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Uncoupling of Retinoic Acid Signaling From Tailbud Development Before Termination of Body Axis Extension

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Summary

During the early stages of body axis extension, retinoic acid (RA) synthesized in somites by *Raldh2* represses caudal fibroblast growth factor (FGF) signaling to limit the tailbud progenitor zone. Excessive RA down-regulates *Fgf8* and triggers premature termination of body axis extension, suggesting that endogenous RA may function in normal termination of body axis extension. Here, we demonstrate that *Raldh2*−/− mouse embryos undergo normal down-regulation of tailbud *Fgf8* expression and termination of body axis extension in the absence of RA. Interestingly, *Raldh*2 expression in wild-type tail somites and tailbud from E10.5 onwards does not result in RA activity monitored by retinoic acid response element (*RARE*)-*lacZ*. Treatment of wild-type tailbuds with physiological levels of RA or retinaldehyde induces *RARE-lacZ* activity, validating the sensitivity of *RARE-lacZ* and demonstrating that deficient RA synthesis in wild-type tail somites and tailbud is due to a lack of retinaldehyde synthesis. These studies demonstrate an early uncoupling of RA signaling from mouse tailbud development and show that termination of body axis extension occurs in the absence of RA signaling.

Keywords

body axis termination; retinoic acid; retinaldehyde; somitogenesis; *Raldh2*; *Fgf8*; *Mesp2*; *RARE-lacZ*

Vertebrate embryos develop in an anterior to posterior direction through the process of body axis extension from a caudal progenitor zone (Cambray and Wilson, 2002). As the body axis extends, the paraxial mesoderm generated is sequentially segmented into somites that generate the vertebrae and other components of the axial skeleton as well as skeletal muscle (Dequeant and Pourquie, 2008). Species-specific differences in the number of somites results in a wide variety of body axis lengths among vertebrate animals. For instance, the final number of somites varies from 62–65 in mouse embryos, whereas human embryos have 38–39 somites, chick embryos have 51–53 somites, and snake embryos have several hundred somites (Richardson *et al*., 1998). Soon after the process of somitogenesis is completed, body axis extension is terminated. The control of segment number across vertebrate embryos has been shown to involve a similar clock-and-wavefront mechanism with variations in the rate of the segmentation clock relative to the developmental rate (Gomez *et al*., 2008).

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The mechanism that terminates body axis extension and hence determines the final segment number is unknown. Somitogenesis and growth of the caudal progenitor zone requires fibroblast growth factor (FGF) signaling (Wahl *et al*., 2007). Thus, the mechanism that terminates body axis extension may involve down-regulation of caudal FGF signaling at a species-specific point in development. Retinoic acid (RA) is a vitamin A derivative that when administered at high doses to mouse embryos can alter vertebral identity and prematurely terminate body axis extension (Iulianella *et al*., 1999; Kessel and Gruss, 1991). As a safeguard against premature termination of body axis extension, the caudal tip of the tailbud expresses *Cyp26a1* which encodes a cytochrome P450 enzyme that can degrade RA (White *et al*., 1996). Genetic loss of *Cyp26a1* function results in the encroachment of RA activity into the caudal progenitor zone after E8.5, resulting in loss of *Fgf8* expression and premature termination of body axis extension including loss of the tail and sometimes lumbar/sacral truncations (Abu-Abed *et al*., 2001; Sakai *et al*., 2001). RA has been shown to antagonize expression of *Fgf8* in the caudal progenitor zone during body axis extension in chick and mouse embryos (Del Corral *et al*., 2003). RA synthesized in presomitic mesoderm and somites by retinaldehyde dehydrogenase-2 (Raldh2) is essential for proper somitogenesis and body axis extension, potentially through its ability to repress *Fgf8* at the anterior border of the caudal progenitor zone during the early stages of somitogenesis (Sirbu and Duester, 2006; Vermot and Pourquié, 2005; Vermot et al., 2005). The requirement for *Raldh2* to repress caudal *Fgf8* was reported to occur only during the early stages of somitogenesis in mouse when the expression domains of these genes overlap or are in very close proximity (Sirbu and Duester, 2006). Thus, during the middle stages of somitogenesis, the *Raldh2* and *Fgf8* expression domains may be too far apart for RA signaling to play a role in *Fgf8* regulation. Examination of corn snake embryos (which develop ~315 somites) demonstrated that *Raldh2* is still expressed in caudal somites at the 260-somite stage in opposition to *Fgf8* expressed in the tailbud caudal progenitor zone; however, a sizable gap exists between the two domains (Gomez *et al*., 2008). It is unclear whether a natural increase in RA activity at a late point in development could decrease *Fgf8* expression sufficiently to trigger the termination of body axis extension.

