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Multiple Pathways Governing *Cdx1* Expression during Murine Development

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Cdx1 **encodes a mammalian homeobox gene involved in vertebral patterning. Retinoic acid (RA) is likewise implicated in vertebral patterning. We have previously shown that *Cdx1* is a direct retinoid target gene, suggesting that Cdx1 may convey some of the effects of retinoid signaling. However, RA appears to be essential for only early stages of *Cdx1* expression, and therefore other factors must be involved in maintaining later stages of expression. Based on function and pattern of expression, Wnt family members, in particular Wnt3a, are candidates for regulation of expression of *Cdx1*. Consistent with this, we confirm prior results which demonstrated that *Cdx1* can be directly regulated by Wnt signaling, and identify functional LEF/TCF response motifs essential for this response. We also find that *Cdx1* expression is markedly attenuated in a stage- and tissue-specific fashion in the *Wnt3a* hypomorph *vestigial tail*, and present data demonstrating that Wnt3a and RA synergize strongly to activate *Cdx1*. Finally, we show that *Cdx1* positively regulates its own expression. These data prompt a model whereby retinoid and Wnt signaling function directly and synergistically to initiate *Cdx1* expression in the caudal embryo. Expression is then maintained, at least in part, by an autoregulatory mechanism at later stages.**

Key Words: **retinoic acid receptor; Cdx; vertebral specification; *Hox*; *Wnt3a*; *caudal*.**

INTRODUCTION

Establishment of positional identities in the developing embryo has been the focus of considerable study. Work in many model systems demonstrates that the cellular phenotype is governed by a network of transcription factors (Blau, 1992). As one such example, the *Drosophila caudal* (*cad*) homeobox gene product is involved in the development of the posterior region of the embryo, a function which appears generally conserved across species (Macdonald and Struhl, 1986; Freund *et al.*, 1998).

In mice, three *caudal* homologues, *Cdx1*, *Cdx2*, and *Cdx4*, have been identified (Gamer and Wright, 1993). *Cdx1* is initially expressed at embryonic day (E)7.5 in the primitive streak and extends posteriorly as development proceeds. Expression is later observed in dermamyotome, limb buds, and hindgut endoderm (Meyer and Gruss, 1993). Targeted disruption of *Cdx1* in the mouse results in homeotic vertebral transformations which correlate with posterior shifts in the anterior boundary of expression of some *Hox* genes (Subramanian *et al.*, 1995). *Cdx2* transcripts are evident at E8.5 in the hindgut, tail bud, and caudal neural tube (Beck *et al.*, 1995). As with *Cdx1*, *Cdx2* is also required for normal axial development since *Cdx2* heterozygotes exhibit vertebral homeosis as well as a shortened or kinky tail (Chawengsaksophak *et al.*, 1997). *Cdx4* is expressed in the primitive streak from E7.0, with transcripts extending over the posterior half of the streak as development proceeds (Gamer and Wright, 1993). Although a role for *Cdx4* in axial patterning is supported by its pattern of expression, more definitive evidence awaits description of the null mutant phenotype.

Based on the above observations, it has been proposed that a functional gradient of Cdx proteins exists in the

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posterior embryo which is important for transducing positional information to the *Hox* genes (Charité *et al.*, 1998). Indeed, a number of *Hox* promoters harbor consensus *Cdx* response elements, some of which can respond to Cdx1 in tissue culture (Subramanian *et al.*, 1995). Consistent with this, in *Xenopus*, the *caudal* homologue *Xcad3* has been implicated in relaying an FGF signal to *Hox* gene expression (Pownall *et al.*, 1996). Moreover, inhibition of Xcad3 function leads to a disruption of posterior development concomitant with inhibition of expression of certain *Hox* genes (Isaacs *et al.*, 1998). A similar role for *Xcad2* has also been proposed (Epstein *et al.*, 1997). These data further support a pathway from Cdx to *Hox* expression and subsequent impact on antero-posterior patterning.

As with *Cdx* members, RA, acting through the RA receptors (RARs), has also been implicated in vertebral patterning (Kessel and Gruss, 1991). RA can also affect the expression of a number of *Hox* genes, some of which have been shown to be direct retinoid targets (Pöpperl and Featherstone, 1993; Langston and Gudas, 1992; Marshall *et al.*, 1994; Frasch *et al.*, 1995; reviewed in Gudas, 1994; Krumlauf, 1994; Deschamps *et al.*, 1999). We have recently shown that *Cdx1* is an RA target gene, suggesting an indirect mechanism by which retinoid signaling may impact vertebral patterning (Houle *et al.*, 2000). However, although exogenous RA induces *Cdx1* over a broad developmental window, RAR loss appears to affect *Cdx1* expression only at late gastrulation (E7.5; Houle *et al.*, 2000). Alternative signaling pathways must therefore be involved in later stages of *Cdx1* expression, among which *Wnts* are potential candidates.

The importance of the Wnt/wingless pathway in diverse developmental processes has been demonstrated in a variety of organisms including *Caenorhabditis elegans*, *Drosophila*, *Xenopus*, and the mouse (reviewed in Wodarz and Nusse, 1998). In the canonical pathway, the Wnt signal is transduced from receptors of the Frizzled family to cytosolic β-catenin. Cytosolic β-catenin is part of a multiprotein complex that includes APC, Axin, and GSK3, among other components. In unstimulated cells, this complex contributes to the rapid degradation of β-catenin. Wnt signaling via Frizzled results in stabilization of β-catenin which then enters the nucleus, forms a complex with transcription factors of the LEF/TCF family, and activates target genes (reviewed in Hlsken and Behrens, 2000; Miller *et al.*, 1999; Polakis, 2000; Cadigan and Nusse, 1997).

A number of Wnt target genes have been characterized, several of which are involved in axial patterning. Among these are *Drosophila ultrabithorax* (Riese *et al.*, 1997), *Xenopus siamois* (Brannon *et al.*, 1997), and the murine *Brachyury* (T) transcription factor (Yamaguchi *et al.*, 1999). Recent work in *C. elegans* has also demonstrated a genetic link between Wnt and the nematode *caudal* homologue *pal1* (Hunter *et al.*, 1999; Zhang and Emmons, 2000), consistent with the finding that Wnt affects the expression of several *Hox* genes in this organism (Hunter *et al.*, 1999; Hoier *et al.*, 2000). In the mouse, Wnt signaling has been shown to affect *Cdx1* expression in murine endoderm, mesoderm, and cultured intestinal cells (Lickert *et al.*, 2000; Ikeya and Takada, 2001).

The pattern of expression of *Wnt-3a* overlaps with that of *Cdx1* in the caudal embryo (Takada *et al.*, 1994). Loss of this gene results in dose-dependent axial truncations varying from the absence of the tail to complete lack of axial structures caudal to the forelimb (Greco *et al.*, 1996; Takada *et al.*, 1994). A similar phenotype is observed in *LEF1/TCF1* compound null mutants (Galceran *et al.*, 1999), suggesting that LEF1 and TCF1 interpret the Wnt3a signal critical to the normal development of posterior mesoderm.

