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Functional Domains of the LIM Homeodomain Protein Xlim-1 Involved in Negative Regulation, Transactivation, and Axis Formation in *Xenopus* Embryos

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The *Xenopus* LIM homeodomain protein Xlim-1 is specifically expressed in the Spemann organizer region and assumed to play a role in the establishment of the body axis as a transcriptional activator. To further elucidate the mechanism underlying the regulation of its transcriptional activity, we focused on the region C-terminal to the homeodomain of Xlim-1 (CT239-403) and divided it into five regions, CCR1–5 (C-terminal conserved regions), based on similarity between Xlim-1 and its paralog, Xlim-5. The role of Xlim-1 CT239-403 in the Spemann organizer was analyzed by assaying the axis-forming ability of a series of CCR-mutated constructs in *Xenopus* embryos. We show that high doses of Xlim-1 constructs deleted of CCR1 or CCR2 initiate secondary axis formation in the absence of its coactivator Ldb1 (LIM-domain-binding protein 1), suggesting that CCR1 and CCR2 are involved in negative regulation of Xlim-1. In contrast, while Xlim-1 is capable of initiating secondary axis formation at low doses in the presence of Ldb1, deletion of CCR2 (aa 275–295) or substitution of five conserved tyrosines in CCR2 with alanines (CCR2-5YA) abolished the activity. In addition, UAS-GAL4 one-hybrid reporter assays in *Xenopus* showed that CCR2, but not CCR2-5YA, with its flanking regions (aa 261–315) functions as a transactivation domain when fused to the GAL4 DNA-binding domain. Finally, we show that none of the known transcriptional coactivators tested (CBP, SRC-1, and TIF2) interacts with the Xlim-1 transactivation domain (aa 261–315). Thus, Xlim-1 not only contains a unique tyrosine-rich activation domain but also contains a negative regulatory domain in CT239-403, suggesting a complex regulatory mechanism underlying the transcriptional activity of Xlim-1 in the organizer.

***Key Words: Xenopus laevis;* Spemann organizer; axis formation; transcription factor; LIM homeodomain protein; LIM domain; negative regulation; transactivation; Xlim-1; Ldb1.**

INTRODUCTION

Specific induction of gene expression by transcription factors is a key event in all developmental processes. Transcription factors are generally composed of multiple functional domains for DNA binding, transactivation or repression, and regulation via protein–protein interactions (Mitchell and Tjian, 1989; Ptashne, 1988). As the specificity of target gene selection is mainly determined by the DNA-binding properties of the DNA-binding domain, combinatorial binding of various transcription factors to regulatory sequences is thought to define temporally and spatially regulated gene expression during embryogenesis (Ptashne and Gann, 1998). In addition, protein–protein interactions between transcription factors directly or through adaptor proteins appear to be important for regulating their target genes as has been exemplified in the case of Hox proteins and their cofactors (Mann and Affolter, 1998) or LIM homeodomain (LIM-HD) proteins and Ldb1, LIM-domain-binding protein 1 (also known as NLI and CLIM2) (Dawid *et al.*, 1998).

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The LIM-HD proteins comprise a subfamily of the homeodomain protein family of transcription factors and are further classified into six highly conserved subclasses according to sequence similarities of their homeodomains (Dawid *et al.,* 1995, 1998). In addition to the DNA-binding homeodomain, the LIM-HD proteins have two highly conserved LIM domains in their N-terminus, which are involved in protein–protein interactions (Agulnick *et al.,* 1996; Bach *et al.,* 1997; Jurata *et al.,* 1996). They have been implicated in a variety of developmental processes such as body axis determination, regional specification, and tissue- or cell-type specification (Curtiss and Heilig, 1998; Dawid *et al.,* 1998).

The *Xenopus* Xlim-1 is a member of the LIM-HD family specifically expressed in the Spemann organizer region (Taira *et al.,* 1992) and assumed to play important roles in the vertebrate organizer function (Shawlot and Behringer, 1995; Taira *et al.,* 1994). As reported previously, Xlim-1 is inactive when ectopically expressed in *Xenopus* embryos, whereas mutations in the LIM domains impart axis-forming ability to Xlim-1 by relieving the inhibitory effect of the LIM domains (Taira *et al.,* 1994). Ldb1 is also capable of suppressing the inhibitory effect by binding to the LIM domains of Xlim-1 (Agulnick *et al.,* 1996). Recently, we have shown that Xlim-1 and Ldb1 form a complex on the *goosecoid* promoter and activate the expression of the *goosecoid* gene (Mochizuki *et al.,* 2000). By analogy to the case of the *Drosophila* LIM-HD member Apterous and the Ldb1 ortholog, Chip, Xlim-1 and Ldb1 are thought to form a tetrameric complex to function as a transcriptional activator (Breen *et al.,* 1998; Jurata *et al.,* 1998; Milan and Cohen, 1999; van Meyel *et al.,* 1999).

Compared to analyses of the LIM domains, the region C-terminal to the homeodomain of Xlim-1 (referred to as CT239-403) has not been fully studied, even though its amino acid sequence is highly conserved among orthologs of Lim1/Lhx1, as is also the case with orthologs of Lim3/Lhx3 (Zhadanov *et al.,* 1995) and Isl-1 (Tokumoto *et al.,* 1995). In many cases, transcriptional activators possess canonical transactivation domains containing proline-rich, glutamine-rich, or acidic amino acid sequences, which are not well conserved at primary sequence levels. In contrast, Xlim-1 is 90% identical to mouse Lim1 in the C-terminal region, which shows transactivation activity in a yeast one-hybrid system (Breen *et al.,* 1998). This high degree of amino acid sequence conservation in the C-terminal region suggests the existence of multiple functional domains, each of which might interact with other nuclear proteins including coactivators, corepressors, or other transcription factors that modulate the activity of Xlim-1. Therefore, to further elucidate the molecular mechanism by which Xlim-1 regulates its target genes in the Spemann organizer, we examined the role of specific domains in the C-terminal region by analyzing the activities of a series of C-terminal mutated constructs as assayed by axis duplication experiments. The results indicate the existence of both a negative regulatory domain and two activation domains, implying the existence of multiple transcriptional cofactors of Xlim-1.

MATERIALS AND METHODS

Embryo Manipulations, RNA Injections, and Whole-Mount Immunostaining

Xenopus embryos were fertilized *in vitro,* dejellied, and incubated in 0.1× Steinberg's solution (Peng, 1991). Embryonic stages were determined according to Nieuwkoop and Faber (1967). RNA (2.5–10 nl/embryo) or DNA/RNA (10 nl/embryo) injections into embryos were done in 3% Ficoll in 1× MBS (Peng, 1991). Injected embryos were kept in 3% Ficoll in 1× MBS for 2–3 h, transferred to 0.1× Steinberg's solution, 50 μg/ml gentamicin sulfate, and incubated until embryos reached the appropriate stages. For axis duplication assays, mRNAs were injected into the equatorial region of two ventral blastomeres at the four-cell stage and scored for axis development at tail-bud stages (stages 32–37/38). For *Xenopus* one-hybrid assays, mRNA and reporter DNA were co-injected into both blastomeres at the two-cell stage in the animal pole region. Animal caps were dissected as similarly in size as possible at stage 8–9, cultured until the equivalent of stage 11, and collected for luciferase assays (Mochizuki *et al.,* 2000). For whole-mount immunostaining, mRNA-injected embryos were fixed at tail-bud stages with MEMFA and stained as described (Hemmati-Brivanlou and Harland, 1989) using the muscle-specific 12/101 antibody (Kintner and Brockes, 1984).

