* and, *hoxa-11* are expressed posteriorly in *syu* mutants, indicating that their activation does not seem to depend on shh activity (Neumann *et al.,* 1999). Pectoral fin buds of *syu* mutants rapidly fail to develop, presumably due to the impaired function of the apical ectodermal fold (Neumann *et al.,* 1999).

Previous analyses have shown that administration of RA to zebrafish embryos for a short period of time at the onset of pectoral fin bud formation leads to the ectopic expression of *shh* at the anterior margin of the bud starting 24 hours after the end of the treatment (Akimenko and Ekker 1995; Bruneau *et al.,* 1997). The similarity of the response to RA with that observed following local RA treatment in the chick limb bud further supports the hypothesis of common antero-posterior patterning mechanisms in the early fin/limb buds. One interesting difference, however, between experiments performed in chick embryos and those in zebrafish is that the duplication of the domain of *shh* expression is obtained following global rather than local application of RA to the zebrafish embryo. These results suggest that only a small subset of cells acquire properties of cells of the polarizing zone. The location of these cells in the vicinity of the apical ectodermal fold suggests that the fold is required to maintain these polarizing properties (Akimenko

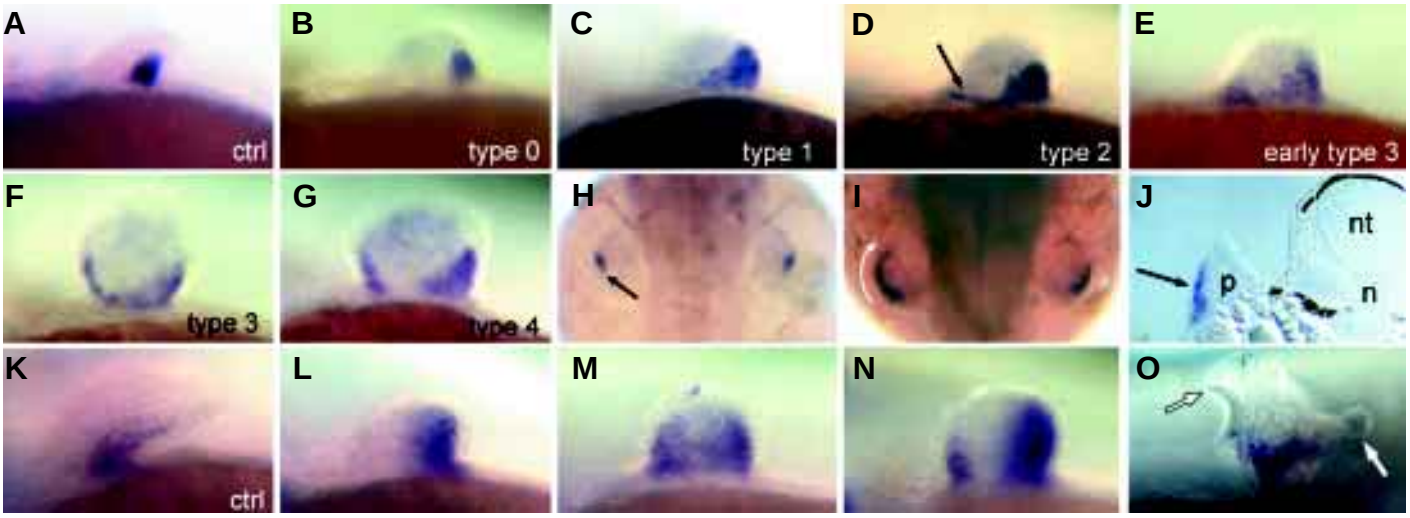

Fig. 1. Progressive anterior extension of *shh* and *ptc1* domains of expression following RA treatment. *Embryos at 30 hpf were exposed for 2 h to* 10^{-6} *M all-trans retinoic acid in 0.5% DMSO (B-G,I,J,L-O). Controls (A,H,K) were incubated in 0.5% DMSO for the same period of time. RA-treated and control embryos were fixed 16 h (A-E, G, K-O) or 24 h (F,H-J) following treatment. (A-G) Lateral views, anterior is to the left. Various* shh *patterns of expression.* **(B)** *Normal expression pattern of* shh *referred to as type 0.* **(C)** *Short anterior extension of* shh *expression (type 1).* **(D)** *Long extension across the antero-posterior axis of the bud indicated by the arrow (type 2).* **(E)** *Small anterior domain representing an early type 3.* **(F)** *Anterior and posterior domains of expression interconnected by a thin stripe of* shh*-expressing cells (type 3).* **(G)** *Discrete anterior and posterior* shh *domains (type 4). (H,I) Dorsal views (anterior is to the top) of a control embryo at 48 hpf showing the normal expression pattern of* shh *restricted to the posterior part of the fin bud* **(H)** *and of an RA-treated embryo exhibiting the anterior extension domain of* shh *on the ventral side of the bud indicated by the white arrow* **(I)**. **(J)** *Transverse section of 48 hpf RA-treated embryo at the level of the central part of a type 3 bud showing that* shh *expression is restricted to mesenchymal cells underlying the ventral ectoderm. (K-N)* ptc1 *expression at 48 hpf in control* **(K)** *and RA-treated embryos (L-N). As for* shh*, various patterns of expression of* ptc1 *are observed following RA treatment. However, due to the large expression domain of* ptc1*, the distinction between the various types of patterns is less clear.* **(L)** *type 1;* **(M)** *type 3;* **(N)** *type 4;* **(O)** ptc1 *expression in a 3 day old embryo presenting a duplicated fin fold. The arrows indicate the anterior and posterior fin folds. nt, neural tube; n, notochord; p, pectoral fin bud.*

and Ekker 1995; Bruneau *et al.,* 1997). Indeed, a similar requirement has been demonstrated for the AER of the chick limb bud (Vogel and Tickle 1993; Helms *et al.,* 1996).

In the present study, we examined the regulation of *shh* in response to RA during the interval of time between the end of RA treatment and the induction of ectopic expression of *shh* in the pectoral fin buds of zebrafish. We also analyzed the developmental time window during which RA has the capacity to respecify the cells of the anterior margin.

