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OTX2 Directly Interacts with LIM1 and HNF-3β

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***Otx2* is a *paired*-class homeobox gene, and its functions in anterior visceral endoderm and/or anterior mesendoderm have been suggested to be vital for head development in mammals. Several transcription factors are expressed in these tissues, and mutant mice analyses have suggested the interactions of the *Otx2* gene cascade with the *Lim1* or *HNF-3β* cascade. Here we show that OTX2 directly associates with LIM1 and HNF-3β; OTX2 binds to the LIM1 homeodomain (HD) with its C-terminal region, whereas both HD and C-terminal regions of OTX2 bind to the HNF-3β fork head domain or OTX2 HD. The luciferase assay with the P3C sequence, a specific DNA binding sequence for *paired*-class homeobox genes, has demonstrated that LIM1 enhances, but HNF-3β represses, OTX2-directed gene expression. Direct interactions of OTX2 with LIM1 or HNF-3β may play important roles in anterior visceral endoderm and/or anterior mesendoderm to constitute transcriptional regulatory networks for head development.** © 2000 Academic Press

Homeoproteins constitute a large family of transcription factors sharing a similar DNA binding motif, homeodomain (HD), and regulate a variety of developmental programs in metazoan (1, 2). Mouse *Otx2*, a cognate of the *Drosophila orthodenticle (otd)*, is a gene that has the *paired*-class HD with lysine at its 50th residue (K50). Mutant analyses in mice have indicated the essential roles of this gene in head development (3–8). *Otx2* is expressed in anterior visceral endoderm before, and in anterior mesendoderm and then in anterior neural plate after, gastrulation (3, 5). Anterior visceral endoderm and anterior mesendoderm have been suggested to play essential roles in mammalian head induction (9–13). Several transcription factors including *Otx2* (3, 5), *Lim1* (14, 15), and *HNF-3β* (16, 17) are expressed in both anterior visceral endoderm and anterior mesendoderm. *Otx2* and *Lim1* mutants exhibit similar headless phenotype. Defects in anterior development by *HNF-3β* mutation also share some common features with those of *Otx2* or *Lim1* mutants (18).

Homeoproteins have been known to function by dimerizing through HD with itself or closely related members or by associating with other transcription factors (19–26). Otx2 HD is, however, too distant from LIM1 HD to expect HD-HD association. No interaction between homeoprotein and fork head protein has been reported, nor has OTX2 interaction with other transcription factors been suggested.

In this paper, we examined the possibility of direct protein-protein interactions between OTX2 and LIM1 or HNF-3β. The study has demonstrated that OTX2 protein interacts specifically with LIM1 or HNF-3β using distinct domains. In the transfection assay with the P3C sequence, a DNA sequence for specific binding of *paired*-class homeobox gene products with K50 (24, 26), LIM1 synergistically transactivated the gene expression directed by OTX2, while HNF-3β repressed it.

MATERIALS AND METHODS

Plasmid construction. *HNF-3β* cDNA was kindly given by Dr. H. Sasaki; *Otx2, Lim1* and *HoxA2* cDNAs were obtained in this study. Each full-length gene construct having *Sal*I site at the 5′ end and *Nhe*I site at the 3′ end was generated by the PCR-based method with appropriate primers. They were placed under the cytomegalovirus promoter by inserting into the *pCMX-PL1* vector (27) at *Xho*I and *Nhe*I sites, yielding *pCMX-Otx2, pCMX-Lim1* and *pCMX-HNF-3β*, respectively. Deletion constructs used for *in vitro* translation were also made by the PCR-based method so as to have the *Bam*HI site at the 5′ end and the *Eco*RI site at the 3′ end. They were inserted into *pSP64polyA* (Promega) at *Bam*H1 and *Eco*R1 sites. To make GST fusion proteins, the *Bam*HI-*Eco*RI PCR fragments were introduced into *pGEX-2TK* (Amersham-Pharmacia). Reporter gene construct, *pP3Ac-Luf*, was generated by cloning three copies of the P3C oligo (top strand: GATCCTCCTGAGTCTAATCCGATTAGTGTGCA; bottom strand: GATCTGCACACTAATCGGATTAGACTCAGGAG) into the *Bam*HI site of *pAc-Luf* which was made by inserting β-actin basal promoter (28, 29) into the upstream of *luciferase* gene of *PGV-B* vector (Promega). The fidelity of all constructs was confirmed by sequencing.