Plasmid Constructs

pCS2+Xlim-1 was constructed with the Xlim-1 coding region flanked by a *Nco*I site at the 5′ end and an *Eco*RI site at the 3′ end (**TAG**CAAGCTTGAATTC, in which the boldface and underline indicate the stop codon and restriction site, respectively) in pCS2+AdN (Mochizuki *et al.,* 2000), a derivative of pCS2+ (Rupp *et al.,* 1994). PCR amplification and subsequent subcloning into appropriate restriction sites were performed to create the following constructs. *Apa*I (Xlim-1)/*Eco*RI (vector) sites of pCS2+Xlim-1 were used to make pCS2+Xlim-1(1-397), pCS2+Xlim-1(1-353), and pCS2+Xlim-1(1-315), in which the last codon was followed by a termination codon and *Eco*RI site (**TAG**TCTTCGAATTC). pCS2+Xlim-1(1-272) was generated by removing the *Apa*I (Xlim-1)/*Xba*I (vector) fragment of pCS2+Xlim-1 followed by Klenow reaction and self-ligation. However, unexpected incomplete blunt-ending of the *Apa*I site by Klenow reaction resulted in the addition of nine unrelated amino acids, ARTIVSRIT, derived from the vector sequence. To fuse various portions of CT239-403 to the homeodomain (aa 178–238), pCS2+Xlim-1(1-240) was made, in which an *Eco*RI site was introduced just after the codon of aa 240, followed by a termination codon and *Xba*I site (GAATTC**TAG**TCTAGA). This construct contains two additional amino acids, EF, corresponding to the *Eco*RI site after aa 240. The *Eco*RI/*Xba*I sites of pCS2+Xlim-1(1-240) were used to make pCS2+Xlim-1(1-240/261-315), pCS2+Xlim-1(1-240/273-305), and pCS2+Xlim-1(1-240/287-293). To make Ldb1/Xlim-1 fusion constructs, the *Bam*HI/*Eco*RI sites of pCS2+ were used to make pCS2+Ldb1(1-291), in which *Cla*I and *Ase*I sites and a stop codon overlapping the *Eco*RI site were introduced after aa 291 (ATCGATATTAAT**TGA**ATTC). *Cla*I/*Eco*RI sites of pCS2+Ldb1(1-291) were used to make pCS2+Ldb-Xlim1, pCS2+Ldb-Xlim1(Δ241-260), pCS2+Ldb-Xlim1(Δ278-292), and pCS2+Ldb-Xlim1(Δ278-303) by in-

serting *Cla*I (Xlim-1)/*Eco*RI (vector) fragments of pCS2+Xlim1 and its mutant constructs. These Ldb-Xlim1 fusion constructs lack most of the LIM domains upstream of aa 107 corresponding to the *Cla*I site. pSP64-Xβm-Xlim-1(Δ241-260), pCS2+Xlim-1(Δ278-292), pCS2+Xlim-1(Δ278-303), pCS2+Xlim-1(5YF), pCS2+Xlim-1(5YA), pCS2+Ldb-Xlim1(5YF), and pCS2+Ldb-Xlim1(5YA) were made by site-directed mutagenesis using the Gene Editor *in vitro* site-directed mutagenesis system (Promega) according to the manufacturer's recommendations. All five tyrosine residues were changed to phenylalanine (5YF) or alanine (5YA) by replacing TAT with TTT or GCT, respectively.

*Bam*HI (blunt-ended)/*Xho*I sites of pCS2+ were used to make pCS2+DBD with GAL4-DBD (aa 1–147) plus multiple cloning sites (*Nde*I through *Bam*HI) from pAS2-1 (Clontech). *Eco*RI/*Xba*I sites of pCS2+DBD were then used to make pCS2+DBD-CT261-403, pCS2+DBD-CT293-403, pCS2+DBD-CT316-403, pCS2+DBD-CT261-315, and pCS2+DBD-CT261-315(5YA). *Nco*I (blunt-ended)/*Eco*RI sites of pCS2+DBD were used for making pCS2+DBD-AD with GAL4AD (aa 768–881) and an HA epitope tag from pACT2 (Clontech). *Nco*I/*Eco*RI sites of pCS2+DBD were used to make pCS2+DBD-CT239-403 with the *Nco*I/*Eco*RI fragment of pAS2-1-DBD-CT239 (Breen *et al.*, 1998), a region encoding aa 239–403 of Xlim-1. To make pCS2+DBD-CT239(Δ278-292), the *Apa*I (Xlim-1)/*Apa*I (vector) fragment of pCS2+DBD-CT239 was replaced with that of pCS2+Ldb-Xlim1(Δ278-292).

pGEX2T-CT261-315 and pGEX2T-CT261-315(5YA) for glutathione *S*-transferase (GST) fusion proteins were constructed by inserting the same *Eco*RI/*Xba*I fragments as used in the pCS2+DBD construct into *Eco*RI/*Xba*I sites of a modified version of pGEX2T (pGEX2T-NEX), which contains *Bam*HI, *Nco*I, *Cla*I, *Eco*RI, and *Xba*I sites upstream of the stop codon. pGEX3X was used to generate the GST protein. All plasmids constructed in this study were verified by sequencing. The following plasmids have been described: pSP64-Xβm-Xlim-1 (Taira *et al.*, 1994), pSP64-Xβm (*Xenopus* β-globin) (Krieg and Melton, 1984), pSP64RI-XLdb1 (Agulnick *et al.*, 1996), pGEX2T-Smad3 (Sano *et al.*, 1999), pcDNA3-mCBP-HA (Sano *et al.*, 1998), pGEX4T1-hERα(DEF) (Endoh *et al.*, 1999), and pcDNA3-hSRC-1 and pcDNA3-hTIF2 (Kobayashi *et al.*, 2000).

Preparation of Synthetic mRNAs

For template preparations, pCS2+ and pSP64 plasmid constructs were linearized with *Not*I and *Sal*I, respectively. Capped mRNAs were synthesized *in vitro* using the MEGAscript SP6 kit (Ambion) with m7-GpppG (New England Biolabs) and dissolved in RNase-free water. The quantities of synthesized mRNAs were determined by comparison with RNA of known concentration on a 1% agarose/formaldehyde gel. mRNA derived from the pCS2+ vector was used for virtually all constructs in order to expect similar efficiency of protein expression. Exceptional use of pSP64 vector for Xlim-1(Δ241-260) is indicated in the figure legends.

Luciferase Reporter Assay

pUAS-Ars194-Luc reporter plasmid (Kiyama *et al.*, 1998) was used for the *Xenopus* one-hybrid assay. Luciferase assays were performed using a luciferase assay system (Promega) according to the manufacturer's protocols at half scale as described (Mochizuki *et al.*, 2000). Cell extracts were prepared from five independent pools each containing three animal caps, and luciferase activity was measured using a Lumat LB 9507 luminometer (Berthold). Means and standard errors (SE) of five independent values were calculated after subtraction of the luciferase activity of uninjected control as background. Relatively small SE (see Fig. 6B) verified reliable assays without internal controls for luciferase activity.