Taken together, these observations suggest that RA and Wnts are intimately involved in patterning the vertebrate axis, potentially through regulation of *Cdx* expression. We investigated these putative interactions by several means, and present data demonstrating that Wnt3a positively affects expression of *Cdx1* in a tissue- and stage-specific manner. We also found that RA and Wnt3a strongly synergize to induce expression from the *Cdx1* promoter. Furthermore, we present evidence that *Cdx1* expression is under some form of autoregulation that functions in a stage-specific manner. Our findings are indicative of an interactive process involving Wnt and retinoid signaling pathways which, together with autoregulatory mechanisms, establishes and maintains expression of *Cdx1* in the caudal embryo.

MATERIALS AND METHODS

Animals and Treatment

Vestigial tail (*vt*) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The *Cdx1* null mice used in this study have previously been described (Subramanian *et al.*, 1995). CD-1 mice (Charles River), or wild-type embryos obtained from crosses between the *vt* or *Cdx1* mutant strains, were used as controls for *in situ* hybridization analysis; no overt differences in gene expression were noted in these controls irrespective of their genetic background. Mice were mated overnight and females examined the following day for the presence of a vaginal plug; noon of the day of plug was considered as E0.5. Pregnant females were sacrificed at E7.5–E9.5 and embryos dissected in phosphate buffered saline (PBS), fixed in 4% paraformaldehyde at 4°C, dehydrated through a methanol series, and stored at −20°C in 100% methanol. For retinoid treatment, pregnant females were dosed by oral gavage with all-*trans* retinoic acid (RA) dissolved in DMSO with corn oil as a vehicle. A final delivery of 10 or 100 mg RA/kg maternal body weight was administered depending on the stage at treatment (10 mg/kg at E7.5, 100 mg/kg at E8.5 or E9.5). Females were sacrificed 4–12 h posttreatment and embryos collected and processed as above.

Embryo Culture

Embryo culture was performed essentially as described (Hogan *et al.*, 1994). Briefly, E8.5 embryos were dissected in PBS containing 10% (v/v) fetal bovine serum. Embryos were cultured in DMEM/rat serum (50:50) equilibrated with 5% O_2/5%CO_2 in N_2 at 37°C in