In vitro protein binding assays. GST fusion proteins were obtained by transforming *E. coli* strain XL1-Blue with a series of *pGEX-2TK* constructs and purified as described (30). The ^{35}S-labeled proteins were generated by *in vitro* translation using TNT rabbit

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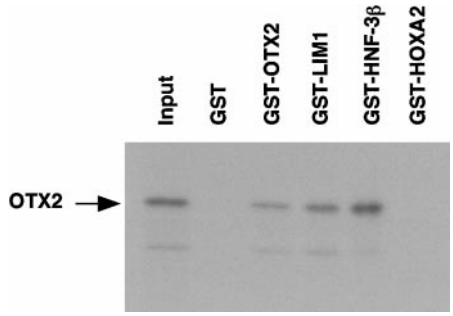

FIG. 1. Protein binding assay for OTX2 interactions with LIM1, HNF-3β, or OTX2 itself. Each protein fused to GST (GST-OTX2, GST-LIM1, GST-HNF-3β and GST-HOXA2) was incubated with ^{35}S-labeled OTX2, the protein complexes were resolved by SDS–PAGE, and then bound OTX2 was visualized by autoradiography. In the input lane, 20% of the ^{35}S-labeled OTX2 used in the assay was loaded.

reticulocyte lysates system (Promega) and ^{35}S-methionine (Amersham-Pharmacia) from the T7 or SP6 promoter present in the *pCMX-PL1* or *pSP64polyA* vectors, respectively. GST fusion proteins (approximately 5 μg) were incubated with 5 μl in vitro translated counterparts in 400 μl binding buffer (50 mM Tris-HCl at pH 7.5, 165 mM KCl, 1 mM $MgCl_2$, 10% glycerol and 1% Triton-X) on a rotating wheel for 2 h at 4°C. Beads were washed five times with binding buffer, and then bound proteins were resolved by sodium doecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and detected by autoradiography.

Transfections and Luciferase assays. HeLa cells were cultured in DMEM (Gibco Lab.) supplemented with 10% fetal bovine serum (Cell Culture Technology). Transfection assays were done using Tfx-20 reagent (Promega) as described (31). Briefly, cells in 16 mm-diameter cell culture plates were transfected with a total of 500 ng DNA containing 100 ng reporter plasmid (*pP3Ac-Luf* or *pAc-Luf*), 5 ng *pRL-TK* (Promega), 200 ng mixture of *pCMX-Otx2* and *pCMX-PL1*, and 195 ng *pGEM-3Z* (Promega). In cotransfection assay with *pCMX-Lim1* or *pCMX-HNF-3β*, 200 or 100 ng each was transfected with 95 or 195 ng *pGEM-3Z*, 100 ng *pCMX-Otx2* or *pCMX-PL1*, 100 ng *pP3Ac-Luf* and 5 ng *pRL-TK* (Promega). In control 300 ng *pCMX-PL1* and 100 ng *pGEM-3Z* were transfected. Cells were harvested 48 h after transfection using lysis buffer (Promega). Transfection efficiency was corrected with *Renilla* Luciferase fluorescence of *pRL-TK* using the Dual-Luciferase reporter assay system (Promega); the fluorescence was measured by TD-20/20 luminometer (Turner Designs). After four separate transfection assays, means and standard errors were calculated.

RESULTS

Direct binding of OTX2 to LIM1 and HNF-3β. *In vitro* protein binding assays were performed to test the direct binding between OTX2 and LIM1 or HNF-3β. ^{35}S-labeled OTX2 protein was incubated with each counterpart protein fused to GST (Fig. 1); the complexes were pulled down and resolved by SDS-PAGE. HOXA2, of which expression *in vivo* does not overlap with that of OTX2 was used to test the specificity of interactions. Under the conditions examined, OTX2 did not bind to HOXA2 or GST. On the contrary, OTX2 was found to associate with LIM1 or HNF-3β. Previously OTX2 was reported to form homodimer on DNA, but not in solution (32). This study, however, demonstrated that OTX2 bound to itself even in solution.

Domain mapping for OTX2/LIM1 and OTX2/HNF-3β interactions. To determine the domains in LIM1 and HNF-3β essential for their interactions with OTX2, protein binding assays were performed with a series of deletion mutants; the mutants were labeled with ^{35}S-methionine and then incubated with GST-OTX2 (Fig. 2A, B). LIM1 mutant that has LIM1 HD (Fig. 2A, LIMΔ2) bound to OTX2 efficiently (lane 7), but other mutants lacking HD did not (lanes 6, 8). HNF-3β mutant containing fork head domain (Fig. 2B, HNFΔ2) interacted with OTX2 (lane 7), but mutants lacking this domain did not (lanes 6, 8). Thus the DNA binding domains of both LIM1 and HNF-3β, namely HD and fork head domain, are involved in the interactions with OTX2.