GST Pull-Down Assay

GST fusion proteins or GST alone were expressed in *Escherichia coli* (DH5α or BL21(DE)) essentially as described (Mochizuki *et al.*, 2000). Expression of proteins with the predicted size was monitored by SDS–PAGE. [^{35}S]Methionine-labeled CBP, SRC-1, and TIF2 were generated by using the TNT T7 coupled reticulocyte lysate system (Promega). Approximately 10 μg of GST fusion proteins or GST alone bound to GST beads and 10 μl of *in vitro*-translated products were mixed in 300 μl of NETN+ buffer (50 mM Tris–Cl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40), incubated on ice for 30 min, washed with NETN+ buffer five times, and subjected to SDS–PAGE and analyzed by BAS-2000 (Fuji Film). For GST-ERα(DEF), binding assays were done in the presence of 10^{-6} M 17β-estradiol.

RESULTS

Division of the C-terminal Region of Xlim-1 into Five Portions Referred to as CCRs (C-terminal Conserved Regions)

In comparison within the Lim1/Lhx1 subclass, Xlim-1 is highly conserved across the entire region sharing 92% identity with the mouse ortholog Lim1 (Barnes *et al.*, 1994; Fujii *et al.*, 1994) and 72% with its *Xenopus* paralog, Xlim-5 (Toyama *et al.*, 1995). To analyze the role of the region C-terminal to the homeodomain of Xlim-1 (CT239-403), we divided it into five regions, CCR1 through CCR5, according to sequence similarity between Xlim-1 and Xlim-5 (Fig. 1). CCR1 (aa 239–260) is the most conserved region within CT239-403. CCR2 (aa 275–295) is also a well-conserved region rich in phenylalanine and tyrosine. Five tyrosine residues in CCR2 are perfectly conserved within the vertebrate Lim1/Lhx1 subclass (Fig. 1). CCR3 (aa 298–350) and CCR4 (aa 353–395) are relatively less conserved regions rich in serine and proline. CCR5 (aa 396–403) is a short stretch of sequence in the most C-terminal region, which is well conserved among vertebrates, sea urchin, and *Drosophila* members of the Lim1/Lhx1 subclass (Kawasaki *et al.*, 1999; Lilly *et al.*, 1999; Tsuji *et al.*, 2000).

CCR1 and CCR2 Are Negative Regulatory Regions

To analyze the role of CT239-403 of Xlim-1 in Spemann organizer function, we adopted the axis duplication assay by mRNA injection into the ventral equatorial region of the *Xenopus* embryo. As described previously, active forms of Xlim-1, in which the LIM domains are point mutated (Xlim-1/3m) or deleted (Xlim-1/ΔNA) (Taira *et al.*, 1994), or wild-type Xlim-1 coexpressed with Ldb1 (Agulnick *et al.*, 1996) gives rise to two typical phenotypes, "secondary axis" and "dorsalized," as shown

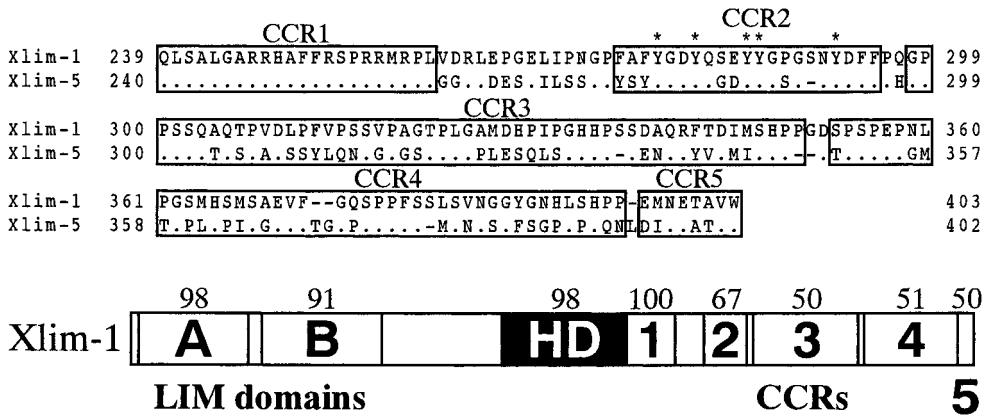

FIG. 1. Sequence alignment of CCR1–5 (C-terminal conserved regions) of Xlim-1 and its paralog, Xlim-5. Dots, identical amino acids; dashes, spaces for alignment; boxes, CCR1 through CCR5; asterisks, five conserved tyrosine residues in CCR2. Shown below is a schematic diagram of Xlim-1. Percentages of amino acid sequence identity between each region compared to Xlim-5 are indicated. Xlim-1 and Xlim-5 are 72% identical overall, 56% identical within the entire C-terminal region. A, B, LIM domains A and B; HD, homeodomain.

in Fig. 2 (upper row). Immunostaining by a muscle-specific antibody, 12/101, verified ectopic muscle formation in embryos with secondary axis and in dorsalized embryos (Fig. 2, lower row). Thus, categorizing the phenotypic effects by morphological appearances as shown in Fig. 2 was used to evaluate the organizer activity of various Xlim-1 constructs as described below.

We first focused on the well-conserved CCR1 and CCR2 and examined their roles by using internal deletion constructs. We found that deletion of CCR1, surprisingly, imparted axis-forming ability to Xlim-1 in the absence of Ldb1 (Fig. 3, Δ241-260). CCR2 deletion constructs, Δ278-292 or Δ278-303, also gave the same result, but to a lesser extent (Fig. 3). These results suggest that, in addition to the LIM domains, CCR1 and CCR2 are also involved in negative regulation of Xlim-1. However, it should be noted that higher doses (1 ng mRNA/embryo) were required for CCR1/CCR2 deletion mutants compared to the LIM domain-mutated active forms of Xlim-1, Xlim-1/3m, and Xlim-1/ΔNA (0.25 ng mRNA/embryo, data not shown), implying smaller contributions of CCR1 and CCR2 to the negative regulation.

