Chicken Ovalbumin Upstream Promoter-Transcription Factor Members Repress Retinoic Acid-induced Cdx1 Expression*

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It is well established that Hox genes are key players in specifying positional identity along the anterior-posterior axis and are targets of diverse transcription factors implicated in axial patterning. Members of the CDX family (CDX1, -2, and -4) are among such effectors of Hox expression as pertains to vertebral patterning in the mouse. Cdx members are themselves targets of signaling molecules that are also implicated in axial patterning, including retinoic acid (RA) and certain members of Wnt and fibroblast growth factor families. In this regard, we have previously shown that, in the mouse, Cdx1 is directly regulated by RA at the late primitive streak stage (embryonic day (E) 7.5) through a RA response element in the proximal Cdx1 promoter. At E8.5, Cdx1 expression remains essentially limited to the posterior embryo. RA, however, is excluded from the caudal embryo at this later stage, but is found in a more anterior domain encompassing the prospective trunk region. These observations suggest the existence of a repressor mechanism that prevents expression of Cdx1 in these anterior domains of retinoid signaling at E8.5. In the present study, we present evidence suggesting that chicken ovalbumin upstream promoter-transcription factor (COUP-TF) members antagonize RA-induced Cdx1 expression by competing with retinoid X receptor-retinoic acid receptor heterodimers for binding to the Cdx1 RA response element. Consistent with this, *in situ* hybridization analysis revealed that COUP-TFs are highly expressed in the anterior embryo in domains where Cdx1 transcripts are excluded. Together with other data, these findings suggest a model by which COUP-TF expression is induced by RA in the trunk region as a negative feedback mechanism to restrict Cdx1 expression to the caudal embryo.

The Cdx family (Cdx1, -2, and -4) encode homeodomain transcription factors. A role for CDX1 and CDX2 in vertebral patterning has been clearly demonstrated by gene targeting studies. Cdx1<sup>-/-</sup> (1) and Cdx2<sup>-/-</sup> (2) mice exhibit homeotic transformations of the cervical and anterior thoracic vertebra. Cdx1<sup>-/-</sup>/Cdx2<sup>-/-</sup> compound mutant offspring exhibit a higher degree of expressivity of these transformations suggesting that both gene products functionally overlap (3). Cdx1 and Cdx2 appear to impact on vertebral patterning at least in part through regulation of Hox gene expression (1, 3). Consistent with this, the vertebral defects seen in Cdx mutant offspring are reminiscent of those observed in certain Hox mutants. Moreover these vertebral homeoses are typically associated with a posterior shift in the anterior expression domains of several Hox genes. A number of Hox promoters also contain consensus CDX binding motifs (1) at least some of which have been demonstrated to be functional *in vivo* (4). Finally Cdx1 transcripts are first detected at embryonic day 7.5 (E7.5) (5) in the mesodermal and ectodermal cells of the primitive streak (5), and this expression is maintained at tail bud stages through E9.5. This pattern of expression is consistent with the onset of Hox gene expression in the caudal embryo.

We have previously demonstrated that, in the mouse, Cdx1 is regulated at E7.5 by retinoic acid (RA) present in the late primitive streak region through an atypical RA response element (RARE) (6, 7). By E8.5, Cdx1 expression is maintained in the caudal embryo through mechanisms involving both Wnt signaling (8) and autoregulation (9). RA, however, is absent in this region at this stage but is found in a more anterior domain in the prospective trunk (10). Despite this, Cdx1 transcripts are not observed (with the exception of later expression in the dermamyotome and limb buds) in the embryonic trunk. This observation suggests the existence of a mechanism that serves to attenuate Cdx1 expression in these more anterior, RA-positive tissues.

Chicken ovalbumin upstream promoter-transcription factors (COUP-TFI and COUP-TFII) are orphan nuclear receptors (11–13). COUP-TFs have been shown to interfere with many nuclear receptor signaling pathways including the RARs (14–18). This antagonism has been suggested to occur by four different mechanisms: (i) competition for RXR heterodimerization; (ii) trans-repression via interaction with the ligand binding domain ofRAR, thyroid hormone receptor, or RXR; (iii) active repression through interaction with co-repressors; and (iv) competition for DNA binding at response elements (19).