Recent studies in chick embryos demonstrated an increase in tailbud *Raldh2* expression and detection of tailbud RA activity near the end of somitogenesis, suggesting that RA may play a role in termination of body axis extension; however, in those same studies, it was reported that mouse tailbuds exhibit much less *Raldh2* expression and RA activity was not detected (Tenin *et al*., 2010). Here, we demonstrated that mouse *Raldh2* expression is found in caudal somites from E9.5–E12.5, but is down-regulated by E13.5 when body axis extension ends (Fig. 1a,d,g,j,m). Similar to previous studies (Tenin *et al*., 2010), we observed that *Raldh2* expression intensified in the caudal-most somites from E11.5–E12.5, plus a small expression domain was observed further caudally in the tailbud during these stages (Fig. 1g,j). We used *Raldh2*−/− mouse embryos to address the issue of whether RA is required for termination of body axis extension in the tailbud which occurs between E12.5–E13.5. Although *Raldh2*−/− embryos normally die at E9.5 (Niederreither *et al*., 1999), we have previously shown that *Raldh2*−/− embryos rescued with a small dose of RA before E9.5 survive early lethality and grow to E13.5, thus allowing analysis of *Raldh2* function in tissues of older embryos (Brade *et al*., 2011; Kumar *et al*., 2011; Zhao *et al*., 2010). First, we analyzed *Raldh2*−/− embryos carrying the retinoic acid response element (*RARE*)-*lacZ* RA-reporter transgene (Rossant *et al*., 1991) to determine where endogenous RA signaling is occurring from time points well before body axis termination up until E13.5 when termination has occurred. In E9.5 wild-type embryos, we observed *RARE-lacZ* expression in caudal lateral plate mesoderm and intermediate mesoderm/mesonephros (although not newly generated somites), while E9.5 *Raldh2*−/− embryos lost most caudal mesodermal *RARE-lacZ* expression (Fig. 1a–c); the remaining *RARE-lacZ* expression has been shown to be due to enzymes other that Raldh2, such as Raldh3 which is responsible for RA activity remaining in the mesonephros (Zhao *et*

al., 2009) although it remains unclear what is responsible for RA activity remaining in the neural tube. Interestingly, in E10.5 wild-type embryos *RARE-lacZ* expression was clearly not observed in the caudal-most somites even though they expressed *Raldh2*; E10.5 *Raldh2*−/− embryos were similar except that they have lost *RARE-lacZ* expression in hindlimb mesoderm (Fig. 1d–f). In wild-type embryos at E11.5-E12.5, *RARE-lacZ* expression was not detected in the tailbud even though a small domain of *Raldh2* expression exists in the tailbud itself; also, despite high *Raldh2* expression in the caudal-most somites we observed only thin stripes of *RARE-lacZ* expression in portions of the caudal-most 6 somites (Fig. 1g,h,j,k). *Raldh2*−/− embryos at E11.5–E12.5 retained the thin stripes of somite *RARE-lacZ* expression, demonstrating that *Raldh2* is not responsible, but these mutants lost *RARE-lacZ* expression in other tissues such as the hindlimb mesoderm (E11.5) and frontonasal mass (E12.5) that are known to receive RA from nearby tissues expressing *Raldh2* (Fig. 1i,l). At E13.5, expression of *Raldh2* and *RARE-lacZ* was not observed in wild-type tails; *RARE-lacZ* expression was nearly eliminated in *Raldh2*−/− tails and was completely lost in the hindlimb interdigital regions associated with *Raldh2* expression (Fig. 1m–o). Our findings demonstrate that the thin stripes of *RARE-lacZ* expression observed in tail somites are due to some unidentified activity since they are unchanged in *Raldh2*−/− tails. Together, these observations suggest that RA signaling does not correlate with termination of body axis extension, and these findings provide the first evidence that tissues expressing *Raldh2* may not always generate RA.

