Hox proteins functionally cooperate with the GC box-binding protein system through distinct domains

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Hox genes encode a transcriptional factor that plays a key role in regulating position-specific cartilage pattern formation. We found that Hoxa-13 and Hoxd-13, which are members of the Abd-B subfamily of Hox genes and are crucial for the autopod development of the limb, stimulate transcription from the Bmp-4 promoter. This stimulation was dependent on the GC box within the promoter and independent of the putative Hox protein binding site. The stimulation by HoxA-13 was remarkably enhanced by cotransfection with members of a family of zinc finger GC box binding transcriptional factors including Sp1. The stimulation was suppressed by another Abd-B Hox protein, HoxA-11, indicating that each Abd-B Hox protein has a different effect on the target genes through the Sp1 system. We have identified multiple functional domains involved in transcriptional regulation, including three independent transcriptional activation domains (ADs) in HoxA-13. AD1 and AD3 in helices 1 and 2 of the homeodomain individually cooperate with Sp1-dependent stimulation. The homeodomain is also required for cooperation of the AD with Sp1. By contrast, AD2 strongly activates transcription in an Sp1-independent manner only when the homeodomain has been removed. These observations indicate that HoxA-13 regulates transcription through multiple pathways. In addition, we found that a helix 3 mutation of the HoxA-13 homeodomain behaves as a dominant negative form.

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Hox genes are an evolutionarily well conserved gene system, which determines positional identity along the body axis during development of bilateral animals (1). In vertebrates, Hox genes are well known to be involved in skeletal pattern formation in the trunk and limbs (2). Among them, Hox genes belonging to the Abd-B subfamily of the HoxA and HoxD cluster are expressed in the limb mesenchyme in a position-specific fashion (3–5) and are known to provide the positional cue for limb cartilage pattern formation (6–8). The Hox genes encode homeodomain proteins that function as transcriptional regulators of downstream target genes through DNA binding by the homeodomain (9). Genes controlling the cell cycle and cell adhesion have been reported to be Hox targets (10–12); however, the target genes of Abd-B homeoproteins during limb cartilage formation have not yet been identified. A significant number of the downstream genes are supposed to be common among Abd-B homeoproteins, since nonparalogous Abd-B Hox proteins showed functional redundancy during cartilage formation (13). It is also proposed that Hox proteins regulate the quantity of transcription of common target genes (14) that are involved in the growth and differentiation of limb cartilage, so that the question is how different Hox homeoproteins result in quantitatively different effects on the transcription of target genes and which domains of the Hox homeoproteins are responsible for such differences. Another important issue concerns which transcriptional system is involved in regulating the quantity of transcription of target genes.

Full-length Hox proteins bind DNA very poorly in in vitro assays and/or exhibit little binding specificity (15). Many lines of evidence suggest that the diversity of Hox function is crucially dependent on cofactors, such as Exd/Pbx (16, 17). Pbx proteins physically interact with the YPWM motif of the Hox proteins belonging to paralog groups 1–8, increasing their DNA binding affinity and defining their sequence specificity (16–18). The biological significance of the formation of this Hox-cofactor complex on transcription has been thoroughly analyzed in Drosophila. For example, a recent study proposes the “activity regulation model” in which Exd cofactors interact with the Dfd homeodomain and unmask the transcriptional activation domain in the Dfd protein through DNA binding (19). Since Abd-B subfamily Hox proteins do not have a typical YPWM motif and Hox proteins of paralog groups 11–13 do not interact with Exd/Pbx, the mechanism of transcriptional regulation by Abd-B Hox proteins is poorly understood. In addition, there have been a few recent papers that describe apparent Hox protein targets, albeit not in the context of cooperative DNA binding with Pbx (11, 20). Hox proteins are also known to interact with other DNA-binding transcription factors, such as BTG2, Maf, and Smad1 (21–24), although the biological relevance is still open for further research. These lines of evidence suggest that Hox proteins function through a variety of pathways.