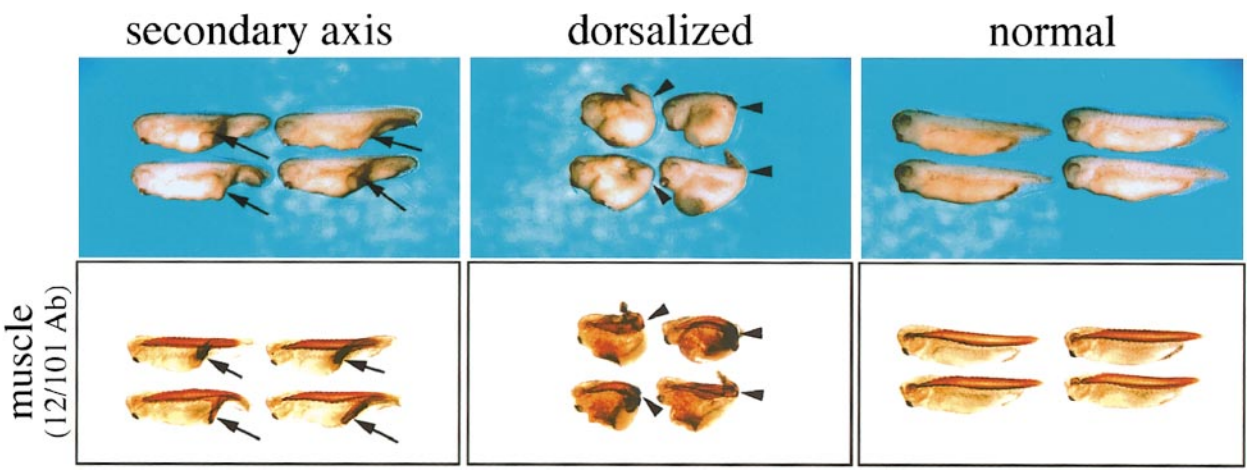

FIG. 2. Phenotypes of mRNA-injected embryos in axis duplication assays. *Xenopus* embryos were injected with mRNA as described under Materials and Methods. Typical morphological appearances elicited by coexpression of Xlim-1 and Ldb1 are shown. Secondary axis, embryos with secondary axes and ectopic muscle formation (arrows); dorsalized, embryos with reduced tail region and ectopic muscle formation (arrowheads) on the ventral side; normal, normal by visual inspection and lack of ectopic muscle formation. Ectopic muscle formation was shown by immunostaining using the muscle-specific 12/101 antibody (lower row).

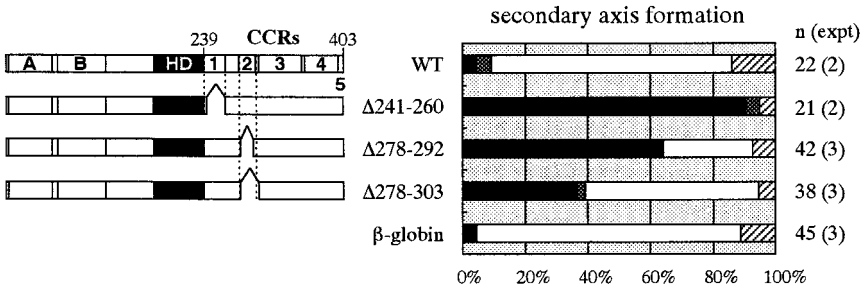

FIG. 3. Axis duplication activities of Xlim-1 deleted of CCR1 or CCR2 in the absence of Ldb1. Xlim-1 constructs deleted of CCR1 (Δ241-260) or CCR2 (Δ278-292, Δ278-303) initiate secondary axis formation at high doses (1 ng/embryo) in the absence of Ldb1 while wild-type Xlim-1 does not. Embryos injected with the mRNAs indicated were scored for axis development at tail-bud stages and categorized as secondary axis (solid bars), dorsalized (dotted bars), normal (open bars), or others (hatched bars). Note that the injected doses were four times higher (1 ng/embryo) than those in Figs. 4 and 5 (0.25 ng/embryo). β-Globin served as a negative control. *n*, total number of injected embryos; expt, number of independent experiments.

CCR2 Is Necessary for the Activity of Xlim-1

Xlim-1 is capable of initiating secondary axis formation in the presence of Ldb1 (Agulnick *et al.*, 1996). To define regions in CT239-403 required for the axis-inducing activity of Xlim-1, we tested the activities of a series of Xlim-1 mutants by coexpression with Ldb1. Stepwise deletion analysis from the C-terminus revealed that deletion of CCR2 and CCR4 dramatically decreased the activity of Xlim-1 (Fig. 4A; compare 1-397 with 1-353 and 1-315 with 1-272). In contrast, deletions of CCR5, CCR3, and CCR1 showed little effects (Fig. 4A; compare 1-403 with 1-397, 1-353 with 1-315, and 1-272 with 1-240, respectively). Furthermore, internal deletion of CCR2 almost completely abolished the activity (Fig. 4A, Δ278-292, Δ278-303), suggesting that the activity of Xlim-1 for the organizer function is mostly dependent on CCR2.

In contrast to CCR2, deletion of CCR1 from wild-type Xlim-1 did not seem to decrease nor enhance the activity (Fig. 4A, Δ241-260), which was also the case when CCR1 was deleted from active forms of Xlim-1, Xlim-1/3m, and Xlim-1/ΔNA (data not shown). Nevertheless, deletion of CCR1 from Xlim-1(1-315) dramatically enhanced the activity, which was comparable to that of wild-type Xlim-1 (Fig. 4A, 1-240/261-315). These data suggest that CCR1 negatively regulates the activity of CCR2 in Xlim-1(1-315) and that in the absence of CCR3–5 (aa 316–403), Ldb1 is incapable of fully suppressing the inhibitory effects of CCR1 on CCR2 (see Discussion).

As we have shown by deletion analysis that CCR2 is an indispensable portion of Xlim-1, we next asked if CCR2 alone was sufficient for the organizer activity of Xlim-1. As shown in Fig. 4A, while Xlim-1(1-240/261-315), which contains CCR2 (aa 275–295) with its flanking regions, was sufficient for maximal activity as described above, further deletion of the CCR2 flanking regions diminished the activity (1-240/273-305), and deletion within CCR2 almost completely abrogated the activity (1-240/287-293). Taken together, these results suggest that CCR2 with its flanking regions (aa 261–315) is necessary and sufficient for exerting maximal organizer activity as measured by secondary axis formation.

Five Conserved Tyrosine Residues in CCR2 Are Essential for the Activity of Xlim-1

To analyze functional amino acid residues in CCR2, we focused on five tyrosine residues in CCR2 that are perfectly conserved among the vertebrate members of the Lim1/Lhx1 subclass. Since tyrosine residues possess the potential to be phosphorylated, site-directed mutagenesis was performed to change all five tyrosines to either phenylalanine (preventing phosphorylation) or alanine (removing aromatic side chain). Figure 4B shows that mutation to phenylalanine (5YF) did not affect the activity of Xlim-1, indicating that phosphorylation of tyrosines is not necessary for the activity. However, mutation to alanine (5YA) abrogated the ability to cause axis duplication (Fig. 4B), suggesting that these tyrosine residues are essential for the activity of Xlim-1 in the Spemann organizer.

CCR2 was shown above to be a negative regulatory region since deletion of CCR2 led to axis duplication at high doses (1 ng/embryo) in the absence of Ldb1 (Fig. 3, Δ278-292, Δ278-303). However, the same constructs (Δ278-292, Δ278-303) showed marked reduction in the ability to cause secondary axis formation compared to wild-type at lower doses (0.25 ng/embryo) in the presence of Ldb1 (Fig. 4A). These results suggest that CCR2 is involved in both positive and negative regulation of Xlim-1 (see Discussion). However, in the absence of Ldb1, neither Xlim-1(5YF) nor Xlim-1(5YA) initiates secondary axis formation at high doses (1 ng/embryo) as opposed to CCR2 deletion mutants (data not shown), indicating that the tyrosine residues in CCR2 are not essential for the negative regulation by CCR2.