To examine more closely whether *Raldh2* is required for termination of body axis extension, we examined embryos from E9.5–E13.5 for expression of *Fgf8* which marks the caudal progenitor zone and *Mesp2* which marks a small region of anterior presomitic mesoderm where each new somite is generated (Morimoto *et al.*, 2005). In both wild-type and *Raldh2*−/− embryos, *Mesp2* expression was observed at high levels from E9.5–E11.5, at a lower level on E12.5, and expression was almost undetectable at E13.5 when body axis extension has terminated (Fig. 2a–j). Caudal *Fgf8* expression was also similar in wild-type and *Raldh2*−/− embryos, with high level expression observed at E9.5, a reduction at E10.5–E11.5, and essentially no expression at E12.5–E13.5 when the caudal progenitor zone disappears (Fig. 2a–j). We can conclude that as early as E9.5 *Raldh2* is not required to limit the anterior extent of the caudal *Fgf8* expression zone. As we observed no difference in *Mesp2* or *Fgf8* expression between wild-type and *Raldh2*−/− embryos (in particular no increase of *Mesp2* expression in the mutant), we conclude that *Raldh2* is not required for termination of either somitogenesis or body axis extension.

To further investigate our earlier observation, suggesting that no correlation exists between expression of *Raldh2* and *RARE-lacZ* in caudal somites and tailbud, we sought to validate the sensitivity of *RARE-lacZ* in these tissues. Previous RA measurements in E10.5–E13.5 mouse embryos using HPLC detection have demonstrated that endogenous RA levels range from 1–100 nM in various tissues (Horton and Maden, 1995) with the average concentration of RA in a whole E10.5 embryo being 25 nM (Mic *et al.*, 2003). We designed experiments to determine whether an absence of *RARE-lacZ* expression in the caudal somites and tailbud can be confidently used to conclude that endogenous RA is absent or below physiological levels. Tails from E10.5–E12.5 wild-type embryos carrying *RARE-lacZ* were cultured in the presence of physiological doses of RA (2.5 nM, 10 nM, 25 nM, or 100 nM), then stained for *RARE-lacZ* expression to detect RA activity. Whereas untreated control tails exhibited no *RARE-lacZ* expression in the tailbud or caudal somites (except for very weak detection between the somites), we found that as little as 2.5 nM RA induced *RARE-lacZ* expression in the caudal somites but not the tailbud of E10.5–E12.5 tails (see Fig. 3). Treatment with 10 nM RA resulted in high *RARE-lacZ* expression in the tailbud at E10.5, and lower expression in the tailbud at E11.5–E12.5; treatment with 25 or 100 nM induced *RARE-lacZ* expression throughout the tail and tailbud at E11.5–E12.5 (see Fig. 3). As treatment with 2.5 nM RA

did not induce *RARE-lacZ* in the tailbud, this level of RA is likely degraded by *Cyp26a1* (known to be expressed in the tailbud), suggesting that it is not a supraphysiological level of RA. These results demonstrate that *RARE-lacZ* can detect low levels of RA in the caudal somites and tailbud when provided in culture, but that endogenous RA is not normally present in these tissues.

To examine why caudal somites and tailbud do not exhibit RA activity despite expressing *Raldh2*, we cultured tails from E11.5–E12.5 wild-type embryos carrying *RARE-lacZ* in the presence of retinaldehyde, the Raldh2 substrate needed to generate RA (Duester, 2008). In comparison with untreated control tails which have no RA activity in the tailbud and either little or no RA activity in caudal somites, tails treated with 25 nM retinaldehyde exhibited robust induction of *RARE-lacZ* in caudal neural tube and thin stripes of expression were observed in somites (see Fig. 4). Treatment with 100 nM retinaldehyde resulted in more robust induction of *RARE-lacZ* expression in the caudal somites, but some stripes were apparent indicating that in some cases the whole somite was not stained); treatment with 200 nM retinaldehyde induced more widespread expression of *RARE-lacZ* with detection in both the caudal somites at E11.5–E12.5 and in tailbud at E11.5 (see Fig. 4). The thin stripes of *RARE-lacZ* expression observed in somites of cultured E11.5–E12.5 tails treated with 25–100 nM retinaldehyde somewhat resemble those observed in tails from E11.5–E12.5 embryos stained immediately (Fig. 1h,k), but they are not as clear possibly due to the inability of cultured embryonic tails to fully recapitulate normal development. These studies demonstrate that functional Raldh2 enzyme is present in the tailbud and caudal somites from E11.5–E12.5, but that RA is not normally synthesized because the substrate retinaldehyde is not being generated in those tissues.