In addition to the Hox Abd-B subfamily genes, bone morphogenetic proteins are signaling molecules that play crucial roles in limb cartilage development (25, 26). During the course of our research analyzing the relationship between Bmp genes and Hox genes, we discovered that each Hox protein in the Abd-B subfamily has a unique effect on transcription from the Bmp-4 promoter in NG108-15 cells. We found that HoxA-13 and HoxD-13 cooperate with GC box-binding proteins such as Sp1 to stimulate transcription from the GC box-dependent promoter. On the other hand, HoxA-11 and HoxD-11 suppress the stimulation by HoxA-13 and HoxD-13. The interaction of Hox protein with the Sp1 system seems to be independent of DNA

* This work was supported by the Monbu Kagakusyo “Priority Areas Research (A) Developmental System” (to A. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists.

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Received for publication, April 15, 2003
Published, JBC Papers in Press, May 27, 2003, DOI 10.1074/jbc.M303922200

This paper is available on line at http://www.jbc.org

Vol. 278, No. 32, Issue of August 8, pp. 30148–30156, 2003
Printed in U.S.A.
binding of Hox proteins. We identified three independent transcriptional activation domains (ADs) in HoxA-13 and propose a novel multifunctional model. Two ADs independently stimulate the GC box-binding protein-dependent transcription system, and the other AD functions in the cytoplasm independently of GC box-binding protein only when the homeodomain is removed. Finally, helix 3 of HoxA-13 is shown to be crucial for both GC box-binding protein-dependent and -independent transcriptional stimulation.

EXPERIMENTAL PROCEDURES

Cell Culture and Transient Transfection—NG108–15 cells, a hybrid cell line of mouse neuroblastoma and rat glioma, were grown and maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum and 2% glucose. For transfection assays, 1.5 × 10⁴ cells were seeded per well of 3.5-cm dishes and transfected at 50% confluence. A solution containing 50 ng of internal control plasmid encoding Rous sarcoma virus sea pansy luciferase in 1 ml of Dulbecco’s modified Eagle’s medium was used for transfection. Three hours after transfection, the cells were washed and supplemented with growth medium. Cells were harvested for luciferase assays 36 h after transfection. Luciferase activities were measured by Lumat LB9501 (Berthold) and normalized to sea pansy luciferase activity. Transfection-reporter assays were performed in duplicate and repeated three times with different preparations of the same plasmid.

Plasmid Construction—To construct expression vectors for transfection, fragments containing full-length coding regions of chick HoxA-13, HoxD-13, Hoxa-11, Hoxa-10, Hoxa-9, and Hoxa-8 were cloned into pmiw control vector, to generate gene expression vector or pmiw control vector. Schematic representations of reporter constructs are shown in the left part of A and B. Nucleotides are numbered from the transcription start site. The activation level of each longest reporter (−2.4 kb in mouse and −2.8 kb in chicken), which was co-transfected with pmiw control vector, was set at 1.0. Corresponding activity levels of each reporter construct in co-transfection experiments with HoxA-13 expression vector are shown in the right part of A and B. The bars indicate S.D. values.

1 The abbreviations used are: AD, activation domain; aa, amino acid(s); PBS, phosphate-buffered saline; HBS, Hox binding site; CRSP, cofactor required for Sp1 activation.

FIG. 1. Stimulation of transcription from mouse and chick Bmp-4 promoters by HoxA-13 and HoxD-13. A, NG108–15 cells were cotransfected with 250 ng of mouse (−2.4 kb construct; B) or chick (−2.8 kb construct; C) Bmp-4 promoter-luciferase reporter plasmids and 500 ng of pmiw control vector or expression vectors of chick HoxA-13, HoxD-13, HoxA-11, HoxD-11, Hoxa-10, Hoxa-9, or Hoxa-4. The bars indicate S.D. B and C, the genomic structures of mouse (B) and chick (C) Bmp-4 genes are shown at the top of each diagram. Boxes labeled 1–4 in mouse and 1–3 in chick indicate exons. The transcription initiation sites are shown by right arrows. The promoter fragment of mouse Bmp-4 exon 1A contains one GC box (B), and the equivalent chick fragment contains six GC boxes (C). Reporter constructs containing fragments of different lengths from mouse and chick Bmp-4 promoters were co-transfected with a HoxA-13 expression vector or pmiw control vector. Schematic representations of reporter constructs are shown in the left part of A and B. Nucleotides are numbered from the transcription start site. The activation level of each longest reporter (−2.4 kb in mouse and −2.8 kb in chicken), which was co-transfected with pmiw control vector, was set at 1.0. Corresponding activity levels of each reporter construct in co-transfection experiments with HoxA-13 expression vector are shown in the right part of A and B. The bars indicate S.D. values.