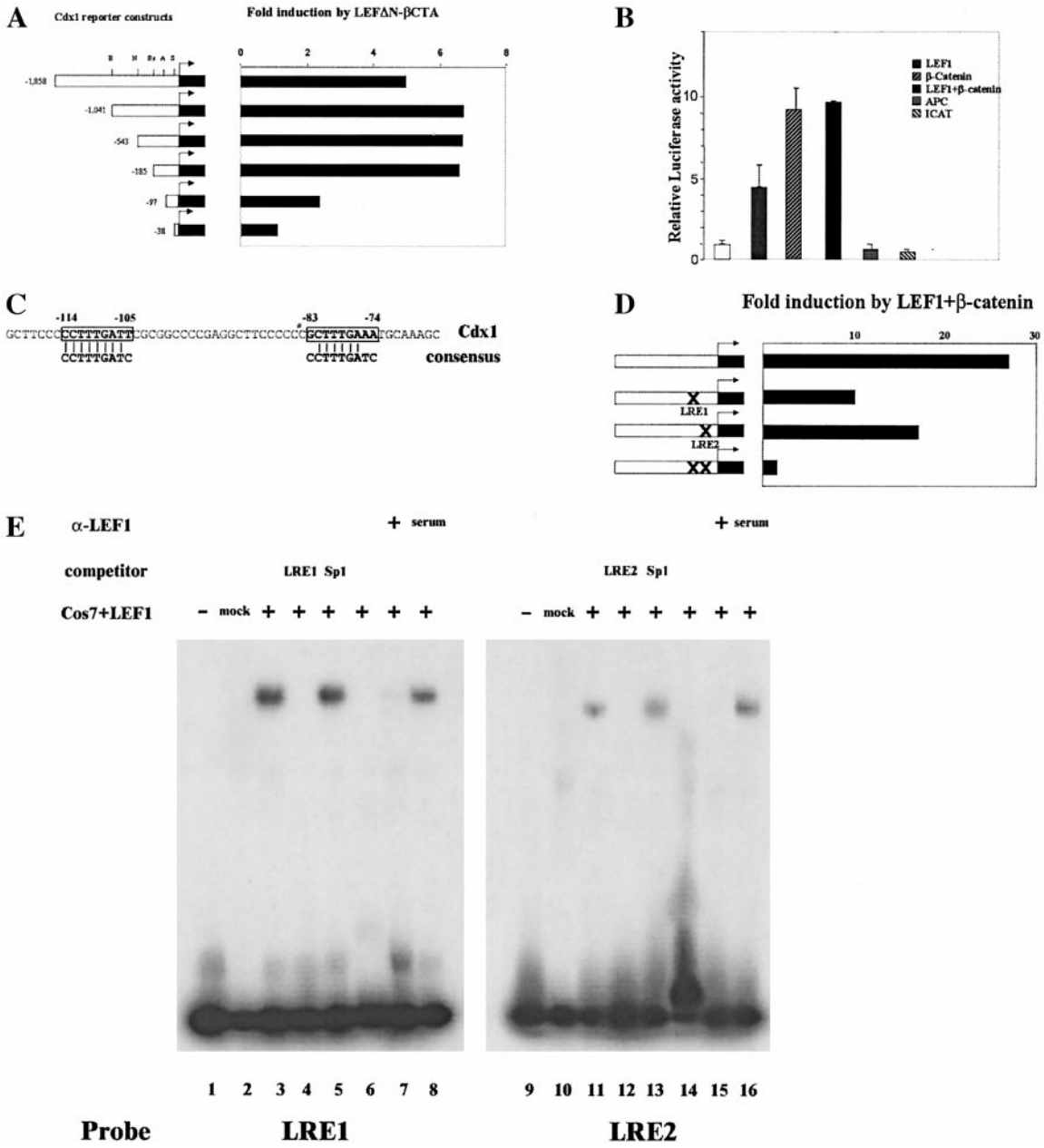

FIG. 1. Wnt signaling induces Cdx1. (A) F9 cells were transfected with each of the indicated *Cdx1* reporter constructs alone or with the *Xenopus* LEFΔN-*β*CTA fusion vector (Vleminckx *et al.*, 1999). Cells were harvested 48 h posttransfection and luciferase activity assessed as in Materials and Methods. Results were expressed as fold induction mediated by LEFΔN-*β*CTA relative to expression of the reporter vector alone. B, *Bam*HI; N, *Nde*I; Bs, *Bst*XI; A, *Ava*I; S, *Sac*II. (B) The wild-type *Cdx1* reporter construct (−1,858 in A) was transfected in F9 cells alone or with expression vectors encoding the indicated Wnt-signaling intermediary. Cells were processed as above and luciferase activity expressed as fold induction relative to the reporter vector alone. (C) The sequence of the proximal *Cdx1* promoter with the two putative LEF/TCF binding sites (LRE1 and LRE2) indicated in bold and compared to the consensus sequence (Clevers and van de Wetering, 1997). Numbering indicates the 5′ nucleotide position relative to the *Cdx1* transcription start site. (D) F9 cells were transfected with the full-length *Cdx1* reporter construct or identical constructs mutated for either the LRE1 or the LRE2 or both elements together. Transfections were performed either with the reporter vector alone or with a LEF1/*β*-catenin expression vector (Labbé *et al.*, 2000) and luciferase activity assessed as above. Data are expressed as fold induction relative to the reporter vector alone. Note that concomitant mutation of both LRE1 and LRE2 were required to completely abolished activation by LEF1/*β*-catenin. (E) Electrophoretic mobility shift assay. LEF1-overexpressing COS cell extracts were incubated with radioactively labeled double-stranded oligonucleotides encoding the LRE1 or LRE2 elements; protein–DNA complexes were then resolved by electrophoresis on a 6% acrylamide gel and revealed by autoradiography. Lane 1, no cell extract; lane 2, mock transfected cells; lanes 3–6, the LRE1 oligonucleotide was incubated with extracts containing LEF1 either alone (lane 3), with an excess of unlabeled LRE1 (lane 4), or an excess of an SP-1 element (lane 5). Lane 6 used a mutated LRE1 sequence as probe. Lanes 7 and 8 were identical to lane 3 but included either a LEF1-specific antibody (lane 7) or preimmune serum (lane 8). Lanes 9–16 are identical to lanes 1–8, except the probe used corresponded to the LRE2 sequences with lane 14 employing a mutated LRE2 as probe.

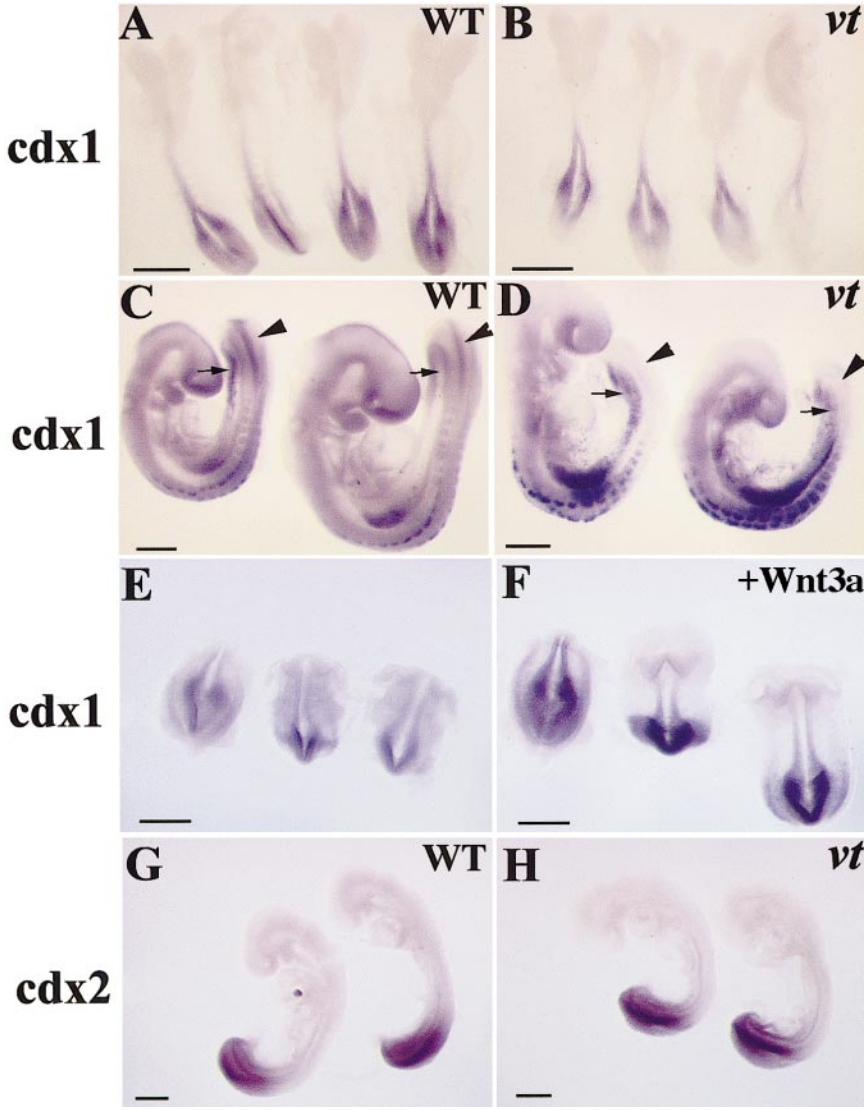

FIG. 2. Wnt3a regulates Cdx1 expression *in vivo*. (A–D) *Cdx1* expression in wild-type (WT; A, C) and *vt* mutant (*vt*; B, D) embryos at E8.5 (A, B) or E9.5 (C, D). Note the reduction of caudal expression of *Cdx1* in the *vt* embryos at E8.5 (compare B to A). Arrowheads in (C) and (D) indicate the residual posterior mesoderm expression of *Cdx1* in wild-type specimens (C) that is lost in the *vt* mutants at E9.5 (D). Note that expression in the adjacent hindgut primordia is unaffected (arrows in C, D) in the mutants, whereas expression in the limb buds and dermamyotome is elevated relative to controls. (E, F) Ventral view of *in situ* hybridization for *Cdx1* from wild-type embryos cultured *ex vivo* for 3 h in the absence (E) or presence (F) of Wnt3a-enriched media. Note the marked increase of *Cdx1* message in the tail bud region in (F). (G, H) Expression of *Cdx2* in E8.5 wild-type (G) or *vt* mutant embryos (H). Bars, 200 μm.

either the absence or presence of supernatant from Wnt3a-expressing cells (Shibamoto *et al.*, 1998). Culture was terminated after 3 h and samples processed as above for *in situ* hybridization analysis.

In Situ Hybridization Analysis

Embryos were pooled according to genotype, developmental stage, and experimental treatment and rehydrated. Digoxigenin-labeled riboprobes were generated from plasmids encoding *Cdx1* (Meyer and Gruss, 1993), *Cdx2* (Suh *et al.*, 1994), or *Wnt3a* (Roelink and Nusse, 1991) and used for whole-mount *in situ* hybridization as previously described (Henrique *et al.*, 1995). Sense riboprobes were used as negative controls in all cases. Samples to be compared were processed simultaneously under identical conditions to control for interexperimental variability in relative signal strength. After *in situ* hybridization, specimens were postfixed in 4% paraformaldehyde/0.2% glutaraldehyde at 4°C for 30 min, rinsed in several changes of PBS containing 0.1% Tween-20, cleared, and photographed.