In summary, our observations have demonstrated that *Raldh2* and RA signaling are unnecessary for termination of body axis extension in the mouse. First, our genetic loss-of-function studies have shown that *Raldh2* is not required for the termination process. Although *Raldh2*−/− embryos were administered a small dose of RA from E6.75–E9.25 to prevent lethality, this RA treatment is unlikely to have played a role in termination of body axis extension as RA treatment ended 4 days before this event which occurs at E13.5, and this RA treatment does not induce *RARE-lacZ* in the tailbud at E8.5–E9.5 (Zhao *et al.*, 2009). Second, our metabolic studies have shown that from E10.5–E12.5 RA is not normally generated in the caudal somites and tailbud due to an absence (or near absence) of retinaldehyde synthesis. Very low metabolism of retinol to retinaldehyde and retinaldehyde to RA may exist in the caudal somites at E11.5–E12.5 as we observe very thin stripes of *RARE-lacZ* expression in the caudal-most somites. However, this very low level of RA detection does not require *Raldh2* and it does not generate RA activity in the tailbud itself where RA generated by Raldh2 has been postulated to function in termination of body axis extension (Tenin *et al.*, 2010). Expression of *Cyp26a1* in the tailbud at E8.5–E9.5 has been shown to be necessary for degradation of RA actively secreted by caudal somites at those stages to prevent teratogenic effects of RA signaling on caudal trunk development (Abu-Abed *et al*., 2001; Sakai *et al*., 2001); caudal defects may be associated with reduced *Fgf8* expression in the tailbud of *Cyp26a1*−/− embryos (Abu-Abed *et al*., 2003). However, our results indicate that *Cyp26a1* expression previously observed in the tailbud at 10.5 (high level) or E11.5 (very low level) (MacLean *et al.*, 2001) is unnecessary for RA degradation as caudal RA synthesis has decreased to a very low level at that stage. Together with our results, we suggest that *Cyp26a1* tailbud expression may persist for some time beyond when it is needed for RA degradation, but it is clearly very much reduced by E11.5 consistent with our hypothesis that RA is not required for termination of body axis extension at approximately E13.5.

Our RA treatment studies have shown that *RARE-lacZ* is competent to detect low physiological RA levels in the tailbud if they exist, but no such RA activity was observed. This finding agrees with studies by others showing a lack of RA activity in mouse tailbuds cultured as explants on RA-reporter cells (Tenin *et al*., 2010). Thus, other mechanisms need to be investigated to determine how the caudal progenitor zone ceases to extend the body axis at a particular point in development. In particular, we demonstrate that *Raldh2* is not required for down-regulation of caudal *Fgf8* expression observed at E10.5–E11.5, indicating that factors other than RA are responsible. Taken together with previous studies (Sirbu and Duester, 2006), the role of RA in repressing caudal *Fgf8* expression in mouse is limited to stages before E9.5. Our studies have also revealed that caution must be exercised when assigning the properties of RA synthesis and RA signaling on tissues expressing Raldh2. We have shown for the first time that a tissue can express Raldh2 but still not generate RA synthesis or RA signaling presumably due to insufficient expression of enzymes that metabolize retinol to retinaldehyde.

METHODS

Generation of *Raldh2* Null Mutant Mouse Embryos

Generation of *Raldh2* null embryos carrying the *RARE-lacZ* reporter transgene were described previously (Mic *et al*., 2002). Following mating, noon on the day of vaginal plug detection was considered embryonic day 0.5 (E0.5). The methods for rescue of *Raldh2*−/− lethality by dietary RA treatment were described previously (Zhao *et al*., 2010). Briefly, a 50 mg/ml stock of all-*trans*-RA (Sigma) in 100% ethanol was mixed with corn oil to obtain a 5 mg/ml RA solution, which was then mixed thoroughly with ground mouse chow to yield a final diet containing 0.1 mg RA/g mouse chow. RA-supplemented food was provided to pregnant mice from E6.75 to E9.25 and was replaced every 12 h to ensure activity; at E9.25 mice were returned to normal mouse chow until collection of embryos at E9.5–E13.5. This low dose of RA provides embryos a physiological amount of RA (Mic *et al*., 2003) and the administered RA is cleared within 12–24 h after treatment ends (Mic *et al*., 2002), thus allowing analysis of later-stage embryos that are RA-deficient. Embryos were genotyped by PCR analysis of yolk sac DNA. All mouse studies conformed to the regulatory standards adopted by the Animal Research Committee at the Sanford-Burnham Medical Research Institute.