Results

Ectopic Expression of shh *and* ptc1 *at the Anterior Margin of the Pectoral Fin Bud is established via a Progressive Extension of the Posterior Domain of Expression Across the Fin Bud*

Previous studies in our laboratory have demonstrated that treatment of zebrafish embryos with 10^{-6} M *all-trans* RA for a period of 2 hours at either 24 or 30 hpf induces the ectopic expression of *shh* at the anterior margin of the pectoral fin bud 24 hours later (Akimenko and Ekker 1995). We initially used the same conditions to examine the effects of RA treatment on *shh* and *ptc1* expression 10, 16, 24 and 36-48 hours following the end of RA treatment.

When zebrafish embryos treated at 30hpf are examined 10 h following RA treatment, we observe a normal pattern of expression of *shh* in the posterior part of the fin bud (Table 1). 16 hours post RA treatment, embryos exhibit various patterns of *shh* expression (Table 1, Fig. 1 A-E). While the normal posterior domain of expression of *shh* is observed in 32.5% of fin buds examined (Fig. 1 A,B), 47.9% present a short anterior extension of this domain at the base of the fin (Fig. 1C), 9.4% reveal an extension of the domain across the bud (Fig. 1D), 8.5% present a small anterior domain of *shh*-expressing cells inter-connected to the posterior domain by a thin stripe of cells (Fig. 1E) and 1.7% present two discrete domains, the normal posterior domain and the ectopic anterior domain (Table 1). By 24 hours post treatment, the same patterns are observed but the percentage of buds showing the various patterns differ from the 16 h time point (Table 1). The most frequent patterns observed at the 24 hour time point are the discrete, duplicated anterior and posterior domains (35%) (Fig. 1G) as well as the two domains inter-connected by a thin stripe of *shh*-expressing cells on the ventral side at the base of the bud (30.5%)(Fig. 1F). However, 12.6% and 15.3% of the buds still present a short or long extension of the posterior domain (Fig. 1 H,I). The normal unique and posterior domain of expression of *shh* is observed in 6.6% of the buds. Later still, between 36 – 48 hours post treatment, *shh* expression is observed only in the two discrete domains of expression (data not shown and Akimenko and Ekker 1995). Altogether, these results suggest that as the observation time point following the end of RA treatment increases, the domain of expression of *shh* is altered from its normal posterior expression to a progressively anterior extension of the posterior domain which subsequently spans the entire antero-posterior axis of the bud. Once the extension has reached the anterior border, the number of *shh*-expressing cells at this level increases, possibly through a mechanism of amplification involving interactions between signals from the apical ectoderm ridge and the newly *shh*-expressing cells. In contrast, *shh* expression is turned off in cells located in the central part of the bud and which are not under the influence of the apical ectoderm, leaving two discrete domains located at the posterior and anterior margin of the bud. The anterior extension of *shh* domain is composed of mesenchymal cells located at the base of the bud and underlying the ventral ectoderm as shown on cross-section of embryos following *in situ* hybridization with *shh* probe (Fig. 1J). As previously described, morphological effects observed following RA exposure includes an overall delay of fin development, a thickening at the base of the fin bud, and a lack of rotation of the bud (Akimenko and Ekker 1995). We also observed that 15 to 30 minutes exposure to RA is sufficient to induce the expression of *shh* in cells anterior to the ZPA 24 hrs later. However, a larger proportion of embryos exhibit an ectopic expression and/or a duplication of the *shh* domain following 1-2 hour exposure (data not shown).