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Hox Proteins Functionally Cooperate with Sp1

RESULTS

HoxA-13 and HoxD-13 Stimulate Transcription from the Mouse or Chick Bmp-4 Promoter—The mouse Bmp-4 gene has two different promoters, followed by the 5'-untranslated region exon 1α or exon 1b (Fig. 1B) (29). The mRNA containing exon 1α is a major product in the mouse limb bud.2 To test the effect of Hox proteins on Bmp-4 transcription, we first constructed a reporter plasmid containing exon 1α and 2.4 kb of 5'-flanking region of the mouse Bmp-4 gene. This construct was co-transfected into NG108–15 cells together with expression plasmids encoding full-length chick HoxA-13, HoxD-13, HoxA-11, HoxD-11, HoxA-10, HoxA-9, or HoxA-4. HoxA-13 and HoxD-13 stimulated reporter gene transcription up to 23-fold and up to 9-fold, respectively (Fig. 1A). Stimulations by HoxA-10, HoxA-9, and HoxA-4 were weak but reproducible. HoxA-11 and HoxD-11, as well as a pMiw control vector, exhibited no stimulation (Fig. 1A). Similar stimulation patterns by each Hox protein were also observed in the chick Bmp-4 promoter. Reporter gene transcription driven by –2.8 kb of chick Bmp-4 promoter3 was stimulated 13–17-fold by HoxA-13 and 6–9-fold by HoxD-13 but not by HoxA-11 or HoxD-11 (Fig. 1A). These results indicate that each Hox protein has a unique effect on transcription from mouse and chick Bmp-4 promoters.

The GC Box but Not the HBS Is Essential for Cooperative Stimulation by HoxA-13 and Sp1—To identify the responsible cis element for HoxA-13-dependent transactivation in the Bmp-4 promoter, we tested deletion constructs of the mouse and chick Bmp-4 promoter. Using the mouse Bmp-4 promoter, the –260 bp construct was transactivated up to 5-fold by HoxA-13, but when deletion extended to –80 bp, stimulation was no longer observed (Fig. 1B). Using the chick Bmp-4 promoter, the –600 bp construct still retained sensitivity to HoxA-13 (Fig. 1C). The minimal regions of both mouse and chick Bmp-4 promoter that exhibit HoxA-13-dependent transactivation have no TATA box. We found one of each Hox binding site

2 M. Suzuki, N. Ueno, and A. Kuroiwa, unpublished results.
3 M. Suzuki, N. Ueno, and A. Kuroiwa, unpublished clone.
transcription factors stimulate the transcription 2–5-fold alone (Fig. 3A). Remarkable enhancement of transcription up to 50-fold was seen following co-transfection of Sp1 together with HoxA-13 (Fig. 3A). Sp3, Sp4, TIEG1, and BTEB1 also exhibited 15–20-fold enhancement of transcription. In addition, HoxD-13 also showed remarkable synergism with Sp1 (Fig. 3B).

HoxA-13 Did Not Alter the Sp1 Binding Profile either at the Quantitative or the Qualitative Level—We found no significant increase in the amount of Sp1, Sp3, and Sp4 proteins by Western blot analysis after transfection of HoxA-13 to NG108-15 cells (data not shown). This indicates that HoxA-13 stimulated the transcription through potentiation of DNA binding activity or alteration of components of the GC box-binding protein. To explore these possibilities, we performed electrophoretic mobility shift assay experiments using double-stranded oligonucleotides containing a GC box (SP1BS) or two HBS (NP2) and nuclear extracts prepared from NG108-15 cells with either transfected pMiw control vector, HoxA-13, or Sp1 alone or both Sp1 and HoxA-13.