Culture of Embryonic Tissues and Retinoid Treatment

Tails were dissected from E10.5–E13.5 wild-type embryos (carrying the *RARE-lacZ* RA-reporter trans-gene) in phosphate-buffered saline (PBS) and cultured in Millicell culture plate inserts (Millipore, Billerica, MA) at 37°C in 5% CO2. Tissues were cultured for 18 h in retinoid-free DMEM/F-12 culture media (Gibco-Life Technologies, NY) containing vehicle only (control) or various concentrations of either all-*trans*-retinoic acid or all-*trans*-retinaldehyde (Sigma Chemical) dissolved in 0.1% dimethyl sulfoxide. After 18 h, tissues were washed twice in PBS then processed to examine RA activity (see below).

Whole-Mount *In Situ* Hybridization

Whole-mount *in situ* hybridization was performed on E9.5–E13.5 mouse embryos or isolated embryonic tails using digoxigenin-labeled antisense riboprobes as described previously (Wilkinson and Nieto, 1993).

Detection of RA Activity

The *RARE-lacZ* RA-reporter transgene, which places *lacZ* (encoding β-galactosidase) under the control of a retinoic acid response element (RARE), was used for in situ detection of RA

activity in embryonic tissues; β-Galactosidase activity was detected by fixation with glutaraldehyde followed by staining with X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) for 24 h at 37°C as described (Rossant *et al.*, 1991).

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Abbreviations

FGF	fibroblast growth factor
RA	retinoic acid
RARE	retinoic acid response element

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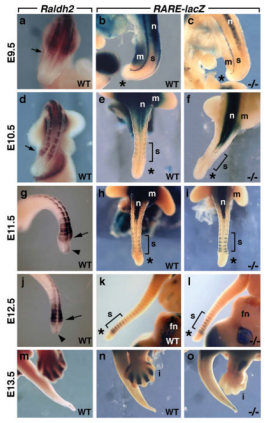

FIG. 1.

RA synthesis and signaling during the late stages of body axis extension. (**a**, **d**, **g**, **j**, **m)** Detection of *Raldh2* mRNA by *in situ* hybridization; arrows mark newly formed somites; arrowheads mark *Raldh2* expression in the tailbud. (**b**, **c**, **e**, **f**, **h**, **i**, **k**, **l**, **n**, **o**) Detection of RA signaling activity in wild-type or *Raldh2*−/− embryos carrying *RARE-lacZ*; brackets mark caudal somites with either no RA activity or very thin stripes of RA activity; asterisks indicate a lack of RA activity in the tailbud; *n* = 3 for all *Raldh2*−/− specimens. fn, frontonasal mass; i, interdigital mesenchyme; m, mesoderm (lateral plate and intermediate-mesonephros); n, neural tube; s, somite.

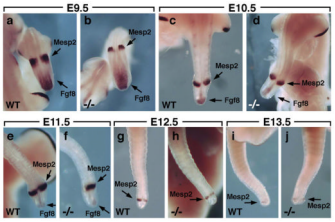

FIG. 2.

Raldh2 is not required for termination of body axis extension. (**a**–**j**) Wild-type and *Raldh2*−/− embryos from E9.5–E13.5 were subjected to double *in situ* hybridization with probes for both *Fgf8* and *Mesp2* as indicated by arrows; *n* = 2 for all *Raldh2*−/− specimens.