Derivation of Plasmids

Isolation of *Cdx1* genomic sequences and generation of reporter vectors has been previously described (Houle *et al.*, 2000). Site-directed mutagenesis of the two putative LEF binding sites on the Cdx1 promoter was performed by using the Transformer kit (Clontech) or PCR (Horton *et al.*, 1994). The primers used were LRE1 5′-GGGCTTCCCCCTTTCGAACGCGGCCCCG-3′, and LRE2 5′-GCTTCCCCCCGCTTGGGCCGGCCAAGCCGCCCG-GC-3′ (mutated sequences are underlined). All constructs were verified by sequencing.

A *Cdx1* expression vector was derived by subcloning the coding sequences into the *Not*I/*Xba*I sites of the pRc/CMV expression vector (Invitrogen). The murine LEF-1 expression vector (pCG mLEF-1-HA) was a gift from Liliana Attisano, and the *Xenopus* LEF-1 DNA binding domain-β-catenin activation domain fusion construct was generously provided by Andreas Hecht. The human β-catenin and APC and the murine ICAT expression plasmids were gifts from David Rimm, Bert Vogelstein, and Tsutomu Nakamura, respectively.

Cell Culture and Transfection Analysis

F9 embryocarcinoma cells were maintained in DMEM (Life Technologies) supplemented with glucose (4.5 g/liter), 10% fetal bovine serum, and gentamicin (10 μg/ml). P19 embryocarcinoma cells were maintained in α-MEM (Life Technologies) under otherwise identical conditions. For routine maintenance, cells were passaged every third day into 100-mm tissue culture plates and cultured at 37°C in 5% CO_2. To generate stable F9 cell lines, 2×10^6 cells were electroporated with 30 μg of linearized reporter vector together with a neomycin expression vector and pools of stable transformants were derived by selection in medium supplemented with 200 μg/ml G418 for 10 days. For RA and Wnt3a regulation studies, approximately 10^5 cells were plated in 6-well plates. The next day, cells were treated with either control medium, medium containing 1 μM RA, Wnt3a-conditioned medium (Shibamoto *et al.*, 1998), or medium containing both Wnt3a and RA for 20 h. Cells were subsequently lysed and the supernatant assayed for luciferase activity as described below.

For transfection analysis, cells were passaged into 6-well cluster plates (approximately 10^5 cells/well) and transfected 24 h later by using the calcium phosphate method or with Superfect (Gibco-BRL). DNA mixes were comprised of 1.0 μg of luciferase reporter construct, 0.5 μg of expression vector (where appropriate), 0.5 μg of a *lacZ* expression vector as an internal control and empty expression vector (where required) to a final concentration of 2 μg DNA per transfection. The following day, transfected cells were washed with PBS, media was replenished, and culture continued for 24 h. Monolayers were then rinsed twice in ice-cold PBS, and cells disrupted by addition of 250 μl of lysis buffer (0.1 M Tris–HCl, pH 8, 1% NP-40, 1 μM DTT). Lysates were collected and assessed for luciferase and β-galactosidase activity as described (Ausubel *et al.*, 2001), and the latter used to correct for transfection efficiency. Results were corrected for background (empty expression vector) and are the mean of three independent transfections. Each experiment was repeated a minimum of three times with similar results.

Electrophoretic Mobility Shift Assays

Nuclear extracts were prepared from COS cells which had been transfected either with an empty vector or with an expression vector encoding murine *LEF1*, *Cdx1*, or *Cdx2*. Binding reactions were performed essentially as described (Houle *et al.*, 2000). The upper strands of each double-stranded probe used were: LRE1, 5′-TTCCCCCTTTGATTCGCGGC-3′; LRE1 mut, 5′-GGGCTTC-CCCCTTTCGAACGCGGCCCC-3′; LRE2, 5′-GCTTTGAAATG-CAAAGCCGC-3′;LRE2 mut,5′-GCTTCCCCCCGCTTGGGCCG-GCCAAGCCGCCCGGC-3′ (mutated sequences are underlined). For antibody competition experiments, 2 μl of anti-LEF/TCF antibody (Maine Biotechnology Services) was added to protein extracts on ice 30 min prior to addition of probe.

Reverse Transcription-PCR (RT-PCR)

E8.5 embryos from $Cdx1^{-/-} \times Cdx1^{+/+}$ or wild-type intercrosses were harvested and total RNA isolated from 5–10 pooled embryos using Trizol (Life Technologies). Reverse transcription-PCR was subsequently performed under standard conditions to amplify either the wild-type or mutant *Cdx1* alleles across the boundary of the *neo* integration site (Subramanian *et al.*, 1995). Amplification using primers *Cdx1*F and *Cdx1*R (see below) was predicted to yield a 370-base pair (bp) product specific to the wild-type allele, whereas primers *Cdx1*F and *neo*R were expected to generate a 236-bp product characteristic of the null allele. Multiplex PCR using all three primers was also performed to simultaneously amplify both the wild-type and null alleles. PCR products were resolved on a 2% agarose gel, transferred to a nylon membrane, and hybridized with the end-labeled internal oligonucleotide GACCACCAACGCCTA-GAGC common to both the wild-type and mutant amplification products. Primer sequences for PCR were: *Cdx1*F, GTAAGAC-CCGAACCAAGGAC; *Cdx1*R, CAGGATATCCTAGGGTAGAA-ACTCCTCCTTGACG; *neo*R, CTTAGCGGCCGCCTTCTATC-GCCTTCTTGACG.

RESULTS

Wnt Signaling Directly Activates the Cdx1 Promoter

A number of Wnts have been shown to be involved in axial development. Of these, *Wnt3a* is expressed in a pattern that overlaps with *Cdx1* at E7.5–E8.5 in the caudal embryo (Takada *et al.*, 1994; Meyer and Gruss, 1993). We first investigated this relationship by assessing the effects of Wnt signaling on expression from a *Cdx1* reporter using transient transfection assays. As the status of Wnt pathway intermediaries are not fully characterized in F9 cells, we employed a chimeric transcription factor consisting of a LEF1 DNA binding domain/β-catenin activation domain fusion protein (LEFΔN-βCTA) which mimics activation of the canonical Wnt pathway (Vleminckx *et al.*, 1999). Transfection of this fusion protein resulted in a significant increase in *Cdx1* reporter activity, with sequences mediating this effect mapping to the proximal region of the promoter (Fig. 1A).