Next, protein binding assays were performed to map the domain in the OTX2 protein essential for the interaction with LIM1, HNF-3β or OTX2 itself (Fig. 2C–F). OTX2 mutants were fused with GST (Fig. 2C) and incubated with each ^{35}S-labeled counterpart. As shown in Fig. 2D, OTX2 mutants containing HD did not bind to LIM1 (lanes 3, 6) while C-terminal region (lane 4) did. The C-terminal region contains sequences conserved among vertebrate *Otx* homologues, called OTX-tail (33). We then tested whether this region is responsible for the interaction with LIM1. The C-terminal region that lacks the OTX-tail region (CTLR), however, bound to LIM1 (lane 7), but the tail region did not (lane 8).

In contrast to OTX2/LIM1 interaction, as shown in Fig. 2E, both HD (lane 6) and CTLR of OTX2 (lane 7) associated with HNF-3β, suggesting that the two independent domains of OTX2 are involved in OTX2/HNF-3β interaction. The participation of CTLR in the interaction with LIM1 or HNF-3β raised a question about OTX2 homo-dimerization by HD-HD interaction (32). Indeed, not only HD (lane 6) but also CTLR (lane 7) bound to OTX2 (Fig. 2F).

Finally, the direct interactions between each fragment of OTX2 and LIM1 or HNF-3β were tested (Fig. 2G, H). As expected, HD of LIM1 bound to the OTX2 CTLR, but not to OTX2 HD (Fig. 2G). The fork head domain of HNF-3β associated with either HD or CTLR of OTX2 (Fig. 2F); the interaction between this domain and CTLR was weaker (Fig. 2H). OTX2 HD interacted with either HD or CTLR of OTX2, but CTLR did not interact with itself (Fig. 2I, J).

Effects of LIM1 and HNF-3β on gene expression by OTX2. The functional significance of the OTX2/LIM1 and OTX2/HNF-3β interactions was tested on transcriptional activity of OTX2. A family of *paired*-class homeoproteins with K50 has been known to bind to P3C site (24, 26). Thus, this sequence was chosen to make Luciferase (Luf) assay in HeLa cells with β-actin