Using SP1BS as a probe, three major shift bands were observed with all extracts (Fig. 3C, top, lanes 1, 4, 7, and 10). Among them, the intensity of one shift band, indicated as Sp1, was increased when Sp1 was transfected (Fig. 3C, top, lanes 7–12) and supershifted in the presence of anti-Sp1 antibody (Fig. 3C, top, lanes 2, 5, 8, and 11 indicated as SS). We observed no quantitative and qualitative change in band shift even in the presence of HoxA-13 (Fig. 3C, top, lanes 4 and 19). In addition, the presence of anti-HoxA-13 antibodies did not alter the band pattern (Fig. 3C, top, lanes 3, 6, 9, and 12), indicating that HoxA-13 was not included in putative transcriptional components bound to the GC box. Productions of HoxA-13 proteins in the transfected cells were monitored by electrophoretic mobility shift assay using NP2 as the probe (Fig. 3C, bottom). One major shift band was detected using the nuclear extract from the transfected cells (Fig. 3C, bottom, lanes 4, 5, 10, and 11), and this band disappeared in the presence of anti-Hoxa-13 antibody (Fig. 3C, bottom, lanes 6 and 12). Surprisingly, these results indicate that HoxA-13 caused no considerable change in GC box binding complex and did not participate in the binding complex.

Identification of Transcriptional Activation Domains in HoxA-13—To determine the functional domain of the HoxA-13 protein, the HoxA-13 protein coding regions were mutated, and the effect of the alternation was analyzed using the transient transfection system. Wild type HoxA-13 and the mutated proteins showed different subcellular localization; however, the level of accumulation of each protein was almost equal as judged by immunostaining with HoxA-13-specific antibodies (Fig. 4A).

The amino acid sequence of helix 3 in the homeodomain is highly conserved, and all Hox proteins carry WFQ residues. As a recognition helix, helix 3 enters the major groove of target DNA, and the Q residue of the WFQ motif interacts with the bases of the target DNA (32). We constructed helix 3 mutants of HoxA-13 and analyzed their effect on transcription. The mutant in which the well conserved WFQ residues of helix 3 in
the homeodomain were replaced with AAA (A13–3A) and a deletion that removes 33 amino acids from the C-terminal including helix 3 (A13-dC33) exhibited no transactivation (Fig. 4B), although the protein localized in the nuclei (Fig. 4A). In contrast, strong enhancement of transactivation was observed when the homeodomain was deleted (A13-dHD) (Fig. 4B). This strong activation was not due to increased accumulation of A13-dHD protein in the transfected cells over the wild type protein (data not shown). This strong activation was no longer observed when the deletion extended another 37 amino acids toward the N-end (A13-N191); furthermore, transactivation itself was abolished (Fig. 4B). These results suggest that amino acid residues in the 192–227 region contain a transcriptional AD, whose function seems to be suppressed in cis by the homeodomain. To identify activation domains of HoxA-13 outside the 192–227 region, this region was internally deleted from HoxA-13 (A13-dAD). Interestingly, A13-dAD still transactivated both mouse and chick Bmp-4-reporter transcription up to 6-fold (Fig. 4B), suggesting that HoxA-13 has additional activation domain(s) independent of the 192–227 region.

The mutant A13-dHD showed unique subcellular localization. The protein product was detected in the nuclei 18 h after transfection, whereas 65% of the expressing cells exhibited cytoplasmic localization of A13-dHD 36 h after transfection (Fig. 5), suggesting that A13-dHD was exported from the nucleus to the cytoplasm. A13-dHD showed no transactivation 18 h after transfection, despite nuclear localization. On the other hand, transcriptional stimulation by wild type HoxA-13 was already detected 18 h after transfection. Transcriptional activation by A13-dHD was detected 36 h after transfection, when most proteins were found in the cytoplasm (Fig. 5). These results indicate that transient localization in the nucleus or localization in the cytoplasm is necessary for transactivation by A13-dHD.

The Domain Responsible for Synergism with Sp1—We then analyzed synergism of these mutated Hox constructs with Sp1. Transfection of Sp1 strongly enhanced transcription in the presence of A13-dAD as well as that of wild type HoxA-13. In contrast, Sp1 exhibited no enhancement of transactivation by A13-dHD (Fig. 4C), although increased amounts of the effector construct were present (data not shown). This indicates that the mechanism of transactivation by A13-dHD, which would be due to the activation domain in the 192–227 region, is independent of Sp1.