We investigated the effects of known modulators of Wnt signaling on *Cdx1* expression in F9 cells and found that either LEF1 or β-catenin elicited an induction that was comparable to the response elicited by the fusion protein (Fig. 1B). Moreover, both basal promoter activity (Fig. 1B) and LEF1- and/or β-catenin-induced reporter activity (data

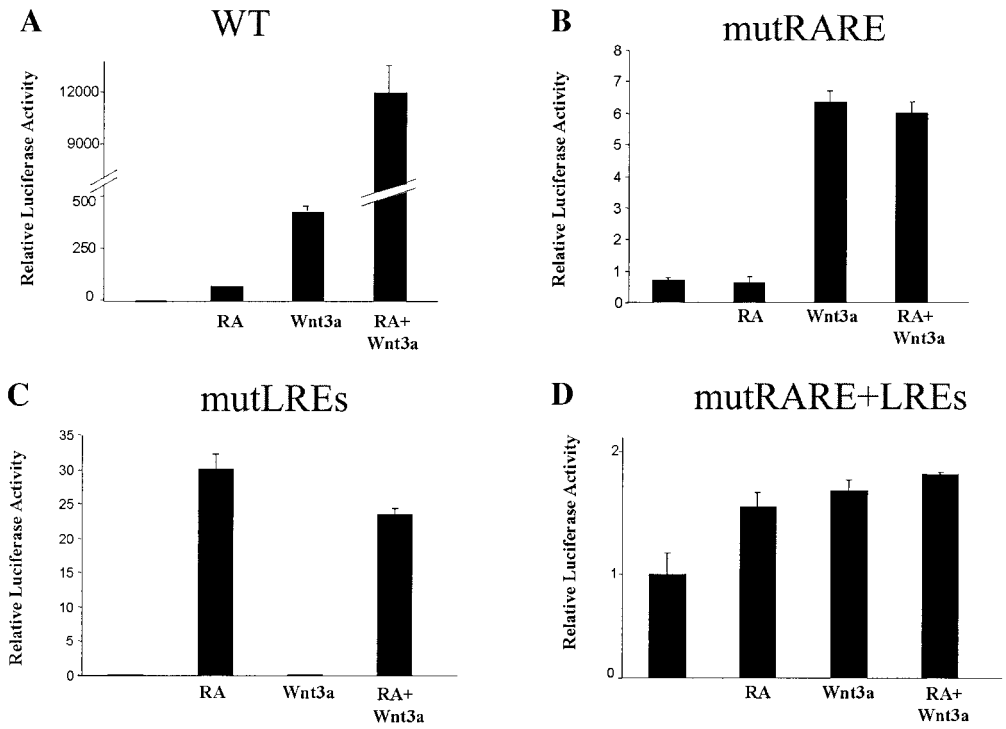

FIG. 3. Retinoic acid synergizes with Wnt3a on the Cdx1 promoter. Stable pools of F9 cells were derived harboring the wild-type 2-kb *Cdx1* reporter (A) or identical reporters mutated for the RARE (B), LRE1/LRE2 (C), or the RARE and LRE1/LRE2 together (D). Cells were cultured in the presence of vehicle, 1 μM RA, Wnt3a-conditioned medium, or RA and Wnt3a together as indicated below each panel. Luciferase activity was assessed 20 h posttreatment as described in Materials and Methods. Note the split scale in (A).

not shown) were attenuated by known negative regulators of the Wnt pathway, APC (Morin *et al.*, 1997) and ICAT (Tago *et al.*, 2000) (Fig. 1B).

The above data suggested that the *Cdx1* promoter responds to Wnt/β-catenin signaling, and that this effect maps to sequences between −185 and −38 nucleotides upstream of the *Cdx1* transcription start site (numbered according to Hu *et al.*, 1993). Examination of these sequences revealed the presence of a near-consensus LEF/TCF binding motif (Clevers and van de Wetering, 1997) at position −113 to −104 (denoted denoted LRE1), with a second potential element (designated LRE2) at position −82 to −70 (Fig. 1C).

Transfection analysis using reporters mutated for either LRE1 or LRE2 or both together demonstrated that both of these motifs contribute to a response to exogenous LEF1-β-catenin in the context of the full-length promoter (Fig. 1D). However, LRE1 seems to have a slightly more pronounced effect on the response of the promoter relative to LRE2, consistent with the higher homology of this element to the consensus LEF/TCF binding motif (Fig. 1C; Clevers and van de Wetering, 1997).

Electrophoretic mobility shift analysis demonstrated that LEF1 can associate with either LRE1 or LRE2 *in vitro* (Fig. 1E), consistent with previous data (Lickert *et al.*, 2000). Interestingly, LRE1 exhibited greater binding compared to LRE2 under identical conditions (compare lanes 3 and 11, Fig. 1E) consistent with their homology to the LEF/TCF consensus (Clevers and van de Wetering, 1997; Fig. 1C) and transfection analysis (Fig. 1D). Mutations in either element that abrogated transcriptional response (Fig. 1D) also abolished binding of LEF1 (Fig. 1E, lanes 6 and 14). Specificity of binding was supported by efficient competition with excess unlabeled LRE1 or LRE2 (Fig. 1E, lanes 4 and 12) but not with the unrelated Sp1 motif (Fig. 1E, lanes 5 and 13). Specific binding was also inhibited by an anti-LEF1 antibody (Fig. 1E, lanes 7 and 15), but not preimmune serum (Fig. 1E, lanes 8 and 16). Taken together, these findings support a direct role for Wnt signaling in regulating *Cdx1* expression.

Wnt3a Regulates Cdx1 Expression in Vivo

Since LEF1/β-catenin mediates the Wnt signal, it is probable that a Wnt protein lies upstream of *Cdx1* expression during embryogenesis; Wnt3a is of particular interest as its expression overlaps with *Cdx1* at late gastrulation and tail bud stages (Takada *et al.*, 1994). We therefore investigated *Cdx1* expression in *vestigial tail* (*vt*) embryos, a hypomorphic *Wnt3a* mutant (Greco *et al.*, 1996). Consis-

tent with our findings in transfection assays, *vt* homozygotes consistently exhibited reduced *Cdx1* expression in the caudal embryo at E8.5 (compare the *vt* mutants in Fig. 2B to wild-type controls in Fig. 2A); note that the affected region overlaps precisely with *Wnt3a* at this stage (Takada *et al.*, 1994). This effect was stage- and tissue-specific as *Cdx1* transcripts were not lost, and in fact appeared more abundant, in the dermamyotome and forelimb buds in *vt* mutants at E9.5 (compare mutants in Fig. 2D to controls in Fig. 2C). However, the residual *Cdx1* message in the tail bud region of the *vt* offspring is lost at this stage (arrowheads in Fig. 2C and Fig. 2D), further emphasizing the tissue-specific nature of this effect.

The *vt* mutant is likely a *Wnt3a* hypomorph (Greco *et al.*, 1996). However, the molecular basis for the *vt* phenotype has not been elucidated, and it remains formally possible that mutation of another closely linked gene is responsible. Therefore, to further establish the relationship between Wnt3a and *Cdx1*, wild-type embryos were cultured in the absence or presence of exogenous Wnt3a (Shibamoto *et al.*, 1998). Subsequent *in situ* hybridization revealed that Wnt3a elicited an increase in *Cdx1* levels in the posterior embryo as early as 3 h postexposure (Fig. 2F compare to control culture in Fig. 2E).