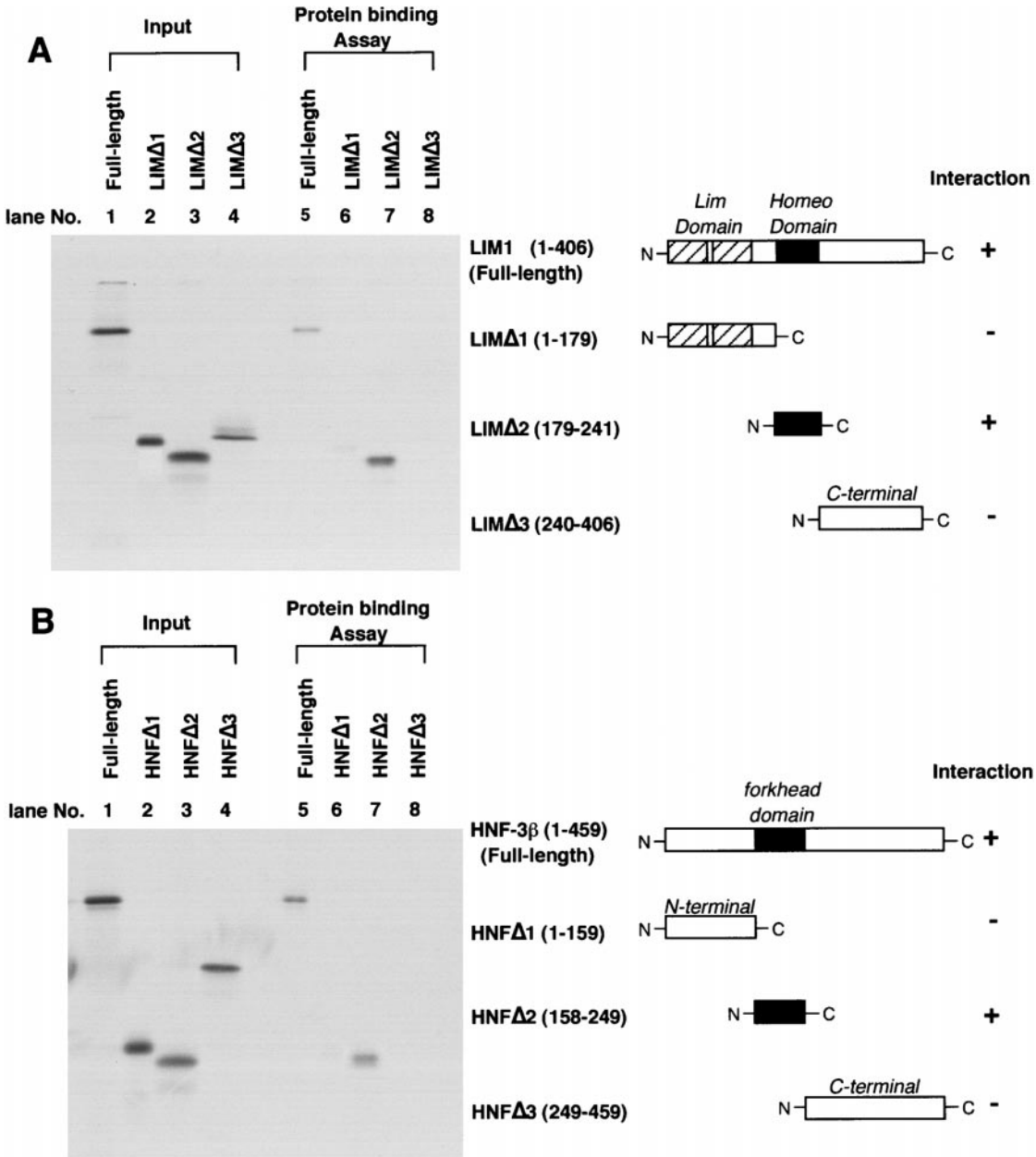

FIG. 2. Domain mapping for protein–protein interactions. The protein binding assay on each LIM1 (A) and HNF-3β (B) domain for the interaction with OTX2; the assay on each OTX2 domain for the interaction with LIM1 (D), HNF-3β (E), and OTX2 (F); the binding assay on each OTX2 domain to LIM1 HD (G), HNF-3β fork head domain (H), OTX2 HD (I), and OTX2 CTLR (J). (C) Each OTX2 fragment (GST-OTX2Δ1–6) and the summary of binding assays of (D–F); black boxes indicate OTX-tail. C-terminal region was divided into two parts, CTLR and tail region. Numbers in parentheses indicate amino acid residues. In (A) and (B) OTX2 was fused to GST, and each deletion mutant of LIM1 (LIMΔ1–3) or HNF-3β (HNFΔ1–3) was labeled with ^{35}S-methionine. Numbers in parentheses indicate amino acid residues. Lanes 1–4 give 20% of the ^{35}S-labeled fragments used in the assay of lanes 5–8. DNA binding domains, LIM1 HD, and HNF-3β fork head domain, participate in the interaction with OTX2. In (D–F) OTX2 mutants were fused to GST, and LIM1 (D), HNF-3β (E), and OTX2 (F) were labeled with ^{35}S-methionine; 20% of the ^{35}S-labeled fragments used in the assay as an input was loaded in lanes indicated by IP in (D–F). For the association OTX2 uses different domains depending on the partners (C). In (G–J) OTX2 HD and CTLR were fused to GST, and LIM1 HD (G), HNF-3β fork head domain (H), OTX2 HD (I), and OTX2 CTLR (J) were labeled with ^{35}S-methionine; 20% of the ^{35}S-labeled fragments used in the assay was loaded in each input lane. OTX2 binds to LIM1 HD with CTLR, whereas both OTX2 HD and CTLR bind to HNF-3β fork head domain or OTX2 HD.

basal promoter (*pP3Ac-Luf*). To express OTX2, LIM1 and HNF-3β, their cDNAs were directed by cytomegalovirus promoter (*pCMX-Otx2*, *pCMX-Lim1* and *pCMX-HNF-3*β). When the cells were transfected with *pP3Ac-Luf* and *pCMX-Otx2*, the maximal Luf expression (about 3-fold) was obtained at 100 ng *pCMX-Otx2*