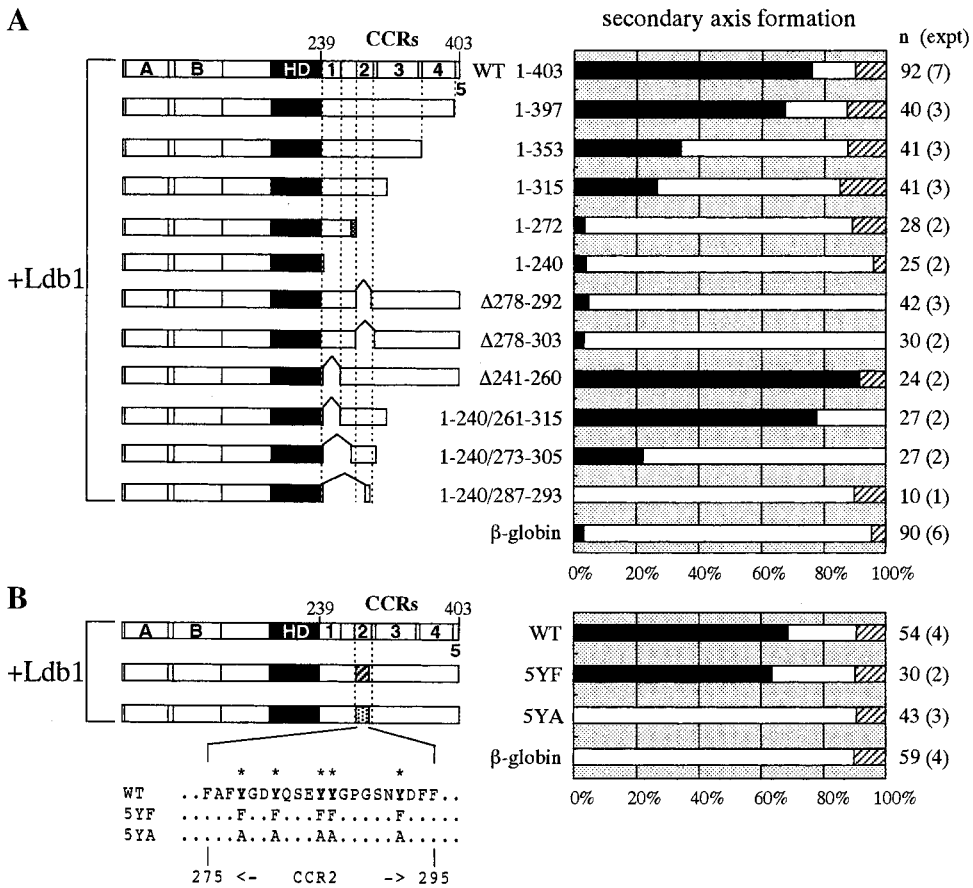

FIG. 4. Axis duplication activities of Xlim-1 with mutated CT239-403 in the presence of Ldb1. (A) Deletion analysis of CT239-403. Ldb1 mRNA together with the mRNA indicated was injected as described under Materials and Methods. Axis development was scored as described in Fig. 2. CCR2 with its flanking regions (aa 261–315) was necessary (Δ278-292, Δ278-303) and sufficient (1-240/261-315) for the axis-inducing activity of Xlim-1 in the presence of Ldb1. CCR4 was also necessary for the full activity of Xlim-1 (compare 1-397 with 1-353) but not sufficient (Δ278-292, Δ278-303). (B) Effects of point mutations of five conserved tyrosine residues in CCR2 on the activity of Xlim-1. Substitution of the five conserved tyrosines with phenylalanines did not affect the activity of Xlim-1 (5YF), whereas substitution with alanines (5YA) abolished the axis-inducing activity of Xlim-1 in the presence of Ldb1. Amount of mRNAs (ng/embryo): Xlim-1 constructs, 0.25 (0.5 for pSP64-Xβm-Xlim-1(Δ241-260)); Ldb1, 0.5; β-globin, 1.0.

CCR2 Is Directly Involved in the Activation Function Rather Than in Interaction with Ldb1

Since the analysis of the activation domains shown above was done in the presence of Ldb1, there remained the possibility that CCR1 or CCR2 affects the activity of Xlim-1 through interactions with Ldb1. To address this issue, we constructed a novel activated form of Xlim-1, Ldb-Xlim1 (Ldb1/Xlim-1 fusion protein), in which the LIM domains were replaced with the N-terminal region of Ldb1 (aa 1–291) to bypass the interaction between Xlim-1 and Ldb1 (Figs. 5A and 5B). This construct is equivalent to the *Drosophila* Apterous and Chip/Ldb1 fusion protein, referred to as ChAp (Milan and Cohen, 1999). Since Ldb-Xlim1 retains the dimerization domain of Ldb1, Ldb-Xlim1 possibly forms a dimer and mimics the Xlim-1/Ldb1 tetrameric complex (Fig. 5A). As expected, Ldb-Xlim1 has proved to be capable of inducing secondary axis as effectively as Xlim-1/3m and Xlim-1 plus Ldb1 (Fig. 5C and data not shown).

Using this construct, we obtained essentially the same results as in Fig. 4. Deletion of CCR1 did not seem to affect the activity of Ldb-Xlim1 (Fig. 5C, Δ241-260), whereas CCR2 deletion (Δ278-292, Δ278-303) as well as mutating five tyrosine residues within CCR2 to alanine (5YA) abolished the activity, but not mutation to phenylalanine (5YF) (Fig. 5C). These results suggest that CCR2 is directly involved in the activation function, and its involvement in interactions with Ldb1 seems unlikely.