In marked contrast to the effects on *Cdx1*, we found that *Cdx2* expression was unaltered in *vt* embryos at E8.5 (Fig. 2H, compare to Fig. 2G). The effects of loss of Wnt3a therefore appear to be specific to *Cdx1*. The expression of *Wnt3a* was likewise not affected in *Cdx1* null embryos (data not shown).

Wnt3a and RA Synergize on the Cdx1 Promoter

Previous studies have demonstrated interactions between retinoid and Wnt pathways (Easwaran *et al.*, 1999; Sasai and de Robertis, 1997; Hecht and Kemler, 2000). We therefore investigated the effects of combinatorial treatment with Wnt and RA on *Cdx1* expression. To this end, we generated pools of stable F9 cells harboring a wild-type *Cdx1* reporter, or identical reporters but mutated for the RARE (Houle *et al.*, 2000), the LRE1/LRE2 elements, or all motifs together. As shown in Fig. 3, treatment of cells harboring the wild-type reporter with RA or Wnt3a resulted in a significant induction of expression. However, treatment with RA and Wnt3a together resulted in a much more profound induction, greatly exceeding the effects seen with either agent alone (Fig. 3A). This synergistic effect appeared to be specific and direct, since it required both the RARE and the LRE1/LRE2 motifs (Figs. 3B–3D). The lower relative values in Figs. 3B–3D may be due to complete loss of retinoid or Wnt signal mediated by mutation of the cognate response element.

Cdx1 Autoregulation

A number of homeobox genes, including *Cdx2*, have been shown to positively regulate their own expression (Xu *et al.*, 1999; Deschamps *et al.*, 1999, and references therein). We determined whether such a mechanism could affect expression of *Cdx1*, using the cognate null mice. At E7.5, *in situ* hybridization revealed comparable expression of *Cdx1* between wild-type and *Cdx1* null embryos (compare the null mutant in Fig. 4B to the control in Fig. 4A), indicating that the mutant mRNA is as readily detectable as the wild-type transcript at this stage. In marked contrast, *Cdx1* message was barely detected in the null mutant background at E8.5 relative to controls (compare mutants in Fig. 4D to controls in 4C; see also Figs. 5A and 5B).

These null mutant mice were generated by homologous recombination resulting in insertion of a neomycin cassette in exon 3 of the *Cdx1* locus (Subramanian *et al.*, 1995). However, as the mutant transcript can be readily detected at E7.5 (Fig. 4B), it would appear that the mutant message is not markedly destabilized by these extraneous sequences. The loss of expression in the *Cdx1* null background therefore appears to reflect a requirement for Cdx1 to maintain its own expression. Moreover, RT-PCR assays revealed readily detectable levels of the mutant allele in E8.5 *Cdx1* heterozygous embryos, further demonstrating that it is not markedly destabilized (Fig. 4E; note that the presence of an additional product from amplification of the null allele is likely due to a cryptic splice site in the neo cassette; Nordstrom and Westhafer, 1986). These data are consistent with an autoregulation mechanism necessary to maintain Cdx1 expression at late (E8.5) but not early (E7.5) stages.

In agreement with the above observations, cotransfection of a Cdx1 expression vector in P19 cells resulted in a sixfold activation of a wild-type *Cdx1* reporter (Fig. 4F). This effect was mapped to proximal promoter sequence (data not shown). However, examination of these sequences did not reveal any consensus Cdx-binding sites, nor have we been able to isolate a variant binding motif from this region. This suggests that this putative autoregulatory loop involves an indirect mechanism.

Although *Cdx1* expression is essentially extinguished in the null background, it was still induced by exogenous RA at E8.5 (Fig. 5D, compare to 5B), demonstrating that retinoid response does not absolutely require Cdx1. However, it is interesting to note that RA induced expression in the mutants to lower levels relative to wild-type controls (compare Fig. 5D to 5C), and that induction did not extend to the same anterior boundaries as either untreated or RA treated controls (compare Fig. 5D to 5A and 5C). This suggests a role for RA in initiation of *Cdx1* expression, with Cdx1 autoregulation reinforcing its own transcription at later stages.

DISCUSSION

We have demonstrated that the mouse homeobox gene *Cdx1* is a direct Wnt target, and specifically responds to Wnt3a *in vivo*. We have identified LEF/TCF binding sites in the *Cdx1* promoter, the presence of which are necessary for