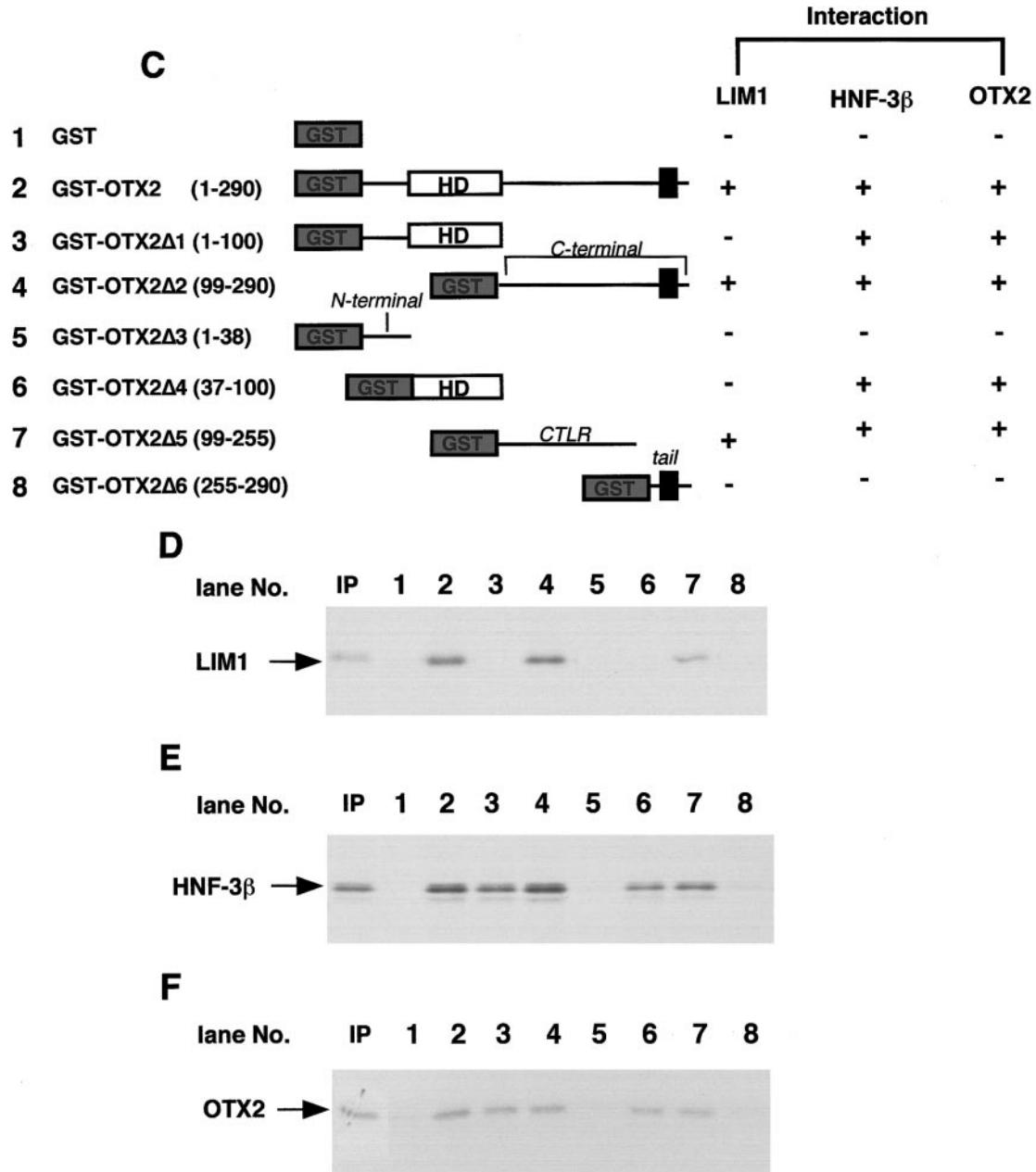

FIG. 2—*Continued*

(Fig. 3A). The *pCMX-Otx2* did not induce the expression of *pAc-Luf* that has no P3C sequence. In addition, with *pP3Ac-Luf* the Luf expression did not take place at all either by *pCMX-Lim1* or *pCMX-HNF-3β* (Fig. 3B, C).

The effects of LIM1 and HNF-3β on the expression of *pP3Ac-Luf* by OTX2 were then investigated with a 100 ng amount of *pCMX-Otx2*. When *pCMX-Lim1* was co-transfected with *P3Ac-Luf and CMX-Otx2*, at either 100 or 200 ng, the Luf expression was further activated up to about 2.5-fold that by *pCMX-Otx2* alone (Fig. 3B and data not shown). In contrast, 200 ng *pCMX-HNF-3β*, when co-transfected with *pCMX-Otx2* and *pP3Ac-Luf*, suppressed the Luf expression (Fig. 3C); the effect was partial by 100 ng *pCMX-HNF-3β* (data not shown).

DISCUSSION

The present study demonstrates that OTX2 protein physically interacts with LIM1 and HNF-3β proteins. DNA binding domain, HD of LIM1 or fork head domain of HNF-3β, participates in each interaction, but OTX2 protein uses different domains depending on its partner. OTX2 binds to LIM1 HD with its CTLR, while