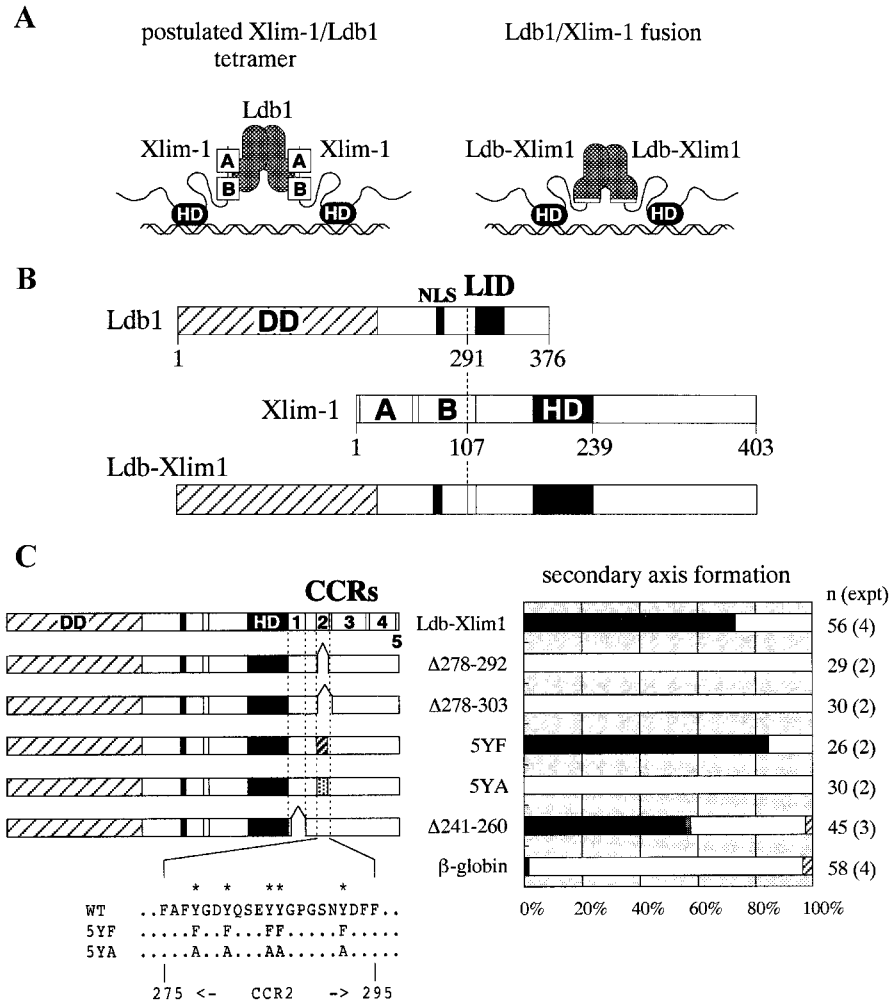

FIG. 5. Analysis of CT239-403 using a novel activated form of Xlim-1, Ldb-Xlim1. (A) Diagrams showing possible DNA binding by Xlim-1 and Ldb1 complex. Left, postulated Xlim-1/Ldb1 tetrameric complex. Right, possible dimeric complex of the Ldb1/Xlim-1 fusion protein, Ldb-Xlim1. (B) Schematic representation of Ldb-Xlim1. Interaction between Xlim-1 and Ldb1 was bypassed artificially by fusing Xlim-1 and Ldb1. The Ldb1 dimerization domain (DD) and the Xlim-1 homeodomain (HD) were retained, while LIM domains A and B of Xlim-1 and LID (LIM interaction domain) of Ldb1, which are responsible for the interaction, were deleted. NLS, nuclear localization signal. (C) Effects of point mutations of five conserved tyrosine residues in CCR2 on the axis duplication activity of Ldb-Xlim1. Ldb-Xlim1 mimics the effects of Xlim-1 plus Ldb1 when ectopically expressed, thus supporting the model depicted in (A). CCR2 deletion (Δ278-292, Δ278-303) as well as substitution of five tyrosines in CCR2 with alanines (5YA) abolished the axis-inducing activity of Ldb-Xlim1. However, neither removal of CCR1 (Δ241-260) nor substitution of the five tyrosines with phenylalanines (5YF) affects the activity of Ldb-Xlim1. Amount of mRNAs (ng/embryo): Ldb-Xlim1 constructs, 0.25; *β*-globin, 1.0.

CCR1 and CCR2 Retain Their Negative and Positive Regulatory Functions, Respectively, When Fused to the GAL4DBD

Above results have shown the importance of CCR2 and CCR4 in the activation function of Xlim-1 and CCR1 and CCR2 in negative regulation of Xlim-1. Therefore, we next examined whether CCRs retain their functions when fused to a heterologous DNA-binding domain. To address this issue, we developed a *Xenopus* one-hybrid system in which a GAL4-UAS system was introduced to *Xenopus* embryos. In this system, we used a UAS-driven luciferase gene construct (pUAS-Ars194-Luc) as a reporter gene, which has five copies of the GAL4 binding element upstream of a minimal promoter from the sea urchin arylsulfatase gene (Kiyama *et al.*, 1998), and GAL4 DNA-binding domain (GAL4DBD) fusion proteins as an effector. We first ascertained that the luciferase activity of this reporter was low enough in *Xenopus* embryos and was strongly activated by GAL4DBD-AD, in which the GAL4 activation domain (GAL4AD) is fused to GAL4DBD, as has been shown in the sea urchin (Kiyama *et al.*, 1998).

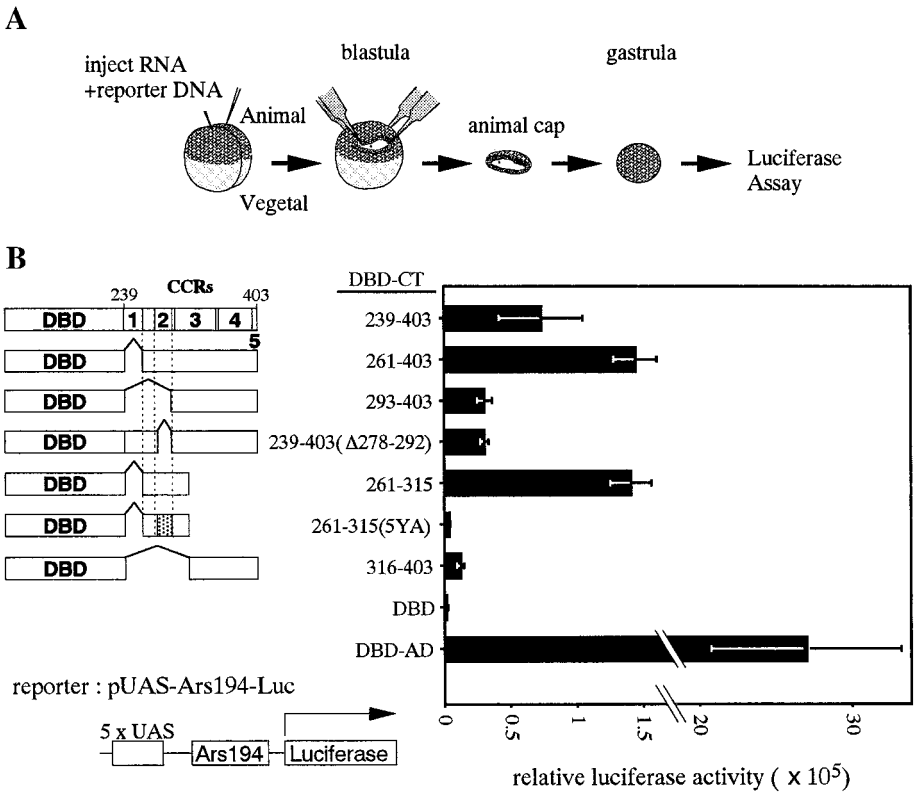

FIG. 6. Transactivation properties of various portions of CT239-403 as assayed by the *Xenopus* one-hybrid system. (A) Diagram of the *Xenopus* one-hybrid assay. Injection of mRNA and reporter DNA and luciferase assay are described under Materials and Methods. (B) Activation of UAS-reporter gene by various portions of CT239-403 fused to GAL4DBD (referred to as DBD-CT constructs). The aa 261–315 region containing CCR2 functions as a transactivation domain when fused to GAL4DBD (see 261-315), and the activity is dependent on the conserved tyrosine residues (261-315(5YA)). CCR1 negatively regulated the transcriptional activity of DBD-CT239-403 (compare 239-403 and 261-403).