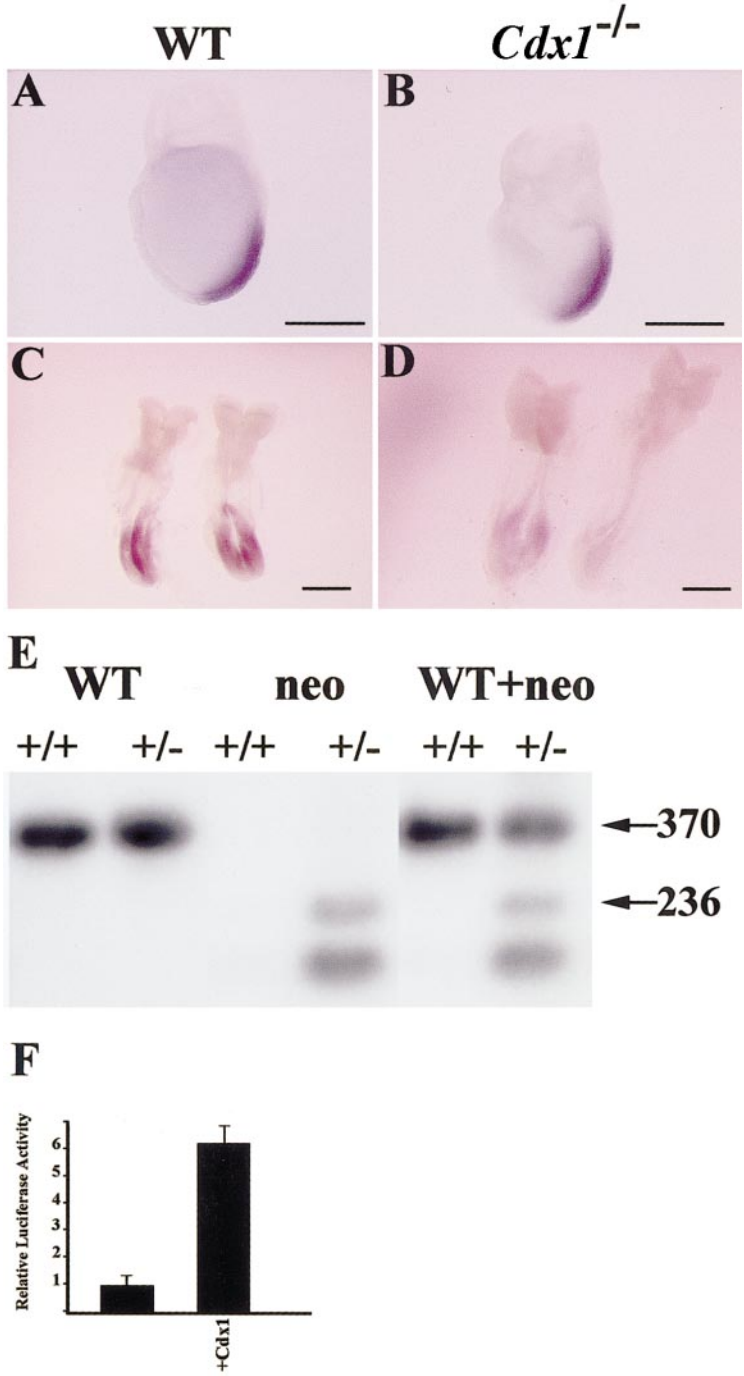

FIG. 4. Evidence for Cdx1 autoregulation. (A, B) Whole-mount *in situ* hybridization for *Cdx1* expression at E7.5 in wild-type (A) or *Cdx1* null mutants (B). (C, D) *Cdx1* expression at E8.5 in wild-type (C) or $Cdx1^{-/-}$ embryos (D). Note the significant reduction in *Cdx1* message in the null mutant background relative to controls at E8.5 (compare D to C) but not E7.5 (compare B to A). Scale bars, 100 μm. (E) RT-PCR analysis of C*dx1* expression from E8.5 wild-type or *Cdx1* heterozygous embryos. Genotypes are indicated above each lane. WT and neo denote PCR designed to amplify the wild-type (370-bp product) or mutant allele (236-bp product), respectively. Note that the smaller product from neo amplification of heterozygous samples was likely produced by a cryptic splice site in the *neo* cassette (Nordstrom and Westhafer, 1986). (F) P19 cells were transfected with the 2-kb *Cdx1* reporter alone or with a *Cdx1* expression plasmid and luciferase activity assessed 48 h posttransfection. Results were expressed as fold induction relative to the reporter vector alone.

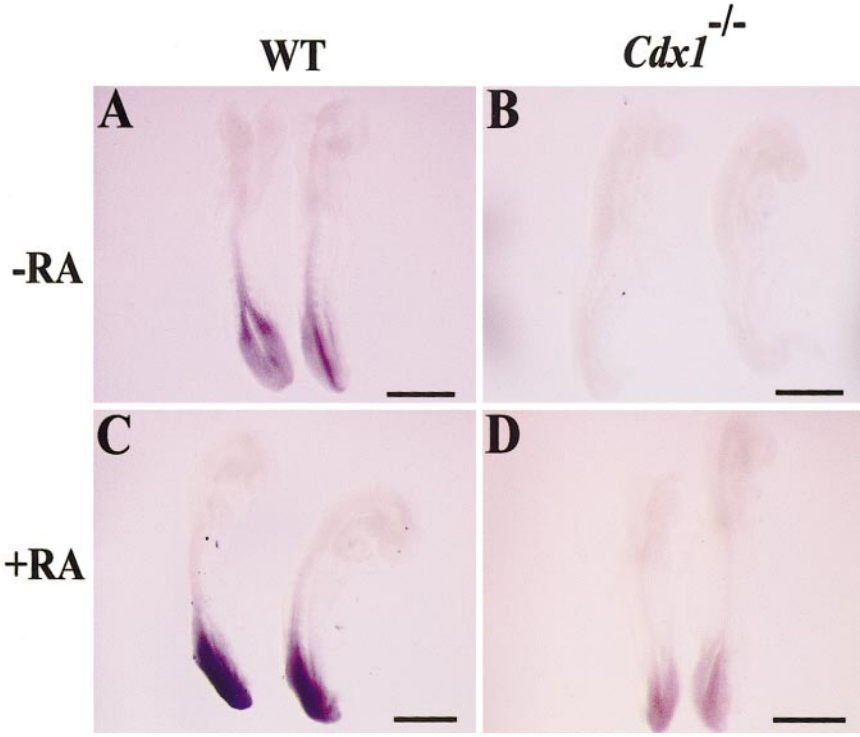

FIG. 5. Cdx1 induction by RA is independent of Cdx1 protein. Wild-type (A, C) or *Cdx1*$^{-/-}$ (B, D) embryos were treated *in utero* with vehicle (A, B) or RA (C, D) at E8.25 and analyzed for *Cdx1* expression by whole-mount *in situ* hybridization 6 h postgavage. Scale bars, 200 μm.

LEF/β-catenin response in F9 cells. The same sites were previously found to mediate Wnt response in epithelial cells (Lickert *et al.,* 2000) while, more recently, others have also documented a role for Wnt3a in affecting *Cdx1* expression *in vivo* (Ikeya and Takada, 2001). We also present evidence indicating that Wnt3a synergizes strongly with RA to activate the *Cdx1* promoter and that Cdx1 is essential to maintain its own expression at later stages. These findings suggest an interactive signaling cascade involving Wnts, RA, and Cdx1, essential to axial patterning.

Wnt Signaling Regulates Cdx1 Expression

Expression of *Cdx1* was at least partially dependent on Wnt3a *in vivo,* as judged by a marked decrease in caudal *Cdx1* transcripts in E8.5 *vt/vt* embryos. This effect was not likely due to a general reduction of nascent mesoderm, as we observed no difference in *Cdx2* expression, which marks this population, in the *vt* background. This latter observation also suggests that *Cdx2* is not a Wnt3a target. In support of this, we did not observe any significant effects of Wnt signaling on a *Cdx2* reporter in P19 cells (P.P. and D.L., unpublished observations) in agreement with prior work (Lickert *et al.,* 2000; Ikeya and Takada, 2001). In contrast, others (da Costa *et al.,* 1999) have found that APC can induce *Cdx2* in a colorectal cancer cell line. This suggests either that APC is acting through means other than the canonical Wnt pathway or that the effects of Wnt signaling on *Cdx2* expression depend on the cell type.

The effect of the *vt* mutation on *Cdx1* appeared to be both tissue- and stage-specific, in agreement with the pattern of expresion of *Wnt3a*. In contrast to the reduction of expression seen in the tail bud of *vt* mutants at E8.5, *Cdx1* transcripts in the presumptive dermamyotome and limb buds were comparable, or higher, in *vt* offspring relative to wild-type controls at E9.5. Given that *Wnt3a* expression has not been reported in dermamyotome or limb buds (Takada *et al.,* 1994), the basis for this increase is speculative, but may be due to reduced Wnt3a signaling from the dorsal neural tube, which has been shown to affect somite patterning (Ikeya and Takada, 1998). In any event, the functional significance of this observation is unclear, as a role for *Cdx1* in dermamyotome or limb development has not been demonstrated.