Based on their ability to interfere with retinoid signaling, we investigated whether COUP-TFs could act as negative regulators of Cdx1 expression. Consistent with such a relationship, *in situ* hybridization analysis in mouse embryos revealed that Cdx1 expression was restricted to regions where COUP-TF transcripts were excluded. Moreover we found that COUP-TFs could attenuate RA-induced transcriptional activation of the Cdx1 promoter in tissue culture and that this effect was dependent upon the DNA binding domain of COUP-TFI. We also demonstrated that COUP-TFs can bind the Cdx1 RARE and...
Regulation of Cdx1 by COUP-TF

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The luciferase reporter vector containing 2 kb of the Cdx1 promoter or a heterologous (TK109 luciferase) basal promoter harboring the Cdx1 RARE have been described previously (6). Murine COUP-TFI and COUP-TFII cDNAs, generously provided by Dr. Ming-Jer Tsai, were subcloned into a modified version of pSG5 (Stratagene) harboring an extended polylinker. The COUP-TFIΔDBD mutant was generated by PCR-mediated deletion of nucleotides 565–621 (NCBI accession number X74134), which encode the first zinc finger of the COUP-TFI DNA binding domain.

Cell Culture and Transfection Analysis—F9 embryocarcinoma cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum in an atmosphere of 5% CO₂ at 37 °C. For transient transfection assays, cells were passed into gelatinized 6-well plates and transfected using the calcium phosphate precipitation method. Each transfection consisted of 1 μg of luciferase reporter construct, 0.05–0.5 μg of relevant expression vector(s), and empty expression vectors (where required) such that all transfections contained a total of 1.5 μg of DNA. The following day media were replenished, cells were treated with 1 μM RA or vehicle (Me2SO; final concentration, 0.01% (v/v)), and culture continued for another 24 h. Monolayers were then rinsed with phosphate-buffered saline, cells were disrupted by addition of lysis buffer (0.1 M Tris, pH 8, 1% Igepal, 1 mM dithiothreitol), and lysates were assessed for luciferase activity as described previously (6). Data are presented as the mean ± S.D. from three independent transfections and are expressed as -fold expression relative to control.

Electrophoretic Mobility Shift Assay—Oligonucleotide probe sequences corresponding to the wild type or a mutated Cdx1 RARE incapable of receptor association, an SP-1 binding motif, and conditions for binding reactions have been described previously (6). In some experiments, 1–2 μg of PEGFP-F (Clontech) was co-transfected with 25 μg of COUP-TFI expression vector, and GFP-positive cells were isolated by fluorescence-activated cell sorting 24 h post-treatment. Cells were harvested, snap frozen, and stored at −80 °C prior to RNA extraction. COUP-TFII was assayed as described previously (6).

In Situ Hybridization—Whole-mount in situ hybridization was performed on E8.0–E8.75 embryos as described previously (20) using riboprobes generated from Cdx1 or COUP-TF cDNAs.

RESULTS

COUP-TFI and Cdx1 Exhibit Complementary Patterns of Expression—To begin to establish whether COUP-TFs could function as negative regulators of Cdx1 expression, we first examined the expression patterns of each gene. Whole-mount in situ hybridization analysis revealed COUP-TFI expression at E8.0 in the anterior prospective nervous system (Fig. 1A) with expression extending caudally as elongation proceeded at E8.75 (Fig. 1C). COUP-TFI transcripts, however, were never detected in the tail bud region. Similar patterns of expression were also observed for COUP-TFII (data not shown). By contrast, expression of Cdx1 was observed exclusively in the prospective neural tube to the level of the caudal hindbrain and in a broad region throughout the posterior embryo at E8.5 with expression restricted to the neural tube and tail bud regions at E8.75 (Fig. 1, B and D). Thus, Cdx1 expression was restricted to regions where transcripts encoding COUP-TFs were excluded in agreement with a possible role for COUP-TFs as negative regulators of Cdx1.