As expected, *ptc1* expression is affected in a manner similar to that of *shh* following RA treatment (Fig. 1 K-O; Table 1). Interestingly, ectopic expression of *shh* and *ptc1* in the anterior mesenchyme of the bud is occasionally accompanied by varying degrees of fin duplication (Fig. 1 O).

Embryos treated at 36 hpf with 10^{-6} M RA exhibit similar extension and eventual duplication of *shh* and *ptc1* expression in the developing fin bud, although the proportion of embryos showing a pattern different from that of untreated controls is considerably less than for embryos treated just prior to or during the early stages of pectoral fin outgrowth (24 –30 hpf) (Table 1).

In contrast, embryos treated at 48 hpf rarely exhibit such anterior extension and/or duplication of *shh* or *ptc1* expression 10 to 48 hours following treatment (Table 1), suggesting that RA at this

TABLE 1

TIME COURSE ANALYSIS OF THE EFFECTS OF A 2 HOUR EXPOSURE TO 10^{-6} M ALL-TRANS RETINOIC ACID ON *shh* AND *ptc1* EXPRESSION IN THE PECTORAL FIN BUDS OF ZEBRAFISH EMBRYOS

				Type of buds					
				0	1	2	3	4	5
Stage	Marker	T	n						
30 hpf	shh	10	14	100.0					
		16	117	32.5	47.9	9.4	8.5	1.7	
		24	151	6.6	12.6	15.3	30.5	35	
	ptc1	16	100	44.0	27.0	22.0	3.0	4.0	
		24	78	10.0	9.0	24.4	31.0	25.6	
		36-48	8				37.5	62.5	
36 hpf	shh	10	20	85.0	15.0				
		16	33	27.3	66.6		6.1		
		24	10	40.0	40.0	20.0			
		36-48	30	36.7	30.0	3.3		30.0	
	ptc1	16	6		100.0				
		24	11	9.1	63.6			18.2	9.1
		36-48	9	22.2	33.3	11.2		33.3	
48 hpf	shh	10	19	5.3					97.4
		16	40	85.0	10.0			5.0	
		24	37	67.6	13.5				18.9
		36-48	14	78.6					21.4
	ptc1	16	10	100.0					
		24	16	100.0					
		36-48	10	80.0					20.0

Type of buds: refers to the different types of expression patterns observed. Type 0: normal expression pattern (similar to control). Type 1: short extension. Type 2: long extension across the bud. Type 3: interconnected anterior and posterior domains. Type 4: discrete anterior and posterior domains. Type 5: no expression. Numbers below the schematic representation of the buds indicate the percentage of the total number (n) of buds showing a specific pattern of gene expression. Stage: developmental stage at which RA treatment started. T, number of hours following RA treatment at which gene expression was determined. n, sample size.

developmental stage can no longer respecify the cells of the fin buds to a more posterior positional identity.

The Immediate Response to RA is a Decrease or Complete Down-Regulation of shh *and* ptc1

Immediately following a 2 hour treatment with 10^{-6} M RA, 30 to 48 hour old embryos exhibit a decrease or complete loss of *shh* expression in the posterior margin of the fin bud (Fig. 2 A-C). *Ptc1* expression is diminished, but not absent from this region under the same conditions (Fig. 2 D-F). Not all gene markers expressed endogenously in the developing pectoral fin buds are similarly affected by the RA treatment. The expression of *dlx3* in the apical ectodermal fold and of *msxC* in the mesenchyme underlying the apical ectodermal fold are unaffected in the buds of 36 and 48 h old embryos following a 2 h RA exposure (Fig. 2 G-I and data not shown), indicating the effects of RA are specific to *shh* and *ptc1* expression.

As described above, several hours (10 to 16hr) post treatment, expression of *shh* and *ptc1* is re-activated in embryos that were treated at 30 hpf. However, in a large proportion of embryos (97.4%) treated at 48 hpf, there is no detectable expression of *shh* (Table 1) 10 h following RA treatment and, even at later stages, *shh* expression is not re-activated in about 20% of the buds.