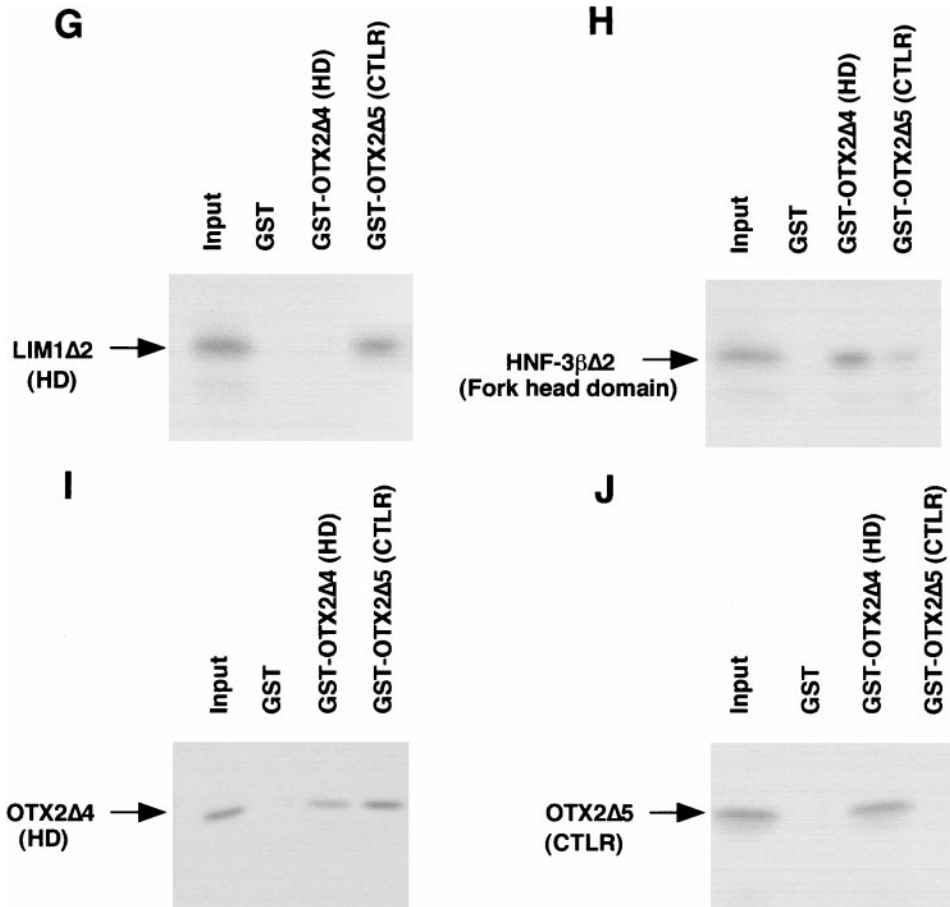

FIG. 2—*Continued*

both HD and CTLR of OTX2 bind to HNF-3β fork head domain or to OTX2 HD. Concomitant with these differences, the OTX2 interaction with LIM1 enhanced, while that with HNF-3β suppressed OTX2-directed transcription with P3C sequence in culture.

Paired-class homeoproteins that have lysine at the 50th residue of their HDs have been known to form homodimers; some only on the DNA, but some also in solution (24, 26, 32). This protein-protein association is generally considered due to HD-HD binding. OTX2 proteins were reported not to make homodimers in solution by gel filtration assay (32), while *Drosophila* OTD to associate with itself in solution by GST pull-down assay (24). No analysis has been made to determine the domain(s) for OTX2 homodimer formation. The present study by the protein binding assay indicated that OTX2 binds to itself in solution independently of DNA binding and that HD binds not only to HD but also to CTLR.

Heteromeric HD interactions have been known between closely related homeoproteins such as HOXD8/HOXD9 and MSX/DLX (20, 25). HD-HD interaction does not take place between those less related; OTX2 does not associate with HOXA2 as shown in this study, nor does MSX does not with HOXC8 (20). *Lim* HD and *paired*-class HD are too distant to expect HD-HD binding. Indeed, OTX2 HD did not associate with LIM1 HD; OTX2 bound to LIM1 HD with its CTLR. P-OTX has also been reported to associate with P-LIM but not by HD-HD interaction; a C-terminal region of P-OTX binds to LIM domain, not to HD, of P-LIM (34). P-OTX has a *paired*-class homeoprotein, but it does not belong to the *Otx/Crx* family, and the P-OTX C-terminal region does not have any homology with OTX2 CTLR. The fact that OTX2 CTLR is involved in all OTX2/OTX2, OTX2/LIM1, and OTX2/HNF-3β interactions suggests that OTX2 CTLR has a domain crucial for protein-protein interactions.

HD has been reported to interact with other types of DNA binding domains. These include zinc finger domain and DNA-binding C domain, as shown by CSX/GATA4 (19), BRN-3a/Estrogen receptor (23), and Oct-1/Retinoid X receptor (21) interactions. HD has also been known to associate with paired domain intramolecularly (35). In addition, the present study has shown that OTX2 HD can bind to HNF-3β fork head domain as the first example of HD-fork head domain interaction.