FIG. 1. Cdx1 and COUP-TFI exhibit complementary patterns of expression. Whole-mount in situ hybridization analysis of COUP-TFI (A and C) and Cdx1 (B and D) expression in E8.0 (A and B) and E8.75 (C and D) mouse embryos. a, anterior; p, posterior.

COPU-TFs Interfere with Induction of Cdx1 by RA and Bind the Cdx1 RARE—To assess whether COUP-TF members could antagonize the induction of Cdx1 by RA, we transfected F9 cells with a reporter gene (pXP2, Ref. 6) under the control of Cdx1 promoter sequences, including the RARE, and cultured the cells in the absence or presence of RA (1 μM). As shown in Fig. 2A, RA treatment resulted in an induction of reporter activity consistent with previous findings (6). Moreover, co-transfection of the reporter with increasing amounts of an expression vector encoding COUP-TFI or COUP-TFII resulted in a complete attenuation of this induction.

COUP-TFs have been shown to exert their repressor function through several mechanisms, including competition for occupancy of hormone response elements. We therefore investigated whether COUP-TFs could bind to the Cdx1 RARE. To this end, nuclear extracts from COS7 cells transfected with COUP-TFI or -II were incubated with a radiolabeled probe consisting of the Cdx1 RARE sequence. Nuclear extracts from cells transfected with RA treated plus IκBα were used as a positive control since RAR-RXR heterodimers have been shown to bind efficiently to this element (6). The results, illustrated in Fig. 2B, indicate that both COUP-TFI and -II formed a complex with the Cdx1 RARE (lanes 6 and 11) that was comparable to or greater than the complex formed using RXR-RAR (lane 2). The presence of COUP-TFI or -II in each complex was confirmed by supershift assays using the appropriate antibodies (lanes 7 and 12). Specificity of binding was further established by efficient competition with an unlabeled RARE probe (lanes 8 and 13), whereas a mutated Cdx1 RARE (lanes 9 and 14) or an SP-1 binding motif (lanes 10 and 15) were unable to compete.

The above results demonstrated that COUP-TFs can bind directly to the Cdx1 RARE. To determine whether this interaction could inhibit Cdx1 expression, we assessed the effect of COUP-TFs on RA induction from a heterologous promoter containing the Cdx1 RARE. As shown in Fig. 2C, both COUP-TFI
and COUP-TFII repressed RA-induced transcriptional activity from this reporter.

**DNA Binding by COUP-TFI Is Necessary to Repress Cdx1**—
Mutation of the RARE abrogates induction of Cdx1 by RA (6).

However, a similar strategy would not be informative as a means to investigate the role of COUP-TFs as these factors attenuate RA induction through this element but do not interfere with basal expression. As an alternative approach, we assessed the ability of a COUP-TFI mutant lacking the first zinc finger motif of its DNA binding domain (COUP-TFI/H9004DBD) to impact on Cdx1 expression.

Electrophoretic mobility shift assay demonstrated that, in contrast to COUP-TFI, COUP-TFI/H9004DBD could not effectively bind the Cdx1 RARE (Fig. 3A, lane 6). This finding is consistent with prior work demonstrating the importance of this zinc finger motif for DNA binding by other members of the steroid hormone receptor superfamily (21). We next tested the ability of the COUP-TFI/H9004DBD mutant to repress RA-induced activation of the Cdx1 promoter in F9 cells. As illustrated in Fig. 3B, the COUP-TFI/H9004DBD mutant failed to inhibit RA-mediated activation of the Cdx1 promoter. Similar findings were also observed with the RARE directing expression from a heterologous promoter (TK109, Ref. 6; data not shown).