We found that a short exposure of 30 min is enough to provoke the decrease or loss of *shh* expression immediately following the treatment, in 30% (n = 66) of the buds of embryos treated at 48 hpf. However, when the duration of RA treatment is 1 or 2 hours, the proportion of affected buds is increased to 50% (n = 84) and 90% (n = 46), respectively (data not shown).

Effects of RA Treatment on Shh *Expression are Dose-Dependent*

To determine the optimal dose required to induce *shh* expression at the anterior margin of the fin bud, zebrafish embryos were treated at 30 hpf with various concentrations of *all trans* RA ranging from 10^{-7} M to 10^{-5} M and *shh* expression subsequently examined 24 h post treatment (Table 2). Embryos treated with concentrations of 10^{-7}M – 5 X 10^{-7}M RA exhibit an anterior extension, but not a complete duplication of the *shh* expression domain. At concentrations of 10^{-6}M – 5 X 10^{-6}M RA, a complete duplication of *shh* expression at the anterior margin of the fin bud is observed consistently, although at the higher of the two concentrations we observed more severe morphological effects. Not surprisingly, at higher concentrations (5 X 10^{-6} and 10^{-5} M RA), RA has severe effects on the development of the entire embryo; little if any fin buds are apparent, and *shh* expression is absent even from the posterior mesenchyme of the bud where it is normally expressed (Table 2). Nevertheless, at all doses tested, RA-treated embryos do not survive longer than 5 to 7 days post fertilization due to the teratogenic effects of RA, preventing us from examining the late consequences of RA treatment on fin development.

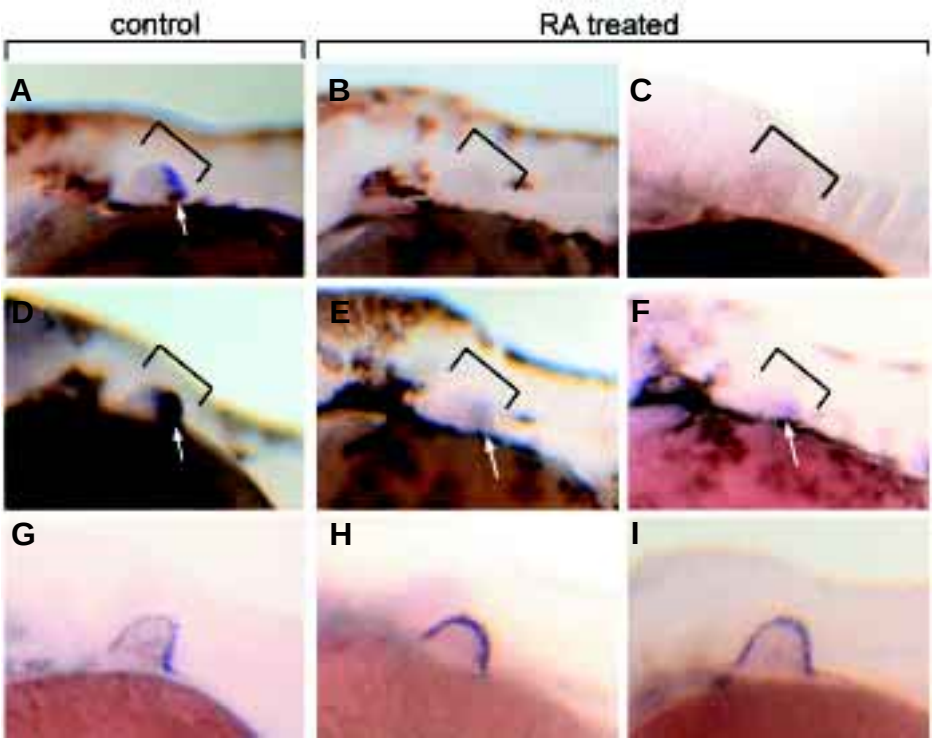

Fig. 2. Decrease or loss of *shh* and *ptc1* expression immediately following RA treatment. *Embryos were exposed for 2 h to 10^{-6} M all-trans retinoic acid in 0.5% DMSO at 36 hpf (B,E,H) or at 48 hpf (C,F,I). Controls at 36 hpf (A,D,G) were incubated in 0.5% DMSO for the same period of time. RA-treated and control embryos were fixed immediately following treatment. (A-I) Lateral views, anterior is to the left.* **(A)** shh *expression in control embryo is restricted to the posterior part of the bud (white arrow). Following RA treatment,* shh *expression is either significantly decreased* **(B)** *or completely lost* **(C)**. **(D)** ptc1 *expression in control embryo; the white arrow indicates the large domain of* ptc1 *expression in the posterior mesenchyme of the bud.* **(E,F)** *Following RA treatment,* ptc1 *expression is significantly decreased.* **(G)** dlx3 *in control embryo is expressed in the apical ectodermal fold.* **(H,I)** *RA treatment has no effect on* dlx3 *expression in the apical ectodermal fold. The brackets in (A-F) indicate the position of the pectoral fin buds.*