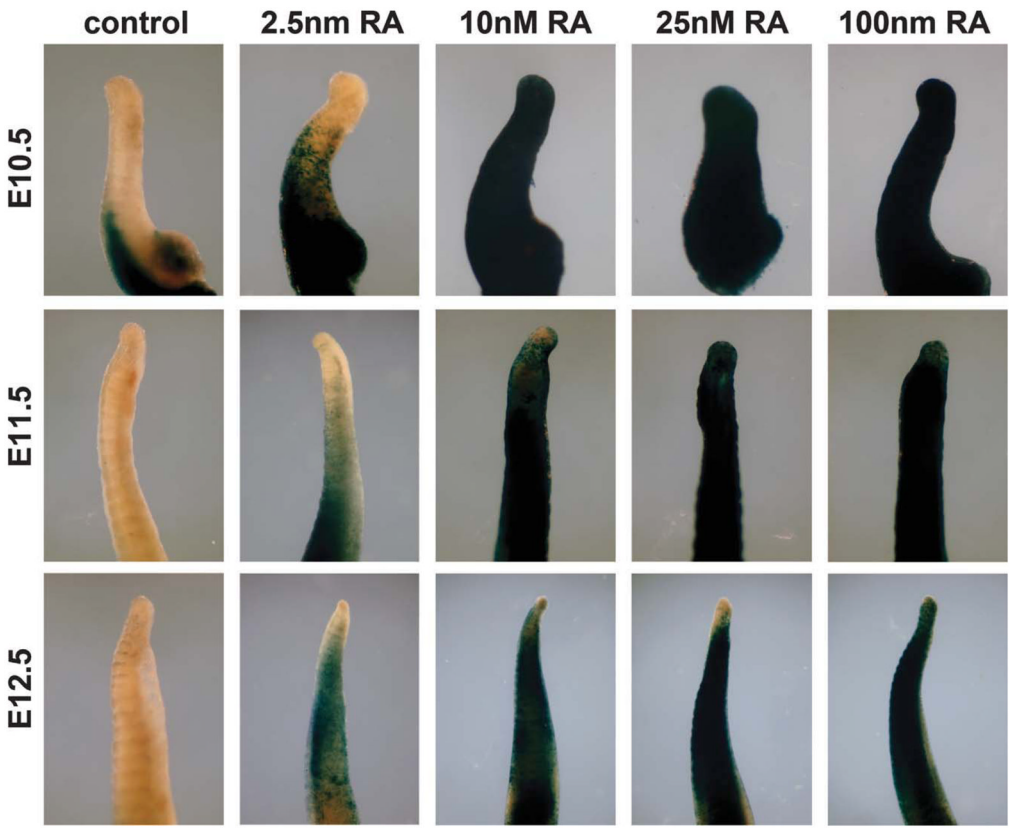

FIG. 3.

RA treatment of embryonic tails validates *RARE-lacZ* as a sensitive RA reporter. Tails from E10.5–E12.5 wild-type embryos carrying *RARE-lacZ* were cultured for 18 h in the absence of RA (control) or presence of physiological doses of RA (2.5, 10, 25, or 100 nM), then stained for *RARE-lacZ* expression to detect RA activity. Results similar to those shown here were observed in several independent experiments; E10.5: control *n* = 5, 2.5 nM *n* = 4, 10 nM *n* = 2, 25 nM *n* = 4, 100 nM *n* = 1; E11.5: control n = 4, 2.5 nM *n* = 4, 10 nM n = 2, 25 nM *n* = 2, 100 nM *n* = 3; E12.5: control *n* = 4, 2.5 nM *n* = 3, 10 nM *n* = 3, 25 nM *n* = 6, 100 nM *n* = 3.

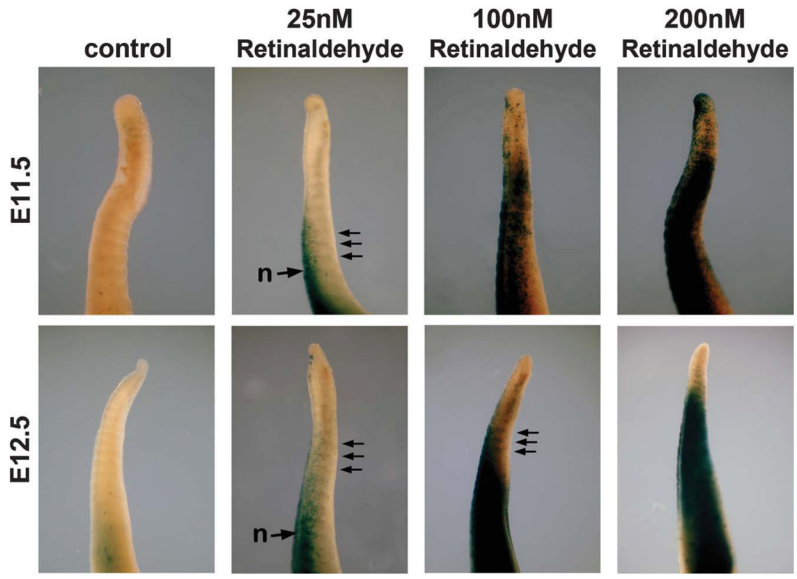

FIG. 4.

Retinaldehyde treatment of embryonic tails stimulates RA activity. Tails from E11.5–E12.5 wild-type embryos carrying *RARE-lacZ* were cultured for 18 h in the absence of retinaldehyde (control) or presence of retinaldehyde (25, 100, or 200 nM), then stained for *RARE-lacZ* expression to detect RA activity. Arrows point out thin stripes of *RARE-lacZ* expression in somites; n, neural tube. Results similar to those shown here were observed in several independent experiments; E11.5: control $n = 4$, 25 nM $n = 4$, 100 nM $n = 2$, 200 nM $n = 3$; E12.5: control $n = 4$, 25 nM $n = 4$, 100 nM $n = 3$, 200 nM $n = 2$.