Interestingly, the *C. elegans caudal* homologue *pal-1* has recently been shown to be regulated by Wnt signaling (Zhang and Emmons, 2000; Hunter *et al.,* 1999). It is therefore tempting to speculate that Wnt-dependent regulation of *caudal* homologues may be a common feature of diverse species.

Retinoid and Wnt Signaling Converge on a Common Target

Cdx1 is expressed only in a subset of tissues known to be dependent on Wnt signaling. This suggests either a specific role for Wnt3a in this event, or that other (tissue restricted) players collaborate with Wnt signaling to affect expression of *Cdx1*. In this regard, we have previously found that endogenous retinoid signaling contributes to *Cdx1* expression at E7.5 (Houle *et al.*, 2000). Our present data indicate a remarkable synergy between Wnt and retinoid signaling on expression from the *Cdx1* promoter. It is notable that this effect was mediated via natural ligands through endogenous receptors, and did not require overexpression of any ancillary components. Furthermore, this synergy was entirely dependent on the presence of intact RA and LEF/TCF response elements in the *Cdx1* promoter, indicating that it is direct and specific.

Wnt and RA signaling are involved in posterior mesoderm patterning and in the specification of the posterior CNS in *Xenopus*. Moreover, these pathways exert synergistic effects on posteriorization processes in this species (reviewed in Sasai and de Robertis, 1997; Altmann and Brivanlou, 2001). Our present findings suggest that this synergism may occur, in part, through *Cdx1*.

Recently, Szeto *et al.* (2001) reported synergistic induction of the Stra6 gene by Wnt-1 and retinoic acid in mammary epithelial cells. Cross-regulation of Wnt and retinoid-signaling pathways has also been reported in breast and colon cancer cells (Easwaran *et al.*, 1999). However, in the latter case, RA was proposed to affect the activity of a LEF/TCF-β-catenin reporter by a direct interaction between β-catenin and RARα. More recently, Hecht *et al.* (2000) have identified CBP/p300 as a β-catenin coactivator. As CBP/p300 is also an RAR coactivator, it is conceivable that RARs and β-catenin may compete for limiting amounts of such ancillary factors, as has been proposed for cross-talk between RARs and other pathways (Glass and Rosenfeld, 2000). Wnt–retinoid interactions may therefore occur through several means and may be synergistic or inhibitory depending on the cellular context and the target gene examined.

Cdx1 Autoregulation

We have found that *Cdx1* expression is dramatically reduced in the *Cdx1* null mutant background at E8.5. There are at least two possible explanations for this: (1) transcript instability due to the disruption of the gene by *neo* sequences; (2) a positive feedback mechanism. We favor the latter for several reasons. First, the down-regulation of *Cdx1* was stage-specific, with expression unperturbed in the primitive streak region at E7.5. Second, cotransfection of a *Cdx1* expressing vector resulted in activation of its own promoter in P19 cells. Third, RA could induce *Cdx1* in the cognate null background, albeit absolute levels were reduced relative to wild-type controls. Finally, RT-PCR analysis demonstrated that both wild-type and *Cdx1* mutant alleles were present at relatively equivalent levels in *Cdx1* heterozygotes at E8.5. Taken together, these observations strongly suggest that a positive autoregulatory loop is critical to maintain expression of *Cdx1* at E8.5.

Autoregulatory loops have been demonstrated for murine *Hoxa1*, *Hoxb1*, *Hoxa4*, *Hoxb4*, and *Hoxd4* and are important to establish *Hox* expression domains (Deschamps *et al.*, 1999, and references therein). Interestingly, these examples also comprise all *Hox* genes that are known to be direct RA targets (reviewed in Deschamps *et al.*, 1999). Moreover, the *Hoxa4* autoregulatory element is required for maintenance of the effects of RA (Packer *et al.*, 1998). This is very similar to our present observations, with *Cdx1* transcripts present at E7.5 in *Cdx1* null mutants when RA signaling is active (discussed in Houle *et al.*, 2000) and subsequent loss of expression at later stages.

In contrast to the eventual loss of *Cdx1* message in the cognate null mutants, expression was reduced but not prematurely extinguished in *vt* embryos. This suggests that Cdx1 autoregulation is absolutely essential for maintenance of expression, whereas Wnt3a may be only one of several factors involved in other facets of expression. Alternatively, residual Wnt3a function in the *vt* hypomorph background may be sufficient to support *Cdx1* expression, albeit at reduced levels. The finding that *Cdx1* expression is more severely attenuated in *Wnt3a* null mutants than in the *vt* background (Ikeya and Takada, 2001) supports the latter possibility. However, the profound effect of *Wnt3a* disruption on posterior development precludes a clear assessment of the requirement for Wnt3a in later stages of *Cdx1* expression.

A Model for Cdx1 Expression

Our findings confirm prior data (Lickert *et al.*, 2000; Ikeya and Takada, 2001), and, together with our findings for a profound interaction between Wnt3a and RA and evidence for Cdx1 auto-regulation, prompt a model for initiation and maintenance of expression of *Cdx1* (Fig. 6). In this model, RA is important for early phases, perhaps initial activation, of *Cdx1* expression in the primitive streak region at E7.5 (Houle *et al.*, 2000) where it may act in synergy with Wnt3a. Once Cdx1 accumulates to a certain level, an autoregulatory loop reinforces and maintains expression which eventually becomes independent of RA at stages corresponding to the exclusion of retinoid signaling in the tail bud around E8.5 (Rossant *et al.*, 1991). Wnt3a could be involved at both early as well as later phases of *Cdx1* expression, perhaps reinforcing the *Cdx1* autoregulatory loop. Other factors, such as FGFs, may also be involved in *Cdx* regulation, as evidenced by data from *Xenopus* (Pownall *et al.*, 1996). However, a role for FGF in *Cdx1* expression in the mouse has not yet been demonstrated.

In conclusion, we present evidence supporting that retinoids, Wnt, and autoregulatory pathways cooperate to activate and maintain *Cdx1* gene expression in the primitive streak/tailbud of the murine embryo. Wnt and retinoid

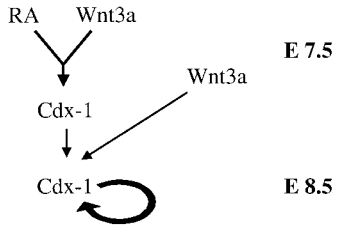

FIG. 6. A model for stage-dependent regulation of *Cdx1*. RA initiates *Cdx1* expression at E7.5 leading to accumulation of Cdx1 and establishment of an autoregulatory loop by E8.5. Wnt3a may both synergize with RA at E7.5 and reinforce the autoregulatory loop at E8.5.

signaling pathways have been postulated to interact to posteriorize the vertebrate axis (reviewed in Altmann and Brivanlou, 2001; Sasai and de Robertis, 1997). Our findings suggest that convergence of these signaling molecules on a common target gene, *Cdx1*, may underlie some of these effects.

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