The Anterior Extension of Shh *and* Ptc1 *Expression is Independent of Shh Signaling*

The progressive extension of the domain of expression of *shh* and its receptor *ptc1* towards the anterior margin of the developing fin bud following RA treatment could be attributed to shh signaling in a cell-cell relay fashion. To explore this possibility, we analyzed the effects of RA treatment on *shh* and *ptc1* expression in the developing fin buds of *syu* ^{tbx392} mutant zebrafish. The *tbx392* allele of the *syu* mutation is a G>A change in the conserved splice donor junction of the first intron that impairs splicing of the first intron of *shh* gene, resulting in a truncated open reading frame and a biologically inactive protein (Schauerte *et al.,* 1998). Phenotypes resulting from this mutation include a failure to form a horizontal myoseptum, the formation of U-shaped rather than V-shaped somites, and a reduction in pectoral fin outgrowth (Schauerte *et al.,* 1998). In support of previous reports by Schauerte *et al.,* (1998), we observed that *shh* is expressed weakly and in a more restricted domain, relative to their wild-type siblings, in the ZPA of the fin buds of 40 hpf *syu* ^{tbx392} mutant zebrafish (Fig. 3 A,B). By 48 hpf, *shh*

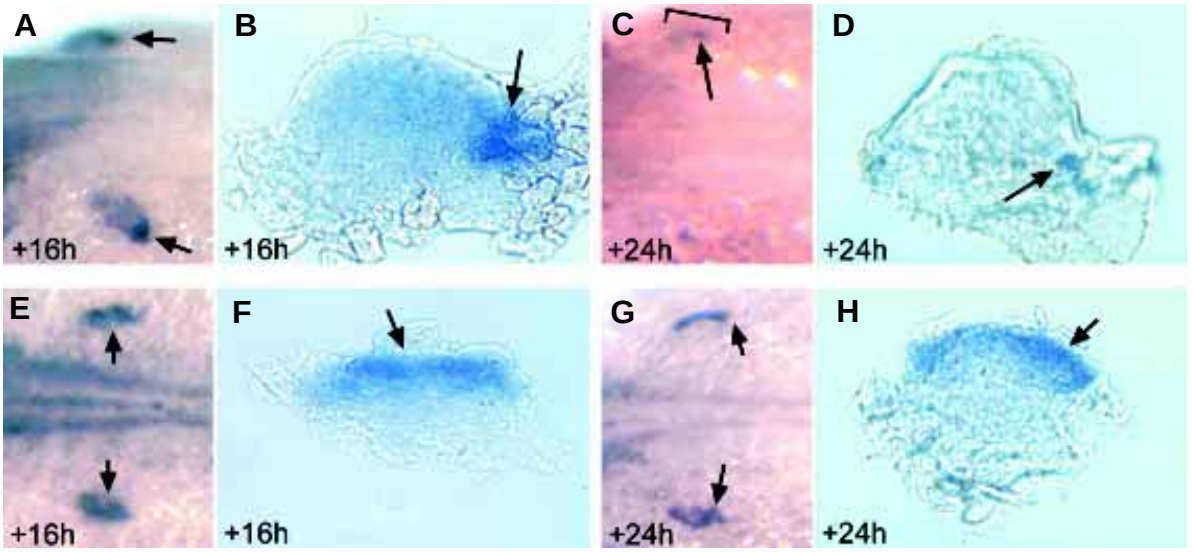

Fig. 3. Effects of RA on *shh* expression in *syu*^{tbx392} mutants. *24 hpf* syu *embryos were exposed for 2 h to 10*$^{-6}$ *M all-trans retinoic acid in 0.5% DMSO (E-H). Controls (A-D) were incubated at 24 hpf in 0.5% DMSO for the same period of time. RA-treated and control embryos were fixed 16 h (A,B,E,F) or 24 h (C,D,G,H) following treatment. (A,C,E,G) Dorsal views of embryos, anterior is to the left. (B,D,F,H) Dissected fin buds, anterior is to the left.* **(A,B)** *At 40 hpf,* shh *expression in control* syu^{tbx392} *embryo is restricted to a small domain of the posterior part of the bud.* **(C,D)** *At 48 hpf* shh *transcripts in* syu^{tbx392} *mutants are found in a small number of cells in the posterior mesenchyme of the bud.* **(E,F)** *16 h following RA treatment,* shh *expression is activated along the antero-posterior axis of the fin buds of* syu^{tbx392} *mutants.* **(G,H)** *Expression of* shh *in* syu^{tbx392} *treated embryos persists in the entire bud 24 h after RA treatment. Black arrows indicate* shh *expression. The bracket in (C) indicates the position of the fin bud.*