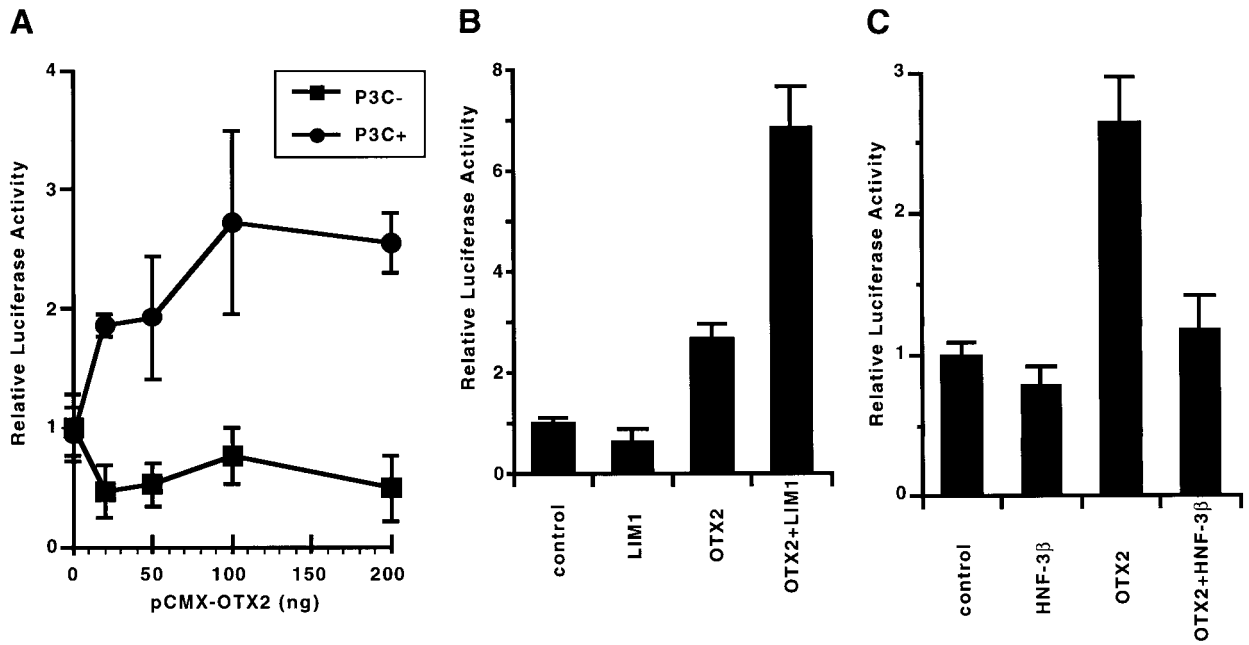

FIG. 3. Effects of LIM1 and HNF-3β on OTX2 transcriptional activity. (A) OTX2-directed transcription with P3C (24, 26). The *luciferase* gene combined with β-actin basal promoter either with or without P3C sequence served as the reporter genes. *Otx2* gene expression was directed by cytomegalovirus promoter in *pCMX-Otx2.* These were co-transfected into HeLa cells, and luciferase (Luf) activity was assayed with cell extracts (see Materials and Methods for details). (B) Effects of LIM1 on the OTX2-directed transcription. *Lim1* gene expression was directed by cytomegalovirus promoter in *pCMX-Lim1.* 200 ng *pCMX-Lim1* was cotransfected either with or without 100 ng *pCMX-Otx2.* Similar results were obtained with 100 ng *pCMX-Lim1.* (C) Effects of HNF-3β on the OTX2-directed transcription. *HNF-3β* gene expression was directed by cytomegalovirus promoter in *pCMX-HNF-3β.* 200 ng *pCMX-HNF-3β* was cotransfected either with or without 100 ng *pCMX-Otx2.* In all panels one unit of Relative Luf Activity is the activity when none of *pCMX-Otx2, pCMX-Lim1,* or *pCMX-HNF-3β* is added (control).

Lim1 and *Otx2* expressions co-localize in anterior visceral endoderm and anterior mesendoderm. *HNF-3β* expression also overlaps in these tissues. *Otx2* and *Lim1* mutants exhibit similar headless phenotype (3–5, 15). *HNF-3β* mutants exhibit shortened and kinky A–P axis, and the most severely affected embryos lack brain structures similar to *Otx2* and *Lim1* mutants (18). Direct interactions of these molecules in anterior visceral endoderm and/or anterior mesoendoderm may constitute regulatory networks for head development in dosage-dependent manners. The identification of target genes of which expressions are regulated by these interactions is eagerly awaited to elucidate the mechanisms of head development in vertebrates.