Using this *Xenopus* one-hybrid system, several C-terminal fragments of Xlim-1 fused to GAL4DBD (referred to as DBD-CT constructs) were tested for transcriptional activity (Fig. 6A). As shown in Fig. 6B, while DBD-CT239-403 possessed a significant amount of transcriptional activity (see 239-403), removal of CCR1 further enhanced the activity (see 261-403), indicating that CCR1 retains its negative regulatory function in this context. Interestingly, aa 293–403 (corresponds to CCR3–5) and aa 239–403 (Δ278–292) (corresponds to CCR1+CCR3–5) showed similar activity when fused to the GAL4DBD (Fig. 6B, 293-403 and 239-403(Δ278-292)). These results suggest that CCR1 negatively regulates the activity of CCR2 but not that of CCR3–5.

CCR2 (aa 275–295) with its flanking regions (aa 261–315) showed strong transcriptional activity comparable to that of aa 261–403 when fused to GAL4DBD (Fig. 6B, 261-403 and 261-315). Deletion of the CCR2 flanking regions diminished the activity (DBD-CT273-305, data not shown), and further deletion within CCR2 completely abrogated the activity (DBD-CT287-293, data not shown), as is also the case when the same fragments were fused to Xlim-1(1-240) (Fig. 4A). Furthermore, consistent with the results from the axis duplication assays, substitution of five conserved tyrosines to alanines abolished the transcriptional activity of DBD-CT261-315 (Fig. 6B, 261-315(5YA)). These results indicate that transcriptional activation function of CT239-403 is mostly dependent on CCR2 and its flanking regions (aa 261–315) and that the conserved tyrosine residues in CCR2 are essential for the activity. However, it should be noted that, although DBD-CT261-403 and DBD-CT261-315 showed the largest transcriptional activity among the DBD-CT constructs tested, much larger transcriptional activity was observed with DBD-AD (Fig. 6B), which contains an acidic activation domain (aa 768–881) of yeast GAL4. One explanation would be that the activation domain of Xlim-1 is qualitatively different from that of yeast GAL4.

DBD-CT316-403 also possessed a weak transcriptional activity (Fig. 6B). This activity may be dependent on CCR4 (aa 353–395), which was shown to be necessary for the full activity of Xlim-1 (Fig. 4A). The fact that DBD-CT293-403, DBD-CT239-403(Δ278-292), and DBD-CT316-403 possess

weak transactivation functions may account for the ability of Xlim-1(Δ278-292) or Xlim-1(Δ278-303) to initiate secondary axis formation at high doses in the absence of Ldb1, despite the lack of CCR2 in these constructs (Fig. 3).

CT261-315 Does Not Interact with CBP, SRC-1, or TIF2, the Known Transcriptional Coactivators

Understanding the transactivating properties of Xlim-1 CT261-315 requires the identification of the cofactor(s) interacting with CT261-315. Thus, we have performed GST pull-down experiments to examine whether CT261-315 could directly interact with the known candidate factors, CBP (CREB-binding protein), SRC-1 (steroid receptor coactivator-1), and TIF2 (transcriptional intermediary factor-2), that function as coactivators for certain classes of transcription factors. CBP as well as its closely related protein p300 serve essential coactivator roles for many classes of sequence-specific transcription factors (Shikama *et al.*, 1997). SRC-1 and TIF2, members of the p160 family, are well-known coactivators of nuclear receptors (NRs) that interact with the NRs in a ligand-dependent manner (Glass and Rosenfeld, 2000). As shown in Fig. 7, ^{35}S-labeled CBP, SRC-1, and TIF2 proteins bind to appropriate positive controls, Smad3 (Feng *et al.*, 1998; Janknecht *et al.*, 1998) or ERα(DEF) (Onate *et al.*, 1995; Voegel *et al.*, 1996). However, we were unable to observe any interactions of CT261-315 with CBP, SRC-1, or TIF2 (Fig. 7). These results suggest that CT261-315 interacts either with other general transcriptional coactivators or with some cofactor(s) specific to Xlim-1.

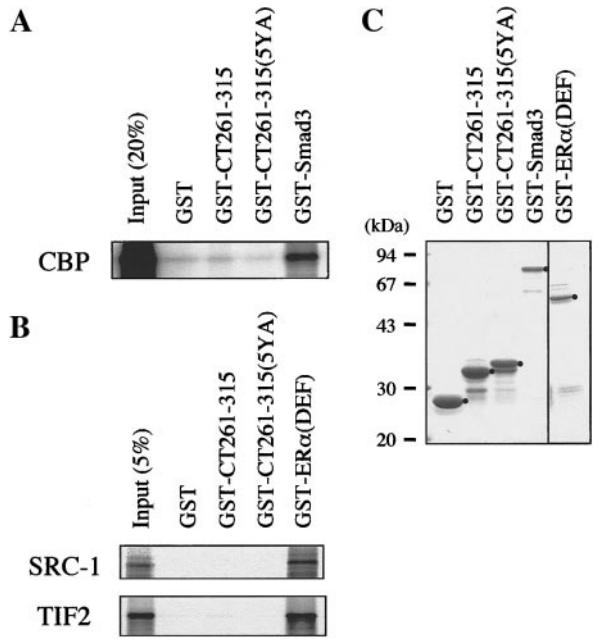

FIG. 7. Xlim-1 CT261-315 does not interact with transcriptional coactivators CBP, SRC-1, or TIF2. (A, B) GST pull-down assays. *In vitro*-translated [^{35}S]methionine-labeled CBP (A), SRC-1, and TIF2 (B) were incubated with GST fusion proteins or GST alone, and the bound proteins were subjected to SDS–PAGE followed by autoradiography. Whereas CBP and SRC-1/TIF2 interacted with GST-Smad3 and GST-ERα(DEF) (in the presence of the ligand 10^{-6} M 17β-estradiol), respectively, none of the three coactivators (CBP, SRC-1, and TIF2) interacted with either GST-CT261-315 or a tyrosine mutant, GST-CT261-315(5YA). (C) Coomassie brilliant blue staining of GST fusion proteins. Comparable amounts of GST fusion proteins (indicated by dots) were used in the assay.

DISCUSSION

The region C-terminal to the homeodomain of Xlim-1 (CT239-403) has been shown to be involved in transcriptional activation based on a yeast one-hybrid system (Breen *et al.*, 1998). In the present study we demonstrate the existence of two independent activation domains and a negative regulatory domain involved in axis formation by Xlim-1 (Fig. 8A). Furthermore, one of the activation domains (aa 261–315 region) including CCR2 contained conserved tyrosine residues critical for transcriptional activation and may define a novel activation motif.