These results indicate the presence of another activation domain(s), which is responsible for cooperative interaction with
Sp1, in the homeodomain or within both the 1–191 region and the homeodomain. The N-terminal domain (N1–191), lacking the Sp1-independent activation domain, showed no transcription (Fig. 4B). However, this does not exclude the possibility that the N1–191 region participates in Sp1-dependent transactivation in cooperation with the homeodomain. We were unable to analyze activity of the construct containing the homeodomain alone (N225HD+Cter) because the protein was very unstable in the transfected cells (data not shown). We therefore fused the N-terminal region of HoxA-11, which exhibited no activity in NG108 cells, and the HoxA-13 homeodomain (A11N-A13HD). A11N-A13HD transactivated both Bmp-4 promoters up to 6-fold (Fig. 4B). This suggests that a novel AD besides the homeodomain. The N-terminal domain (N1–191), lacking Sp1, in the homeodomain or within both the 1–191 region and the homeodomain. The N-terminal domain (N1–191), lacking the Sp1-independent activation domain, showed no transcription (Fig. 4B). However, this does not exclude the possibility that the N1–191 region participates in Sp1-dependent transactivation in cooperation with the homeodomain. We were unable to analyze activity of the construct containing the homeodomain alone (N225HD+Cter) because the protein was very unstable in the transfected cells (data not shown). We therefore fused the N-terminal region of HoxA-11, which exhibited no activity in NG108 cells, and the HoxA-13 homeodomain (A11N-A13HD). A11N-A13HD transactivated both Bmp-4 promoters up to 6-fold (Fig. 4B). This suggests that a novel AD besides

**Figure 5.** A13-dHD, lacking the entire homeodomain, worked in the cytoplasm. A, three typical patterns of A13-dHD protein subcellular localization. N, nucleus; C, cytoplasm. N > C indicates that proteins were uniformly detected throughout the nucleus and cytoplasm. N < C indicates that most proteins were seen in the cytoplasm. B, the ratio of each localization pattern of A13-dHD and its activities are shown at both 18 and 36 h after transfection. Activities of A13-dHD are presented by -fold induction. “1-fold” induction indicates no difference in luciferase activities between A13-dHD and pmw control. Mean ± S.D. of three independent experiments is shown. Values in parentheses indicate the average transcriptional stimulation ratio by wild type HoxA-13. Note that A13-dHD did not show any transcriptional stimulation at 18 h after transfection, whereas wild type HoxA-13 had already shown stimulation.

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**Activation Domain in N1–191**—We then examined the function of the N1–191 region. For this purpose, the opposite fusion construct, in which the N-terminal region of HoxA-13 was followed by the HoxA-11 homeodomain (A13N-A11HD) was prepared. This fusion protein transactivated both Bmp-4 promoters up to 5-fold and A11N-A13HD (Fig. 4B). In addition, both A11N-A13HD and A13N-A11HD exhibited significant synergism with Sp1 as well as with wild type HoxA-13 and A13-dAD (Fig. 4C). These results indicate that transactivation by A13N-A11HD may be due to another AD, which is independent of the AD in the 192–227 region. This possibility is strongly supported by the following two pieces of evidence. The first is that A13N-A11HD proteins were localized in the nucleus (Fig. 4A), although the AD in the 192–227 region of A13-dHD also functioned when the proteins were exported to the cytoplasm (as shown in Fig. 5). The other piece of evidence is the result of competition of stimulation by N1–191. When the N1–191 construct was cotransfected with wild type HoxA-13, stimulation was suppressed. This suppressive effect of N1–191 was observed when transfected with either A13N-A11HD or A13-dAD. On the other hand, suppression was not seen with A13-dHD and A11N-A13HD (Fig. 4D). These results suggest that an additional AD is present in N1–191 and that this domain is responsible for the activities of A13-dAD and A13N-A11HD and partly for wild type HoxA-13 but not for A13-dHD.

**Msx-1 and HoxA-11 Suppressed Sp1-dependent Transactivation by HoxA-13**—A previous study has shown that the homeodomain protein Msx-1 suppressed Sp1-dependent transcription through cooperative interactions with general transcriptional components for Msx-1 gene autorepression (33). To test the effect of Msx-1 protein on Sp1-dependent transactivation by HoxA-13, we performed co-transfection experiments of minimal mouse Bmp-4-reporter and Msx-1 together with HoxA-13 or Sp1. Transactivations both by HoxA-13 and Sp1 were suppressed by Msx-1 in a dose-dependent manner (Fig. 6A).

As shown above, HoxA-11 exhibited no stimulation of transcription from the Bmp-4 promoter, but the HoxA-11 homeodomain functioned when fused to the N-terminal domain of HoxA-13. We therefore tested whether full-length HoxA-11 was nonfunctional or suppressive. Interestingly, like Msx-1, HoxA-11 not only repressed Sp1-dependent transcription (Fig. 6C) but also suppressed enhancement of transcription by HoxA-13, A11N-A13HD, and A13N-A11HD. These observations suggest that Msx-1 and HoxA-11 suppress Sp1-dependent transactivation by HoxA-13 in our system.