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REFERENCES

1. Gehring, W. J., Affolter, M., and Burglin, T. (1994) *Annu. Rev. Biochem.* **63,** 487–526.
2. McGinnis, W., and Krumlauf, R. (1992) *Cell* **68,** 283–302.
3. Acampora, D., Mazan, S., Lallemand, Y., Avantaggiato, V., Maury, M., Simeone, A., and Brulet, P. (1995) *Development* **121,** 3279–3290.
4. Matsuo, I., Kuratani, S., Kimura, C., Takeda, N., and Aizawa, S. (1995) *Genes Dev.* **9,** 2646–2658.
5. Ang, S. L., Jin, O., Rhinn, M., Daigle, N., Stevenson, L., and Rossant, J. (1996) *Development* **122,** 243–252.
6. Suda, Y., Matsuo, I., and Aizawa, S. (1997) *Mech. Dev.* **69,** 125–141.
7. Acampora, D., Avantaggiato, V., Tuorto, F., and Simeone, A. (1997) *Development* **124,** 3639–3650.
8. Suda, Y., Nakabayashi, J., Matsuo, I., and Aizawa, S. (1999) *Development* **126,** 743–757.
9. Thomas, P. Q., Brown, A., and Beddington, R. S. (1998) *Development* **125,** 85–94.
10. Rhinn, M., Dierich, A., Shawlot, W., Behringer, R. R., Le Meur, M., and Ang, S. L. (1998) *Development* **125,** 845–856.
11. Storey, K. G., Crossley, J. M., De Robertis, E. M., Norris, W. E., and Stern, C. D. (1992) *Development* **114,** 729–741.
12. Shawlot, W., Wakamiya, M., Kwan, K. M., Kania, A., Jessell, T. M., and Behringer, R. R. (1999) *Development* **126,** 4925–4932.
13. Ang, S. L., Conlon, R. A., Jin, O., and Rossant, J. (1994) *Development* **120,** 2979–2989.
14. Belo, J. A., Bouwmeester, T., Leyns, L., Kertesz, N., Gallo, M., Follettie, M., and De Robertis, E. M. (1997) *Mech. Dev.* **68,** 45–57.
15. Shawlot, W., and Behringer, R. R. (1995) *Nature* **374,** 425–430.

16. Filosa, S., Rivera-Perez, J. A., Gomez, A. P., Gansmuller, A., Sasaki, H., Behringer, R. R., and Ang, S. L. (1997) *Development* **124,** 2843–2854.
17. Weinstein, D. C., Ruiz i Altaba, A., Chen, W. S., Hoodless, P., Prezioso, V. R., Jessell, T. M., and Darnell, J. E., Jr. (1994) *Cell* **78,** 575–588.
18. Ang, S. L., and Rossant, J. (1994) *Cell* **78,** 561–574.
19. Lee, Y., Shioi, T., Kasahara, H., Jobe, S. M., Wiese, R. J., Markham, B. E., and Izumo, S. (1998) *Mol. Cell Biol.* **18,** 3120–3129.
20. Zhang, H., Hu, G., Wang, H., Sciavolino, P., Iler, N., Shen, M. M., and Abate-Shen, C. (1997) *Mol. Cell Biol.* **17,** 2920–2932.
21. Kakizawa, T., Miyamoto, T., Ichikawa, K., Kaneko, A., Suzuki, S., Hara, M., Nagasawa, T., Takeda, T., Mori, J., Kumagai, M., and Hashizume, K. (1999) *J. Biol. Chem.* **274,** 19103–19108.
22. Mann, R. S., and Affolter, M. (1998) *Curr. Opin. Genet. Dev.* **8,** 423–429.
23. Budhram-Mahadeo, V., Parker, M., and Latchman, D. S. (1998) *Mol. Cell Biol.* **18,** 1029–1041.
24. Mailhos, C., Andre, S., Mollereau, B., Goriely, A., Hemmati-Brivanlou, A., and Desplan, C. (1998) *Development* **125,** 937–947.
25. Zappavigna, V., Sartori, D., and Mavilio, F. (1994) *Genes Dev.* **8,** 732–744.
26. Wilson, D., Sheng, G., Lecuit, T., Dostatni, N., and Desplan, C. (1993) *Genes Dev.* **7,** 2120–2134.
27. Umesono, K., Murakami, K. K., Thompson, C. C., and Evans, R. M. (1991) *Cell* **65,** 1255–1266.
28. Matsuo, I., Kitamura, M., Okazaki, K., and Yasuda, K. (1991) *Development* **113,** 539–550.
29. Murata, T., Nitta, M., and Yasuda, K. (1998) *Genes Cells* **3,** 443–457.
30. Catron, K. M., Iler, N., and Abate, C. (1993) *Mol. Cell Biol.* **13,** 2354–2365.
31. Schenborn, E., Olter, L., Goiffon, V., and Balasubramaniam, R. (1995) *Promega Notes* **52,** 2–7.
32. Briata, P., Ilengo, C., Bobola, N., and Corte, G. (1999) *FEBS Lett.* **445,** 160–164.
33. Chen, S., Wang, Q. L., Nie, Z., Sun, H., Lennon, G., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and Zack, D. J. (1997) *Neuron* **19,** 1017–1030.
34. Bach, I., Carriere, C., Ostendorff, H. P., Andersen, B., and Rosenfeld, M. G. (1997) *Genes Dev.* **11,** 1370–1380.
35. Jun, S., and Desplan, C. (1996) *Development* **122,** 2639–2650.