is expressed in a very limited number of cells in the posterior mesenchyme (Fig. 3 C,D). The diminished *shh* expression in the ZPA is indicative that shh is indeed not functional in these mutants since maintenance of shh signaling in this region depends on the presence of an operational feedback loop with factors (e.g. FGFs) from the overlying apical ectodermal ridge which itself requires shh signaling for its maintenance (Laufer *et al.,* 1994; Niswander *et al.,* 1994). Furthermore, *ptc1* expression which is normally induced in response to shh in wild-type embryos, is absent in the developing fin buds of *syu* ^{tbx392} mutants (data not shown). We hypothesized that if shh was indeed signaling in a relay fashion, RA treatment would induce the expression of *shh* in *syu*^{tbx392} mutant zebrafish only in the posterior margin of the fin buds where it is normally expressed in wild-type embryos. We observed, however, that as in wild-type embryos treated with RA, *syu*^{tbx392} mutant zebrafish embryos treated at 24 hpf with 10^{-6} M RA exhibit a progressive anterior expansion of *shh* expression 16-24 hours post treatment (Fig. 3 E-H). These results suggest that shh activation in cells composing the anterior expansion does not depend on shh signaling. It is interesting to note that *shh* is strongly expressed at 48 hpf in RA-treated *syu* embryos while its expression in parallel control *syu* embryos has diminished considerably, suggesting that RA treatment may maintain or prolong *shh* expression at later developmental stages (compare Fig. 3 D,H).

Discussion

The present analysis has revealed some aspects of the early response of shh to a short retinoic acid (RA) exposure that ultimately leads to the induction of an ectopic domain of expression of *shh* at the anterior border of the pectoral fin bud in zebrafish.

The immediate effects of RA treatment on the pectoral fin buds of 30 hpf and 48 hpf zebrafish embryos is a decrease or complete loss, respectively, of *shh* expression in its posterior domain of the fin bud. Expression of *ptc1*, however, is only diminished following RA treatment of embryos at both 30 hpf and 48 hpf. This rapid response to RA suggests that RA may directly exerts its effects on *shh* expression. Indeed, a similar downregulation of *shh* expression following RA treatment has also been reported in certain craniofacial prominences of the chick and in the chick limb buds (Helms *et al.,* 1997), in fin regenerates (Laforest *et al.,* 1998), and in regenerating axolotl limbs (Torok *et al.,* 1999).

TABLE 2

EFFECTS OF VARIOUS CONCENTRATIONS OF ALL-TRANS RETINOIC ACID ON *shh* EXPRESSION IN PECTORAL FIN BUDS OF 30HPF ZEBRAFISH

	Type of buds					
	0	1	2	3	4	6
RA concentration						
1 x 10^{-7} M	3	3	3	5		
5 x 10^{-7} M		1	3	12		
1 x 10^{-6} M	1	1		10	5	1
5 x 10^{-6} M	1			3	5	8
1 x 10^{-5} M						16

Type of buds: refers to the different types of expression patterns observed. Type 0: normal expression pattern (similar to control). Type 1: short extension. Type 2: long extension across the bud. Type 3: interconnected anterior and posterior domains. Type 4: discrete anterior and posterior domains. Type 6: no expression in small underdeveloped bud.Numbers below the schematic representation of the buds indicate the numbers of buds exhibiting the specific pattern of expression.

Further evidence that RA may be acting directly on *shh* expression comes from the identification of a functional retinoic acid response element (RARE) of the DR5 type in the 5' region of the zebrafish *shh* gene which is capable of activating the *shh* promoter in cultured cells (Chang *et al.,* 1997). It is currently unknown whether this RARE may activate or downregulate *shh* expression in response to the different combinations of factors (e.g. RARs and RXRs) present in various cell types. To date, no such element has been identified in chick or in mouse *shh* gene regulatory region. Alternatively, a destabilization effect on *shh* mRNA which would lead to its degradation would also explain the rapid decrease of *shh* expression immediately following RA exposure.