The relative mobility of the complex formed between FIG. 2. COUP-TFs antagonize induction of Cdx1 by RA and bind the Cdx1 RARE. A, F9 cells were transfected with a luciferase reporter gene under the control of the 2-kb Cdx1 promoter with or without 50–500 ng of COUP-TFI or -II expression vector and cultured in the absence or presence of RA (1 μM) for 24 h. Luciferase values are the mean ± S.D. and are expressed as -fold induction by RA relative to the reporter vector alone. B, nuclear extracts from COS7 cells, transfected with the indicated expression vectors, were incubated with a radiolabeled oligonucleotide probe corresponding to the Cdx1 RARE, and complexes were resolved by PAGE. Lane 1, mock-transfected cells; lanes 2–5, RAR and RXR; lanes 6–10, COUP-TFI; lanes 11–15, COUP-TFII. Specific complexes (lanes 2, 6, and 11) were not affected by inclusion of an SP-1 nonspecific competitor (lanes 5, 9, and 14) or a mutated Cdx1 RARE (lanes 9, 13, and 14) but were competed for by wild type Cdx1 RARE (lanes 8 and 13). Supershifts were observed after incubation with anti-COUP-TFI (lane 7) and anti-COUP-TFII (lane 10) or with COUP-TFI or -II expression vector. Cultures were treated with vehicle or RA, and luciferase activity was assessed 24 h post-treatment. Values are the mean ± S.D. from three independent transfections and are expressed as -fold induction by RA relative to the reporter vector alone. DMSO, Me2SO; Ab, antibody; mut, mutant; WT, wild type.

**FIG. 3.** COUP-TFI inhibition of Cdx1 is dependent on DNA binding. A, nuclear extracts from COS7 cells were incubated with a radiolabeled oligonucleotide probe corresponding to the Cdx1 RARE. Lanes 1–4, transfected with COUP-TFI; lanes 5–8, transfected with COUP-TFI/H9004DBD. Note that, unlike COUP-TFI (lane 1), COUP-TFI/H9004DBD was unable to form a specific complex with the RARE (lane 5). B, F9 cells were transfected with a luciferase reporter gene under the control of the 2-kb Cdx1 promoter in the absence or presence of RA (1 μM) 50, 100, or 500 ng (amounts indicated by relative line or triangle thickness) of COUP-TFI and/or COUP-TFI/H9004DBD expression vector(a) were used in co-transfection experiments. Results are the mean ± S.D. of independent triplicate transfections and are expressed as -fold induction by RA relative to the reporter vector alone. DMSO, Me2SO; Ab, antibody; mut, mutant; WT, wild type.
COUP-TF and the Cdx1 RARE, compared with the RXR-RAR-RARE complex, suggested that COUP-TFs bind this motif as homodimers, although this has not been formally assessed. However, consistent with this possibility, we found that co-expression COUP-TFIΔDBD was able to relieve the transcriptional repression mediated by its wild type counterpart in a dose-dependent manner (Fig. 3B). This suggests that the interaction of COUP-TF with COUP-TFIΔDBD prevented DNA binding and inhibition of RA-induced transactivation by the former and further supports the contention that COUP-TFs inhibit RA induction of Cdx1 by direct association with the RARE.

RA Induction of COUP-TFI Leads to Down-regulation of Cdx1 Expression—In addition to negatively regulating the expression of certain RA target genes, COUP-TFs have themselves been demonstrated to be RA targets (12, 22, 23). This finding has led to the hypothesis that COUP-TFs are involved in a negative feedback mechanism that serves to repress the expression of RA target genes (23). To examine this relationship, P19 cells were cultured in the continual presence 1 μM RA, and expression of COUP-TFI and Cdx1 were assessed from 1 to 7 days. Northern blot analysis (Fig. 4A) revealed that COUP-TFI was first detected 2 days after RA treatment with transcripts accumulating to a maximum by day 5. By contrast, Cdx1 transcripts were already detectable 24 h post-treatment with a subsequent decline in expression from day 2 onward. These complementary kinetics of expression are consistent with retinoid induction of COUP-TFI leading to down-regulation of Cdx1 despite the continued presence of RA.

To further investigate the relationship between COUP-TF and Cdx1, we transfected P19 cells with an expression vector encoding either COUP-TFI or COUP-TFIΔDBD together with a GFP expression plasmid. Cdx1 is induced by RA in P19 cells in a manner indistinguishable from F9 cells (see Fig. 4A).2 P19 cells were used for these experiments as a higher transfection efficiency was necessary to obtain sufficient cells for Northern blot analysis. Cultures were then treated for 24 h with RA or vehicle, and GFP-positive cells were isolated by fluorescence-activated cell sorting. Nuclear proteins were extracted, and expression of Cdx1 was assessed by Northern blot analysis using β-actin as a loading control.