**Helix 3 Is Essential for Sp1-dependent and -independent Transactivation by HoxA-13**—The well conserved helix 3 of the homeodomain has been reported to perform a crucial role during embryonic development (34). We analyzed the effect of the helix 3 mutants, A13–3A and A13–4C33, on wild type HoxA-13 or on several deletion clones, A13-dAD, A11N-A13HD, and A13N-A11HD, that showed Sp1-dependent transcriptional stimulation. Both helix 3 mutant forms suppressed stimulation in a dose-dependent manner (Fig. 7). Surprisingly, both helix 3 mutant forms also suppressed stimulation by A13-dAD, whose stimulation was Sp1-independent. Furthermore, the same helix 3 mutation constructs also suppressed transactivation by HoxD-13 (Fig. 7). These results suggest that the helix 3 mutation suppressed both Sp1-dependent and -independent transactivation and that helix 3 mutants act as dominant negative forms not only on HoxA-13 itself but also on HoxD-13.

**DISCUSSION**

In this study, we showed that Hox homeoproteins modulate transcription that is dependent on GC box-binding proteins. Among five GC box-binding proteins examined in this study, Sp1 showed remarkable enhancement of both HoxA-13- and HoxD-13-dependent transactivation. The ubiquitously expressed Sp1 protein has been implicated in the activation of a very large number of genes including housekeeping genes and tissue-specific genes (31, 35). Sp1 is thought to be involved in cellular process, such as cell cycle regulation, chromatin remodeling, and the propagation of methylation-free islands (36–41). Loss of function mutation of Hox Abd-B exhibited retardation of growth and differentiation of the limb cartilage, indicating that Hox genes are involved in the simultaneous quantitative transcriptional control of the genes crucial for these processes (6–8, 13, 14). Our results suggest that interaction with Sp1 is involved in Hox homeodomain-dependent quantitative transcriptional control mechanism.

We found no change in the gel shift band profile in the presence of HoxA-13 using both Bmp-4 promoter fragments (~260bHBS; result not shown) and GC box oligonucleotide (Fig. 3C) probes. These results indicate that the interaction of HoxA-13 and Sp1 is indirect. In this case, this activation may be mediated by non-DNA binding type transcriptional mediator(s) induced or activated by HoxA-13. Alternatively, HoxA-13 is a component of Sp1-interacting transcriptional mediator complex or a cofactor whose interaction is too unstable to detect...
by electrophoretic mobility shift assay. CRSP is a multiprotein complex that mediates Sp1 and TFIID in an in vitro transcription system (42). However, no homeodomain proteins were listed as the basic component of CRSP (42). It is possible that the Hox homeodomain protein is a variable regulatory component of the basic complex of CRSP. HoxA-13 is unlikely to be a component of TFIID, because HoxA-13 was not detected in the immunoprecipitate recovered from transfected nuclear extract using anti-TATA-binding protein antibody (results not shown).

CBP/p300 is reported to interact with both Sp1 and Hox proteins (43–47). We tested the effect of p300 by transfection analysis and found no functional participation of p300 in this Hox-Sp1 system (results not shown). Thus, Hox homeodomain proteins participate in the GC box-dependent transcriptional modulation in a novel manner.

Multiple ADs of HoxA-13 Activate Transcription Individually through GC Box-binding Protein-dependent and -independent Transcription Systems—We demonstrated that HoxA-13 contains three independent transcription stimulation domains (AD1 to AD3) (Fig. 8A). AD1 (amino acid residues 1–191) and AD3 (the region around helices 1 and 2) exhibited cooperation with Sp1 (Fig. 4) and AD1 functions only when connected with the homeodomain. Co-expression of isolated AD1 (N1–191) with full-length HoxA-13 or a construct carrying AD1 and the homeodomain suppressed transcriptional stimulation (Fig. 4D). This indicates the following two possibilities. One possibility is that AD1 and the homeodomain interact with a cofactor at the same time. The other possibility is that AD1 or the homeodomain interact with the first cofactor, and the remaining domain interacts with the second cofactor. The fusion construct A11N-A13HD showed cooperation with Sp1; however, suppression by AD1 was not observed (Fig. 4D). This evidence supports the hypothesis that both AD1 and the homeodomain simultaneously contact one cofactor.