Subsequent to the early response to RA, we observe that in 30 hpf zebrafish embryos, the retinoid induces a reactivation of *shh* expression in the ZPA that is followed by progressive anterior extension of *shh* and *ptc1* domains of expression that, over time, reaches the opposite, anterior margin of the fin bud. Evidence of two completely separate domains of expression, however, is seen only several hours later. These results suggest the possibility that *shh*, once activated by exogenous RA, may be signaling in a cell-cell fashion towards the anterior end of the fin bud. Indeed, it has been postulated that once Shh has "primed" limb mesenchyme cells, making them competent to form digits, it then acts in a short-range, relay fashion to induce downstream effectors such as Bmp2 (Drossopoulou *et al.,* 2000). To address this possibility, the effects of RA treatment on *shh* expression in *syu*^{tbx392} mutant zebrafish were analyzed. Surprisingly, we observed that RA exerts similar effects in mutant embryos as in wild-type embryos, ie., RA also induces the progressive extension of *shh* expression towards the anterior margin of the fin bud. These results indicate that RA-mediated induction of *shh* expression during the initial stages of pectoral fin development occurs independently of a shh relay mechanism across the fin bud.

Evidence that RA may be acting indirectly during *shh* domain extension is provided by our observation that the effects of RA treatment are only observed several hours following a short exposure to the retinoid. The differential effects of RA on *shh* expression immediately following exposure and 16-24 hours later may be explained by the observation that *shh* expression is diminished or downregulated when levels of RA are high, and that as these levels decrease, *shh* expression is first reactivated in its normal domain, then progresses anteriorly.

A time delay in *shh* activation suggests that RA's effects on *shh* expression may involve intermediate factors. Candidate molecules include members of the *Hox* gene family since they are expressed in distinct domains along the A-P axis of the embryo in a manner consistent with their role in specifying A-P identity (Duboule 1994). *Hoxb-8*, in particular, is not only expressed in regions of the flank and wing bud of the developing chicken embryo that exhibit polarizing activity, but it has also been shown to display its own polarizing activity (Lu *et al.,* 1997; Stratford *et al.,* 1997). Moreover, it has been demonstrated that *Hoxb-8* is rapidly induced by RA treatment, prior to the induction of *Shh* (Lu *et al.,* 1997; Stratford *et al.,* 1997). 5' members of the *HoxD* gene cluster may also be good candidates for playing key roles in the regulation of *shh* following RA exposure. Misexpression of *Hoxd-12*, for example, has been shown to induce a duplication of *Shh* expression at the anterior limb margin of the mouse (Knezevic *et al.,* 1997). Shh, in turn, is known to activate 5' members of the *HoxD* cluster, some of which, including *Hoxd-11* and *Hoxd-12*, operate in a positive feedback loop with Shh to reinforce polarizing signals during limb outgrowth (reviewed in Johnson and Tabin 1997; Knezevic *et al.,* 1997). In zebrafish, the transient posterior expression of *hoxd-11* and *hoxd-12* in the pectoral fin buds of *syu* mutant suggests that activation of these genes occurs independently of shh, but maintenance of their expression requires shh (Neumann *et al.,* 1999). Treatment with RA activates the expression of both *Shh* and 5' members of the *HoxD* complex and consequently induces the formation of mirror-image digit duplications at the anterior margin of the limb bud (Johnson and Tabin 1997). Interestingly, while RA can activate *hoxd-11* and *hoxd-12* expression throughout the entire fin bud of wild-type zebrafish, RA is not sufficient to induce an ectopic expression of *hoxd-11* and *hoxd-12* in *syu* mutants (Neumann *et al.,* 1999), suggesting that these genes are probably not mediating RA effects on *shh* in the pectoral fin buds of zebrafish. Thus, given the current literature, detailed spatiotemporal analyses of *Hox* gene expression in response to RA in the developing fin buds of the zebrafish may help further elucidate the mechanisms underlying *Shh* activation across the bud.

Another mechanism by which RA may be inducing the progressive anterior extension and eventual duplication of *shh* and *ptc1* expression in the pectoral fin buds is the inhibition of a repressor of shh at the anterior margin of the fin bud. The Gli3 transcription factor is a plausible candidate for such a repressor since it has been shown to restrict *Shh* expression to its posterior domain (Wang *et al.,* 2000). Shh, in turn, acts to inhibit Gli3 processing, thereby producing a gradient of Gli3 expression across the A-P axis of the developing limb, with Gli3 being expressed at high levels anteriorly and at low levels posteriorly (Wang *et al.,* 2000). Furthermore, disruption of Gli3 results in the production of a polarizing region anteriorly (Buscher *et al.,* 1997). Given this evidence, it seems plausible that RA may exert its effects on *shh* expression via an inhibition of *Gli3* expression. Indeed, Gli3 has recently been shown to be required to restrict expression of the dHand transcription factor to the posterior mesenchyme of the early stage limb bud (Te Welscher *et al.,* 2002). dHand has previously been demonstrated to act as an intermediate between Hoxb-8 and Shh in establishing the ZPA (Charite *et al.,* 2000; Fernandez-Teran *et al.,* 2000), and has been shown to be inducible by RA (Fernandez-Teran *et al.,* 2000).