DISCUSSION

The results presented in this study provide evidence that COUP-TFs can effectively antagonize the induction of Cdx1 by RA. The inability of the COUP-TFIΔDBD mutant to repress this induction indicates that COUP-TF DNA binding is essential to this outcome. This observation also rules out the possibility that this inhibition results from the interaction between the ligand binding domains of COUP-TFs and RAR as such a mechanism does not require COUP-TF binding to DNA (17). Moreover others have shown that COUP-TFs can interact with silencing mediator of retinoic acid and thyroid hormone receptor, nuclear receptor co-repressor, and histone deacetylase 1 (24, 25). However, trichostatin A did not abrogate the effects of COUP-TF on the Cdx1 promoter (data not shown) suggesting either that histone deacetylase activity is not implicated in this event or that trichostatin A-insensitive histone deacetylases are involved (26, 27).

Further evidence for competition for RARE binding comes from the finding that COUP-TFI and COUP-TFIΔDBD heterodimerized and that COUP-TFIΔDBD reversed the inhibitory effects of COUP-TFI on the Cdx1 promoter. In this regard, the Cdx1 RARE is closely related to a palindromic thyroid hormone receptor response element, and COUP-TF homodimers have been demonstrated to bind efficiently to such motifs (14, 18, 28). Taken together, these data support a model whereby COUP-TFs attenuate Cdx1 expression in the presence of RA by competition with RXR-RAR heterodimers for binding to the Cdx1 RARE.

In addition to the primitive streak and tail bud, Cdx1 is also

2 M. Be´land and D. Lohnes, unpublished data.
expressed in the endoderm of the developing intestine starting at E14 and eventually forming a rostral-caudal gradient with higher levels in the colon (29). In this regard, the Cdx1 RARE was first identified as a silencer element in a human colon carcinoma cell line (30). Moreover several studies have also reported the presence of COUP-TFI and COUP-TFII transcripts along the developing gut endoderm and in the adult intestine (31–33). Taken together with our present findings, it is interesting to speculate that negative regulation by COUP-TFs may also contribute to the graded expression of Cdx1 in the intestinal tract. Although the role of Cdx1 in this tissue is presently unknown, it has been suggested that it may be involved in specifying identity along the anterior-posterior axis of the gut (34) as has been demonstrated for CDX2 (2, 35). A similar function may be masked in CDX1 null mutants by functional redundancy with CDX2 (3).

COUP-TFs are involved in numerous developmental processes (36, 37). Of particular relevance is a potential role for COUP-TF1 in vertebral patterning as suggested by the high incidence of fusions between the exoccipital and basioccipital bones in the cognate null mutant. Based on our present findings, it is conceivable that this phenotype could be due to derepression of Cdx1. The extensive homology between COUP-TFI and COUP-TFII together with their overlapping expression profiles also suggests redundancy between these transcription factors as has also been indicated from gene targeting studies. Such redundancy could account for the relatively weak vertebral phenotype observed in COUP-TFI null mice relative to the anticipated effect of misexpression of Cdx1.

The impact of RA on vertebral patterning has been well documented. Although many Hox genes respond to RA, only a limited number have been demonstrated to be direct targets. We have previously suggested that Cdx1 serves as an intermediate between RA and mesodermal Hox gene expression (6). Thus, an understanding of means by which Cdx1 expression is controlled is essential to resolving the mechanisms impacting on vertebral patterning. The results presented in the present study led us to propose the following model (Fig. 5). Cdx1 expression is induced by RA generated in the primitive streak region at E7.5. At E8.5, when RA production ceases in the tail bud Cdx1 expression is maintained in the caudal embryo by Wnt signaling and an autoregulatory loop (9). To attenuate ectopic expression of Cdx1 in the trunk, which is retinoid-positive, RA induces COUP-TF expression, which blocks access to the RARE by competition. This results in a restriction of Cdx1 transcripts to the caudal embryo, contributing to appropriate Hox gene expression and patterning of the vertebral anterior-posterior axis.

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