Five tyrosine residues in CCR2 were shown to be essential for the axis duplication activity of Xlim-1 in whole embryos as well as the transcriptional activity in *Xenopus* one-hybrid assays. A number of transcription factors have been shown to be activated by phosphorylation (Karin and Hunter, 1995). In the case of CREB or CREM (De Cesare *et al.*, 1999), serine phosphorylation in their activation domains allows recruitment of CBP (Shikama *et al.*, 1997), a large coactivator that contacts the general transcriptional machinery. Another example is tyrosine phosphorylation in STATs (Chatterjee-Kishore *et al.*, 2000; Darnell, 1997), which is hypothesized to trigger translocation of STATs into the nucleus. The involvement of tyrosine phosphorylation in transactivation function of Xlim-1, however, is unlikely because only substitution of the conserved tyrosines with alanines and not with phenylalanines abrogated the activity (Fig. 4B). Nevertheless, the possibility remains that tyrosine phosphorylation is involved in negative regulation instead of positive regulation, as has been exemplified in the case of the nuclear receptor PPARγ (Adams *et al.*, 1997), in which phosphorylation of a critical serine residue in PPARγ results in significant reduction of transcriptional activity. Previous work also suggests the existence of posttranslational modification, possibly phosphorylation, of Xlim-1 in *Xenopus* embryos (Karavanov *et al.*, 1996).

Several types of activation domains have been reported, which consist of acidic amino acid residues or are rich in glutamine or proline (Mitchell and Tjian, 1989; Triezenberg, 1995). Although an activation domain that contains tyrosine residues has been reported previously in the C-terminal region III of the winged helix transcription factor HNF3β (Pani *et al.*, 1992), or in a nuclear protein,

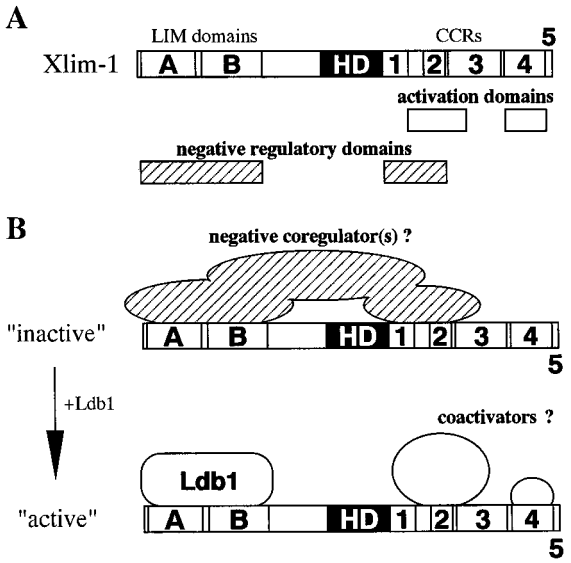

FIG. 8. Models of CCR1–CCR5 functions for the activity of Xlim-1. (A) Xlim-1 has two activation domains and a negative regulatory domain in CT239-403. Activation domains (open box): CCR2 and its flanking regions (aa 261–315) and region containing CCR4. Negative regulatory domains (hatched box): LIM domains and region stretching across CCR1–2. See Discussion for more detail. (B) Possible molecular mechanisms for changing between inactive and active states of Xlim-1. In the absence of Ldb1, Xlim-1 binds corepressor(s), or negative coregulator(s) through LIM domains and CCR1-2, and stays in an inactive state. Once Ldb1 is present, Xlim-1 shifts to an activated state as transcriptional coactivators take the place of negative coregulator(s) through binding to CCR2. Although a negative coregulator complex that binds both LIM domains and CCR1–2 is shown, there is no evidence showing simultaneous binding of a single corepressor complex to LIM domains and CCR1–2 at present.

Npw38 (Komuro *et al.*, 1999), tyrosine-rich CCR2 does not possess motifs in common with any other transcription factors yet reported. Thus, this type of tyrosine-dependent transactivation domain appears to be unique. The phenylalanine residue, an aromatic amino acid similar to tyrosine, has been shown to be critical for the transactivation function of the VP16 activation domain (VP16AD) of an acidic type (Cress and Triezenberg, 1991; Regier *et al.*, 1993). Several global coactivators with histone acetyltransferase activity such as p300/CBP were shown to interact with the VP16AD through the phenylalanine 442 as an essential residue (Utley *et al.*, 1998; Wang *et al.*, 2000). In contrast, none of the known coactivators tested interacted with the Xlim-1 transactivation domain, aa 261–315 (Fig. 7). Furthermore, activity of aa 261–315 is much weaker than that of GAL4AD which is an acidic type of activation domain similar to VP16AD (Fig. 6B). These results lead to the possibility that Xlim-1 may not directly interact with global coactivators and instead may require a specific adaptor protein(s) or transcription factor(s) to exert transactivation function. This possibility is supported by a report that Lhx2, another member of the LIM-HD proteins, binds to a nuclear protein, MRG1, to recruit p300/CBP (Glenn and Maurer, 1999). Existence of such a specific cofactor(s) might overcome the requirement of relatively high doses of Xlim-1 and Ldb1 mRNAs for secondary axis formation when coexpressed in the ventral region compared to the level of the endogenous mRNAs in the dorsal mesoderm. It might also overcome the inability of Xlim-1 plus Ldb1 to form a complete secondary axis which includes a head structure (Agulnick *et al.*, 1996). Future work deals with the characterization of cofactors that interact with the activation domains of Xlim-1, CCR2, and CCR4.

Our results also suggest the existence of a negative regulatory domain stretching across CCR1 and CCR2 (Figs. 3 and 7A). Two possible models for the mechanism of repression of Xlim-1 activity by this negative regulatory domain are proposed: (1) an intramolecular interaction model in which CCR1 and CCR2 interact with the LIM domains to form an inactive conformation or (2) an intermolecular interaction model in which these CCRs bind to a transcriptional corepressor or a certain protein to prevent CCR2 from binding to a coactivator. Previous work done by Breen *et al.* (1998) suggests the latter possibility since removal of CCR1 from GAL4DBD-CT239-403 led to enhancement of transcriptional activity in a yeast one-hybrid system. Interestingly, while our *Xenopus* one-hybrid analysis is generally consistent with their data, our additional axis duplication data revealed that removal of CCR1 does not enhance the activity of Xlim-1 in the presence of Ldb1. Deletion of CCR1 also did not seem to enhance the activity of active forms of Xlim-1, Ldb-Xlim1, Xlim-1/3m, and Xlim-1/ΔNA (Figs. 4A and 5C and data not shown). However, removal of CCR1 from Xlim-1 (1–315) dramatically enhanced the axis-inducing activity (Fig. 4A). Taken together, we prefer the view that Xlim-1 might interact with a putative corepressor(s) through LIM domains and CCR1–2 and stay in an inactive state when Ldb1 is absent (Fig. 8B). However, once Ldb1 is present, Xlim-1 probably shifts to an activated state and the transcriptional coactivator(s) takes the place of the corepressor(s) through binding to CCR2 (Fig. 8B). When CCR3 through CCR5 are absent, Ldb1 possibly fails to fully activate Xlim-1; that is, Ldb1 is incapable of fully replacing a putative corepressor(s) with a coactivator(s). There is also a possibility that corepressors that bind to LIM domains and CCR1–2 are parts of a single corepressor complex as depicted in Fig. 8B. Recently, RLIM, a novel LIM-domain-binding corepressor, has been isolated (Bach *et al.*, 1999). Further analysis is required to see whether a corepressor complex containing RLIM interacts with CCR1 and CCR2 together with the LIM domains. In the future, identification of a corepressor(s) and a coactivator(s) interacting with CCR1–2 and CCR2/CCR4, respectively, will provide us with deeper insight into the molecular mechanisms underlying the organizer functions that are mediated by Xlim-1.

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