RA can posteriorize, ventralize, and proximalize blastemal cells of the regenerating limb of axolotls (Maden 1982) and induce a proximalization of cell identity in the developing limb (Tamura *et al.,* 1997; Mercader *et al.,* 2000). When RA-treated distal cells of a chick limb bud are transplanted to a host bud, they promote the formation of more proximal limb structures than those resulting from control distal cells (Tamura *et al.,* 1997). Furthermore, RA bead implantation in the distal mesenchyme of chick limb buds induces the ectopic expression of proximal limb determinants, *Meis1* and *Meis2* homeobox genes, in the distal part of the limb bud, indicating a proximalization of the positional identity of the distal cells (Mercader *et al.,* 2000). It is therefore surprising to observe that the expression of *shh* and *ptc1* towards the anterior margin of the pectoral fin buds following RA treatment appears to be restricted to the ventral side and to the base of the fin bud. This suggests that there is an active mechanism that restricts *shh* expression on the ventral and proximal region of the bud. Given the above evidence, addressing the mechanism(s) by which RA may

promote the proximal expression of *shh* in the fin bud mesenchyme warrants further analysis including a time course analysis of zebrafish *meis* gene expression in response to RA. In addition, it will be important to analyze the effects of RA treatment on various dorsoventral markers such as *en-1* (ventral ectoderm marker) (Hatta *et al.,* 1991; Ekker *et al.,* 1992), *Wnt7a* (dorsal ectoderm marker) (Dealy *et al.,* 1993; Parr *et al.,* 1993), and *Lmx1* (dorsal mesoderm marker) (Riddle *et al.,* 1995; Vogel *et al.,* 1995), especially, since it has been shown that Shh and Wnt7a are dependent on the activity of each other (Parr and McMahon 1995; Yang and Niswander 1995).

The present study demonstrates that, following RA exposure, ectopic expression of *shh* and *ptc1* at the anterior margin of the pectoral fin buds is established via a progressive extension of the posterior domain of expression across the fin bud. The activation of ectopic *shh* expression appears to be independent of shh signaling. We have also demonstrated that RA exerts its effects in a stage-dependent manner since the posteriorization of anterior fin bud mesenchyme occurs only in zebrafish embryos treated at the initial stages of pectoral fin development, but not in older embryos. Lastly, the results of this study indicate that an early response to RA is a rapid decrease or loss of *shh* expression in its posterior domain. Further analyses of the regulation of *shh* by RA should help to further elucidate the mechanism(s) underlying limb patterning and outgrowth.

Materials and Methods

Animals

Zebrafish embryos were obtained from the wild type colony raised in our facility. *Syu*^{tbx392} mutant zebrafish were obtained from the Tubingen strain (Haffter *et al.*, 1996). Adults and embryos were maintained at 28°C using standard methods (Westerfield 1995). To prevent the formation of pigment, 18-24 hpf embryos were treated with a 1:5,000 dilution of 200 mM PTC (Sigma P5272, grade II, in 70% DMSO) in system water.

RA Treatment

All-trans retinoic acid (RA) was purchased from Sigma (R2625), and a 0.1 M stock solution prepared in dimethyl sulfoxide (DMSO). For treatment of dechorionated embryos at either 24, 30, 36, or 48 hpf (see individual experiments), the RA stock was further dissolved in 0.5% DMSO to a final concentration of 10^{-7} M RA –10^{-5} M RA. Following RA treatment that varied in duration from 15 minutes to 2 hours (see individual experiments), embryos were washed extensively with system water and then collected at various time points (2, 10, 16, 24, 36-48 hours). Control embryos were immersed in 0.5% DMSO for the same periods of time.

Gene Expression Analysis

In situ hybridization analyses on whole-mount embryos were performed as previously described (Akimenko *et al.,* 1995). cDNAs used to generate antisense RNA probes: *shh* (Krauss *et al.,* 1993; Roelink *et al.,* 1994), *ptc1* (Concordet *et al.,* 1996), *msxC* (Akimenko *et al.,* 1995), *dlx3* (Akimenko *et al.,* 1994). Following *in situ* hybridization, selected embryos were cut into 16 µm sections following standard cryosectioning procedures (Westerfield 1995).

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