The fusion construct A11N-A13HD has Sp1-dependent transcriptional enhancement activity (Fig. 4B). Since the amino-terminal domain of HoxA-11 does not have a transcriptional activation function, transactivation would be dependent on the HoxA-13 homeodomain. The HoxA-13 portion of this construct consists of the homeodomain itself, 14 amino acid residues upstream of the homeodomain, and 7 amino acid residues downstream (Fig. 8B). All of these three regions are present in the homeodomain exon. As shown in Fig. 8B, there are patch-like regions that are conserved within the same paralog and
unique between different paralogs in these three regions. These patchlike regions are candidate subdomains for carrying different functions between paralog 11 and 13 homeodomains. Recently, it was reported that the Ultrabithorax protein, encoded by one of the Drosophila Hox genes, carries crucial regulatory domains that interact with other parts of the same protein at a site downstream of the homeodomain (48, 49). Identification of such a functional subdomain in the HoxA-13 homeodomain exon remains a subject for future study.

With regard to a GC box-binding protein-independent transactivation domain, we identified a novel AD in the region N192–227 (AD2; see Fig. 8A). AD2 showed hyperactivation when the homeodomain was removed. Li et al. (19) found that Dfd protein exhibited hyperactivation when the homeodomain was separated, and they proposed that the N-terminal activation domain was persistently masked by the homeodomain. In our observation, however, hyperactivation by AD2 (homeodomain-deleted form) has different characteristics from that of wild type HoxA-13, since hyperactivation was GC box protein-independent. We found that transient localization in the nucleus or localization in the cytoplasm is necessary for transactivation by AD2. This suggests that transactivation by AD2 is not mediated by the general transcriptional machinery; for example, AD2 may interact with negative regulators in the nucleus and export them to the cytoplasm.

Cytoplasmic expression of non-homeodomain isoforms has been observed in some homeodomain proteins, such as HoxA-1, HoxA-5, and HoxB-6 (50–53). It is possible that AD2 functions in vivo as a non-homeodomain isoform of HoxA-13. This is further supported by the fact that HoxA-13 proteins are detected as three isoforms in transfected cells (54) and four isoforms in limb bud, one of which is detected in the cytoplasm fraction (55). Although most Hox genes are detected as multiple, differentially spliced isoforms (56), little is known about the functional difference between each isoform. HoxA-13 may have multiple functions through individual isoforms and may regulate transactivation through multiple steps.

The Third Helix of the Homeodomain Plays a Crucial Role for Transactivation by HoxA-13—In this study, we demonstrated that the region conserved between HoxA-13 and HoxA-11 homeodomains plays a crucial role in cooperation with Sp1-dependent transcriptional enhancement (Fig. 8). We also found that helix 3 mutants of HoxA-13 strongly suppress transactivation by HoxA-13 in a dominant negative manner (Fig. 7). It is possible that helix 3 mutants of HoxA-13 can bind to the cofactor, whereas they cannot proceed to the following transactivation as HoxA-11 cannot. Alternatively, helix 3 mutants may no longer bind the cofactor but instead may interact with an unknown molecule(s), which is responsible for the subsequent interaction step. One of the helix 3 mutants, A13-cD3, is similar to the human HoxA-13 mutant, which causes hand-foot-genital syndrome. Hand-foot-genital syndrome is a dominantly inherited condition affecting the distal limbs and genitalia (57). The associated phenotypes are more severe in hand-foot-genital syndrome than in targeted disruption or deletion of mouse HoxA-13 (58). These phenotypic differences raise the possibility that the mutant protein may act in a dominant negative manner toward not only itself but also neighboring homeodomain proteins. Our dominant negative data strongly support this possibility and further suggest that HoxA-13 and HoxD-13 activate transcription via a similar regulatory mechanism, including cooperation with the GC box binding protein-dependent transcription system.

Acknowledgments—We are grateful to Drs. G. Suske, M. Subramaniam, and Y. Fujii for the gift human Sp3 and Sp4 cDNA clones, human TIEG1 cDNA clone, and human BTEB1 cDNA clone, respectively, and Dr. T. Suzuki for helpful discussion.

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